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Raphaële Gresse

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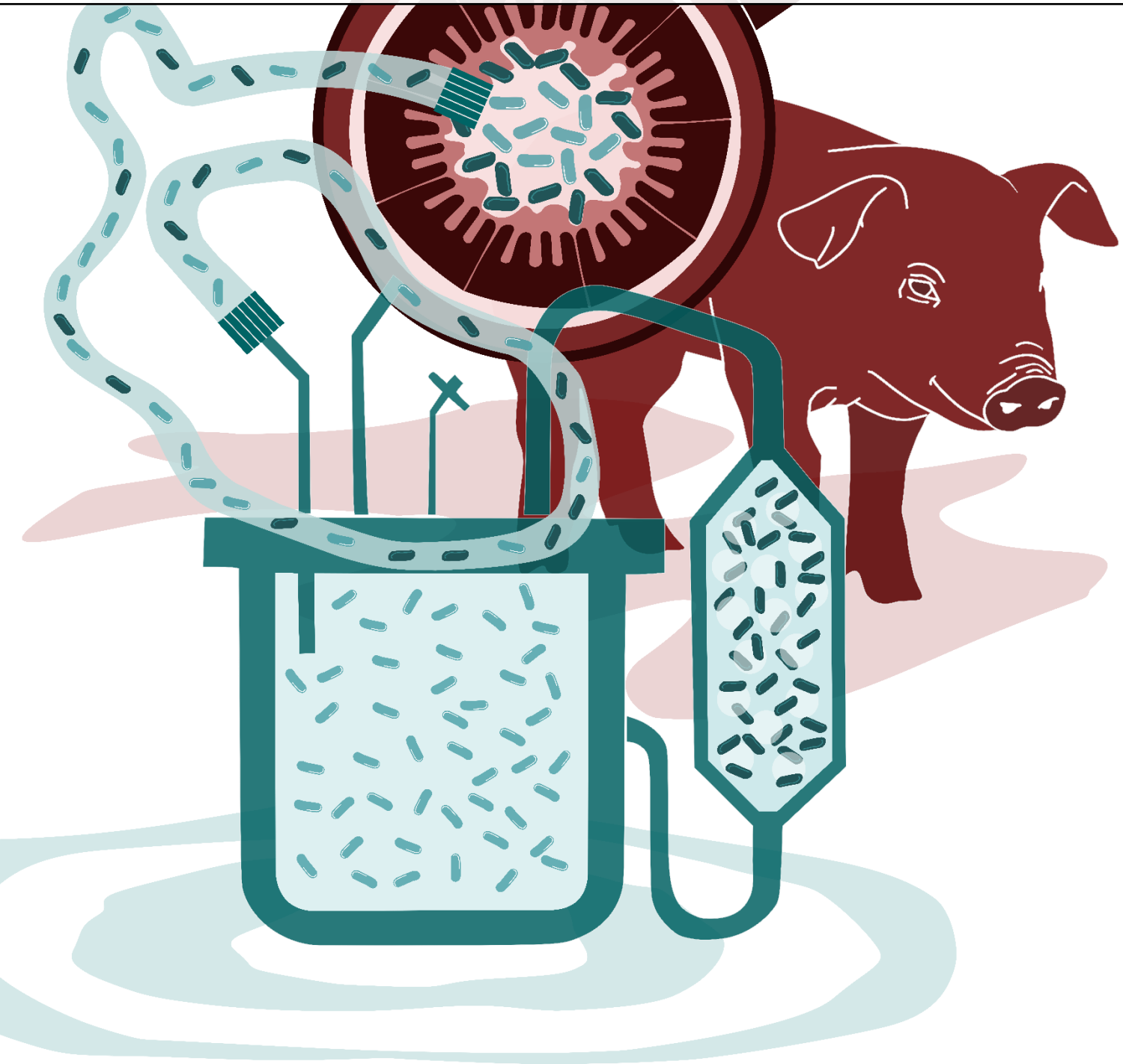
PhD Thesis
Defended the 17th of June 2020

Raphaële Gresse

DEVELOPMENT OF AN *IN VITRO* MODEL OF GUT DYSBIOSIS IN WEANING PIGLETS. APPLICATION TO THE STUDY OF PROBIOTICS TO FIGHT AGAINST POST-WEANING DIARRHEA.



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Résumé du manuscrit en français

Contexte scientifique et objectifs principaux de la thèse

Le porc est la viande la plus consommée au monde. On recensait en effet 767.6 millions de porcs vivants dans le monde en 2019. En conséquence, le rythme de production très élevé influence le bien-être des animaux en imposant par exemple des conditions de vie en confinement ou un sevrage précoce des animaux. Dans l'industrie moderne, les porcs sont élevés en « bandes » qui correspondent à des lots d'animaux à un même stade physiologique tels que les truies en insémination, les truies en gestation, les truies en lactation et leurs porcelets, et les porcelets sevrés. Le sevrage est la période la plus critique dans la vie du porc. Il est pratiqué généralement à 3 ou 4 semaines alors qu'un sevrage naturel aurait lieu à 17 semaines chez le porc. Cette période est associée à de nombreux stress d'ordre environnementaux, sociaux et à un changement parfois brusque d'alimentation. Les porcelets en allaitement se nourrissent du lait maternel couplé à un aliment pré-sevrage sous la mère appelé « creep feeding » contenant majoritairement des produits lactés mais aussi des sucres, fibres et protéines qui servent à habituer l'animal à ingérer et digérer des aliments solides et d'origine végétale. Au sevrage, les animaux reçoivent un aliment premier âge contenant un taux de protéines végétales et de céréales plus importantes. Bien qu'aidé par l'aliment pré-sevrage, le sevrage a notamment des conséquences comportementales néfastes pour les porcelets comme une période d'anorexie. L'anorexie associée au sevrage peut toucher jusqu'à 50% des porcelets et dure entre 12h et 48h dans la majorité des cas. Les conséquences physiologiques associées au sevrage et à cette période d'anorexie sont nombreuses telles qu'un impact négatif sur la croissance associée à une perte de poids, des modifications néfastes de la morphologie de l'épithélium intestinal impactant les fonctions d'absorption et de perméabilité de l'intestin, une inflammation accrue et des modifications du microbiote intestinal.

Le microbiote intestinal, correspondant à l'ensemble des microorganismes présents dans le tractus digestif, est responsable de nombreuses fonctions essentielles pour son hôte notamment la production de vitamines et divers métabolites énergétiques ou de signalisation cellulaire comme les acides gras à chaine courtes (AGCC), la régulation des fonctions intestinales et immunitaires ou la protection contre les pathogènes. La composition du microbiote du porcelet varie en fonction des compartiments du tube digestif et aussi entre la lumière intestinale et la muqueuse. Elle est influencée par divers facteurs comme le pH, le gradient d'oxygène, le temps de transit et les sécrétions digestives en fonction des segments. La majorité des recherches sur le microbiote des porcelets au sevrage se concentre sur la composition bactérienne des fèces. Le microbiote fécal est composé majoritairement des phyla *Bacteroidetes*, *Firmicutes* et

Proteobacteria. Suite au sevrage, des modifications du microbiote, parfois contradictoires, ont été relevées par des nombreuses études. La majorité rapporte une augmentation des familles *Prevotellaceae* et *Clostridiaceae* et une réduction des genres *Lactobacillus, Bacteroides* et *Fusobacterium.* Très peu d'informations sur la composition des archées et des levures sont disponibles chez le porcelet autour du sevrage, néanmoins un shift des espèces au sein du du genre *Methanobrevibacter* a été décrit. Chez les porcelets, le microbiote associé au mucus du colon contient généralement une proportion plus importante de protéobactéries que le microbiote de la lumière digestive, dont les familles *Campylobacteriaceae* et *Enterobacteriaceae*. Jusqu'à présent peu étudié, le microbiote associé au mucus pourrait, en raison de sa localisation au contact des cellules épithéliales de l'intestin, impacter la santé des porcelets via, par exemple, une sur activation du système immunitaire en cas de modifications de sa composition ou son activité. De plus, les bactéries du microbiote interagissent avec les mucines composant la muqueuse intestinale en les dégradant ou en modulant leur structure via la production d'acides gras à chaines courte.

Les modifications du microbiote au sevrage, également appelées dysbiose, sont associées aux diarrhées colibacillaires survenant quelques jours après le sevrage des porcelets. Les diarrhées post-sevrages représentent un problème majeur dans les élevages et sont responsables de 17% des pertes totales des porcelets nés en Europe. Généralement, ces infections sont majoritairement dues à des *Escherichia coli (E. coli)* pathogènes, les *E. coli* entérotoxinogènes (ETEC). Une fois entrés dans le système digestif des porcelets par voie orale, les ETEC sont capables de coloniser et proliférer au niveau de la lumière digestive et de l'épithélium intestinal par le biais de facteurs de virulence qui sont différents selon les souches d'ETEC. Les ETEC responsables des diarrhées post-sevrage possèdent majoritairement des fimbriae de type F4 qui leur permettent d'adhérer aux entérocytes. Ces bactéries sont capables de secréter plusieurs entérotoxines telles que les entérotoxines thermostables a et b (STa et STb), les « heat labile » entérotoxines (LT) ou les EAEC entérotoxines thermolabiles (EAST1). Si ces toxines agissent via des mécanismes cellulaires différents, leur effet principal est de déclencher de violentes diarrhées aqueuses provoquant une déshydratation sévère parfois suivie de la mort de l'animal.

Les infections à ETEC sont généralement combattues à l'aide de traitements antibiotiques curatifs et préventifs. Ce recours systématique aux antibiotiques a favorisé l'expansion de gènes de résistance parmi les communautés bactériennes, ce qui représente maintenant un réel danger pour la santé publique. En conséquence, il est urgent de rechercher d'autres stratégies pour prévenir et traiter les diarrhées post-sevrage chez le porcelet comme l'utilisation de

probiotiques. Parmi les probiotiques déjà commercialisés dans la production porcine, certaines levures vivantes sont d'ores et déjà utilisées sous forme sèche active chez les truies gestantes ou allaitantes pour limiter la constipation et le stress à la mise bas, améliorer la digestibilité des aliments, la qualité du lait, et la croissance des porcelets. Chez les jeunes porcelets, les levures de types *Saccharomyces cerevisiae* (SC) ou *Saccharomyces cerevisiae* var *Boulardii* (SB) sont recommandées pour lutter contre les diarrhées. Différents potentiels modes d'action des levures pour lutter contre les pathogènes responsables de troubles digestifs chez les porcelets ont été identifiés via des études *in vivo* ou *in vitro*. Ces mécanismes pourraient impliquer une modulation du microbiote intestinal favorisant les populations bénéfiques pour l'hôte, une dégradation des toxines sécrétées par le pathogène ou de leurs récepteurs situés sur les entérocytes, la stimulation du système immunitaire de l'hôte ou une régulation de l'inflammation ou encore le blocage des sites adhésions du pathogène aux entérocytes.

Les études *in vivo* restent le « gold standard » pour étudier ces problématiques. Néanmoins, la directive de l'Union Européenne 2010/63/EU visant à remplacer, réduire et raffiner l'utilisation des animaux dans la recherche encourage fortement les expérimentations impliquant des alternatives *in vitro.* L'utilisation de modèles *in vitro* reproduisant l'environnement digestif du porc permet d'économiser du temps, offre une flexibilité technique appréciable et une reproductibilité grâce à des conditions plus standardisées par rapport à l'*in vivo,* représente un gain financier non négligeable et est éthiquement mieux toléré du grand public. A ce jour, plusieurs modèles *in vitro* de l'environnement colique du porc ou du porcelet, tel que le modèle PolyFermS où le baby-SPIME, ont été mis au point à partir de fèces afin d'en reproduire la composante microbienne. A notre connaissance, le baby-SPIME est le seul modèle *in vitro* visant à reproduire le microbiote du porcelet lors du sevrage. Néanmoins, en plus de reproduire exclusivement le microbiote de la lumière intestinale en excluant celui du mucus, plusieurs paramètres de ce modèle manquent d'adaptation à l'environnement colique des porcelets de cet âge tels qu'un milieu nutritif reproduisant la composition des effluents iléaux, un pH ou un temps de transit adaptés.

Dans ce contexte, ce projet de thèse avait pour principaux objectifs d'apporter plus d'informations sur la structure des communautés microbiennes des porcelets en âge d'être sevrés au niveau de l'ensemble du tractus digestif et plus précisément de la lumière et de la muqueuse colique, mais aussi sur l'activité métabolique du microbiote et les paramètres physico-chimiques du colon afin de les reproduire le plus fidèlement possible dans le modèle *in vitro.* L'objectif suivant a été de développer le modèle *in vitro* du colon des porcelets

implémenté de billes de mucines dans un compartiment externe relié au fermenteur principal permettant de maintenir l'anaérobiose par la seule activité du microbiote résident. Ce modèle a ensuite été utilisé pour simuler l'anorexie transitoire associée au sevrage et évaluer les effets d'une souche de SB. L'objectif suivant étant de simuler complétement les effets du sevrage sur le microbiote du porcelet, un changement alimentaire suivant la période de jeûne alimentaire a donc été ensuite introduit dans le modèle. L'impact de l'inoculation d'une souche pathogène porcine d'ETEC sur le microbiote et sur des cellules intestinales porcines juste après la simulation du sevrage a enfin été étudié ainsi que les effets d'une supplémentation avec la souche de levure probiotique.

Organisation de la thèse

Ce projet de thèse CIFRE s'est déroulé au sein de l'UMR 454 « Microbiologie Environnement Digestif et Santé » (MEDIS), unité mixte entre INRAE (site de Theix en Auvergne-Rhône-Alpes) et l'Université Clermont Auvergne, en partenariat étroit avec la société Lallemand Nutrition Animale. L'UMR MEDIS étudie principalement les interactions entre l'hôte, le microbiote intestinal, son environnement, les pathogènes et les probiotiques dans le but de développer des stratégies innovantes pour améliorer la santé humaine et animale. De plus, l'UMR MEDIS possède 25 ans d'expérience dans la simulation de l'environnement digestif et a développé une plateforme unique en France qui combine différents modèles *in vitro* de l'environnement digestif humain et animal. La société Lallemand Nutrition Animale figure quant à elle parmi les leaders mondiaux de la fermentation microbienne et développe des stratégies à base de levures et bactéries probiotiques pour améliorer la santé, la nutrition et le bien-être des animaux ruminants ou monogastriques. Le développement des billes de mucines et de leur compartiment s'est fait en collaboration avec le Centre d'Ecologie Microbienne et de Technologie (CMET) de l'Université de Gand (Belgique) qui collabore avec l'UMR MEDIS depuis plus de 10 ans et qui possède également une forte expertise sur les modèles de digestion artificiels complémentaire de celle de l'UMR MEDIS. Enfin, une collaboration avec le Département de Génétique Animale de l'Université de Cordoba en Espagne (Dr Garrido) et comprenant une période expérimentale de 2 mois sur site en automne 2019 a été mise en place afin de coupler des effluents du modèle *in vitro* développé avec des cellules intestinales porcines en culture.

Organisation du manuscrit

Le Chapitre 1 consiste tout d'abord en une revue de la littérature publiée dans le journal Trends in Microbiology en juin 2017 rassemblant les problématiques liées au sevrage des porcelets et les possibles stratégies non pharmaceutiques pour lutter contre l'incidence des diarrhées post-sevrage. Les Chapitres 2 à 7 de ce manuscrit sont dédiés à une exploration plus précise de l'état de l'art sur l'élevage porcin, le sevrage et ses conséquences, le microbiote du porcelet, les infections à ETEC et les modèles *in vitro* de l'environnement digestif. Les chapitres 8 à 11 sont consacrés à la partie expérimentale. Enfin le Chapitre 12 discute les résultats obtenus dans la partie expérimentale et décrit les perspectives futures pour continuer à explorer l'étiologie de la dysbiose post-sevrage et des infections associées à ETEC chez les porcelets ainsi qu'à évaluer les stratégies de prévention basées sur l'utilisation de probiotiques à l'aide du modèle *in vitro* développé au cours de ce travail de thèse.

Description des résultats de la partie expérimentale

Etude du microbiote du tractus digestif de porcelets

Le **Chapitre 8** de ce manuscrit, valorisé par une publication dans le journal « Microorganisms » (doi:10.3390/microorganisms7090343), décrit la première partie de ce travail de thèse qui a consisté à étudier le microbiote de porcelets de 28 jours provenant d'un élevage commercial qui sera également le lieu de prélèvement des inocula fécaux pour le démarrage des fermentations. Cet élevage, la ferme Porc Aubènes, est situé en Haute-Loire (France) et produit des porcelets élevés en bandes de manière classique. La composition du microbiote de 6 porcelets males de 28 jours a été étudiée de l'estomac aux fèces grâce aux techniques de PCR quantitative (qPCR) et de séquençage partiel du gène 16S via une plateforme Illumina MiSeq. La quantité de bactéries totales a été mesurée de 7 à 9 Log_{10} de gène 16S par g d'échantillon digestif pour l'estomac et les différentes parties de l'intestin grêle. Au niveau du cecum, du colon et des fèces les bactéries totales ont été quantifiées à plus de 10.5 Log_{10} de gène 16S par g d'échantillon digestif et à 5.8 Log_{10} de gène 16S par g de mucus colique en moyenne. Les archées n'ont pas pu être détectées dans la partie haute du tube digestif mais ont été quantifiées entre 8.5 et 10 Log_{10} de gène 16S par g d'échantillon digestif dans le gros intestin et entre 7 et 8 Log_{10} de gène 16S par g de mucus colique. Au niveau des phyla, les *Firmicutes* ont été détectés globalement majoritaires dans tout le tube digestif suivis des *Proteobacteria* essentiellement dans l'estomac, le duodénum, le jéjunum et l'iléon et des *Bacteroidetes* au

niveau du cecum, colon proximal, colon distal et des fèces. Les principales différences entre le microbiote du mucus du colon par rapport à celui de la lumière colique consistaient à des proportions plus importantes des phyla *Proteobacteria, Spirochaetes* et *Epsilonbacteraeota.* A plus bas niveau taxonomique, les échantillons de la partie haute du tube digestif étaient majoritairement composés des familles *Lactobacillaceae, Enterobacteriaceae, Pasteurellaceae* et *Peptostreptococcaceae* alors que la partie basse du tractus digestif, du cecum aux feces, arborait préférentiellement les familles *Ruminococcaceae, Lachnospiraceae, Prevotellaceae* et *Bacteroidiaceae.* Une des principales observations au niveau de la composition du microbiote des porcelets, et ceci tout le long du tube digestif, a été la présence de genres comprenant des pathogènes opportunistes tels que *Escherichia/Shigella, Campylobacter, Acinetobacter, Romboutsia* ou *Terrisporobacter.* Ces pathogènes opportunistes pourraient en cas de stress au cours du sevrage être avantagés et entrainer potentiellement des pathologies digestives. Les communautés archéennes, détectées en quantité importante dans le gros intestin, sont peu diversifiées et composées très majoritairement du genre *Methanobrevibacter.* L'activité microbienne tout le long du tube digestif des porcelets a été analysée via des prédictions fonctionnelles grâce à l'outil Tax4Fun et la mesure des concentrations des principaux AGCC grâce à la chromatographie en phase gazeuse dans le cecum, colon proximal et distal et les fèces. Les 3 principaux AGCC détectés dans les contenus digestifs étaient l'acétate, le propionate et le butyrate. L'analyse des prédictions fonctionnelles basées sur le gène 16S a révélé une clusterisation de la partie haute de l'intestin, arborant principalement des fonctions liées aux mécanismes cellulaires ainsi qu'à la communication entre bactéries, de la partie basse du tube digestif comprenant une proportion plus importante des fonctions bactériennes liées à la production de métabolites, et des fèces où la totalité des fonctions bactériennes détectées appartenaient au métabolisme de divers composés et à la production d'énergie. Les principaux résultats de ce chapitre sont résumés dans la Figure A.

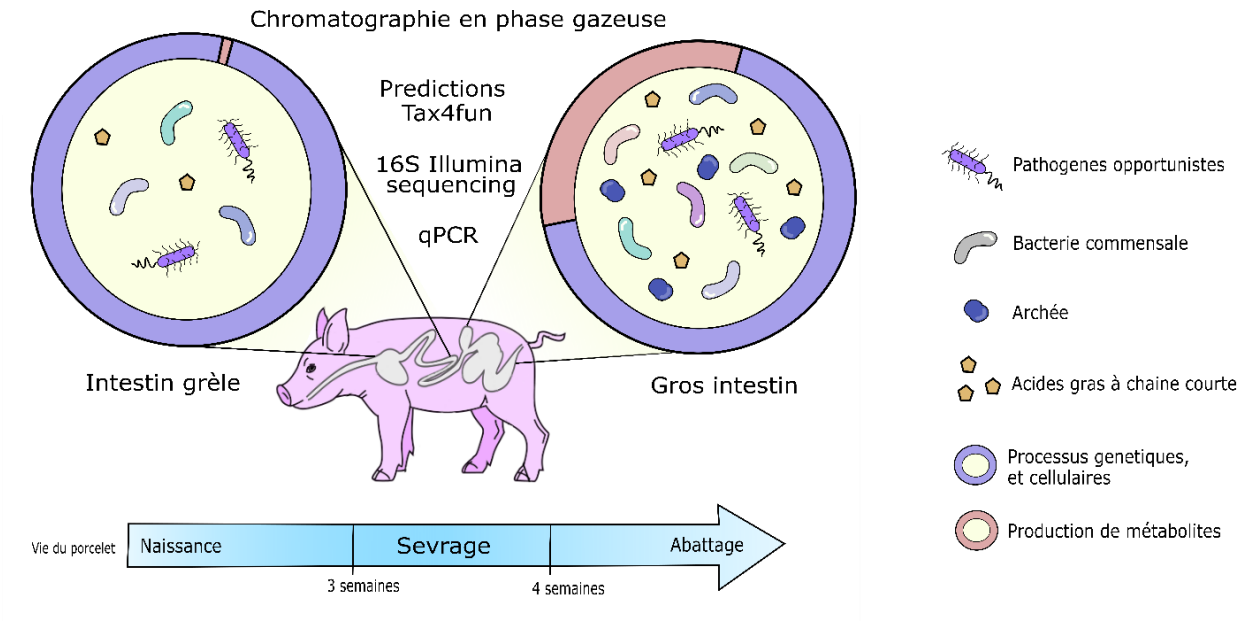

Figure A. Résumé des principaux résultats du chapitre 8 de ce manuscrit.

Développement du modèle* in vitro *du colon du porcelet et application d'un jeûne alimentaire et d'un traitement probiotique

Le **Chapitre 9** décrit le développement du modèle *in vitro* ainsi que l'application d'un stress de jeûne alimentaire dans le but de mimer l'anorexie des porcelets associée au sevrage. Enfin, dans une dernière partie, les résultats de l'étude de l'effet d'une supplémentation quotidienne en levure probiotique avant et après le stress de jeûne alimentaire sont présentés. Le modèle *in vitro*, nommé MPigut-IVM (« Mucosal Pig Gut In Vitro Model ») pour « modèle *in vitro* du colon du porcelet contenant des mucines », a été développé en utilisant des mini bioréacteurs en fermentation continue. Plusieurs paramètres sont contrôlés et mesurés en continu grâce à des sondes dans la cuve du bioréacteur, ont été adaptés à la physiologie du côlon du porcelet, tels que le pH fixé à 6 et la température fixée à 39°C. Le temps de rétention a quant à lui été fixé à 18h grâce au réglage du débit entrant de milieu nutritif, apporté en continu, du débit du soutirage de milieu fermentaire et du maintien constant du volume de la cuve par une sonde de niveau (Figure BI). Le potentiel d'oxydo-réduction n'est que mesuré dans le bioréacteur. Le milieu nutritif du MPigut-IVM reproduit la composition moyenne des effluents iléaux du porcelet. Il a été formulé en calculant la digestibilité des aliments et la quantité de nutriments non absorbés par l'intestin grêle et qui donc arrivent au côlon des animaux, comme précédemment décrit dans les modèles *in vitro* de MacFarlane *et al.* (1998) et Tanner *et al.* (2014). Les pratiques courantes d'élevage telles que le « creep feeding » ont aussi été prises en compte pour la définition de la

composition de ce milieu. Le milieu du MPigut-IVM contient donc une grande proportion de produits lactés, ainsi que des fibres et des protéines de nature végétale comme le soja et le blé. Les mini bioréacteurs ont été associés à des compartiments faits sur mesure d'un volume de 50 ml, contenant des billes de mucines, formées de mucines gastriques de porc et d'alginate de sodium, dans lesquelles le milieu de fermentation circule en continu pour faire le lien avec la cuve du bioréacteur (Figure BII). Les billes de mucines, dégradées continuellement par le microbiote, doivent être remplacées toutes les 48 heures. Une particularité du MPigut-IVM est le maintien de l'anaérobiose grâce à la seule activité du microbiote, le modèle étant flushé à l'azote seulement au démarrage des fermentations. Le MPigut-IVM a été inoculé au début de chaque run de fermentation avec un pool de fèces fraiches provenant de 6 à 8 porcelets males de 28 jours prélevés à la ferme et maintenus sous atmosphère anaérobie jusqu'à l'inoculation dans la cuve des fermenteurs. Afin de vérifier si le MPigut-IVM était en mesure de reproduire le microbiote luminal et mucosal du colon des porcelets de 28 jours, des fermentations contrôle comprenant 2 réplicats biologiques et 1 réplicat technique ont été réalisées sur une période 15 jours. Pendant la fermentation, des échantillons du milieu du bioréacteur et des billes de mucines (lavées 3 fois en PBS stérile) ont été prélevés quotidiennement. Les analyses de la composition du microbiote du MPigut-IVM ont été réalisées par des techniques de qPCR et de séquençage haut débit *via* la plateforme Illumina MiSeq ; la production de gaz et d'AGCC a été déterminée par chromatographie en phase gazeuse. Enfin, le microbiote adhérent aux billes de mucines a été observé par microscopie électronique à balayage (MEB).

Une phase de 7 jours a été nécessaire pour stabiliser la composition et l'activité du microbiote du MPigut-IVM. La présence d'un microbiote adhérent sur les billes après 48h de fermentation a été confirmée par MEB. Les principaux résultats de ce Chapitre 9 sont présentés en Figure C. Les analyses de séquençage haut débit ont mis en évidence une bonne reproductibilité du microbiote du colon des porcelets de 28 jours grâce à l'adaptation des populations fécales à l'environnement colique simulé par les paramètres physico-chimiques du modèle, par exemple, une augmentation de l'abondance relative des phyla des *Bacteroidetes* et *Proteobacteria.*

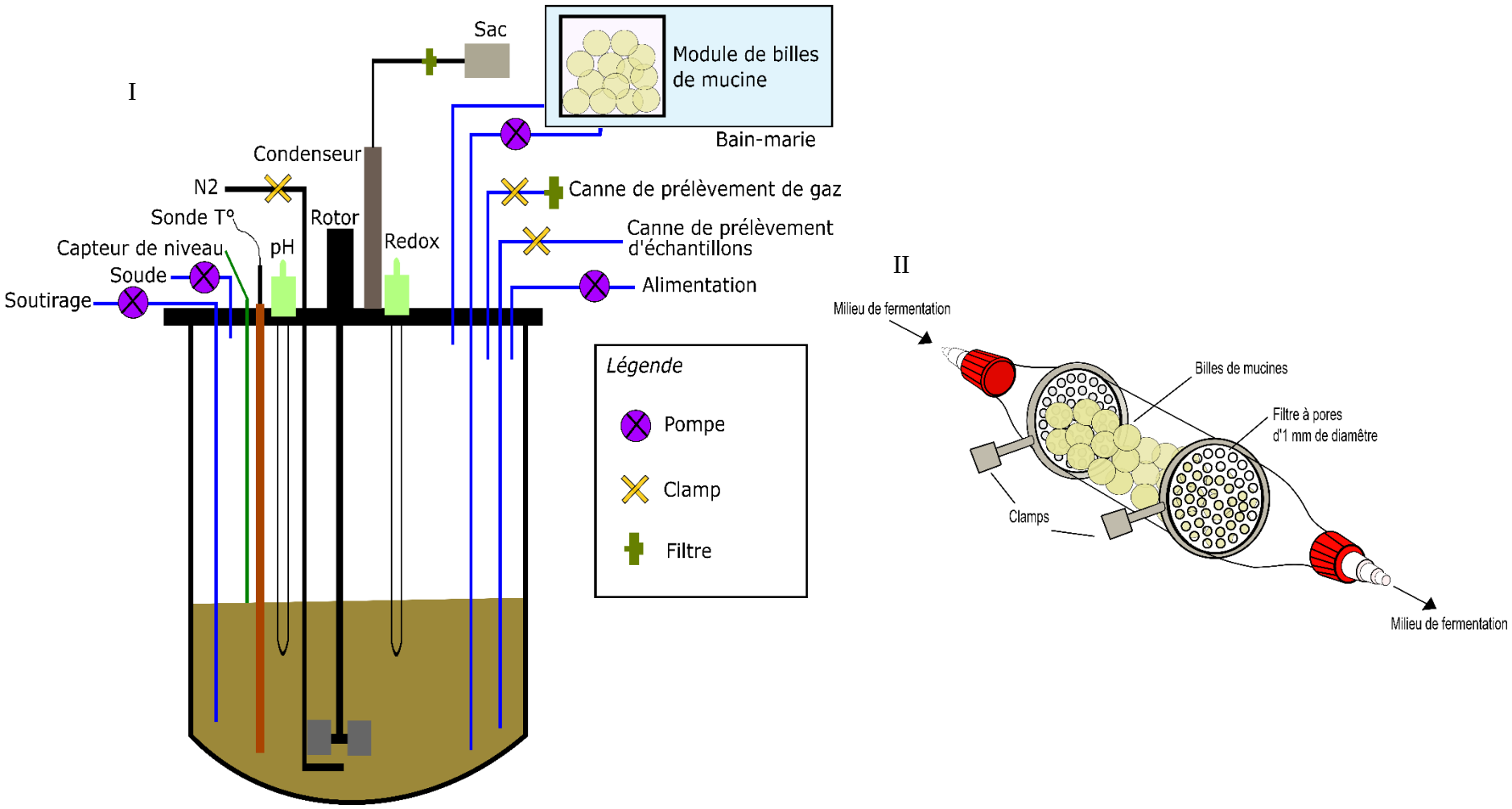

Figure B. (I) Schéma du MPigut-IVM et (II) focus sur le compartiment des billes de mucines.

Dix familles bactériennes sur les 15 principales ont été détectées dans le milieu du bioréacteur en comparaison avec des contenus digestifs de colon de porcelets de 28 jours, et 14 familles sur 15 ont été retrouvées sur les billes de mucines en comparaison avec du mucus colique de porcelets de 28 jours. En accord avec les données *in vivo,* le microbiote des billes de mucines est clairement distinct de celui du milieu du bioréacteur. Les principales familles bactériennes dans le milieu du bioréacteur étaient les *Bacteroidiaceae, Prevotellaceae, Lachnospiraceae* et les *Acidaminococcaceae* tandis que les billes de mucines comprenaient majoritairement des *Clostridiaceae* 1, *Lachnospiraceae, Peptostreptococcaceae, Desulfovibrionaceae* et *Clostridium* Family XIII. De plus, le MPigut-IVM a permis de maintenir les populations d'archées après la période de stabilisation avec, comme pour l'*in vivo,* un microbiote quantitativement important mais presque exclusivement composé du genre *Methanobrevibacter*. Les différences de population provenant des 2 réplicats biologiques ont été maintenues suite à la période de stabilisation montrant que le MPigut-IVM est capable de préserver les différences inter-inocula. Concernant l'activité fermentaire du microbiote, du dioxyde de carbone, de l'hydrogène et du méthane ont été détectés confirmant la présence de populations microbiennes métaboliquement actives et, notamment, des archées méthanogènes. La proportion d'acétate, butyrate et propionate s'est révélée être en concordance avec la proportion d'AGCC dans les échantillons coliques *in vivo.* Enfin, le potentiel redox moyen de -250 mV au cours des fermentations s'est révélé être en accord avec les données de la littérature. Une fois le modèle caractérisé, un stress de jeûne alimentaire de 48h a été appliqué au MPigut-IVM à la suite de la période de stabilisation afin d'en étudier l'impact sur le microbiote, sur 4 réplicats biologiques. Cette période de jeûne a eu plusieurs conséquences, pour la plupart transitoires, sur la composition des populations bactériennes du modèle, notamment une augmentation des familles *Prevotellaceae* et *Enterobacteriaceae* (pour 2 répétitions sur 4) ainsi qu'une diminution des *Bacteroidiaceae, Ruminococcaceae* et *Clostridiaceae* (sur les billes de mucines). Ces informations sont en accord avec les rares études *in vivo* ayant étudié l'impact de l'anorexie associée au sevrage sur le microbiote.

Concernant les conséquences sur l'activité du microbiote, le jeune alimentaire a induit également de légères modifications transitoires d'AGCC et des proportions de gaz et surtout une augmentation conséquente du potentiel redox traduisant un ralentissement important de la fermentation. Le métabolome lors des fermentations reproduisant l'effet du jeune alimentaire a été étudié par la technique de résonance magnétique nucléaire (RMN) en collaboration avec l'équipe GenPhySe de l'INRAE de Toulouse.

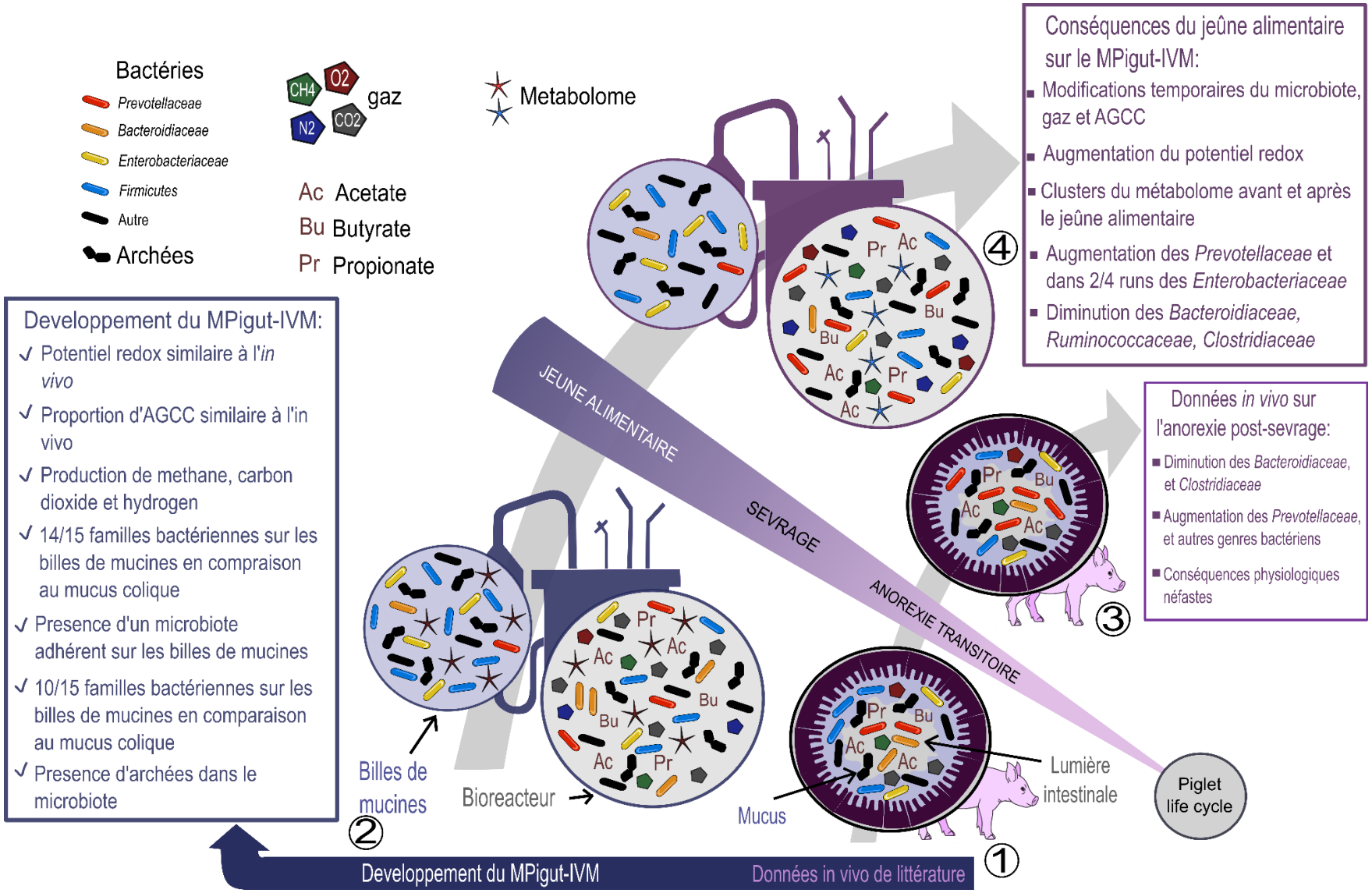

Figure C. Résumé graphique des principaux résultats obtenus dans le chapitre 9 de ce manuscrit.

Le principal résultat de cette analyse RMN s'est traduit par une séparation distincte du métabolome selon les trois phases de fermentations : stabilisation, fin du jeune alimentaire et récupération (dès la reprise de l'alimentation). Il est important de noter que l'analyse métabolomique des échantillons du MPigut-IVM a révélé la présence d'une large diversité de métabolites tels que des amines biogènes (ex : cadavérine, 5-aminovalérate, et putrescine) et des composés aromatiques (ex : 3-phénylpropionate, phénylacétate, *p*-crésol) ce qui démontre que la fonctionnalité du microbiote est maintenue *in vitro* dans le MPigut-IVM.

Dans la deuxième partie du Chapitre 9, l'effet d'un traitement préventif avec une supplémentation quotidienne de 10^6 UFC/ml de SB CNCM I-1079 sur le microbiote du MPigut-IVM a été étudié. La dose de probiotiques a été choisie grâce à des données de Lallemand Animal Nutrition et publiées rapportant la concentration de levures dans le colon de porcelets après une supplémentation de $3x10^9$ UFC/porcelet. La dose quotidienne de SB CNCM I-1079, bien que plutôt faible comparée à la majorité des études *in vitro* impliquant des levures, a démontré quelques effets significatifs sur le microbiote du MPigut-IVM et notamment une augmentation des *Escherichia coli* dans le milieu du bioréacteur, et une augmentation de la famille des *Streptococcaceae* et une diminution des *Veillonellaceae* sur les billes de mucines. Concernant l'activité du microbiote, le traitement probiotique n'a pas eu d'effet sur les AGCC ni sur la composition des gaz mais a tout de même permis une atténuation significative de l'augmentation du potentiel redox pendant le jeûne alimentaire ce qui semblerait indiquer un meilleur maintien de l'activité fermentaire du microbiote pendent le manque de nutriments.

Impact de l'introduction d'un pathogène et d'un stress alimentaire dans le MPigut-IVM

Le **Chapitre 10,** dont les principaux résultats sont présentés en Figure D, a consisté en l'étude des effets d'une simulation du sevrage plus complète avec l'introduction d'un changement alimentaire suite au jeûne de 48h suivi ou non d'un challenge par une souche d'ETEC porcine dans le MPigut-IVM. Le changement alimentaire a majoritairement consisté en une diminution conséquente des ingrédients lactés et une augmentation et une diversification de la proportion de céréales et protéines végétales. La souche d'ETEC de type F4 a été isolée d'un porcelet diarrhéique en Espagne et a été mise à disposition par le Dr. Juan J. Garrido. Cette expérimentation a été réalisée avec 3 réplicats biologiques pour chacune des deux conditions (ETEC et CTRL). Une fois encore la composition du microbiote a été étudiée par qPCR et Illumina MiSeq et l'activité du microbiote par chromatographie en phase gazeuse et RMN. Le changement de régime alimentaire dans le modèle *in vitro* a engendré plusieurs modifications

du microbiote du MPigut-IVM, qui ont cette fois-ci perduré jusqu'à la fin des fermentations, et notamment une quantité plus importante d'*Escherichia/Shigella,* une augmentation conséquente de l'abondance relative des genres *Prevotella* et *Methanosphaera,* et une diminution des genres *Bacteroides* et *Methanobrevibacter* dans le milieu du bioréacteur et sur les billes de mucines, ainsi qu'une diminution des *Ruminococcus* sur les billes de mucines. Le changement d'alimentation a également induit des modifications de l'activité du microbiote telles qu'une augmentation de la concentration totale d'AGCC, des modifications d'abondance relatives d'acétate, butyrate, valérate et caproate différentes entre le milieu du bioréacteur et le milieu du compartiment des billes de mucines, une augmentation du potentiel redox et une augmentation de plusieurs métabolites tels que l'éthanol, le phénylacetate et le 3-phényl-propionate. L'inoculation de la souche ETEC F4 a été réalisée à une dose de 10^7 UFC/ml, choisie selon une estimation de la dose infectieuse chez les porcelets, et 24h après la fin du stress de jeune alimentaire et le changement d'alimentation. Concernant la composition du microbiote du MPigut-IVM, l'exposition à la souche d'ETEC a induit une augmentation des genres *Prevotella, Enterococcus* et une diminution des genres *Bacteroides* et *Megasphaera* en comparaison de la condition contrôle n'ayant pas été exposée au pathogène. De plus, une augmentation de l'abondance relative de différents composés comme le valérate, l'éthanol et le 3-phenyl-propionate a également été mesurée dans la condition ETEC. De manière intéressante, le suivi qPCR de la souche d'ETEC F4 par quantification du gène de toxine LT, a montré qu'elle s'est préférentiellement maintenue, à hauteur de 8 Log de copies du gène LT/mL, dans le milieu du compartiment des billes de mucines alors qu'elle a rapidement diminué dans le bioréacteur. Enfin, la quantification par RT-qPCR de l'expression de gènes de virulence de la souche ETEC (STb, F4, LT et EAST1) a révélé que la souche a persisté de manière métaboliquement active dans le milieu du bioréacteur et du compartiment des billes de mucines MPigut-IVM.

De plus, afin d'étudier les interactions hôte-microbiote, des surnageants du milieu du compartiment du MPigut-IVM ont été appliqués à des cellules intestinales porcines IPI-2I en culture. Cette partie du travail a été effectuée dans le laboratoire du Dr. Juan J. Garrido à l'Université de Cordoba. L'incubation des surnageants du jour 15 de fermentation du MPigut-IVM ayant été exposés à la souche ETEC F4 a entrainé une augmentation de l'expression des gènes cellulaires TNFα et MyD88 impliqués dans la réponse pro-inflammatoire, du gène MUC1 impliqué dans la production de mucines de type 1 et du gène CLDN4 impliqué dans la structure des protéines de jonction de l'épithélium.

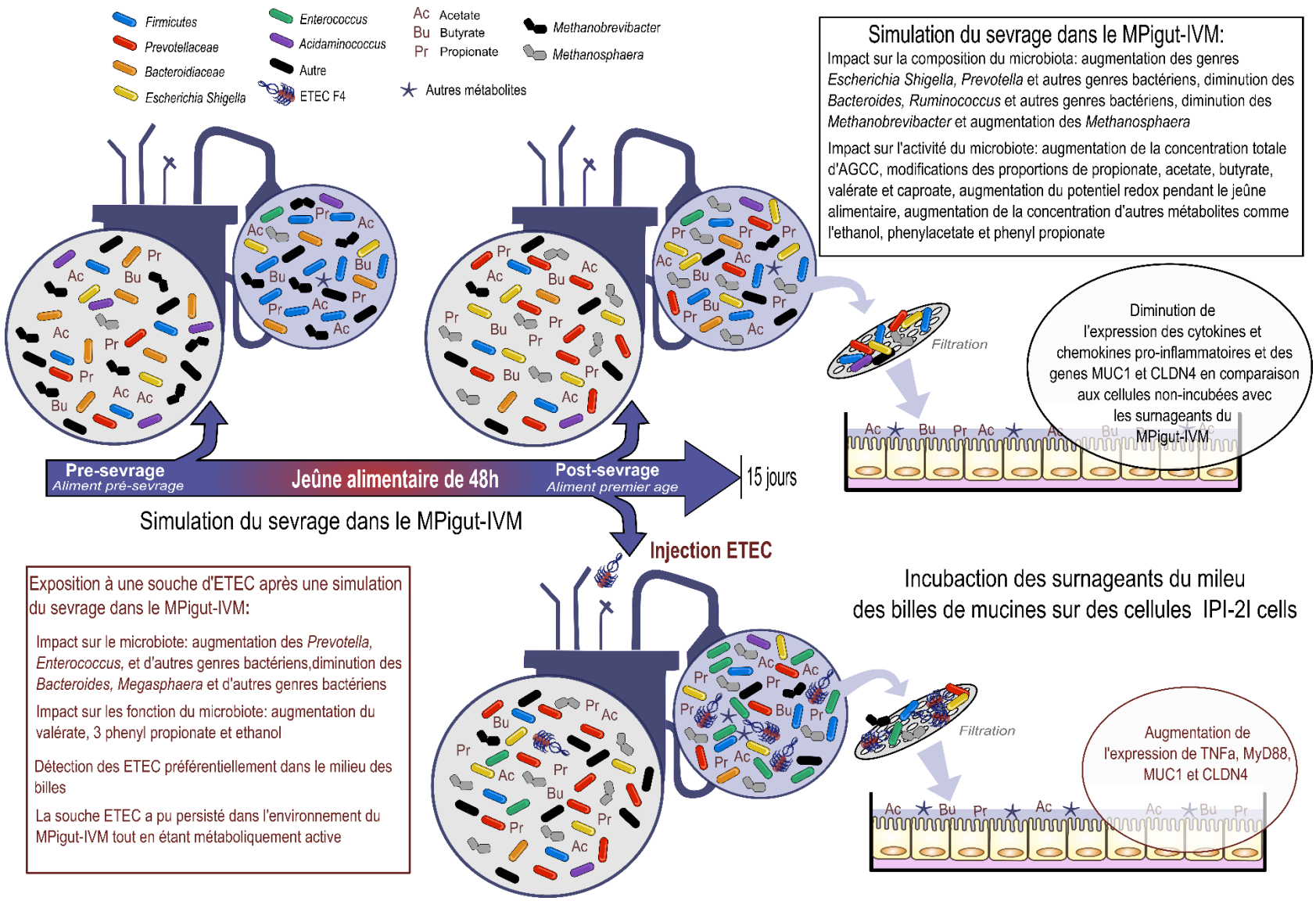

Figure D. Résumé graphique des résultats obtenus dans le Chapitre 10.

Effets de la levure SB CNCM I-1079

Le **Chapitre 11** décrit les résultats obtenus (Figure E) en rapport avec l'effet d'un traitement préventif quotidien de SB CNCM I-1079 à hauteur de 10^7 UFC/mL dans le MPigut-IVM simulant le sevrage des porcelets grâce à un jeune et une transition alimentaire ainsi qu'une exposition à la souche d'ETEC F4, comme décrit dans le Chapitre 10. La dose de probiotique journalière pour cette expérimentation a été augmentée par rapport à celle utilisée dans le chapitre 9, afin de s'aligner sur la dose de 10^7 UFC/mL de la souche d'ETEC et de permettre un ratio 1/1 entre probiotique et pathogène inoculés dans les fermenteurs. Cette expérimentation a été réalisée avec 2 réplicats biologiques avec comme contrôle des fermentations sans supplémentation en SB mais également exposées à la souche d'ETEC. Les mêmes techniques d'analyses que celles utilisées au Chapitre 10 ont été appliquées. Les résultats préliminaires ont permis l'observation d'une diminution du genre *Prevotella* dans le milieu du bioréacteur (comparativement au contrôle sans probiotiques) qui était particulièrement favorisé à la suite de la transition alimentaire et de l'exposition au pathogène. L'abondance relative d'autres genres bactériens tels que *Succiniclasticum* et *Olsenella* a été diminuée alors que les genres *Bacteroides, Escherichia Shigella, Ruminococcus* et *Enterococcus* ont été favorisés par le traitement probiotique. Aucun effet du traitement probiotique n'a été observé sur les paramètres fermentaires du microbiote (gaz, AGCC).

Deux expérimentations de culture cellulaire ont ensuite été réalisées à l'Université de Cordoba. La première a consisté à évaluer l'efficacité de la souche SB CNCM I-1079 sur l'adhésion de la souche porcine d'ETEC F4 aux cellules intestinales porcines IPEC-J2, qui possèdent le récepteur au fimbriae F4. Cette expérimentation a été effectuée avec trois réplicats biologiques et 3 réplicats techniques par réplicat biologique. L'effet d'un prétraitement de SB CNCM I-1079 sur l'expression de gènes impliqués dans la réponse immunitaire et la sécrétion de mucines des cellules IPEC-J2 incubées avec la souche d'ETEC F4 a été étudié. Sur ces cellules IPEC-J2, trois doses de SB de 10^6, 10^7 et 10^8 UFC correspondant respectivement à 625, 62.5 et 6.25 UFC de SB par cellule IPEC-J2, ont été testées tandis que la souche d'ETEC F4 a été inoculée à une dose de 100 UFC par cellule IPEC-J2. Un effet dose de SB a été clairement mis en évidence sur l'adhésion de la souche ETEC aux cellules IPEC-J2. En comparaison avec les cellules n'ayant pas reçu de prétraitement probiotique et correspondant à 100% d'adhésion, les doses de 10^6, 10^7 et 10^8 UFC de SB ont respectivement permis de réduire à 69, 53 et 13% l'adhésion des ETEC aux cellules IPEC-J2. Un effet dose sur la réduction de l'expression des gènes TNFα, TRL4, CCL20, CXCL2, IL1α, IL6 et IL8 impliqués dans l'expression de

cytokines et chemokines pro-inflammatoires a également été observé ainsi qu'une légère augmentation de l'expression de MUC1.

Enfin, comme pour le Chapitre 10, la deuxième expérimentation a consisté à incuber des cellules IPI-2I avec des surnageants de milieu du compartiment des billes du MPigut-IVM ayant reçu un traitement journalier de SB et une dose de souche d'ETEC après la simulation du sevrage. Cette condition a été comparée avec la condition ayant été exposée à la souche pathogène mais n'ayant pas reçu de traitement probiotique. Comme pour la fermentation, cette expérimentation a été réalisée avec 2 réplicats biologiques et 3 réplicats techniques par réplicat biologique. Le même jeu de gènes que celui suivi dans le chapitre 10 a été sélectionné. Nos résultats ont montré que les surnageants de la condition ayant reçu des probiotiques et exposés aux pathogènes l provoquaient une atténuation de l'expression des gènes TNFα, MyD88, MUC1 et CLDN4 des cellules IPI-2I, dont l'expression avait été activée par les surnageants provenant de la condition ayant été exposés aux pathogènes sans probiotiques.

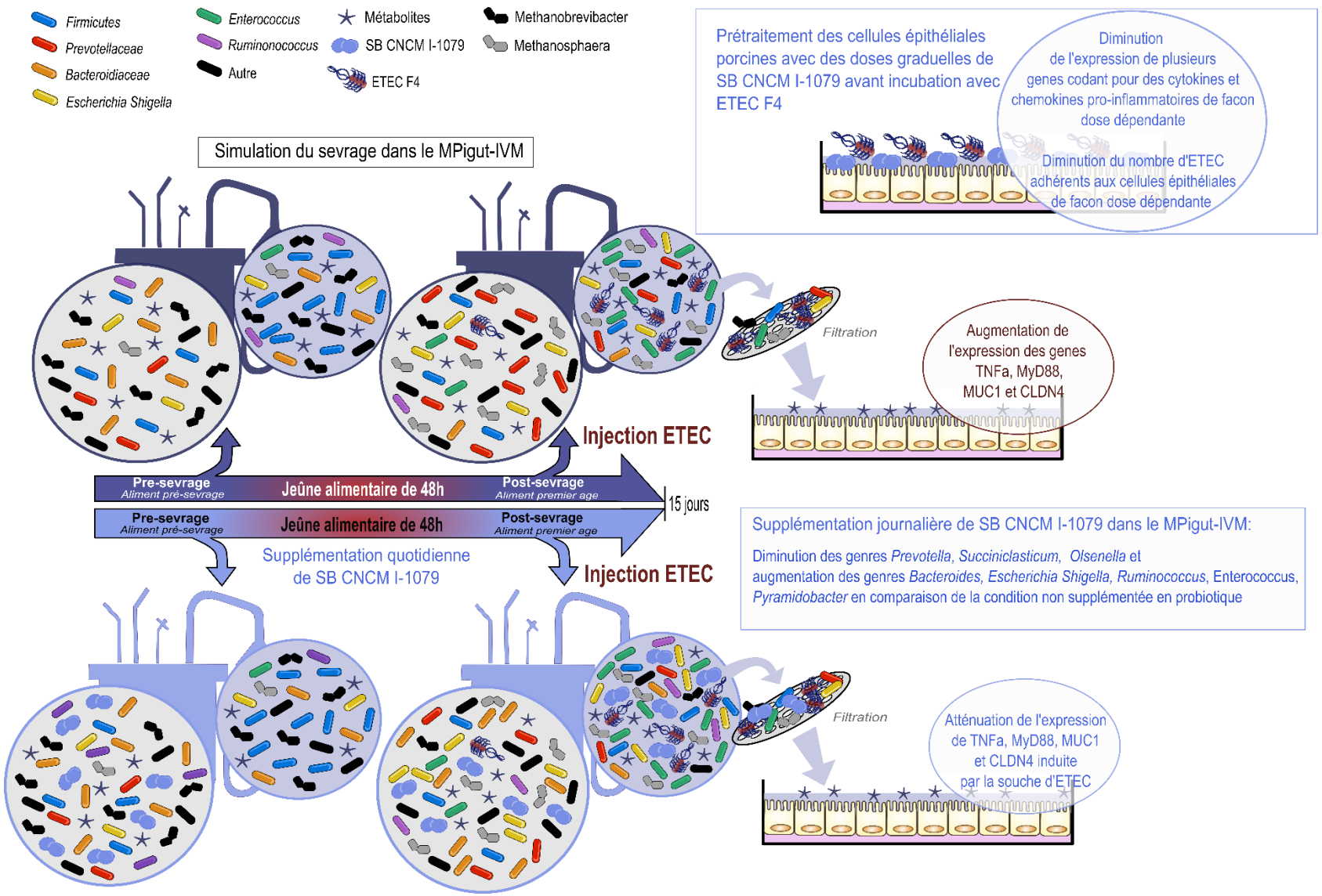

Figure E. Résumé graphique de résultats principaux obtenus dans la Chapitre 11 de ce manuscrit.

Discussion et perspectives

Le **Chapitre 12** de ce manuscrit discute les résultats obtenus et décrit les perspectives de cette thèse. Pour rappel, ce travail de thèse a été réalisé dans un contexte où les diarrhées post-sevrage, causant une mortalité importante chez les porcelets, devraient être soignées avec une quantité limitée d'antibiotiques afin d'enrayer l'expansion inquiétante des gènes de résistance parmi la population bactérienne. Le développement d'autres traitements non pharmaceutiques apparait comme essentiel et urgent. En parallèle, les nouvelles directives européennes, engendrées par une pression sociétale forte, encouragent la mise en place de modèles *in vitro* afin de limiter considérablement l'utilisation des animaux dans la recherche. Les modèles *in vitro* de digestion/fermentation artificielle, continuellement optimisés pour se rapprocher au mieux des conditions *in vivo*, se présentent comme une stratégie avantageuse tant en termes de coût que de standardisation des conditions expérimentales et de reproductibilité. Le but de ce projet était donc de développer un modèle *in vitro* du côlon du porcelet au sevrage présentant l'originalité d'intégrer une surface de mucines, afin d'être utilisé pour mieux comprendre l'étiologie de la dysbiose intestinale associée au sevrage et de rechercher des solutions préventives ou curatives alternatives aux antibiotiques pour lutter contre les infections à ETEC et les diarrhées associées. Dans ce cadre-là, un probiotique de la société Lallemand Animal Nutrition, la souche de levure SB CNCM I-1079 a fait l'objet de premiers tests.

Développement du MPigut-IVM : caractéristiques et paramètres

Le développement d'un modèle *in vitro* complexe de l'environnement colique du porcelet nécessite l'adaptation de nombreux paramètres tels que le pH, le temps de rétention, la composition des effluents iléaux, approche qui s'avère souvent limitée par le manque de données *in vivo* dans la littérature. En amont du développement du MPigut-IVM, une étude *in vivo* a donc été réalisée pour récupérer les paramètres nécessaires au développement du modèle. Cette étude nous a permis d'acquérir de précieuses données sur les profils d'AGCC dans le côlon du porcelet, la composition du microbiote colique de la lumière digestif et du mucus et le pH colique de porcelets vivant dans les mêmes conditions que ceux utilisés pour inoculer le MPigut-IVM. Seul le temps de transit colique n'a pu être déterminé dans cette étude et a été fixé par rapport aux données de la littérature. Comparé à l'autre modèle *in vitro* colique existant du porcelet au sevrage, le baby-SPIME décrit dans une publication datant de 2019, le MPigut-IVM a de nombreux avantages tels que : une adaptation du milieu nutritif pour qu'il reproduise au mieux la composition des effluents iléaux des porcelets, une prise en compte du « creep feeding » pour mettre au point la composition du milieu nutritif « pré-sevrage », un pH

physiologique basé sur nos propres données *in vivo,* une adaptation du temps de rétention, une anaérobiose maintenue par la seule activité du microbiote résident, la mesure du potentiel redox et de la composition des gaz produits dans le modèle et surtout une surface adhérente composée de mucines pour mimer le microbiote associé au mucus. Ces paramètres sont repris un par un et discutés par rapport à la littérature dans le Chapitre 12. Les billes de mucines utilisées dans le MPigut-IVM ont permis l'établissement d'un microbiote adhérent, comme démontré par l'observation en MEB, diversifié et fortement similaire à celui observé dans le mucus colique de porcelets. Pour finir, il faut souligner que l'on observe dans le MPigut-IVM une bonne reproductibilité des essais entre réplicats techniques (même pool de fèces) tout en captant la variabilité due à l'utilisation de différents inocula (réplicats biologiques, pools de fèces différents), et ce malgré l'utilisation d'un pool de fèces et non de fèces provenant d'un unique individu. Ce dernier point est important afin de reproduire les variabilités interindividuelles observées *in vivo* entre animaux.

Effet d'un stress de jeûne alimentaire sur le microbiote colique

Dans le MPigut-IVM, un jeûne alimentaire de 48h a provoqué des changements transitoires du microbiote tels qu'une augmentation des *Prevotella* et une diminution des *Bacteroides* surtout dans le milieu du bioréacteur. Dans les billes de mucines, des changements distincts ont été remarqués, notamment une diminution des *Clostridium*. *In vivo,* la diminution de ces populations sécrétrices de butyrate dans le microbiote associé au mucus des porcelets pourrait diminuer la quantité de butyrate absorbé par les entérocytes et contribuer aux modifications physiologiques néfastes observées lors du sevrage, comme les modifications de la morphologie de l'épithélium intestinal. Si le jeune a eu relativement peu d'effets sur les proportions d'AGCC, potentiellement à cause de leur accumulation dans le bioréacteur, une augmentation drastique du potentiel redox a caractérisé de manière reproductible la période de jeûne alimentaire. En condition contrôle, l'activité fermentaire diminue le potentiel redox grâce à la dégradation des sucres et rend ainsi le milieu plus réducteur. L'augmentation du potentiel redox pendant le jeûne traduit une réduction du métabolisme des sucres et des protéines. Une augmentation des genres *Escherichia Shigella* sur les billes de mucines dans 2 des 4 réplicats biologiques a été observée. Les espèces du genre *Escherichia* sont connues pour être particulièrement résistantes au stress oxydant. Une hypothèse concernant cette expansion dans certains réplicats seulement serait que dans ces réplicats le microbiote présenterait plus d'espèces sensibles aux stress oxydatifs, le genre *Escherichia Shigella* pourrait alors tirer un avantage compétitif de la situation pour se développer.

Impact de la transition alimentaire après un jeûne de 48h

Un changement de milieu nutritif consécutivement à la période de jeûne alimentaire a essentiellement conduit à une réduction du ratio *Bacteroides/ Prevotella* dans le milieu du bioréacteur et sur les billes de mucines qui a perduré jusqu'à l'arrêt des fermentations. Cette observation a été raportée dans de nombreuses études *in vivo* chez les porcelets en cours de sevrage. Le genre *Prevotella* pourrait mieux s'adapter aux changements alimentaires par rapport à d'autres populations en raison de ses capacités métaboliques très diverses. Dans les billes de mucines, les populations *Erysipelotrichaceae, Megasphaera, Olsenella* et *Ruminococcus gauvreauii* ont été augmentées suite au jeûne alimentaire et au changement d'alimentation alors que *Romboutsia* et *Ruminococcus* 2 ont été négativement impactés. En raison de la similitude entre le microbiote associé aux billes de mucines et celui associé au mucus colique de porcelets, ces modifications sur les billes de mucines après la simulation du sevrage dans la MPigut-IVM pourraient être une bonne indication des changements survenant *in vivo* et ces populations devraient être considérées comme des populations d'intérêt pour les études à venir. Ces modifications, *in vivo*, pourraient impacter directement les interactions avec l'hôte en raison de la proximité du mucus avec les colonocytes.

Importance de la variabilité individuelle

Une des principales idées mises en lumière dans le Chapitre 12 est le rôle de la variabilité individuelle dans les changements de la composition du microbiote induits par la simulation du sevrage dans le MPigut-IVM. En effet, ce modèle *in vitro* permettant de conserver les variabilités entre inoculum, a permis de mettre en évidence que les changements des abondances relatives des populations *Prevotella, Bacteroides* et *Escherichia Shigella* étaient particulièrement dépendants des réplicats biologiques. Or, plusieurs études *in vivo* ont souligné que ces populations pourraient être considérées comme des biomarqueurs de la susceptibilité des porcelets à développer une dysbiose intestinale et donc à être plus sensibles à la transition alimentaire et aux stress du sevrage. En pratique, ces biomarqueurs pourraient être quantifiés dans les fèces des porcelets d'élevage par une technique de qPCR afin de prendre des mesures préventives nécessaires si les animaux ont un profil à risque.

Colonization des ETEC dans le MPigut-IVM

Chez les porcelets, 9 à 10 Log_{10} sont nécessaires pour provoquer des diarrhées chez les porcelets. En conséquence, 2x 10^9 CFU d'une souche ETEC F4, précédemment isolée d'un porcelet diarrhéique en Espagne, a été inoculée au MPigut-IVM 24h après la simulation du

sevrage. La survie de la souche d'ETEC dans le MPigut-IVM a été déterminée par qPCR en suivant le gène LT afin de différencier la souche des *Escherichia/Shigella* commensaux présents en grande nombre dans le modèle. Après quelques jours dans le MPigut-IVM, la souche d'ETEC a été détectée presque exclusivement dans le milieu du compartiment des billes de mucines. Ce résultat confirme l'affinité des ETEC pour les milieux riches en mucines, et est cohérent avec leur capacité à dégrader le mucus grâce à deux protéases EatA et YghJ. Dans 2 des 3 répétitions, la souche d'ETEC a été maintenue à son niveau d'inoculation dans le MPigut-IVM. Cependant, dans un des réplicats biologiques, la souche d'ETEC a été pratiquement éliminée du modèle en fin de fermentation. Là encore, il semble donc que certains profils de microbiote soient plus susceptibles d'être colonisés par des pathogènes que d'autres. Afin de compléter nos connaissances sur ces points, différentes doses d'ETEC ainsi que différents modes d'inoculation, pour simuler par exemple une exposition chronique de faible dose de pathogène, pourraient être testés dans le MPigut-IVM. De manière intéressante, la présence de la souche d'ETEC dans le MPigut-IVM a été associée à une forte croissance du genre *Enterococcus* au niveau des billes de mucines. L'introduction d'une nouvelle espèce, ici la souche d'ETEC, pourrait provoquer des changements au niveau des interactions entre espèces et modifier l'organisation au sein des niches écologiques de l'écosystème. La possibilité de s'implanter au sein d'une niche écologique serait susceptible, *in vitro* comme *in vivo,* de moduler la colonization des espèces microbiennes pathogènes ou non dans le tractus digestif. Dans le tube digestif les niches écologiques dépendent de facteurs environnementaux tels que la disponibilité en nutriments et pourraient être majoritairement responsables de la composition du microbiote. Le MPigut-IVM étant un environnement fermé, continuellement mélangé et supplémenté en nutriments, il apparaît comme idéal pour étudier les modifications des niches écologiques. Enfin, si des lignées de cellules intestinales porcines d'origine colique ne sont encore pas disponibles, le couplage des surnageants du MPigut-IVM avec les cellules IPI-2I provenant de l'iléon a permis de démontrer que la présence du pathogène dans le microbiote modifie assez le métabolome pour engendrer des différences significatives dans l'expression de gènes liés à l'inflammation.

Etude de la stratégie probiotique dans le MPigut-IVM

Pour lutter contre les pathogènes, les souches probiotiques peuvent utiliser plusieurs modes d'action. Au cours de ce projet de thèse, la capacité de la souche SB CNCM I-1079 à moduler le microbiote intestinal dans des fermenteurs soumis à un stress de jeûne alimentaire seul puis à une simulation du sevrage complet (jeûne et changement alimentaire) avec exposition à une

souche d'ETEC a été étudié dans le MPigut-IVM. Si la plupart des études *in vitro* en fermenteurs utilisent de fortes doses de probiotiques telles que 10^{10} UFC/ml afin d'en exacerber les effets, notre souhait était de préserver le coté réaliste de notre projet en fixant une dose de probiotique journalière basée sur des données *in vivo.* Des doses de 10^6 et 10^7 UFC/ml ont été donc utilisées respectivement pour l'étude des effets sur le jeûne alimentaire seul et la simulation du sevrage complète avec exposition au pathogène. Malgré la dose relativement faible de 10^6 UFC/mL, le traitement journalier avec SB CNCM I-1079 lors du jeûne alimentaire a pu améliorer le métabolisme général du microbiote comme mis en évidence par un maintien d'un potentiel redox fortement négatif dans le MPigut-IVM. Dans les deux expérimentations, les effets du SB sur la composition du microbiote ont été particulièrement remarqués sur les genres *Streptococcus* et *Escherichia/Shigella.* Si un traitement avec des levures probiotiques a déjà été associé à une augmentation du genre *Streptococcus* dans la littérature, l'association avec les genres *Escherichia/Shigella,* souvent apparentés à des pathogènes opportunistes n'a jamais été soulignée. Toutefois, il est important de rappeler que si certains individus de ces genres bactériens sont effectivement pathogènes, la majorité de ces espèces sont commensales. De plus, plusieurs études *in vivo* effectuées chez des porcelets au cours du sevrage ont relevé que les animaux arborant une plus grande proportion d'*E. coli* avant le sevrage étaient moins susceptibles de développer des diarrhées post-sevrage. De plus, la souche probiotique *E. coli* Nissle 1917 s'est déjà montrée efficace chez des jeunes porcelets pour prévenir les infections à ETEC. L'effet protecteur des *E. coli* commensaux pourrait s'expliquer par l'occupation de la niche écologique des *Escherichia/Shigella* empêchant ainsi une implantation d'une souche pathogène d'*E. coli* au sein du microbiote. L'effet du SB conduisant à augmenter le ratio *Bacteroides/Prevotella* a été particulièrement remarqué lors de l'étude de l'effet probiotique lors d'une simulation du sevrage avec exposition au pathogène (Chapitre 11), et pourrait représenter une modulation bénéfique supplémentaire du microbiote pour prévenir la dysbiose post-sevrage.

Comme pour le Chapitre 10, les surnageants du milieu des billes de mucines des fermenteurs ayant reçu des probiotiques et ayant été exposés à une simulation du sevrage et à la présence d'une souche ETEC F4 ont été incubés avec des cellules IPI-2I. Les résultats ont été comparés à ceux obtenus avec les surnageants provenant des fermenteurs ayant été exposés à la simulation du sevrage et au pathogène sans avoir reçu de probiotiques. Les résultats ont montré une atténuation de l'expression des gènes TNFα, MyD88, MUC1 et CLDN4 par les surnageants du jour 15 de la condition SB par rapport au témoin sans SB. Hypothétiquement, les levures pourraient être en mesure de lutter contre les effets d'une infection à ETEC soit en réduisant

l'inflammation, soit par effet direct par exemple en dégradant les toxines secrétées par le pathogène. Ces deux mécanismes ont été largement documentés dans la littérature chez différentes souches de levures avec cependant des effets souche-dépendants. Les propriétés immunomodulatrices d'un prétraitement avec différentes doses de SB CNCM I-1079 ont été également étudiées sur des cellules épithéliales porcines IPEC-J2 incubées avec la souche d'ETEC F4. Une diminution dose dépendante de l'expression de gènes de cytokines et chemokines pro-inflammatoires a été observée. Ainsi, la souche SB semble montrer des propriétés d'immunomodulation ou de compétition directe contre la souche d'ETEC F4. La capacité de la souche SB à réguler des gènes de la souche d'ETEC F4 n'a pas pu être étudiée au cours de ce projet de thèse faute de temps mais une telle étude pourra être effectuée dans un futur proche. L'expérimentation, réalisée sur les cellules IPEC-J2, a également montré que SB était en mesure de diminuer l'adhésion des ETEC sur les cellules épithéliales avec un effet dose dépendant, ce qui pourrait être dû à un encombrement stérique des récepteurs du fimbriae F4 sur les cellules intestinales du fait de la présence des cellules de levures dans l'environnement cellulaire.

Perspectives futures

La dernière partie du Chapitre 12 présente les perspectives réalisables avec le MPigut-IVM.

A court terme, les perspectives pourraient se concentrer sur une optimisation technique du compartiment des billes de mucines en mettant au point par exemple une fermeture par bouchon à vis plus facile à manipuler, une enveloppe chauffante sur mesure évitant l'utilisation d'un bain marie et un système permettant de prélever du milieu du compartiment des billes sans ouvrir le compartiment. Enfin, les autres perspectives à court terme décrites dans ce Chapitre 12 sont :

- la répétition de conditions SB et pathogène afin de pouvoir appliquer des analyses statistiques à cette partie du travail
- des tests de différentes doses de probiotique ainsi que différents modes d'inoculation afin de caractériser au mieux ses effets et de définir le mode d'administration le plus performant.
- la comparaison des effets du SB avec les effets des antibiotiques et de l'oxyde de zinc classiquement utilisés au sevrage, ce dernier étant efficace dans plusieurs études *in vivo* pour lutter contre les ETEC. Néanmoins l'oxyde de zinc provoque une baisse de la

diversité du microbiote et présente de forts risques environnementaux, donc son utilisation est actuellement très controversée.

- le couplage de surnageants du MPigut-IVM avec des modèles cellulaires plus complexes tels que les organoides, en utilisant aussi des approches omiques telles que le RNA-seq afin de faire des corrélations entre les métabolites présents dans les surnageants du MPigut-IVM, l'expression de facteurs de virulence chez le pathogène et la réponse des cellules intestinales de l'hôte.

Les perspectives à long terme développées dans la discussion de ce manuscrit se concentrent sur l'étude d'autres pathogènes digestifs associés au sevrage des porcelets tels que *Salmonella enterica* serovar Typhimurium et l'effet du SB CNCM I-1079, qui est le premier probiotique autorisé en Europe pour lutter contre les Salmonelles chez les volailles, ou d'autres souches probiotiques sur ce pathogène, l'inoculation des fermenteurs avec des feces individuelles de porcelets pour déterminer des biomarqueurs de susceptibilité individuelle chez les animaux, et enfin, la mise au point d'un modèle de l'iléon.

Les expérimentations *in vivo* restent la solution optimale pour étudier les interactions hôte – microbiote, ou les effets d'un composé ou d'un pathogène sur le système immunitaire ou neuroendocrine ou sur la physiologie de l'hôte que les alternatives *in vitro* ne peuvent reproduire. Néanmoins, les modèles *in vitro,* continuellement optimisés pour se rapprocher au maximum de l'*in vivo,* possèdent plusieurs avantages scientifiques et pratiques. En plus d'une bonne reproductibilité, d'un coût avantageux et d'une mise en place rapide ne nécessitant pas de formalités administration ou de comité d'éthique, ils sont particulièrement adaptés pour étudier la dynamique du microbiote et sa réponse à divers évènements et composés sans influence de facteurs pouvant biaiser ces résultats tels que le comportement et la physiologie de l'animal.

Pour conclure, ce travail de thèse a mis en évidence que le modèle *in vitro* développé, le MPigut-IVM, a permis l'obtention de résultats intéressants sur les modifications du microbiote associées aux stress du sevrage, à la présence d'un pathogène entérique et aux effets antagonistes d'une souche de levure probiotique. De plus, le MPigut-IVM est un modèle reproduisant de manière performante le microbiote des porcelets et pourra être utilisé dans des nombreuses perspectives de recherches.

General Introduction

Pork is the world's most consumed meat from terrestrial animals [http://www.fao.org/ag/againfo/themes/en/meat/background.html]. Swine industry, such as other livestock production, is confronted with the increasing pressure to improve efficiency and sustainability while feeding the expanding human population (Garnett et al., 2013). Over the last decades, the commercial production in industrialized countries has initiated considerable changes from small family-run farms to very large production units (Pedersen, 2018). Worldwide, the number of living pigs reached 767.6 million in 2019 (https://www.statista.com/statistics/263963/number-of-pigs-worldwide-since-1990/). The high rhythm of production in swine industry induced practices impacting animal welfare such as the use of indoor confinement housing systems or early weaning (Kingston, 1989; Lallès et al., 2007a; Park et al., 2017; Pedersen, 2018). Weaning is the most critical and stressful event in pig's life (Gresse et al., 2017; Lallès et al., 2007a). Newly weaned piglets encounter numbers of stresses having extensive behavioural and physiological consequences such as transient anorexia or disruptions of the intestinal microbiota, also called dysbiosis, leading to a high prevalence of colibacillar diarrhea (Gresse et al., 2017; Lallès et al., 2007a; Pié et al., 2004). Post-weaning diarrhea account for approximately 17% of death of piglets born in Europe and substantial economic losses (Lallès et al., 2007a). In the last decades, principal strategies consisted in metaphylactic treatments of infected animals with antimicrobials but, before the ban of antibiotics as feed additives in 2006, also prophylactic treatments with in-feed antibiotics (Barton, 2014). The massive antimicrobial use has favoured the bloom of multi-resistant bacterial strains which now represents a real threat for human and animal health (Gross, 2013). Research is now more and more oriented towards finding non-pharmacological treatments against PWD. Finding effective non-pharmacological strategies goes through a better understanding of the role of intestinal microbiota in the etiology of post-weaning diarrhea. In parallel, increasing concerns are raised by public opinion about animal welfare. The 2010/63/EU directive now consequently aims to replace, reduce and refine the use of animal in research (the "3R" principle) and encourages investigations using *in vitro* alternative experiments.

In this context, this PhD project aimed to develop an innovative *in vitro* model of the piglet colon at weaning implemented with mucin beads in order to simulate the microbiota of the mucus environment which is thought to be an important regulator of health. The second goal of this doctorate was to use this model to try to better understand the etiology of post-weaning intestinal dysbiosis and evaluate the use of probiotics, such as live yeast additives, to prevent or circumvent this dysbiosis and the establishment of intestinal pathogens such as Enterotoxigenic *Escherichia coli* (ETEC).

The PhD was conducted in the Microbiology, Digestive Environment and Health (UMR 454 MEDIS) team, a joint research unit between Clermont Auvergne University and the INRAE Centre of Auvergne-Rhône-Alpes, in which are studied interactions between gut microbes, host, diet, environmental factors and foodborne pathogens with the aim to develop nutritional strategies to improve health of humans and animals. MEDIS research unit possesses more than 25 years of expertise in the *in vitro* simulation of the digestive environment and has developed a unique platform in France combining different dynamic gut models simulating the upper and lower digestive tract of humans or monogastric animals. This doctorate was conducted under industrial research partnership with Lallemand Animal Nutrition, a global leader in microbial fermentation. Its Animal Nutrition business unit develops yeasts and bacteria products such as probiotics, silage inoculants and yeast derivative solutions to improve positively animal nutrition, health and well-being. Lallemand Animal Nutrition boasts a long-lasting partnership with the MEDIS unit by means of a research group integrated in MEDIS. This Lallemand group is focusing on gut microbiota in animal nutrition and welfare, and studies the effects of probiotics, primarily on ruminant health and, more recently, on monogastric animals. The development of the mucin phase of the *in vitro* model was performed in collaboration with the Center for Microbial Ecology and Technology (CMET) of Ghent University in Belgium (Dr Van de Wiele) which has a strong, and complementary to the MEDIS research unit, expertise in *in vitro* gut simulators. Finally, a collaboration was settled with the Department of Genetics of Cordoba University in Spain (Dr Garrido) which works on the interactions between *Salmonella* and pig host cells, and has a recognized expertise in animal epithelial cell lines. A PhD secondment of two months, during fall 2019 was coordinated to couple the newly developed *in vitro* model with porcine intestinal cell lines.

The first part of this manuscript is dedicated to a literature review focusing on the critical steps of weaning in swine industry, piglet microbiota and post-weaning diarrhea, the use of live yeasts in the context of piglet gut health at weaning and, finally, *in vitro* gut models. The first chapter

of the literature review was valorised by a publication in the journal *Trends in Microbiology* in June 2017. Briefly, this review focuses on the consequences of weaning in piglets, the possible mechanisms leading to post-weaning diarrhea, alternatives to avoid the use of antibiotics and the interests of using *in vitro* models for investigating the etiology of post-weaning diarrhea and screening non-pharmacological alternatives. The following chapters of the literature section complete the published review article by adding information about the practices in swine industry, pig gastrointestinal tract, ETEC pathogenesis, and the use of live yeasts in pig nutrition and health. In the chapters 2 to 6 of the literature review part, several updates have been also introduced especially on piglet microbiota around weaning and *in vitro* colonic models. The chapter 7 finally presents the research questions and objectives of this PhD project.

Then, chapters 8 to 11 report the experimental part of the PhD project presented under the form of publications. The chapter 8 provides an overview of the microbiota in the gastrointestinal tract of commercial weaning piglets and is exclusively composed of an article in the journal *Microorganisms* published in September 2019. The chapter 9 is focused on the development of an *in vitro* model of the piglet colon implemented with mucin beads and its validation towards our *in vivo* data, especially our *in vivo* study. In this chapter 9, the impact of a weaning-associated feed deprivation period on microbiota composition and function was then evaluated as well as the impact of a daily supplementation with active dry yeasts provided in a distinct unsubmitted part. The chapter 10 gathers results on the impact of a dietary change from a pre-weaning to a post-weaning diet associated to a pathogen challenge on the microbiota of the *in vitro* piglet colonic model in order to simulate more closely the conditions encountered by piglets at weaning. The chapter 11 reports the effects of an active dry yeast on porcine intestinal cell lines and on microbiota composition and functions of the *in* vitro piglet colonic model subjected to the feed deprivation period, a dietary change and the presence of pathogens.

Finally, chapter 12, the last part of the manuscript, discusses the results of the work described in this thesis as well as future perspectives to respond to the needs to find effective and sustainable solutions to prevent post-weaning diarrhea in weaning piglets.

Literature Review

Chapter 1. Gut Microbiota Dysbiosis in Post-Weaning Piglets: Understanding the Keys to Health

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Review

Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health

Raphaële Gresse,[1,2] Frédérique Chaucheyras-Durand,[2] Mickaël Alain Fleury,[3] Tom Van de Wiele,[4] Evelyne Forano,[1] and Stéphanie Blanquet-Diot[1,*]

Weaning is a critical event in the pig's life cycle, frequently associated with severe enteric infections and overuse of antibiotics; this raises serious economic and public health concerns. In this review, we explain why gut microbiota dysbiosis, induced by abrupt changes in the diet and environment of piglets, emerges as a leading cause of post-weaning diarrhea, even if the exact underlying mechanisms remain unclear. Then, we focus on nonantimicrobial alternatives, such as zinc oxide, essential oils, and prebiotics or probiotics, which are currently evaluated to restore intestinal balance and allow a better management of the crucial weaning transition. Finally, we discuss how *in vitro* models of the piglet gut could be advantageously used as a complement to *ex vivo* and *in vivo* studies for the development and testing of new feed additives.

Trends

Weaning is the most critical phase in pig production, generally associated with enteric infections, and it requires substantial use of antibiotics.

Overuse of antibiotics raises serious public health concerns due to the increasing emergence of multidrug-resistant bacteria.

Gut microbiota dysbiosis has emerged as a leading cause of postweaning diarrhea and associated infections in piglets.

A current challenge is finding new effective nonantibiotic alternatives to restore gut microbial balance in weaning piglets.

In vitro models of the piglet gastrointestinal tract at weaning would accelerate the understanding of dysbiosis etiology and the development of new feed additives.

The Swine Industry and Antibiotic Resistance

Pork is the world's most consumed meat from terrestrial animals [http://www.fao.org/ag/againfo/themes/en/meat/background.html]. In modern swine breeding conditions, weaning is the main critical period in the course of the pig's life due to sudden dietary, social, and environmental changes [1]. Multiple stressors encountered at piglet weaning induce transient anorexia, intestinal inflammation, and unbalanced gut microbiota [1,2]. The circumstances of weaning transition generally cause gastrointestinal (GI) infections, mainly colibacillosis diarrhea [3], that have been associated with the death of around 17% of piglets born in Europe [1]. Despite the ban on antibiotic growth promoters in the European Union since 2006, antimicrobials are still massively used in the swine industry for therapeutic purposes, but are also used as prophylactic or metaphylactic treatments to prevent GI infections in farms and their associated economic losses [4]. The overuse of antibiotics is closely related to the growing number of antimicrobial-resistant agents and raises important concerns about animal and also human health (Box 1). For instance, a new *Clostridium difficile* resistant strain causing life-threatening diarrhea is estimated to provoke 14 000 deaths per year in the USA [5]. Additionally, some bacterial families, such as Enterobacteriaceae, are resistant to all, or nearly all, antibiotics, including last-resort drugs [5]. Regarding this pessimistic context, finding antibiotic alternatives to both maintain piglet health at the critical weaning period and preserve public health becomes a real emergency.

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Box 1. Antimicrobial Resistance: A Major Public Health Concern

Antibiotics have been recognized as one of the most successful therapies in medicine but are now compromised by the growing number of antibiotic-resistant bacteria. Antibiotics are administered in both human and veterinary medicine and lead to the development of resistant bacterial strains within human and animal gut microbiota (Figure I). Bacteria become resistant to antibiotics when a mutation affects (i) the production of enzymes that can inactivate the antibiotic molecule, (ii) the recognition pathway between antibiotics and bacterial cells, or (iii) the mechanisms of entry and transport of the antibiotic molecule through the cells. When resistant strains are extracted from their host, they are disseminated into the environment, and increase the spread of resistant genes among bacterial populations [116,117].

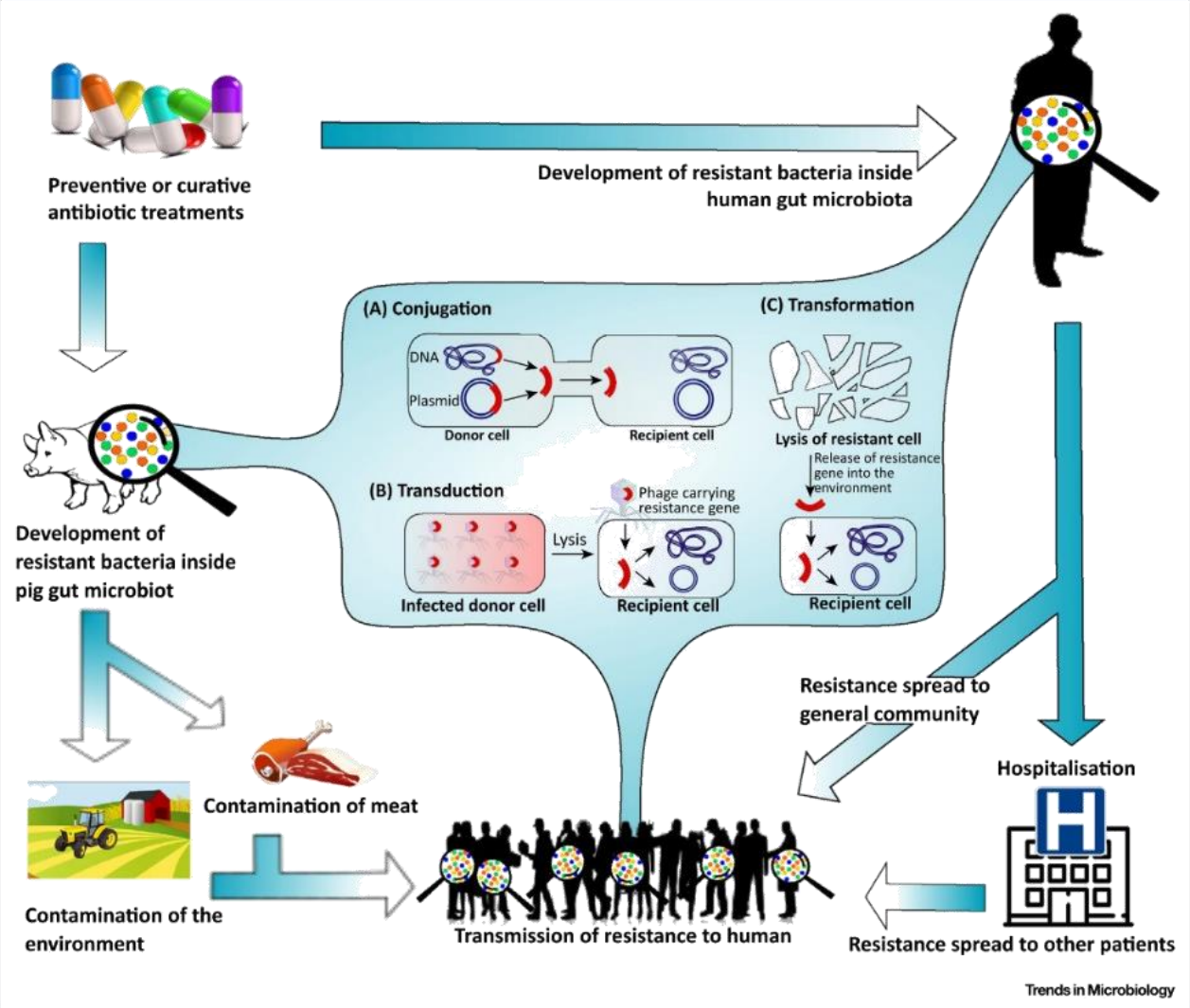

Figure I. Mechanisms of Antibiotic Resistance Dissemination. Several mechanisms allow bacteria to acquire antimicrobial resistance such as (A) conjugation or cell fusion occurring through cell contact between donor and recipient, (B) phage transduction, or (C) transformation from DNA fragments taken up directly from the environment.

In this review, we first describe the main characteristics of weaning in the swine industry with a focus on diet and intestinal physiology. Secondly, we discuss the hypothesis surrounding the disruption of piglet intestinal microbial balance at weaning and its possible involvement in postweaning infections. Then, we examine nonantibiotic solutions to maintain gut ecosystem integrity in newly weaned piglets – such as zinc oxide, essential oils, prebiotics or probiotics. Finally, the usefulness of pig gut models in the development and testing of such nonantibiotic solutions is developed.

Weaning As a Critical Part of Pig Life

In the modern swine industry, weaning is generally practiced at around 3–4 weeks of age, although natural weaning would occur around 17 weeks after birth [6]. Weaning is a sudden, stressful, short, and complex event characterized by changes in diet, social, and environmental life conditions, which profoundly impacts piglet health and leads to decreased performance and sometimes mortality [7]. In intensive production, piglets can even be weaned at only 1 or 2 weeks of age, which consistently amplifies the physiological and behavioral effects of weaning [8–11]. Social and environmental stresses are generated by separation from the mother, handling, transport, different physical environments, and mixing litters [7,9]. These diverse factors induce vocalizations and even fighting, and are associated with elevated heart rates and the secretion of stress-linked factors [9,12]. The switch from highly digestible liquid milk to a less-digestible, more complex solid feed has also critical consequences on piglet behavior and the physiology of their still-immature GI tract [1]. Dietary change is associated with low and erratic feed and water intake, resulting in a fasting period of mostly 24 to 48 h – and thus a transiently reduced growth rate [1,9]. Weaning anorexia has been shown to contribute to local inflammation in the piglet's small intestine [13]. Numbers of other GI changes have been associated with the overall weaning transition, such as activation of pathways related to inflammatory responses, changes in hormonal activity, reduction in gastric motility, induction of small-intestine atrophy and reduced villous height, reduction in nutrient, fluid and electrolyte absorption, and increased permeability to antigens and toxins [14–17]. Finally, it is now more and more accepted that dietary transition and environmental changes at weaning are linked to modifications in piglet intestinal microbiota which could be involved in the etiology of post-weaning diarrhea and enteric infections [15].

Gut Microbiota Dysbiosis and Postweaning Enteric Infections

The gut microbiota of mammals has numerous roles benefiting the host – such as digestion and fermentation of carbohydrates, production of vitamins, maintenance of normal functions of the intestinal villi, regulation of the immune responses, and protection from pathogenic bacteria [18,19]. Among the physiological and GI factors impacted by the weaning transition, gut microbiota disruption is likely to be recognized as one of the keys leading to postweaning diarrhea. The pig gut microbiota is a very complex ecosystem showing dynamic composition and diversity which shifts over time and along the entire GI tract [20]. Colonization is initiated at birth and is shaped by consumption of the sow's milk – which provides nutritional advantages to the population of lactic acid bacteria, building a milk-oriented microbiome [21]. *Escherichia coli* and *Streptococcus* spp. create an anaerobic environment favoring the establishment of other colonizers such as *Bacteroides, Lactobacillus, Bifidobacterium*, and *Clostridium* [22]. During the suckling period, the breed and nursing mother lead to further differentiation of the fecal microbiota of piglets [23]. A study by Bian *et al.* [23], conducted in suckling animals, highlighted that the switch of nursing mother milk and host genetics strongly influenced the development of the gut bacterial community. Therefore, the suckling period offers a peculiar window of gut microbiota modifications.

At weaning, piglets are suddenly fed with a diet containing cereals and a relatively high concentration of crude proteins, although modern diets tend to pay more attention to amino acid balance. In addition, a prestarted diet is generally proposed for suckling piglets with the aim of attenuating the abrupt change of food encountered at weaning. Most of the studies conducted during the weaning transition have reported a decrease in bacteria of the *Lactobacillus* group and a loss of microbial diversity, whereas *Clostridium* spp., *Prevotella* spp. or facultative anaerobes such as Proteobacteriaceae, including *E. coli*, were positively impacted (Table 1). Levels and sources of proteins or fibers shape the diversity and composition of the gut microbiome of weaning piglets [24,25]. For instance, in weaning pigs, enriched pectin diets as well as a soybean meal decreased the relative abundance of *Lactobacillus* and increased that

Table 1. Influence of Weaning Transition on the Characteristics of Piglet Gut Microbiota

Age of piglets when sampling	Diet and breeding conditions	Origin of samples	Method of microbiota analysis	Qualitative modifications of gut microbiota	Quantitative composition of gut microbiota		Refs
					Before weaning	After weaning	
Healthy piglets							
19 days	32 piglets weaned with a cereal and protein-based diet	Ileum Colon	PCR-DGGE qPCR 16S amplicon sequencing	Decrease in *Lactobacillus* in the ileum	No clones of *E. coli* in the ileum and colon $7.1 \pm 1.3 \times 10^7$ of total *Lactobacillus* in ileal samples of unweaned piglets	30% of clones highly similar to *E. coli* and *Shigella flexneri* $4.4 \pm 0.5 \times 10^5$ of total *Lactobacillus* in ileal samples 2 days postweaning	[31]
7 to 35 days	~30 piglets from 3 different litters, fed with cereal-based diet and no antibiotics	Stomach Jejunum Ileum	PCR-DGGE qPCR 16S amplicon sequencing	Decrease in *Lactobacillus* spp. relative abundance in the stomach, jejunum and ileum	*Streptococcus suis* not detected	10^7 copies/g of potentially harmful *Streptococcus suis*	[118]
4 to 6 weeks	15 piglets weaned at 28 days and housed in controlled environmental conditions	Feces	16S amplicon sequencing (454- pyrosequencing)	Increase in *Prevotella* relative abundance Increase in Shannon-Weaver diversity index Shift from Firmicutes to Bacteroidetes New species of *Clostridium* detected	54.0% of Firmicutes 38.7% of Bacteroidetes 4.2% of Proteobacteria 0.7% of Spirochates	35.8% of Firmicutes 59.6% of Bacteroidetes 1.0% of Proteobacteria 2.0% of Spirochates	[119]
7 to 32 days	Piglets weaned at 25 days, randomly divided into 4 litters	Stomach Ileum Colon	PCR DGGE qPCR	Decrease in diversity in the ileum and colon No change in the stomach	ND[a]	ND	[28]
14 to 70 days	31 piglets weaned at 28 days fed with carbohydrate-based concentrate	Feces	16S amplicon sequencing (454-pyrosequencing)	Increase in *Acetivibrio*, *Dialister*, *Oribacerium* and *Prevotella*	At 14 days: ~9% of *Bacteroides* ~2% of *Escherichia/Shigella* ~3% of *Lactobacillus* ~0.5% of *Prevotella*	At 36 days: ~0% of *Bacteroides* ~0% of *Escherichia/Shigella* ~0.5% of *Lactobacillus* ~28% of *Prevotella*	[120]
21 days to 3 weeks	36 crossbred castrated male pigs weaned at 21 days and fed with mash diet	Feces	qPCR 16S amplicon sequencing (Illumina MiSeq)	Increase in Proteobacteria relative abundance at 1 week postweaning and decrease at 3 weeks postweaning Increase in anaerobic fiber-fermenting Firmicutes	ND	40.8–45.8% of Bacteroidetes 35.8–45.1% of Firmicutes 0.9–12.9% of Proteobacteria 0.7–5.2% of Tenericutes 1.4–3.1% of Spirochaetes 0–1.3% of Planctomycetes	[121]
0 to 7 weeks	40 Meishan and Yorkshire piglets weaned at 28 days and separated in mixed groups at birth	Feces	16S amplicon sequencing (454-pyrosequencing)	Disappearance of *Fusobacterium* Lower relative abundance of *Lactobacillus* *Prevotella*, Ruminococcaceae, Spirochaetaceae more abundant	~45–70% of Firmicutes ~20–40% of Bacteroidetes ~3–20% of *Fusobacterium*	~55–70% of Firmicutes ~20–30% of Bacteroidetes ~0–10% of *Fusobacterium*	[23]
35 days	120 piglets weaned at 2 days and fed with basal diet for 14 days	Jejunum	qPCR	Decrease in *Lactobacillus* Increase in *Enterococcus* and *Escherichia coli*	ND	ND	[46]

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Table 1. (continued)

Age of piglets when sampling	Diet and breeding conditions	Origin of samples	Method of microbiota analysis	Qualitative modifications of gut microbiota	Quantitative composition of gut microbiota		Refs
					Before weaning	After weaning	
Healthy piglets treated with antibiotics							
3 to 5 weeks	6 piglets weaned at 3 weeks, 3 of the 6 supplemented with 100 g/ton of chlortetracycline + 100 g/ton of sulfamethazone + 100 g/ton of penicillin	Feces	16S amplicon sequencing (454-pyrosequencing)	Dramatic increase in *E. coli* populations and Proteobacteria Increase in *Succinivibrio* and *Ruminococcus* Decrease in Bacteroidetes, *Anaerobacter*, *Barnesiella*, *Sporacetigeniuma* and *Sarcina*	ND	ND	[32]
3 to 5 weeks	48 piglets weaned at 3 weeks and fed with high complexity diet with 2.73 g of chlortetracycline	Ileal mucosa	16S amplicon sequencing (454-pyrosequencing)	Dramatic decrease in *Lactobacillus* Increase in *Clostridium* and Proteobacteriaceae, including *E. coli*	ND	88.5% of Firmicutes 21.9% of *Lactobacillus* 49% of *Clostridium* 9.9% of Proteobacteriaceae 8.4% of *Escherichia*	[33]
0 to 7 weeks	27 piglets housed in pathogen- free facilities and weaned at 21 days with oat-based diet + 444 mg/kg chlortetracycline + 39 mg/kg tiamulin	Feces	16S amplicon sequencing (Illumina MiSeq)	Decrease in *Lactobacillus*, Bacteroidaceae and Enterobacteriaceae Increase in Prevotellaceae, Lactobacillaceae, Ruminococcaceae, and Veillonellaceae	0.3% of Prevotellaceae 15.4% of Bacteroidaceae 1.7% of Ruminococcaceae	14.8% of Prevotellaceae 1.4% of Bacteroidaceae 9.6% of Ruminococcaceae	[21]
Infected weaning piglets							
25 days	24 piglets weaned at 21 days and fed with commercial diet, among them 2 experimentally infected and 2 naturally infected with *S. typhimurium*	Ileum	16S amplicon sequencing (FLX pyrosequencing)	Top genera of infected piglets: *Clostridium*, *Staphylococcus*, *Helicobacter* and *Lactobacillus*	ND	ND	[122]
Piglets suffering from postweaning diarrhea							
7 to 47 days	20 piglets weaned at 21 days housed in poor conditions, among them 13 suffered from postweaning diarrhea (D) and 7 were healthy (H)	Feces	qPCR 16S amplicon sequencing (Illumina MiSeq)	High relative abundance of Enterobacteriaceae in D pigs after weaning High relative abundance of Bacteroidetes in H pigs after weaning Lower Simpson diversity index in D than H pigs Decrease in evenness index before weaning in both group, and after weaning in D pigs Higher evenness in D pigs before weaning	ND	ND	[29]

[a]ND, not determined.

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of *Prevotella* in the colon [26], and a fish protein source was linked to a large expansion of the *Escherichia/Shigella* group [27]. Such disturbances of the gut microbial ecosystem and loss of diversity at early times of life [28,29] can dramatically increase the risk of GI diseases [30]. In particular, as *Lactobacillus* spp. are major players in disease prevention, their abrupt decrease during weaning transition can contribute to an increase in the risk of disease [31]. In-feed antibiotics can also introduce differences in piglet gut microbiota at weaning [21,32,33] due to their wide spectrum activity and thus their potential ability to kill or prevent the growth of both pathogenic and beneficial microbes. The diversity of the microbiota may be even more decreased [32,34]. Prolonged use of subtherapeutic doses of antibiotics, but also therapeutic treatments, can increase opportunities for pathogenic microorganisms to colonize and trigger diseases [30,35]. The two major pathogens impacting the swine industry are *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *E. coli* [36]. Among pathogenic *E. coli*, enterotoxigenic *E. coli* (ETEC) is the main infectious agent of postweaning diarrhea in piglets, being responsible for 50% of piglet deaths worldwide per year [37]. The genetic background of the host, which has a key role in driving the settlement of the gut microbiota, also represents a predisposing factor to infections in piglets. Indeed, F4 ETEC and $F18^+$ pathogenic *E. coli*-high susceptibility phenotypes have been recently detected, for instance in Flemish pigs [38]. On the other hand, a polymorphism within an intron of the *Mucin 4* gene on the porcine genome can invalidate the production of the specific ETEC F4 receptor and subsequently protect these pigs from ETEC F4 infections [39]. ETEC-susceptible pigs (based on *Mucin 4* gene polymorphism) also display a reduced gut bacterial diversity compared to nonsusceptible pigs [40].

Hence, in piglets, weaning transition has been associated with a disrupted state of the microbiota that can be referred to as 'dysbiosis' [1]. The characteristics of such a state are not completely clear, although dysbiosis has been defined in mammals as a gut microbial imbalance identified by a marked decrease in the representation of obligate anaerobic bacteria, such as members of the classes Clostridia and Bacteroidia, and an increased relative abundance of facultative anaerobic bacteria such as members of Enterobacteriaceae [41]. The process leading to dysbiosis and GI infections is, at this moment, poorly documented in piglets. However, there are several hypotheses which can be translated from *in vitro*, mice, or human studies (Figure 1). The decrease in gut microbial diversity at weaning makes the glycans which compose the mucus layer protecting the gut epithelium more available for pathogenic microorganisms [42]. The degradation of mucus polysaccharides by commensals can release sugars, such as fucose, galactose, or mannose, which can promote the growth of pathogenic species. For instance, an *in vitro* study has revealed that the commensal species *Bacteroides thetaiotaomicron* produces fucose from mucus carbohydrates, used by enterohaemorrhagic *E coli* to activate type III secretion system (T3SS) gene expression [43]. T3SS is the system deployed by some pathogenic *E. coli* and *Salmonella* to sense and adhere to host enterocytes [36]. Besides a loss of gut microbiota diversity, weaning transition is also associated with an increase in permeability which could favor the crossing of toxins and pathogens through the epithelium [14]. Also, change of diet and stresses occurring at weaning promote intestinal inflammation that can be exacerbated by enteric infections [42]. The inflamed gut appears to provide a favorable environment for expansion of Enterobacteriaceae [44]. Indeed, gut inflammatory host-response produces reactive species such as nitric oxide (NO) which shows antimicrobial properties [42,44]. However, NO released into the intestinal lumen is rapidly transformed into nitrate [42,44]. The nitrate-rich environment of the inflamed gut confers growth advantages on strains of *E. coli* which possess nitrate reductase genes that are absent in species of Clostridia or Bacteroidia [41,45]. Interestingly, a recent study in piglets supports this hypothesis by reporting an increased concentration of reactive oxygen species in the intestine coupled with an expansion of the *E. coli* population 7 days after weaning [46]. Additionally, the concentration of oxygen in the inflamed intestine, which is enhanced by the higher blood flow, may favor the bloom of facultative anaerobes such as members of the Enterobacteriaceae,

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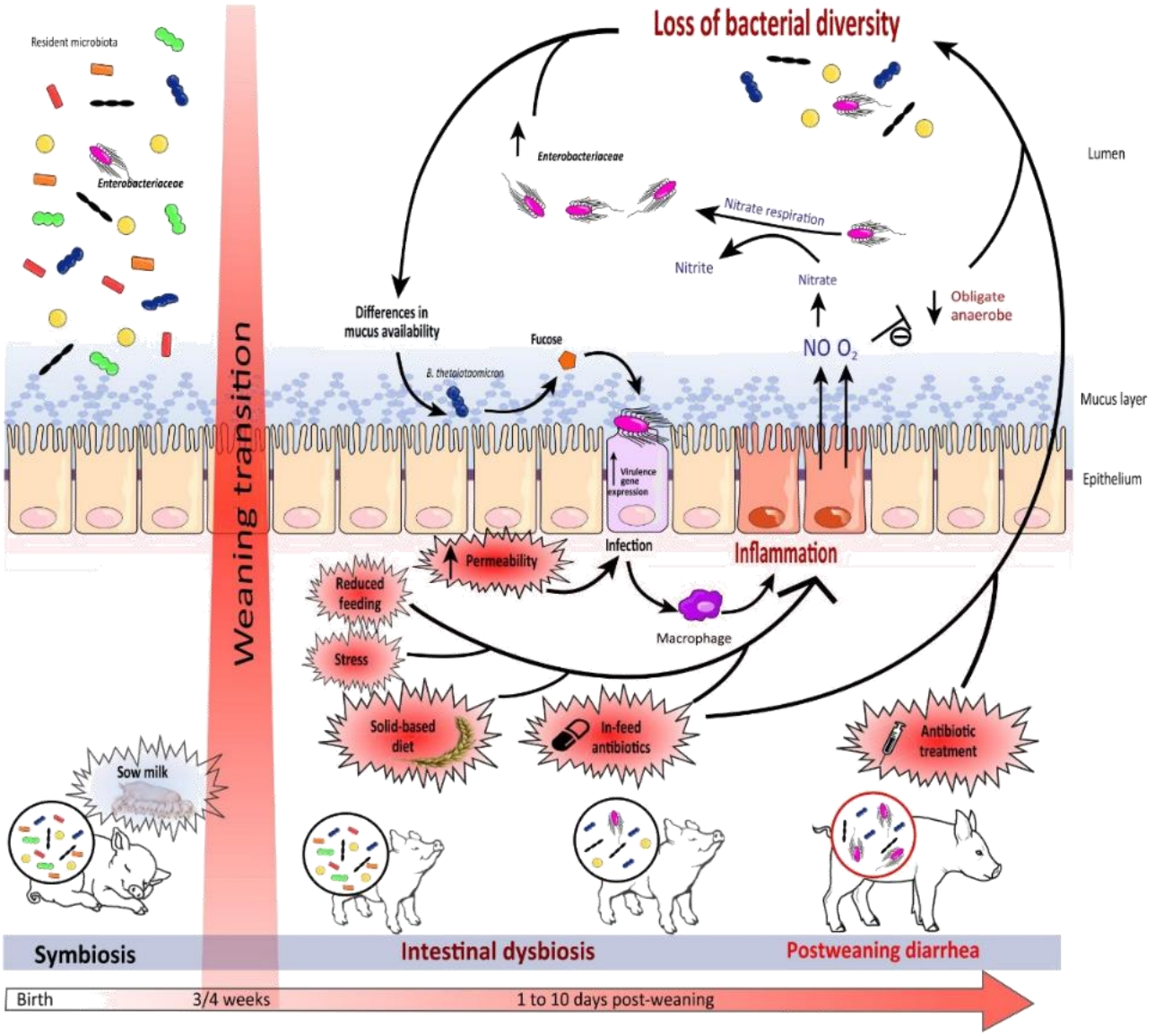

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Figure 1. Impact of Weaning Transition on Piglet Gut Microbiota and Expansion of Infectious Agents. At weaning, piglets undergo the abrupt change from sow's milk to solid feed as well as social and environmental stresses. These modifications result in disruption of gut microbiota composition and intestinal inflammation that can lead to the expansion of enteric pathogens and postweaning diarrhea. Some hypotheses have been raised to explain underlying mechanisms. During weaning, the nutritional landscape of the piglet gut is modified, and mucus polysaccharides may be more available for commensal bacteria (such as *Bacteroides thetaiotaomicron*). Long-term feed antibiotics and therapeutic doses of antimicrobials may contribute to this vicious circle by decreasing bacterial diversity and increasing intestinal inflammation.

decrease obligate anaerobes, and consequently induce a loss of bacterial diversity [44]. Thus, the succession of postweaning events in piglets could drive a real vicious circle, leading to enteric infections (Figure 1). As antibiotics can promote intestinal inflammation [44,47], and are associated with decreased microbiota diversity, antimicrobial compounds may fuel and exacerbate the vicious circle process during weaning transition.

Nonantibiotic Alternatives to Restore Gut Microbial Balance and Prevent Infections

Given the major role of antibiotics in gut microbiota dysbiosis, and the rise in important public health concerns about the spread of multiresistant bacteria, there is an urgent need for developing nonantibiotic alternative strategies to restore microbial balance and control GI infections associated with weaning transition in piglets. Several kinds of solution exist to fight against dysbiosis and infections at weaning.

For instance, a live oral vaccine against ETEC F4 is already commercialized and recommended for healthy piglets from 17 days of age [48]. Weaned piglets have already been successfully vaccinated using this product. The vaccine, administered in drinking water, resulted in a significant reduction in the incidence of diarrhea, ileal colonization, and fecal shedding of ETEC F4 in challenged postweaning piglets [49]. However, protection occurs several days after weaning, meaning that piglets are not protected during the short period in which ETEC infections strongly arise [48]. Vaccination should ideally take place during the suckling period, but vaccines would be susceptible to neutralization by the high level of maternal IgA antibodies present in the suckling piglet gut [48]. Then, there is a need for clarifications regarding the use of vaccination, which, in addition, has for now been developed mostly to target one particular strain or species of pathogen.

Another solution is the use of phages which generally attack a specific bacterium or a narrow group of bacteria without negatively affecting autochthonous bacteria [50]. The oral administration of a bacteriophage cocktail in postweaning piglets challenged with ETEC K88 has been proposed to alleviate the symptoms of infection [51]. In another recent study, a bacteriophage cocktail increased the relative abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. and decreased coliforms and *Clostridium* spp. in weaning piglets [52]. Nonetheless, the disadvantages of this therapy are (i) the requirement for a large number of target bacteria, (ii) the need for rapid administration of the phages after infection, (iii) neutralization of the phages by the host immune system, and (iv) the possible development of resistance [50].

The nutritional components of the animal diet can also be adjusted using various feed additives showing specific properties. Up to now, most of the nutritional studies conducted in weaning piglets have focused on increased feed intake, daily gain weight, improved immune function, or enhanced digestive functions and metabolism [16]. Indeed, rather few *in vivo* studies have investigated the effects of feed additives on piglet intestinal microbiota at weaning, as reviewed in Table 2 and described below.

Zinc Oxide

Zinc oxide (ZnO) has been proposed as one of the most effective feed additives to replace antibiotics and is already widely commercialized in several countries. High levels of ZnO have shown antimicrobial properties and are used to fight against postweaning infections [53]. Nevertheless, the effect of high levels of ZnO on resident microbiota of newly weaned piglets remains controversial. Starke *et al.* [53] showed a reduction in *E. coli* and Enterobacteriaceae in the stomach and small intestine, while Vahjen *et al.* [54] and Højberg *et al.* [55] noticed opposite trends. In addition, all of these studies reported a reduction in health-associated lactic acid bacteria such as *Lactobacillus* spp. [53–56]. Thus, the beneficial effects of ZnO are not universally acknowledged, especially since it can negatively impact animal health due to the accumulation of Zn in the liver, pancreas, and kidney [57], as well as human health by increasing the proportion of multiresistant *E. coli* in the GI tract of piglets [58]. Moreover, the European legislation limits the use of ZnO in animal production to a maximum of 150 mg/kg because of suspected environmental pollution [53].

Table 2. Effects of the Main Nonantibiotic Alternatives on the Gut Microbiota of Healthy or Pathogen-Challenged Piglets at Weaning

Age of piglets when sampling	Diet and rearing conditions	Origin of samples	Method of microbiota analysis	Additives	Dose in the diet	Modification of intestinal microbiota or effect on pathogen colonization	Other observations	Refs
Zinc oxide								
42 days	32 piglets weaned at 28 days housed individually	Caecum Colon	Culture	Zinc oxide	2500 mg/kg	Reduction in lactic acid bacteria Increase in coliforms and enterococci	Reduction of ATP accumulation	[55]
42 days	208 piglets weaned at around 22 days with a basal diet and housed in pens	Ileum	Culture	Zinc oxide	3100 mg/kg	Reduction of anaerobic and lactic acid bacteria No effects on *Escherichia coli*	No effect on performance	[56]
40 to 42 days	12 piglets weaned at 28 days and housed in controlled conditions	Ileum	16S amplicon sequencing (454-pyrosequencing)	Zinc oxide	3042 mg/kg	Increase in Enterobacteriaceae relative abundance and diversity Reduction of *Lactobacillus reuteri* relative abundance Increase in *Weisella cibaria, Weisella confuse, Leuconostoc citreum, Streptococcus equinus and Streptococcus lutetiensis* relative abundances	ND[a]	[54]
25 to 53 days	Piglets weaned at 25 to 26 days and housed in pens	Stomach Jejunum Ileum Colon	qPCR	Zinc oxide	2420 mg/kg	Reduction of Enterobacteriaceae, *E. coli* and *Lactobacillus* spp. especially in the stomach and small intestine 1 week postweaning	Reduction of bacterial metabolites such as SCFA[b] Lower lactate concentrations	[53]
Essential oils								
Healthy piglets								
3 to 5 weeks	6 pens of 6 piglets per treatment, weaned at 16 to 19 days	Ileum Colon feces	Culture PCR DGGE	Herbal extract containing cinnamon, thyme and oregano extract	0.75%	Reduction of coliforms at day 14 No difference in the carriage in *E. coli* K88	No effect on intestinal morphology	[60]
0 to 35 days post-weaning	96 piglets weaned with a basal diet and housed in pens with controlled conditions	Caecum Colon Rectum	Culture	Essential oil blend with 18% thymol and cinnamaldehyde	0.01%	Reduction of *E. coli* number in the caecum, colon and rectum *Lactobacilli* to *E. coli* ratio increased in the colon	Lower level of Interleukin 6 Higher level of tumor necrosis factor-α Higher villous height to crept depth ratio in the jejunum	[61]
8 weeks	14 piglets weaned at 28 days receiving a control diet of 3400 kcal/kg for 28 days	Caecum Colon Rectum	Culture	Essential oil blend with 4.5% cinnamaldehyde and 13.5% thymol	0.025%	Decrease in *E. coli* and total anaerobes in the colon and rectum	Increase in average daily gain Greater jejunum villus height	[59]

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Table 2. (continued)

Age of piglets when sampling	Diet and rearing conditions	Origin of samples	Method of microbiota analysis	Additives	Dose in the diet	Modification of intestinal microbiota or effect on pathogen colonization	Other observations	Refs
35 days	120 piglets weaned at 21 days with the basal diet + essential oil for 14 days	Jejunum	qPCR	Carvacol-thymol blend	100 mg/ kg	Increase in *Lactobacillus* Decrease in *Enterococcus* spp. and *E. coli*	Decrease mRNA levels of TNF-α	[46]
Piglets challenged with pathogens								
32 to 59 days	64 weaned piglets of 24 days challenged with 10^9 CFU of *S.* Typhimurium	Feces	Culture	Thymol	1%	No change in fecal excretion in *S.* Typhimurium	Reduction of feed intake Higher immunoglobulin concentrations in serum before challenge	[62]
Organic acids								
Healthy piglets								
3 to 5 weeks	6 pens of 6 piglets per treatment, weaned at 16 to 19 days	Ileum Colon Feces	Culture PCR DGGE	Acid 1: Acetic, formic, propionic, phosphoric, and citric acids Acid 2: 50% of lactic acid and 50% of acid mixture	1.1% for each mixture	Lower fecal counts of coliforms at day 4 for both treatment No difference in the carriage of *E. coli* K88	No effect on intestinal morphology	[60]
4 weeks	Piglets weaned at 16 to 19 days and housed in standard pens	Ileum	PCR DGGE qPCR 16S amplicon sequencing (454-pyrosequencing)	Acid blend	1.1 to 2.1%	No change in bacterial diversity Increase in *Lactobacillus* relative abundance	ND	[67]
53 to 55 days	96 male piglets of 25 days old castrated and housed in pens	Jejunum Ileum Colon	qPCR	Organic acids (OA) Medium chain fatty acids (MCFA)	OA: 0.416% fumaric acid and 0.328% lactic acid MCFA: 0.15% caprylic and capric acids	OA: Increase in *Bacteroides, Porphyromonas, Prevotella* and *Clostridium* cluster XIVa, I and IV in the stomach Decrease in *Streptococcus* in the colon MCFA: Increase in *Escherichia, Hafnia, Shigella* groups in the jejunum	OA[c]: decrease in intestinal pH	[68]
Piglets challenged with pathogens								
26 to 37 days	27 piglets weaned at 19 to 23 days and challenged with 10^{10} CFU of ETEC K88	Stomach Jejunum Caecum Colon Feces	Culture	Fumaric or citric acids	1.5%	No effect on *E. coli* carriage or commensal microbiota	Higher volatile fatty acids in the jejunum	[69]

Table 2. (continued)

Age of piglets when sampling	Diet and rearing conditions	Origin of samples	Method of microbiota analysis	Additives	Dose in the diet	Modification of intestinal microbiota or effect on pathogen colonization	Other observations	Refs
Prebiotics								
Healthy piglets								
3 to 6 weeks	128 piglets weaned at 18 to 22 days and housed in pens	Jejunum	Culture qPCR	Mannan-oligosaccharides	0.2%	Decrease in Enterobacteriaceae in the jejunum No difference for *Lactobacillus* spp.	No difference in ileum weight or crypt depth	[73]
24 to 49 days	240 piglets weaned at 24 days and housed in pens	Feces	Culture	Lactose (L) Seaweed (SWE) containing laminarin and fucoidan	L: 150 or 250 g/kg SWE: 2.8 g/kg	Reduced counts of *E. coli* with SWE or high L diet	Higher average daily gain with high L diet Increase in nitrogen digestibility and gross energy with SWE	[74]
3 to 5 weeks	144 piglets weaned at 21 days and housed in pens	Jejunum	Culture	Cello-oligosaccharides	1.5, 3.0 and 4.5 g/kg	Increase in *Lactobacillus* Decrease in *Clostridium* No effect on *Bifidobacterium* and *E. coli*	Increase in villus height Increase in villus surface area	[72]
Piglets challenged with pathogens								
21 to 49 days	Piglets weaned at 12 days orally challenged with 10^7 CFU of *S.* Typhimurium	Ileum Colon	PFGE	Fructooligosac charides (FOS)	1% in water or feed	Tendency of reduction of *S.* Typhimurium shedding in feces when FOS administered in water only	ND	[75]
27 to 37 days	105 piglets weaned at 25 to 32 days kept in level 2 biocontainment facilities and orally challenged with 10^9 CFU of *S.* Typhimurium	Ileum Caecum Colon	qPCR	Sugar beet pulp (SBP) Wheat distillers dried grains with solubles (DDGS)	15% of DDGS 6% of SBP	No effect on infection or *S.* Typhimurium shedding	ND	[76]
4 to 7 weeks	~11 piglets weaned at 28 days challenged with 10^9 CFU of *S.* Typhimurium	Ileum Colon	qPCR	Beta glucans hulless barley	ND	No prevention of *Salmonella* colonization or persistance	No change in SCFA concentrations	[77]
4 to 6 weeks	72 weaned pigs of 23 to 27 days challenged with 10^9 CFU of ETEC K88	Ileum Colon Feces Ileal mucosa	qPCR	Lactulose	10 g/kg	Increase in total lactobacilli No effect on ETEC carriage	Increase in daily weight gain Increase in the amount of butyrate in the colon	[78]
Probiotics								
Healthy piglets								
52 to 54 days	360 piglets weaned at 24 to 26 days and housed in pens	Feces	PCR DGGE Real-time PCR	*Enterococcus faecalis*	0.5×10^9, 1.0×10^9 or 2.5×10^9 CFU/kg of feed	Increase in *Lactobacillus*	Lower incidence of diarrhea Higher average daily gain Higher feed conversion efficiency	[81]

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Table 2. (continued)

Age of piglets when sampling	Diet and rearing conditions	Origin of samples	Method of microbiota analysis	Additives	Dose in the diet	Modification of intestinal microbiota or effect on pathogen colonization	Other observations	Refs
42 days	24 piglets weaned at 21 days fed with a basal diet and probiotic and housed in controlled conditions	Feces	Culture	*Lactobacillus johnosii* or *Lactobacillus mucosae*	5×10^7 or 10^8 CFU/g/piglet/day of each strain	Increase in *Lactobacillus* Decrease in *E. coli*	Higher feed intake Higher body weight gain	[82]
10 to 37 days	Piglets receiving from birth probiotic treatment diluted in 2 mL of peptone. At weaning, piglets continued to receive probiotic in a basal diet	Ileum Colon Feces	T-RFLP profiling	*Pediococcus acidilactici* (PA) or *Saccharomyces cerevisiae* (SC)	2×10^9 CFU/kg of feed of each strain	Decrease in ileal diversity with PA treatment Increase in Firmicutes with PA treatment Increase in Porphyromonadaceae and *Ruminococcus* in the colon with SC treatment	ND	[123]
28 to 180 days	36 piglets weaned at 28 days	Feces	Culture	*Lactobacillus acidophilus* or *Pediococcus acidilactici*	10^9 CFU/g of fermented feed	Increase in fecal counts in LAB and bifidobacteria Decreased in *E. coli* and clostridia	Higher average daily weight gain Higher average dry matter intake Higher gain to feed ratio Higher lactic acid concentration in feces Decrease in incidence of diarrhea Increase in villus height Decrease in crypt depth	[83]
50 days	8 piglets weaned at 28 days but a cereal-based diet with probiotic mixture was offered from 10 days	Caecum Colon	RT-PCR	Probiotic mixture of 10^9 CFU of *Lactococcus lactis*, *Carnobacterium divergens*, *Lactobacillus casei* and *Lactobacillus plantarum*	0.5 g of probiotic mixture/kg of feed	No effect on the relative amount in selected bacterial population	Increase in acetic acid concentrations and total SCFA in the caecum	[84]
40 days	30 piglets weaned at 30 days supplemented either with a probiotic or an antibiotic	Jejunum Caecum Colon	16S amplicon sequencing (Illumina MiSeq)	*L. reuteri* (LAB) or chlortetracycline (ATB)	2×10^9 CFU/mL (LAB) 100 mg/kg (ATB)	Increase in the species richness with LAB compared to ATB where several taxa were eliminated Decrease relative abundance in Firmicutes and *Prevotella* in the colon and caecum with LAB compared to ATB	ND	[85]

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Table 2. (continued)

Age of piglets when sampling	Diet and rearing conditions	Origin of samples	Method of microbiota analysis	Additives	Dose in the diet	Modification of intestinal microbiota or effect on pathogen colonization	Other observations	Refs
Piglets challenged with pathogens								
3 to 4 weeks	16 piglets weaned at 21 days challenged with 5×10^{10} CFU ETEC F4	Feces	qPCR	*Lactobacillus sobrius*	10^{10} CFU/piglet/day	Significant reduction in ETEC levels in the ileum and colon	Improvement in daily weight gain	[86]
26 to 36 days	18 piglets weaned at 18 days challenged with 10^{9} CFU of ETEC K88	Stomach Ileum Caecum Colon	Culture	*Lactobacillus rhamnosus*	10^{11} CFU/piglet/day	Decrease in fecal coliforms Increase in lactobacilli and bifidobacteria	Attenuation of serum IL-6 induced by *E. coli* Higher concentrations of TNF-α Decrease in diarrhea incidence	[87]
28 to 35 days	36 piglets weaned at 21 days challenged with 1.5×10^{8} CFU of ETEC F4	Colon Feces	Culture	*Lactobacillus rhamnosus*	10^{10} CFU/piglet/day	Tendency to increase ETEC in the feces No effect on lactic acid bacteria, enterobacteria, yeasts in the colon	No effect on villus to crypt ratio Tendency to decrease villus height Reduction of total IgA in the blood serum	[91]
4 to 6 weeks	72 weaned pigs of 23 to 27 days challenged with 10^{10} CFU of ETEC K88	Ileum Colon Feces Ileal mucosa	qPCR	Lactulose (L) with *Lactobacillus plantarum* (LP)	10 g/kg of feed (L) 2×10^{10} CFU/piglet/day (LP)	Increase in *Lactobacillus plantarum* in the ileum and colon and in total lactobacilli in the colon No effect on ETEC levels	Reduction of diarrhea incidence	[78]
36 days	Piglets weaned at 21 days challenged with 10^{10} CFU of ETEC F4	Feces	qPCR	*Bacillus subtilis* and *B. lichenformis* mixture	3.9×10^{8} or 7.8×10^{8} CFU/piglet/day	Reduction of *E. coli* abundance in feces	Upregulation of the expression of TLR4, NOD2, iNOS, IL-8 and IL-22	[88]
4 to 7 weeks	50 piglets weaned at 24 days challenged with 1.5×10^{8} CFU of ETEC F4	Feces	Culture	*Saccharomyces cerevisiae*	5×10^{10} CFU/kg of feed	Reduction of ETEC excretion	No effect on other parameters	[89]

[a]ND, not determined.
[b]SCFA, short-chain fatty acid.
[c]OA, organic acid.

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Essential Oils

Essential oils are a complex mixture of volatile organic compounds obtained from many diverse plants which have antimicrobial, antioxidant, or antiviral properties [59]. In all available *in vivo* studies, the supplementation of piglet diet with essential oils has been associated with an increase in the *Lactobacillus* group and a decrease in *E. coli* or total coliforms [46,59–61]. Li *et al.* [61] have even reported a decrease in *E. coli* carriage in piglets fed with thymol and cinnamaldehyde at a similar level to that obtained with in-feed antibiotics, suggesting that essential oils could be a candidate to replace traditional antibiotics. However, in piglets, herbal extracts and thymol have shown no effect on the colonization of pathogenic strains of *E. coli* and *S.* Typhimurium, respectively [60,62]. The characterization of active principles as well as the mode of action of essential oils remain relatively unclear, and there is no real agreement about their effects against Gram-negative or Gram-positive bacteria [63]. Therefore, more investigations are needed to develop essential oils with adapted antimicrobial properties. Because of their lipophilic characteristics, essential oil compounds may raise concerns about their potential toxicity and possible negative impact on animal and human health [64]. At least, individual constituents of carvacol, citral, and limonene oxide at subinhibitory concentrations were shown to favor the emergence of a hyper-resistant *E. coli* strain [65].

Organic Acids

Short- and medium-chain organic acids (OAs), such as citric, propionic, lactic, or fumaric acids, are already used in high dose in piglet diet for feed preservation because of their bacteriostatic and bactericidal effects and very low cost [66]. The use of OAs in newly weaned piglets has been mostly related to a decrease in coliforms [60] and an increase in *Lactobacillus* [67], although they seem to impact the microbiota composition from the stomach to the colon in a broader manner [68]. Despite their reviewed antimicrobial properties, citric and fumaric acids demonstrated a lack of efficiency toward an ETEC K88 challenge [69], and mixtures of OAs had no influence on the natural carriage of this pathogenic bacterium [60]. Conversely, they have a demonstrated antimicrobial effect against Gram-positive bacteria [66]. Besides, it has been reported that the pathogenic *E. coli* strain O157:H7 may display a mechanism of resistance against an acetic acid treatment [70]. Further investigations are thus needed to endorse the interest in OAs, fully understand their mode of action, and avoid inhibition of the resident microbiota.

Prebiotics

Prebiotics are fibers defined as selectively fermented dietary ingredients that allow specific changes both in the composition and/or activity of the GI microbiota that confer a beneficial physiological effect on the host [71]. Most prebiotics belong to nonstarch oligosaccharides such as fructooligosaccharides (FOS) or galactooligosaccharides (GOS) [71]. They can stimulate short-chain fatty acid (SCFA)-producing bacteria, such as butyrate-producing bacteria, which provide substrates and promote normal proliferation and differentiation of intestinal cells [30]. Recent investigations reported interesting effects of prebiotics on the intestinal microbiota of weaning piglets, such as an increased proportion of *Lactobacillus* [72] and a decreased amount of potentially harmful groups such as *Clostridium* [72] and Enterobacteriaceae [73,74]. Unfortunately, no study has yet reported a significant effect of prebiotics on the carriage of ETEC K88 or *S.* Typhimurium in orally challenged weaning piglets [75–78]. Future research should confirm the potential interest of prebiotics in the management of the weaning phase and outline the specificity of the different available fibers.

Probiotics

Probiotics are defined as living microorganisms that, when administered in adequate amounts, confer a health benefit to the host [79]. The effects of probiotics on weaning piglets are widely documented. Lactic acid bacteria such as *Lactobacillus, Bifidobacterium, Enterococcus*, or *Streptococcus*, and yeasts from the genus *Saccharomyces*, are the most frequently used

microorganisms [80]. Several recent studies with lactic acid bacteria, run in newly weaned piglets, noted an increased abundance of *Lactobacillus* or *Bifidobacterium* spp., a decrease in *E. coli*, or a higher production of SCFA [81–85]. However, a consensus cannot be established yet as there are noticeable differences between bacteria and yeasts regarding their effects, which, in addition, remain strain-dependent. The ability of probiotics (lactic acid bacteria and *Saccharomyces cerevisiae*) to inhibit colonization by ETEC K88/F4 has been also demonstrated in a number of studies [78,86–89], as recently reviewed by Roussel *et al.* [90]. Again, the effect of probiotics on ETEC seems to be strain-specific as Trevisi *et al.* [91] found that *Lactobacillus rhamnosus* GG (*L. rhamnosus* GG) impairs the health of ETEC K4-challenged piglets. A study from Li *et al.* [92] also reported that a high dose of *L. rhamnosus* in piglets negated the preventive effect against ETEC F4 compared to the administration of a 100-times lower dose.

Among the available alternatives, probiotics seem to have the highest potential as they constitute the only feed additive that is efficient towards pathogenic strains in piglets. Then, probiotics could represent a safe opportunity to fight against postweaning dysbiosis and enteric infections in the swine industry. Although the underlying mechanisms are not fully understood, protection of piglets from postweaning infections by probiotics could notably occur through inhibition of pathogen growth and adhesion to intestinal mucosa, stimulation of the piglet immune system, or modulation of the composition and activity of the resident microbiota (Figure 2). Regarding the last mechanism, the composition of a healthy and disturbed microbiota needs to be more accurately defined to select relevant probiotics able to restore gut microbial balance at weaning. The composition of the healthy piglet gut microbiota is not well established yet. In a broader manner, the early definition of a healthy gut microbiota generally focused on sets of taxa that might be expected to be found in a healthy individual [93]. Investigations in the last decade tended to modify this definition towards a set of functional core which should include housekeeping functions necessary for individual microbial life and processes that are not carried out by host cells, thus building the symbiotic host–microbiota relationship [93]. Yet, an important part of gut microbial gene families remains functionally uncharacterized, which represents a huge knowledge gap [93]. Taken together, these findings suggest that further research is needed to investigate the actual effectiveness and dose for probiotic treatments in weaning piglets.

In Vitro Modelisation of the Pig Gut Ecosystem: A Key Towards Better Understanding

The extreme complexity of the pig's gut favors the use of animal experiments – which remain the best strategy for evaluating phages, feed additives, or probiotic effectiveness. *Ex vivo* experiments, consisting in the isolation of a living organ from the animal to carry out analyses, are an alternative to *in vivo* studies. Such practices would take the immaturity of the piglet GI tract and immune system into account, being representative of the weaning phase conditions. For instance, *ex vivo* models of the small intestine or colon were considered as particularly suitable for investigations on the immune response of piglets toward some feed additives [94] or ETEC infections [95]. Nevertheless, *ex vivo* techniques do not maintain the integrity of the gut microbiota, and they are still invasive because they require the sacrifice of the animals. For technical, cost, and ethical reasons, especially when pathogenic strains are involved, *in vitro* models can be advantageously used as an alternative to *ex vivo* and *in vivo* assays [96,97]. Such *in vitro* techniques can save labor and time, offer flexibility, and allow a good reproducibility between experiments due to standardized conditions, and getting rid of inter- or intra-individual variability. In addition, *in vitro* alternatives are fully in line with the 2010/63/EU directive which aims to replace, reduce, and refine the use of animals in research.

Hitherto, a restrictive number of *in vitro* models of the pig's gut have been developed to mimic the upper or lower GI tract of pigs (Table 3). Currently, the most relevant model of the upper GI

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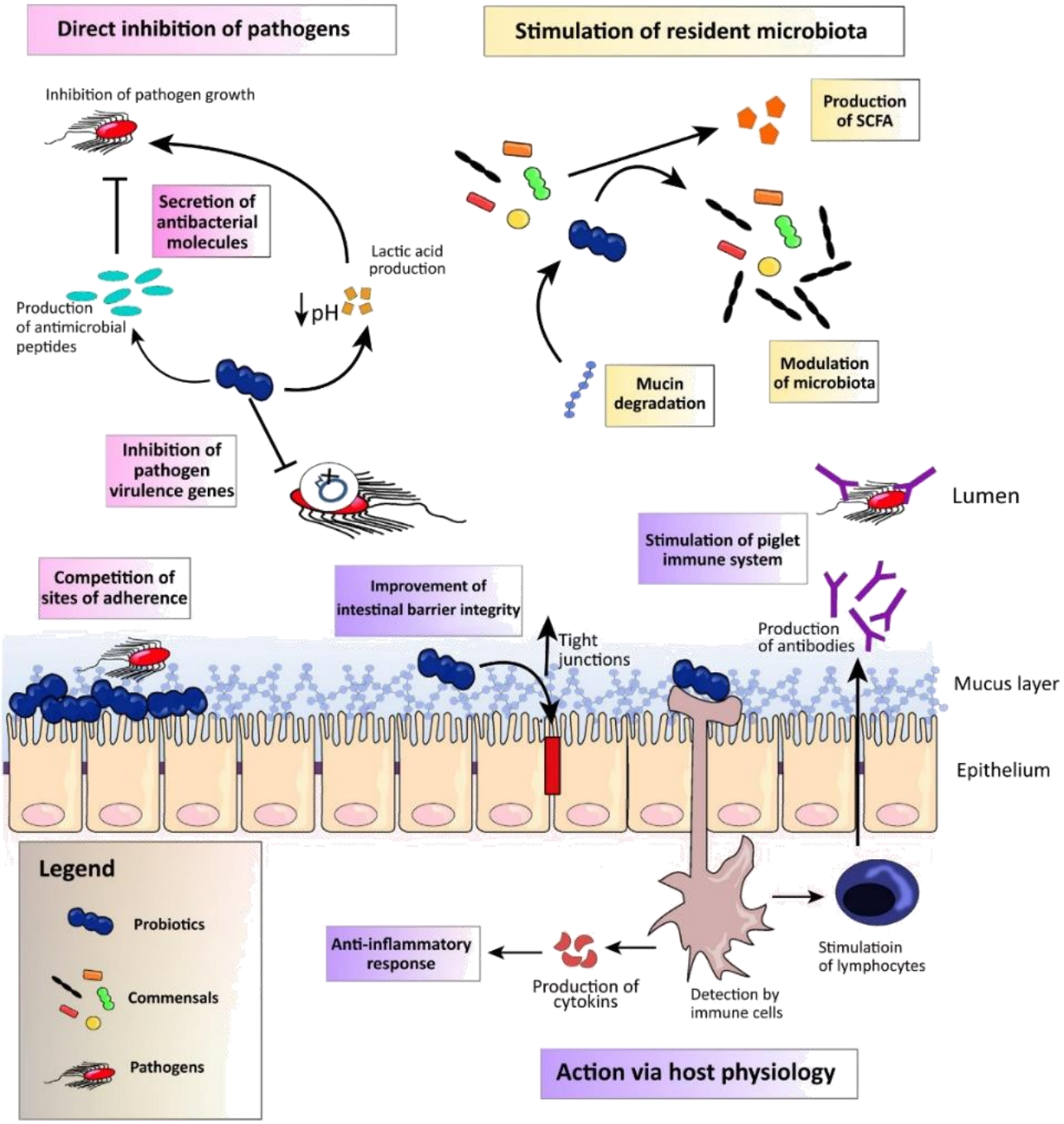


Figure 2. Possible Mechanisms of Probiotic Strains to Fight against Postweaning Infections in Piglets. Several *in vivo* studies have established the beneficial effects of probiotics in healthy and pathogen-challenged piglets. Probiotics may act through three different mechanisms: (i) direct inhibition of pathogen growth and virulence by secretion of antimicrobial substances, (ii) modulation of resident microbiota composition and activity, and (iii) stimulation of the host immune system and improvement in intestinal barrier function. SCFA, short-chain fatty acid.

Table 3. Main *In Vitro* Models of the Pig Digestive Tract and Their Characteristics*In Vitro* [a,b,c]

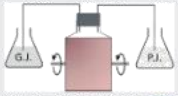
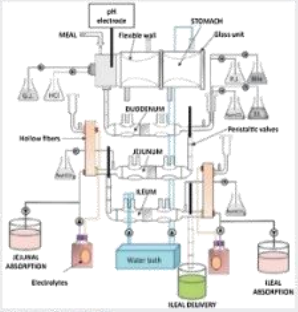
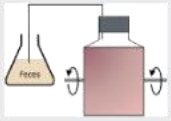
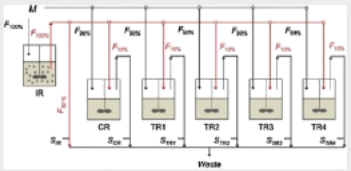
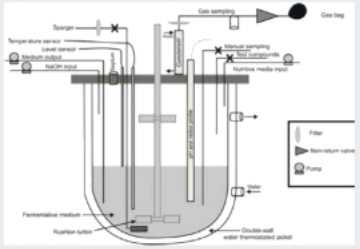
Types of model	Simulated parameters											Refs
	Body T°	Gastric pH	Intestinal pH	Gastric emptying	Intestinal transit	Digestive secretions	Chyme mixing	Intestinal microbiota	Intestinal absorption	Anaerobic conditions	Simulated age	
In vitro models of the pig upper GIT												
Batch systems												
GJ : gastric juice, PJ : pancreatic juice  Figure adapted from [64]	39 °C	pH 2	pH 6.8	–	–	Porcine pepsin Porcine pancreatin	Stirring	–	–	–	ND	[100,124]
Multicompartmental dynamic systems												
TIM (TNO Gastro-Intestinal Model)  Figure from [64]	39 °C	Kinetic of gastric pH fall (addition of hydrochloric acid) pH/time (min) 6.0/5 3.5/30 3.0/120 2.5/180 2.0/240	Intestinal pH controlled with sodium bicarbonate duodenum: pH 5 jejunum: pH 6.5 ileum: pH 6.5	Half-time of stomach emptying: 150 min	Half-time of ileal emptying: 650 min	Saliva mixed with solid food Gastric juice Panceatic juice Bile Electrolytes	Water pressure	–	Use of hollow fibers	–	ND	[98,99]
In vitro models of the pig lower GIT												
Batch systems												
	39 °C	NA	–	NA	–	–	Stirring	Microbiota from pig feces	–	Flushing with CO_2 or N_2	ND	[100,101, 111]

Table 3. (continued)

Types of model	Simulated parameters											Refs
	Body T°	Gastric pH	Intestinal pH	Gastric emptying	Intestinal transit	Digestive secretions	Chyme mixing	Intestinal microbiota	Intestinal absorption	Anaerobic conditions	Simulated age	
Continuous fermentation models												
PolyFermS  Figure from [90]	38 °C	NA	Controlled pH at 6.0 with sodium hydroxyde	NA	Retention time of 9 h (swine proximal colon)	–	Stirring at 120 rpm	Immobilized microbiota from sow feces	–	Flushing with CO_2	5 months	[102,109]
PigutIVM (Piglet Gut *in Vitro* Model)  Figure from [92]	39 °C	NA	Controlled pH at 6.2 with sodium hydroxide	NA	Retention time of 24 h (entire colon)	–	Stirring at 400 rpm	Microbiota from piglet feces	–	Anaerobiosis self-maintained by the activity of resident microbiota	8 weeks	[103]

[a]NA, not applicable.
[b]ND, not determined.
[c]–, not reproduced in the model.

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tract is the TNO Gastrointestinal Model (TIM) that has been adapted by Meunier *et al.* [98,99] to simulate the stomach and small intestine of growing pigs. The major feature of this dynamic porcine digestion model is its ability to simulate the main digestive parameters of the stomach, duodenum, jejunum, and ileum, namely body temperature, kinetics of pH, peristaltic mixing and transit, salivary, gastric, biliary, and pancreatic secretions, as well as absorption of small molecules and water. It should be emphasised that, even though the TIM is a very pertinent model for studying physicochemical parameters of the GI tract, it does not include resident microbiota. In favor of their low cost and simple use, batch systems have been largely used to reproduce the pig colonic environment and related microbial fermentations [36,100,101]. However, batch cultures are restricted by substrate depletion and the continuous change in pH and redox potential which limit the experimental duration to only several hours [96]. Continuous *in vitro* fermentation models can more closely mimic pig colonic physiology due to integration of essential digestive parameters, such as realistic transit time and the supply of nutrient medium simulating ileal/caecal effluents while maintaining a functional microbiota [102–105]. Such kinds of model are designed for long-term experimental periods – up to 54 days for the PolyFermS model developed by Tanner *et al.* [102] – which allows increased microbiota stability through immobilization on gel beads. None of these models reproduces the specific colonic conditions of the piglet, except for the PigutIVM (Piglet Gut *In vitro* Model) recently set up by Fleury *et al.* [103]. The PigutIVM has been validated compared to *in vivo* data in 8-week-old piglets under untreated conditions, and when colistin, an antibiotic widely used to treat colibacillosis diarrhea, was administered. The distinctive feature of this model is the reproduction of anaerobiosis by the sole activity of resident microbiota, and not by flushing with CO_2 or N_2 as is usually done.

In vitro models of the pig gut have been used in a large number of nutritional applications aiming to study carbohydrate and protein digestibility [99,106] or follow microbial fermentation of starch and fibers (including prebiotics) [101,104,107]. Regarding microbiological applications, pig digestion models have been used to assess the effect of probiotic yeast strains on pig [108] or piglet [103] microbiota, as well as to study pathogen inhibition by medium-chain fatty acids [105] or probiotics [109]. For instance, in the PigutIVM, the addition of *Saccharomyces boulardii* led to a significant decrease in *E. coli* in the resident *in vitro* microbiota, as observed during colistin treatment [103]. In the PolyFermS, *Bifidobacterium thermophilum*, combined with FOS and GOS, inhibited colonization of S. typhimurium in the swine proximal colon [109].

To keep the peculiarity of the lumen and mucosal environment and facilitate host–microbiota interactions under representative conditions of the GI–microbe interface, piglet *in vitro* models could be optimized to integrate a mucosal microenvironment [110,111]. A study by Tran *et al.* [111] revealed that adding mucins to a pig *in vitro* batch fermentation model increased the proportion of some genera, such as members of the Proteobacteriaceae, which rely on the mucus layer to increase their activity. In addition, to include the interactions between the host, the immune system, and the gut microbiota, future *in vitro* digestion models should be coupled with intestinal porcine cells in culture such as the IPEC-J2 cell line, isolated from neonatal piglet jejunum [112] and porcine immune cell lines [113]. It would be of great interest to adapt *in vitro* models of the pig GI tract to the particular conditions encountered during the critical weaning stage. Such development would allow a better understanding of dysbiosis etiology by testing, in an independent manner, the different parameters that are supposed to be involved (e.g., anorexia, dietary changes, and antibiotics). Such *in vitro* models of the weaning phase could be also advantageously used by researchers, feed producers, or veterinarians for an efficient and cost-effective development and testing of nonantibiotic alternative strategies such as phages, feed ingredients, prebiotics, or probiotics. They could provide additional information on the cellular and molecular mechanisms of action of new in-feed compounds and their appropriate conditions of use (dose and mode of administration), which are questions that need to be addressed before commercialization.

Besides, due to the high similarity between the porcine and human gut, the pig can be used as a disease model for humans, especially for infants [114]. For instance, a piglet model of intestinal failure, a state of inadequate intestinal surface area following the removal of necrotic intestine, has already been used for studying the impact of prebiotics on ileal mucosa [115]. Thus, it is conceivable that a piglet model of dysbiosis could also bring translatable knowledge to the field of pediatric nutritional research.

Concluding Remarks

Weaning is a critical event in the swine industry which frequently leads to severe intestinal disorders and the overuse of antibiotics, raising serious economic and public health concerns. Gut microbiota dysbiosis induced by changes in diet and environment of piglets is prone to be one of the main causes of postweaning diarrhea and enteric infections. However, further research should be undertaken to better understand the succession of events leading to dysbiosis, as well as the relative involvement of influencing factors (see Outstanding Questions). For a better management of the weaning transition, it is of the utmost importance to find nonantibiotic strategies that are able to restore a balanced gut microbiota – such as prebiotics and probiotics. Even though *in vivo* experiments are the gold standard for evaluating such compounds, *in vitro* digestion models of the piglet gut constitute first-choice alternatives for ethical, technical, cost, and regulatory reasons. The forthcoming development of a 'dysbiosis' model – integrating the key parameters encountered in the field at weaning and host dimension – would be a great help for a better understanding of the etiology of postweaning disorders and evaluation of new in-feed additives.

Outstanding Questions

What is the relative importance of gut microbiota dysbiosis in the etiology of postweaning diarrhea?

Would restoration of microbial balance in the piglet gut be sufficient to counter gastrointestinal disorders at weaning?

Would new nonantibiotic solutions be efficient enough to replace antimicrobials in the management of weaning transition?

Can *in vitro* models accurately reproduce the specific gastrointestinal conditions encountered at weaning?

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Regarding this published literature review, the following chapters aim to develop, update or complete several points which needed to be further reviewed for a better understanding of the questions and objectives of this PhD project.

Chapter 2. Piglets at the Critical Time of Weaning

1. General practices in conventional swine industry

In intensive swine industry, animals are mostly reared indoor (Park et al., 2017) and housed in individual stalls or in groups depending on the usual practice of each production unit, specific label requirements, country legislation and the step of production (Pedersen, 2018). Most of the time, the housing systems consist in slurry based floors which are partly slatted providing limited bedding (Kemp and Soede, 2012; Park et al., 2017; Pedersen, 2018). Usually, few days after weaning, sows are placed in a specific mating unit allowing contact with a mature boar then they are artificially inseminated (Kemp and Soede, 2012; Pedersen, 2018). After the mating period, lasting approximately 1 month, sows are moved to the gestation units until 5 or 7 days prior to the estimate date of birth, the gestation period lasting on average 3 months, 3 weeks and 3 days (Kemp and Soede, 2012; Pedersen, 2018). In the farrowing unit, sows give birth to a litter of 10 to 25 piglets (Pedersen, 2018). The farrowing crate is a confined environment where sows are individually housed and cannot turn around to limit accidental crushing of piglets (Barnett et al., 2001). Suckling piglets stay in the farrowing unit with their mother until the artificial weaning date which mostly occurs around 3 to 4 weeks of age when animals weight around 6 to 8 kg (Pedersen, 2018). At weaning, piglets are separated from the mother and relocated in a nursery called weaner unit by groups sharing approximatively the same weight is order to adapt to the diet (Sutherland et al., 2014). In the weaner unit, atmospheric conditions such as temperature, humidity, air speed are precisely controlled to limit the transmission of pathogens. When weaner pigs reach around 25 to 30 kg they are considered as rearing, fattening or finisher pigs (Pedersen, 2018). Slaughter is mostly performed when pigs weight from 80 to 120 kg (Pedersen, 2018), but the age for slaughtering may vary depending on the production method and can even sometimes occur right after weaning. Sows can be culled, generally after having several litters, at certain steps of production, such as after weaning or after the mating unit due to failures to breed, decrease in productivity, lameness, low

farrowing and weaning performances or behavioural issues (Dagorn and Aumaitre, 1979; Friendship et al., 1986).

There are three types of commercial pig production units: a breeding only unit which houses only the inseminated sows, suckling piglets and weaned piglets up to 25 kg; a rearing/finishing only unit which houses pigs from 25kg to slaughter and a combined unit which includes both a breeding and a finishing unit (Marchant-Forde, 2008). The cycle of production of sows and piglets is presented in figure 3.

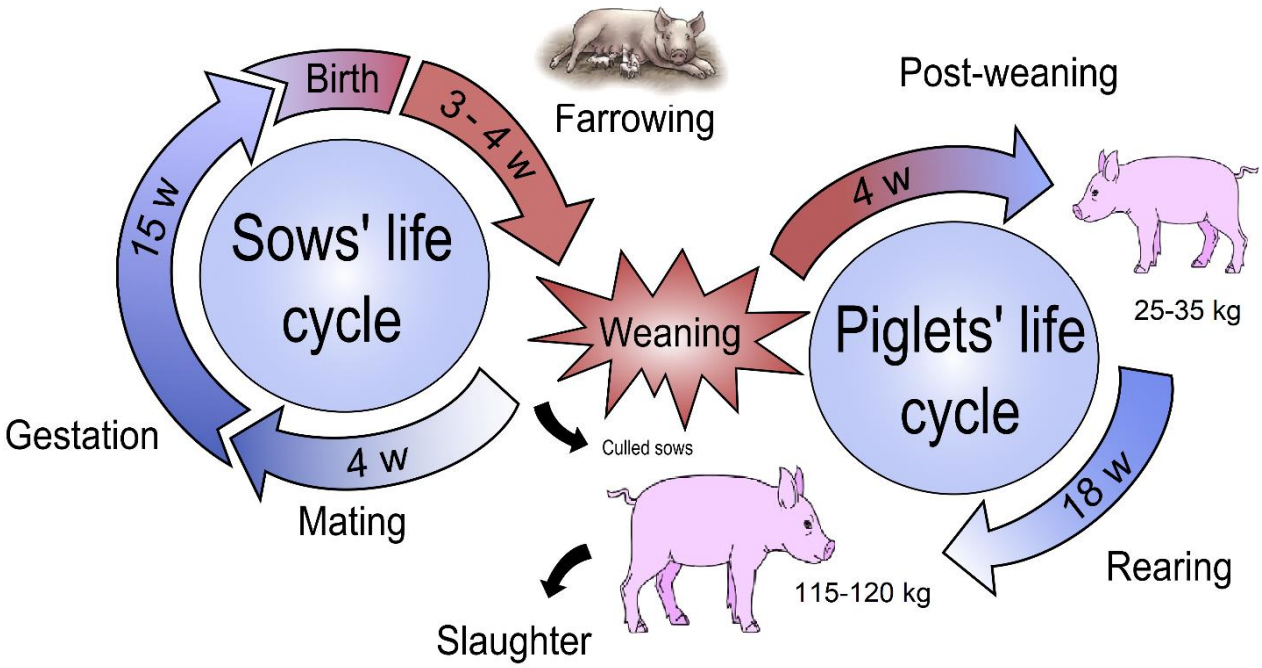


Figure 3. Illustration of life of sows and piglets in conventional swine industry (w = weeks) (© Raphaële Gresse).

2. Social, environmental and dietary stresses in weaning piglets

2.1. Social and environmental conditions

In modern swine industry, weaning is generally practiced around 3 to 4 weeks of age although natural weaning would occur around 17 weeks after birth (Jensen, 1986). For piglets, weaning is a sudden, stressful, short, and complex event characterized by changes in diet, social and

environmental life conditions, which profoundly impacts piglet health and leads to decreased performance and sometimes mortality (Campbell et al., 2013). In intensive production, piglets can even be weaned at only 1 or 2 weeks of age which consistently amplifies the physiological and behavioral effects of weaning (McLamb et al., 2013; Sutherland et al., 2014; Worobec et al., 1999). Social and environmental stresses are generated by abrupt separation from the mother, handling, transport, different physical environments, and mixing litters (Sutherland et al., 2014; Worobec et al., 1999). These diverse factors induce behavioral consequences in piglets such as vocalizations, aggressiveness, fighting or tail biting (Campbell et al., 2013; Tallet et al., 2019).

2.2. Nutritional management and feed intake

At weaning, piglets switch from highly-digestible liquid milk, essentially composed of lactose, proteins and lipids, associated with a high meal frequency to a less-digestible, more complex solid feed (Lallès et al., 2007a; Le Dividich and Sève, 2000). For an increased familiarity to solid feed, suckling piglets of around 3 weeks are frequently offered *ad libitum* a highly palatable and digestible diet called creep feed (Bruininx et al., 2002; Collins et al., 2013; Muns and Magowan, 2018). Creep feed is generally formulated with milk based products and plasma proteins to maximize nutrient uptake from the undeveloped piglet digestive system and is presented under a pelleted form which can be humidified (Heo et al., 2018; Okai et al., 1976). Consumption of creep feed has usually a positive impact on feed intake and body weight of piglets (Bruininx et al., 2002), although this can be variable between individuals (Middelkoop et al., 2019; Muns and Magowan, 2018; Wattanakul et al., 2005). Creep feed eaters, representing approximatively 40 to 65% of the litter, displayed better post-weaning behaviors, feed intake and performance than non-eaters (Pluske et al., 2018). After weaning, a first starter diet containing up to 20% of milk derived products and meeting the nutritional needs of piglets is provided during 1 or 2 weeks (Gao et al., 2019; Muns and Magowan, 2018; Wu et al., 2018). Special attention is given to a good balance of proteins, fibers, electrolytes and starch to improve piglets performance (Gao et al., 2019). Most of the time, starter diets are adapted to piglet weight and change regularly post-weaning. Modifications are made towards a reduction of milk derived products and an increased level of cereals and proteins (table 1).

Table 4. Example of diets for weaned piglets in European pig farms (adapted from www.pigprogress.net).

	Body weight range (kg)				
Ingredients (%)	**4-6**	**6-8**	**8-12**	**12-20**	**20-30**
Cereals Wheat, corn, barley, oat, tapioca…	20-30	30-40	40-50	50-60	60-70
Oils Soybean oil, sunflower oil, coconut oil	0-10	0-8	0-6	0-4	0-2
Sugar beet products Molasses, sugar beet pulp	0-10	0-10	0-10	0-10	0-10
Lactose sources Skim milk, condensed milk, whey, lactose	20-40	10-20	5-15	0-5	0
Animal proteins Milk proteins, fish meal, blood products	5-15	5-10	0-10	0-5	0
Plant proteins Refined proteins from soy, potato, wheat, soybean meal, peas, sunflower meal	10-30	15-30	20-30	20-30	20-30

Despite optimized nutritional strategies, dietary change is associated with low and erratic feed and water intake in newly weaned piglets (Lallès et al., 2007a; Sutherland et al., 2014). In a study of Bruininx et *al.*, 50% of newly weaned piglets refused feed during the first 12 hours and 20% during the 24 hours following weaning transition (Bruininx et al., 2002). This fasting period can last up to 48h and considerably impacts the physiology and the growth performance of weaning piglets (Lallès et al., 2007a; Le Dividich and Sève, 2000; Orgeur et al., 2001; Spreeuwenberg et al., 2001; Sutherland et al., 2014; Worobec et al., 1999).

3. Piglet gastrointestinal tract

3.1. Morphology and functions

The gastrointestinal tract (GIT) of piglets encompasses several functions, the principal being digestion of feed into nutrients absorbed by the host for energy intake, and is composed of

several successive organs (figure 4). The development of the morphology and segmentation of piglet GIT starts during the prenatal period (Pluske et al., 2003). At birth, ingestion of colostrum which contains various nutrients and growth factors induces a fast growth of the intestine with an increase of 75% and 33% of total weight during the first 24 hours of life respectively for the small and large intestine (Xu et al., 1992; Zhang et al., 1997). Digestion starts in the mouth *via* mechanical action of mastication and chemical actions of digestive enzymes from saliva (Hudman et al., 1957). After transit through the oesophagus, digestion continues in the stomach. The stomach of weaning piglets possesses a low pH of around 4 (Wilson and Leibholz, 1981), and a variety of digestive enzymes such as lipase, pepsin or trypsin (Hedemann and Jensen, 2004). The small intestine, following the stomach, is composed of 3 parts: duodenum, jejunum and ileum and is the principal location of digestion of lipids, proteins and soluble carbohydrates helped by the release of pancreatic enzymes such as lipase or amylase and bile salts production by the liver. Retention time in piglets from 2 to 5 weeks was estimated to be around 30 min in duodenum and jejunum and 2h in the ileum while the pH gradually increases from 5.9 in the duodenum until 6.7 in the ileum (Wilson and Leibholz, 1981). The large intestine of pigs is composed of four sections: the cecum being an appendix possessing a blind end, the proximal colon, distal colon and the rectum. The pH of 2 to 5 week-old piglets was previously measured at 6 in the cecum and 6.3 in the large intestine (Wilson and Leibholz, 1981). Mixing of the digestive content continues in the large intestine due slow contractions of the colonic border (Wilson, 2010). The large intestine is also characterized by high capacities for microbial degradation and fermentation of fibers, proteins and complex carbohydrates, non-digested in the small intestine. An important role of proximal colon of mammals is the absorption of water and electrolytes while the distal colon is specialized in the storage of digestive content and evacuation of waste from nutrient digestion (Wilson, 2010). The large intestine also plays a role in ionic composition of feces by absorbing sodium and chloride while releasing potassium and bicarbonate (Wilson, 2010). The mean retention time of 2 and 5 week-old piglets was previously estimated at respectively 3 and 6h in the cecum and 18 and 21h in the colon (Wilson and Leibholz, 1981).

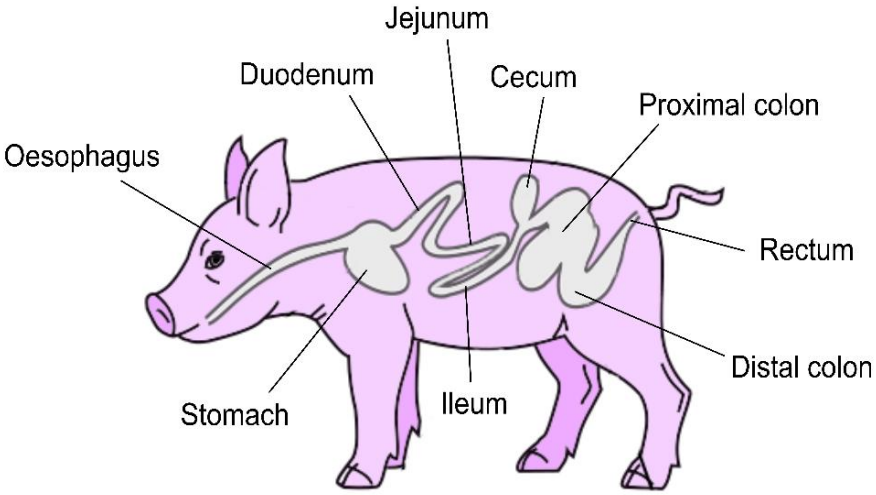


Figure 4. Gastrointestinal tract of pigs (© Raphaële Gresse).

3.2. The mucus layer

The luminal content of the GIT is separated from host intestinal epithelial cells by the mucus layer, a highly hydrated gel found from the stomach to the large intestine (Etienne-Mesmin et al., 2019; Leal et al., 2017). The mucus layer has numerous functions in the GIT such as serving as a lubricant for digestive content, a biological habitat for various microorganisms, keeping intestinal homeostasis, exchanging gases and nutrients and protecting the epithelium like a biological, physical and chemical barrier against invaders, including pathogens (Etienne-Mesmin et al., 2019; Johansson et al., 2013; Leal et al., 2017). The mucus layer displays several physicochemical properties such as pore size, viscoelasticity or a neutral or acid pH which regulates the permeability to molecules and particles (Leal et al., 2017). Mucus thickness varies depending on species (Varum et al., 2012). In adult pigs, the mucus layer gradually increases from around 25 µm in the duodenum to 60 µm in the distal colon (Varum et al., 2012). Mucus is secreted by the specialized goblet cells located in the crypt on the intestine epithelial layer (figure 5) (Etienne-Mesmin et al., 2019; Johansson et al., 2013; Sicard et al., 2017). In the small intestine of mammals the single mucus layer is unattached and easy to remove while the colon harbours a two-layered mucus including an outer loose layer and an inner thick layer (Johansson et al., 2013). Mucus is composed of unattached mucins harbouring different glycosylation patterns, water, globular proteins, salts, DNA, cells and cellular debris (Bansil and Turner, 2006; Leal et al., 2017; Thornton and Sheehan, 2004). Mucins are the main components responsible for the viscoelasticity of the mucus layer (Leal et al., 2017). They can be differentiated between bound and secreted mucins (figure 6) and are large and extracellular

glycoproteins containing around 80 % of carbohydrates such as y N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, and sialic acid (Bansil and Turner, 2006; Leal et al., 2017). The principal type of mucins in the whole GIT of mammals is MUC2, an endogenous protease-resistant large glycoprotein (Johansson et al., 2013; Strugala et al., 2003). At birth, the mucus layer of piglets is generally composed of a high percentage of acidic mucins acting like a real defence against pathogens which is of a particular importance due to their immature immune system (Deplancke and Gaskins, 2001). The ratio of neutral to acidic mucins generally increases until weaning (Macierzanka et al., 2014). Finally, weaning piglet mucus layer in the jejunum is less elastic and concentrated than the mucus layer of mature pigs making it much more permeable (Macierzanka et al., 2014).

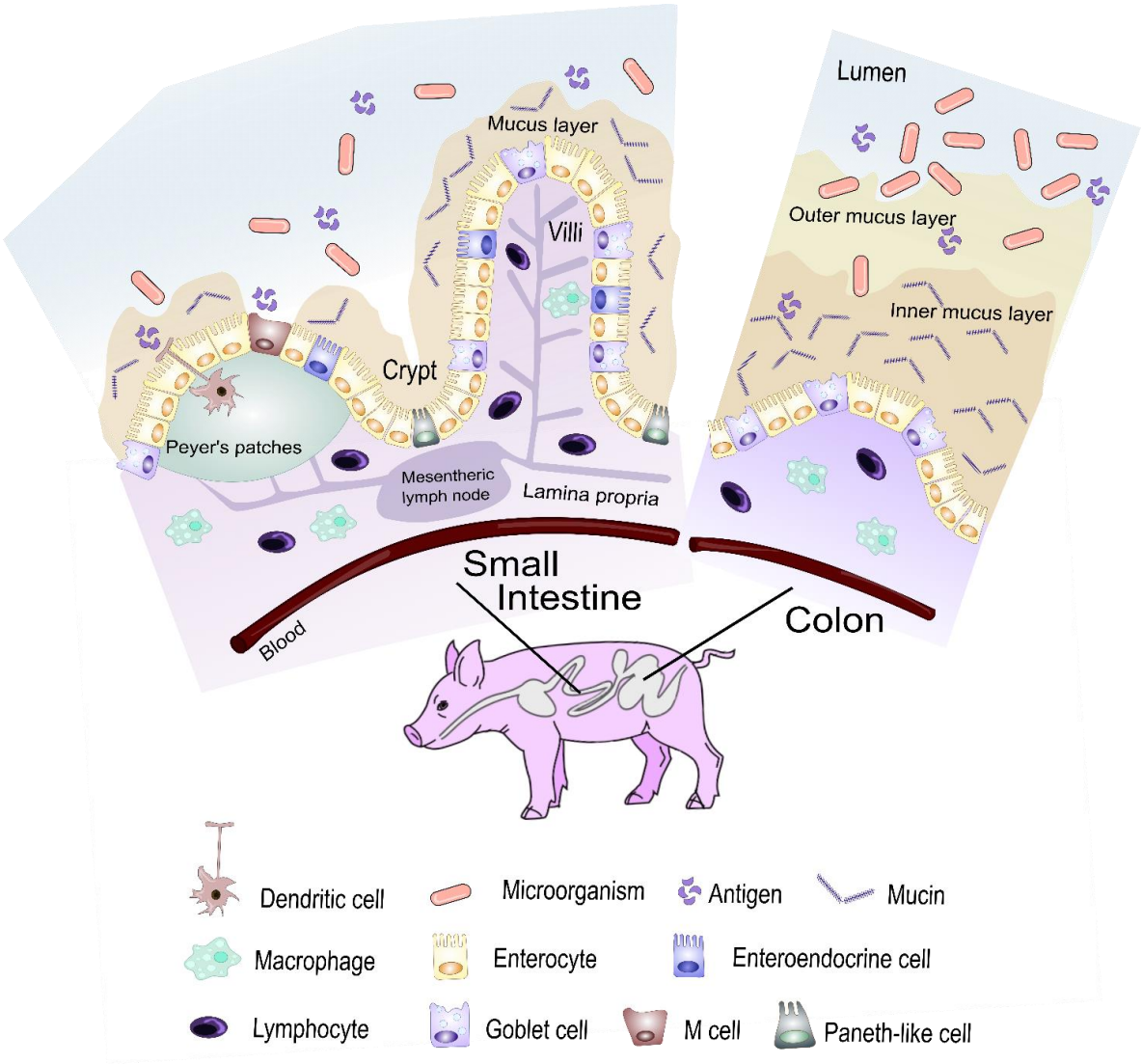


Figure 5. Organisation of the GIT epithelium in the small intestine and in the colon (© Raphaële Gresse).

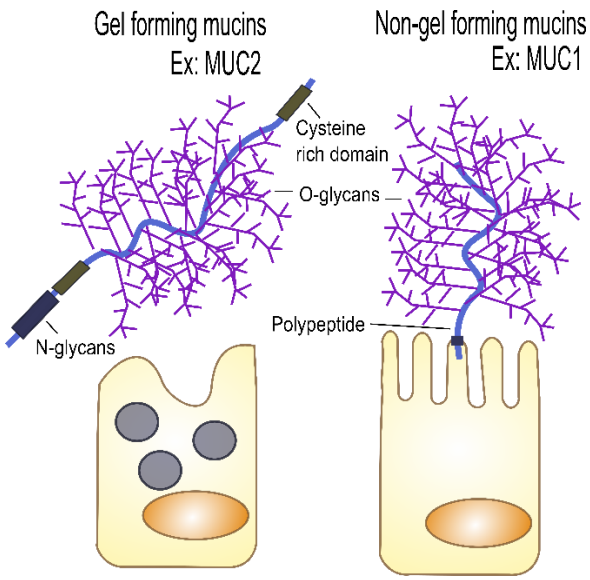


Figure 6. Structure of bound and secreted mucins in the gut. Inspired from (Moran et al., 2011).

3.3. The GIT epithelium

At birth, the development of intestinal epithelium and intestinal epithelial cell proliferation are the principal reasons of the consequent augmentation of GIT volume and weight (Xu et al., 1992; Zhang et al., 1997). The intestinal epithelium consists in a monolayer of polarized epithelial cells forming a barrier against toxic substances and microorganisms (Yang et al., 2016). The principal cell type are enterocytes, covering over 80% of the villi, which secrete hydrolytic enzymes, help for the digestion of soluble polysaccharides and peptides, regulate pH through the secretion of Cl^- and HCO_3^- ions, and immunoglobulins and play an important role in nutrient absorption (Moeser et al., 2017; Yang et al., 2013). Apart from the previously evocated goblet cells secreting mucus, GIT epithelium is also composed of Paneth cells, secreting antimicrobial peptides, and enteroendocrine cells releasing hormones or molecules into the blood stream and acting like chemoreceptors (figure 5) (Kong et al., 2018). However, in piglets of 27 days, only Paneth-like cells were could be identified due to the lack of Paneth cell typical acidophil granules (Verdile et al., 2019). In the small intestine, the epithelium is organized into crypts and villi (Yang et al., 2016). The length, circumference and size of villi and crypt are especially growing during the first days of life in order to maximize contact with nutrients (Xu et al., 1992; Zhang et al., 1997) and are progressively shortened and widened during farrowing especially in the large intestine (Cera et al., 1988). At birth, the cecum and

proximal colon epithelium (figure 7) of piglets are composed of villus-like structures which are considerably reduced three days after birth (Brunsgaard, 1997; Xu et al., 1992). Located between epithelial cells, tight junctions are structures composed of numerous intracellular and apical intercellular membrane proteins such as *zonula occludens*, occludins and claudins (Edelblum and Turner, 2009; Turner, 2009). Tight junctions regulate the permeability of the intestinal barrier and play a critical role in the establishment of epithelial polarity (Moeser et al., 2017).

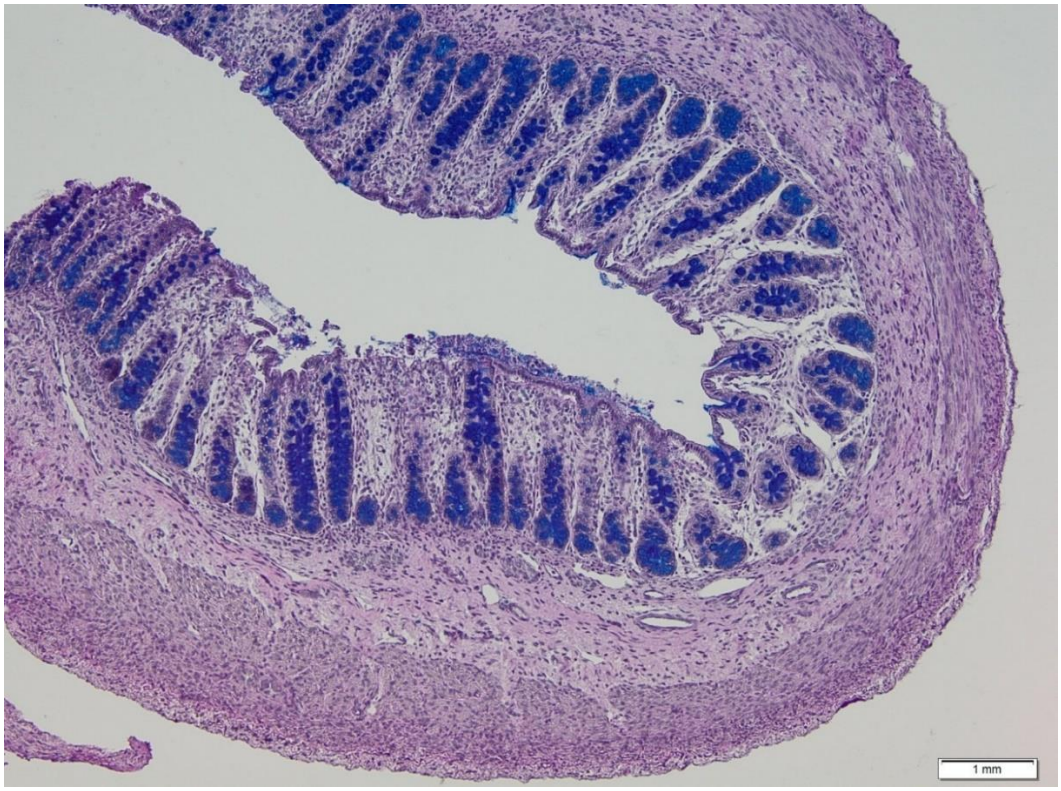


Figure 7. Transversal section of the proximal colon of 27 day old female piglets coloured with paraformaldehyde and observed by microscope (©Raphaële Gresse). On this picture mucins located in Goblet cells are coloured in blue.

3.4. The intestinal immune system

An important mucosal immune system (figure 5) is associated with intestinal epithelium throughout the GIT and is in charge of the recognition of pathogenic and non-pathogenic antigens (Stokes, 2017). Intestinal immune cells are called Gut Associated Lymphoid Tissue

(GALT) and constitute the largest immune organ in the body (Pluske et al., 2018). GALT is composed of a part made of diffuse cells, lymphocytes or macrophages, allocated inside the GIT epithelium and beneath, in the thin layer of connective tissue, called *lamina propria*. The other part of the GALT is concentrated under organized lymphoid follicles made of lymphoid nodules named Peyer's patches and mesenteric nodules located under the enterocytes of the small intestine (Stokes et al., 1994). Epithelial cells located on top of Peyer's patches contain only few digestive enzymes and contain M cells specialized in the translocation of antigens to immune cells (Stokes et al., 1994). Dendritic cells are immune cells present in sub epithelial area and can directly capture antigens from the lumen (Forchielli and Walker, 2005). Newborn piglets are profoundly immunocompromised and need the immunoglobulins present in the colostrum to develop passive immunity responses (Lallès et al., 2004b). Regarding active immunity, piglets are able to induce limited lymphocyte responses against pathogens (Lallès et al., 2004b). The development of piglet GALT is highly dependent of stimuli encountered during the first weeks of life such as immunoglobulins contained in sow milk (Lallès et al., 2004b).

4. Physiological consequences of weaning transition

4.1. Impact on growth performance and metabolism

The abrupt reduction of feed intake after weaning leads to a slowdown of growth and even a reduced body mass index (Le Dividich and Sève, 2000). Piglets loose up to 100 to 250 g on the first day following the weaning transition (Le Dividich and Sève, 2000). If the loss of body weight is generally recovered in a few days, some studies reported that piglets gaining body mass rapidly during the first week post-weaning could reach market weight much earlier than light weight piglets (Kats et al., 1992; Le Dividich and Sève, 2000). The transient fasting period also induces modifications of piglet metabolism. In a study of Whittemore et *al.*, the concentration of lipids in the body of piglets decreased from 80 g during the first 8 days after weaning (Whittemore et al., 1981). Reduced feed intake obviously induces a reduction in the plasma concentration of glucose and thus an increased consumption of hepatic and kidney glycogen stock (Xie et al., 2016). The high protein needs of growing piglets cannot be fulfilled due to the reduced feed intake which negatively impacts the development of muscle mass (Pluske et al., 2003). Finally, a link was established between weaning and oxidative stress due

to a reduced activity of antioxidant enzymes and an increased concentration of reactive oxygen species in the intestine (Sauerwein et al., 2007; Wang et al., 2008; Zhu et al., 2012).

4.2. Impact on intestinal morphology

Numbers of gastrointestinal changes have been associated with weaning transition. Spreeuwenberg et *al*. demonstrated that the weight of the small intestine decreased to more than 20% in the first few days following weaning (Spreeuwenberg, 2002). Several impairments of gut structure were previously highlighted in post-weaning piglets. Depending on studies, the height of intestinal villi was reduced from 40 to 65 % of their pre-weaning height in the duodenum and jejunum and from 10% to 20% in the ileum in post-weaning piglets (Hedemann et al., 2003; Pluske et al., 1997; Spreeuwenberg et al., 2001; van Beers-Schreurs et al., 1998; Vente-Spreeuwenberg et al., 2003, 2003; Wiese et al., 2003). Villus atrophy persisted as villi recovered only 70% of their initial height between 8 to 15 days post-weaning (Pluske et al., 2003). The length of villi is responsible for optimal digestive and absorption functionality of the gut (Pluske et al., 1997). The principal cause of the disruption of intestinal barrier morphology could be the non-fulfilment of the nutritional needs of piglets at weaning induced by the transient period of reduced feed intake which under stimulates the GIT epithelium (Pluske et al., 2003; Spreeuwenberg et al., 2001). Additionally, while a 48h weaning anorexia is associated with villi atrophy, a positive relationship exists between feed intake and the height of villi in the GIT of weaning piglets (Boudry et al., 2004; Pluske et al., 2003).

4.3. Impact on intestinal functions and the immune system

Functional changes also occur in the GIT of post-weaning piglets such as a failure in the intestinal epithelial barrier. Indeed, in the few days following weaning, a reduction of transepithelial resistance (Boudry et al., 2004; Hu et al., 2013; Wijtten et al., 2011) due to tight junction opening and reduced gene expression of occluding and claudin proteins (Hu et al., 2013) was observed which provokes increased permeability and leaky gut. Spreeuwenberg *et al*. also highlighted an augmentation of transepithelial transport in the jejunum of piglets at 4

days post-weaning (Spreeuwenberg et al., 2001). A reduction in gut digestive functions due to decreased enzyme activity, such as lactase, and sucrase, was observed in several studies (Boudry et al., 2004; Pié et al., 2004; Pluske et al., 1997), as well as decrease in absorbing capacity and general enterocyte activity (van Beers-Schreurs et al., 1998; Vente-Spreeuwenberg et al., 2004) and a high level of apoptosis (Zhou et al., 2018). An abrupt weaning transition suddenly deprives piglets from the passive immunity delivered by sow milk (Moeser et al., 2017, 2007; Wagstrom et al., 2000). The immature immune system of weaning piglets is not yet able to fully regulate immune and inflammatory responses towards antigens from pathogenic, commensal or dietary origins (Lallès et al., 2007b, 2004a). The increased intestinal permeability allows the translocation of more antigens to the lamina propria and thus could induce hyperactivation of the immune system (Boudry et al., 2004). Weaning was indeed associated with induction of antimicrobial and inflammatory responses (Bomba et al., 2014). At gene level, an overexpression of genes encoding proinflammatory cytokines such as IL-1, IL-6 or TNFα was reported 24h after weaning in the small intestine and several days post-weaning in the colon (Pié et al., 2004). Several studies also reported an accumulation of immune cells such as lymphocytes or macrophages in the gut mucosa few days post-weaning (B. A. McCracken et al., 1999; McLamb et al., 2013).

To conclude on this chapter, consequences of the critical weaning period such as transient anorexia, loss of body weight, oxidative stress, activation of pathways related to inflammatory responses, changes in hormonal activity, induction of small intestine atrophy, reduced villous height, reduction of nutrient digestion, fluid and electrolytes absorption and increased intestinal permeability to antigens, have been extensively reviewed. The complex interactions between gut health, nutrition and immunity is, in addition, in close relation with the gut microbiota which further impacts weaning associated physiological changes.

Chapter 3. The Piglet Gut Microbiota

1. Major functions of the gut microbiota

The gut microbiota is the total population of bacteria, archaea, protozoa, viruses and fungi inhabiting the GIT (Fouhse et al., 2016). Beside the degradation of diet components arriving in the intestine, the gut microbiota of mammals has other numerous roles benefiting their host such as production of vitamins, maintenance of normal functions of the intestinal villi, regulation of the immune responses, communication with enteric nervous system and protection from pathogenic microorganisms (Buffie and Pamer, 2013; Cryan et al., 2019; Kamada et al., 2013).

1.1. Production of metabolites

In the GIT, nutrients from the diet that have not been digested and absorbed in the upper tract reach the lumen of the lower gut where they are fermented by the microbiota into various metabolites. In pig GIT, the highest concentration of bacterial metabolites is found in the caecum and the colon due to the high fermentative activity of the microbiota in these segments (Fouhse et al., 2016). Gut microorganisms are involved in the breakdown of plant cell wall polysaccharides, polyphenols, proteins and the synthesis of vitamins (Macfarlane and Macfarlane, 2003; Rist et al., 2013). One of the major functions of the pig intestinal microbiota is to provide energy to the host through the synthesis of short chain fatty acids (SCFA) through digestion and fermentation of carbohydrates by microorganisms called SCFA-producers, such as *Clostridiaceae* or *Ruminococcaceae* families (Fouhse et al., 2016). For example, SCFA are thought to provide 30% of the maintenance energy requirements of enterocytes for growing pigs, (Morita et al., 2004; Rist et al., 2013). The fermentation activity of the microbiota can also lead to the production of lactic, formic, succinic acids and ethanol which are generally rapidly transformed into acetate, propionate and butyrate, the principal SCFA in the gut, by a number of cross-feeding organisms (Figure 8) (Macfarlane and Macfarlane, 2003; Morrison and

Preston, 2016). A wide diversity of bacterial groups can produce acetate while the pathways for butyrate and propionate production appear more conserved and substrate specific (Morrison and Preston, 2016). Butyrate is the preferred source of energy for intestinal epithelial cells which use it for normal proliferation and differentiation (Fouhse et al., 2016; Macfarlane and Macfarlane, 2003). Potentially toxic compounds for the host such as ammonia, phenols, p-cresol, indols or biogenic amines can also be synthetized by the microbiota via deamination of amino acids or proteins (A. Williams et al., 2005; Rist et al., 2013) and play important roles in the initiation or progression of a leaky gut or inflammation (Marchesi et al., 2016; Windey et al., 2012). The fermentation of dietary substrates by the gut microbiota also leads to the production of gases such as carbon dioxide, hydrogen, methane or hydrogen sulphide which varies between GIT segments by their concentration and volumes (Figure 8) (Kalantar-Zadeh et al., 2019). The relative proportion of gases in the gut of mammals is predominantly influenced by the composition of the microbiota and their related metabolism and, in case of imbalance, can generate abdominal symptoms and discomfort (Kalantar-Zadeh et al., 2019). Microbial activity and production of metabolites influence two principal physico-chemical parameters of the GIT: pH and redox potential (Fonty and Chaucheyras-Durand, 2007). Gut redox potential represents the capacity of the intestine for reducing chemical reactions and is influenced by various factors such as the presence of oxygen, reactive oxygen or nitrate species, differential microbial metabolism and host interactions (Reese et al., 2018). The redox potential evolves throughout the entire GIT and is associated to the presence of strictly anaerobic bacteria (figure 9) (Fonty and Chaucheyras-Durand, 2007). A disruption of redox homeostasis can impact the physiology of the gut epithelium via self-renewal, proliferation, migration and differentiation processes and the immune system (Pérez et al., 2017).

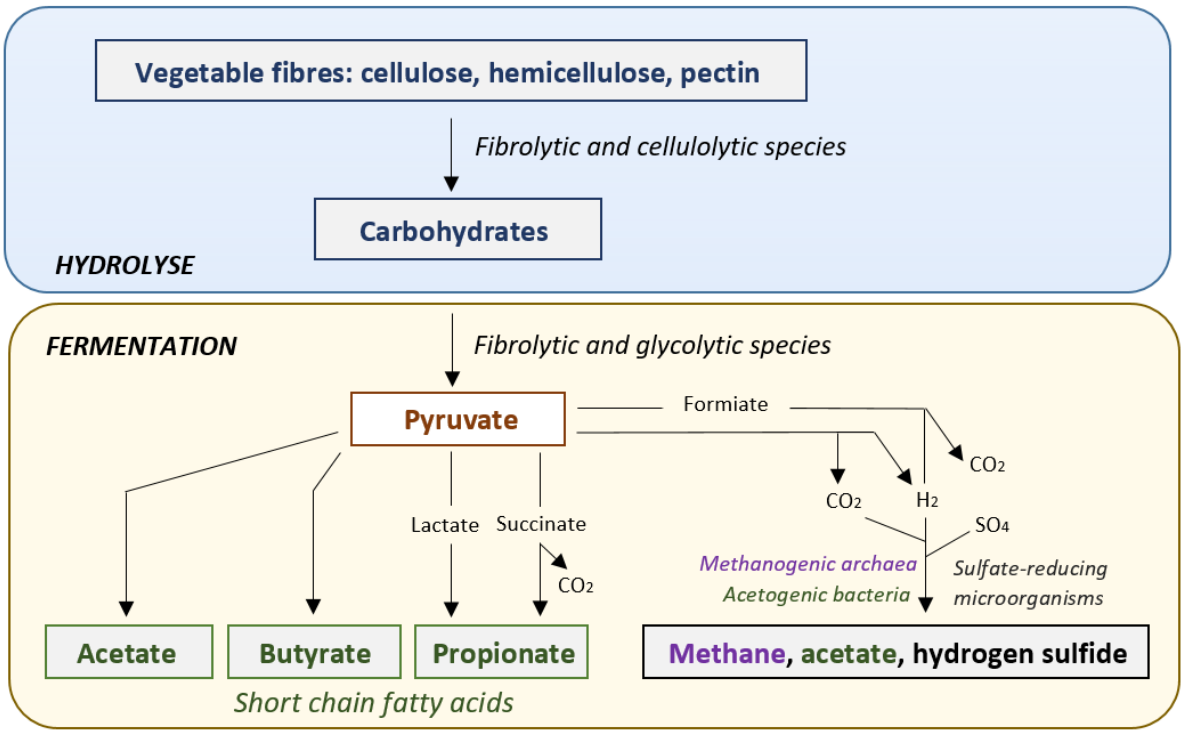


Figure 8. SCFA and gas production by fermentative activity of the colonic microbiota.

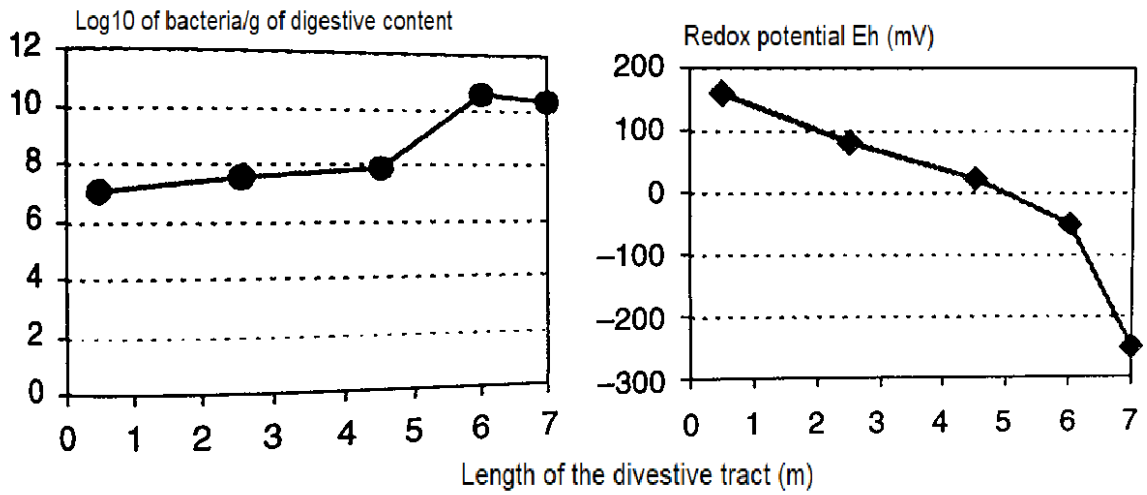


Figure 9. Relation between the presence of strictly anaerobic bacteria and redox potential throughout the GIT of piglets (Modified from (Fonty and Chaucheyras-Durand, 2007); 0-5 m correspond to small intestine; 6 m = cecum and 7m = colon).

1.2. Interactions with the GALT

The gut microbiota of mammals shapes the development and functions of the immune system since birth (Stokes, 2017). Germ free animal studies have shown that the development of Peyer's patches, mesenteric lymph nodes and lymphoid follicles as well as induction of immune responses in the gut are enhanced by the microbiota (Round and Mazmanian, 2009). The mucosal immune system is indeed continuously challenged by the massive antigenic luminal environment and tightly regulated via wide molecular pathways (Pluske et al., 2018). Microbial molecular patterns (MAMPs) produced by bacteria are recognized via Toll Like receptors (TLRs) or myeloid differentiation primary responses 88 (MyD88) harboured by intestinal epithelial cells and GALT cells (Pickard et al., 2017; Pluske et al., 2018). The recognition of pathogenic signal molecules among commensals induces the secretion of interleukins , molecules having immunomodulatory properties, which trigger immune responses (Pluske et al., 2018).

1.3. Resistance to pathogen colonization

A stable symbiotic microbial community is known to resist to the colonization of autochthonous bacteria via direct and indirect mechanisms. First, commensal microorganisms can compete for the same limited nutrients which makes them unavailable for pathogens (Pickard et al., 2017). Some commensal bacterial populations possess the ability to secrete bacteriocins, small antimicrobial peptides able to kill or inhibit the growth of specific other bacteria (Hou et al., 2015; Pickard et al., 2017; Pultz et al., 2005). The secretion of SCFA in the lumen can also inhibit indirectly the growth of pathogens by decreasing the gut pH (Morrison and Preston, 2016).

2. The piglet luminal gut microbiota

2.1. Generalities

The GIT microbiota varies in population density and diversity across the successive gut organs and between the lumen and the mucosa (Holman et al., 2017; Leser et al., 2002; Pluske et al.,

2018; Zhao et al., 2015). The number of culturable bacteria was previously reported to range from 10^3 per gram of content in the stomach to 10^{11} in the colon of pig (Pluske et al., 2002). However, the fecal microbiota is much more investigated, despite its differences with other GIT segments, due to its advantageous accessibility. Microbiota composition is indeed directly influenced by gut intrinsic factors such as pH, oxygen gradient, transit time, digestive secretions varying between compartments (Holman et al., 2017; Leser et al., 2002; Pluske et al., 2018; Zhao et al., 2015). The colonization of piglet GIT also highly depends on the environment, allowance and composition of creep feed, genetics or feed additives in sow diet (Fouhse et al., 2016). Many recent reviews have reported the composition of the adult pig GIT microbiota and its interactions with the diet and the host, as well as its relationship with the animal health, feed efficiency and diseases (Aluthge et al., 2019; Maltecca et al., 2020; Patil et al., 2019). In the literature review presented below we will only focus on what is known about the piglet microbiota.

2.2. Colonization of piglet GIT

2.2.1. Bacterial microbiota

In piglets, colonization is initiated at birth by contact with skin, feces or vagina of the sow and shaped by the consumption of sow milk which provides nutritional advantages to lactic acid bacterial population, building a milk oriented microbiome (Frese et al., 2015). *Escherichia coli* (*E. coli*), *Lactobacillus* and *Clostridium* are the major genera detected in the feces of newborn piglets (Chen et al., 2018; Konstantinov et al., 2006). Petri et *al.* hypothesized that the facultative anaerobes *E. coli* and *Streptococcus* spp. create an anaerobic environment favoring the establishment of other colonizers such as *Bacteroides, Lactobacillus, Bifidobacterium* and *Clostridium* (Petri et al., 2010). During the suckling period, the fecal microbiota is more and more diversified and predominantly contains *Firmicutes, Bacteroidetes, Proteobacteria* and *Fusobacteria* at phylum level and *Lactobacillus, Fusobacterium,* or *Bacteroides* at genus level (Chen et al., 2018; Hasan et al., 2018; Poulsen et al., 2018; Schokker et al., 2018; Wang et al., 2016). De Rodas et *al.* investigated the microbiota of newborn and suckling piglets along the GIT. Right after birth, the microbiota of newborn was similarly composed between all segments of a great majority of *Bacilli* followed by *Clostridia* and *Gammaproteobacteria* (De Rodas et al., 2018). Ten days after birth, the major changes consisted in the augmentation of *Clostridia* and *Bacteroidia* relative abundance in the ileum, cecum and colon microbiota of suckling piglets (De Rodas et al., 2018).

2.2.2. Fungal and archaeal microbiota

The fungal part of the microbiota is considered as the "rare biosphere" due to its low quantity in the GIT and has been only recently investigated (Huffnagle and Noverr, 2013). Colonization of the yeast microbiota starts at birth with *Netricaceae* and *Cladosporicaeae* families, though at a low and unstable level, which rapidly disappear during the suckling period possible due to the contamination of creep feed by fungi (Summers et al., 2019). Very few data are available about the archaeal microbiota of piglets (and even of adult pigs). Yet, archaea represent a functionally important community within the microbiota which notably converts bacterial - metabolites into methane (Luo et al., 2017; Moissl-Eichinger et al., 2018). Up to date, no studies reported the colonization of archaeal microbiota in piglets.

2.3. Piglet microbiota at weaning

2.3.1 Bacterial microbiota

At weaning, piglets are suddenly fed with protein or cereal-based diet containing more complex nutrient sources which may influence small intestine absorption capacity, piglet growth and feed efficiency (Guevarra et al., 2018). Consequently to the change of the diet, the piglet gut microbiota also encounters numerous changes at weaning which are considered as a real microbial shift (Guevarra et al., 2018; Kim and Isaacson, 2015). The establishment of adult-like bacterial species into early-life is of particular importance due to potential lifelong effects on piglet immune system and health (Dou et al., 2017; Guevarra et al., 2018; Kim et al., 2011). Increase or decrease in bacterial richness and diversity were reported in feces or intestine content of weaned piglets compared to suckling period (Alain B. Pajarillo et al., 2014; Chen et al., 2017; Frese et al., 2015; Poulsen et al., 2018; Slifierz et al., 2015; Tao et al., 2015). We have previously reviewed the changes in microbiota of piglets at weaning, as reported in Gresse et al., 2017 presented in chapter 1. The changes in microbiota composition of weaning piglets reported in the literature after 2017 are thus presented in Table 5. Briefly, sharp increases in *Prevotellaceae*, increases in *Clostridiaceae*, reductions in *Lactobacillus*, *Bacteroides* and complete disappearance of *Fusobacterium* have been reported both in fecal samples and large intestine contents (Adhikari et al., 2019; Pajarillo et al., 2014; Bian et al., 2016; De Rodas et al., 2018; Guevarra et al., 2018; Konstantinov et al., 2006; Li et al., 2018; Mach et al., 2015; Su

et al., 2008; Summers et al., 2019; Wang et al., 2017). In several studies, the *Firmicutes/Bacteroidetes* ratio was modified, though inconsistently, in the feces or in the colon, suggesting an even more profound impact of weaning on bacterial communities at the phylum level (Alain B. Pajarillo et al., 2014; Bian et al., 2016; Li et al., 2018). However, diet composition (amount and sources of the dietary ingredients) shape the diversity and composition of the gut microbiome of weaning piglets and can thus further impact the bacterial shifts at weaning (Rist et al., 2013). This may explain the differences observed between studies as well as the different techniques used, genetic factors, environmental conditions, age of animals and the sampling methods. For instance, enriched pectin diets as well as a soybean meal decreased the relative abundance of *Lactobacillus* and increased that of *Prevotella* in the colon (Tian et al., 2017) whereas a fish protein source was linked to a high expansion of *Escherichia/Shigella* group (Cao et al., 2016). Also, weaning before the recommended 28 days of age could have a stronger impact on bacterial composition and could favor the post-weaning expansion of *Escherichia/ Shigella* group (Konstantinov et al., 2006; C. C. Xu et al., 2014; G.-Y. Yang et al., 2016).

2.3.2. Fungal and archaeal microbiota

Recently, two studies investigated the mycobiome of newborn to post-weaning piglets using culture independent techniques (Arfken et al., 2019; Summers et al., 2019). Both studies reported Shannon diversity indices below 2 for fungal microbiota in the GIT or feces of all piglets (Arfken et al., 2019; Summers et al., 2019). Arfken et al. described the mycobiome of piglets two weeks post-weaning and reported, overall, that the genera *Kazachstania* from family *Saccharomycetaceae, Hyphopichia* from family *Debaryomycetaceae* and *Wallemia* from family *Wallemiaceae* were the most abundant with mean relative abundances of respectively 50, 23 and 6% (Arfken et al., 2019) Summers et al. highlighted that weaning induced a great increase of *Saccharomycetaceae* relative abundance from around 10 to around 90 % in piglet feces as well as a smaller increase of the *Dipodascaceae* (Summers et al., 2019). However, knowledge about the functionality of fungal microbiota in weaning piglets are very limited.

Regarding archaeal microbiota around weaning, Federici et al. analysed the archaeal microbiota in the feces of commercial piglets and detected from 10^9 to 10^{10} 16S gene copy number/ g of

feces of total archaea, which slightly decreased after weaning (Federici et al., 2015). Additionally a shift from *Methanobrevibacter smithii* to *Methanobrevibacter boviskoreani* was observed in piglet feces throughout the weaning transition (Federici et al., 2015).

The figure 10 summarizes the early-life major microbial shifts reported in feces of healthy piglets.

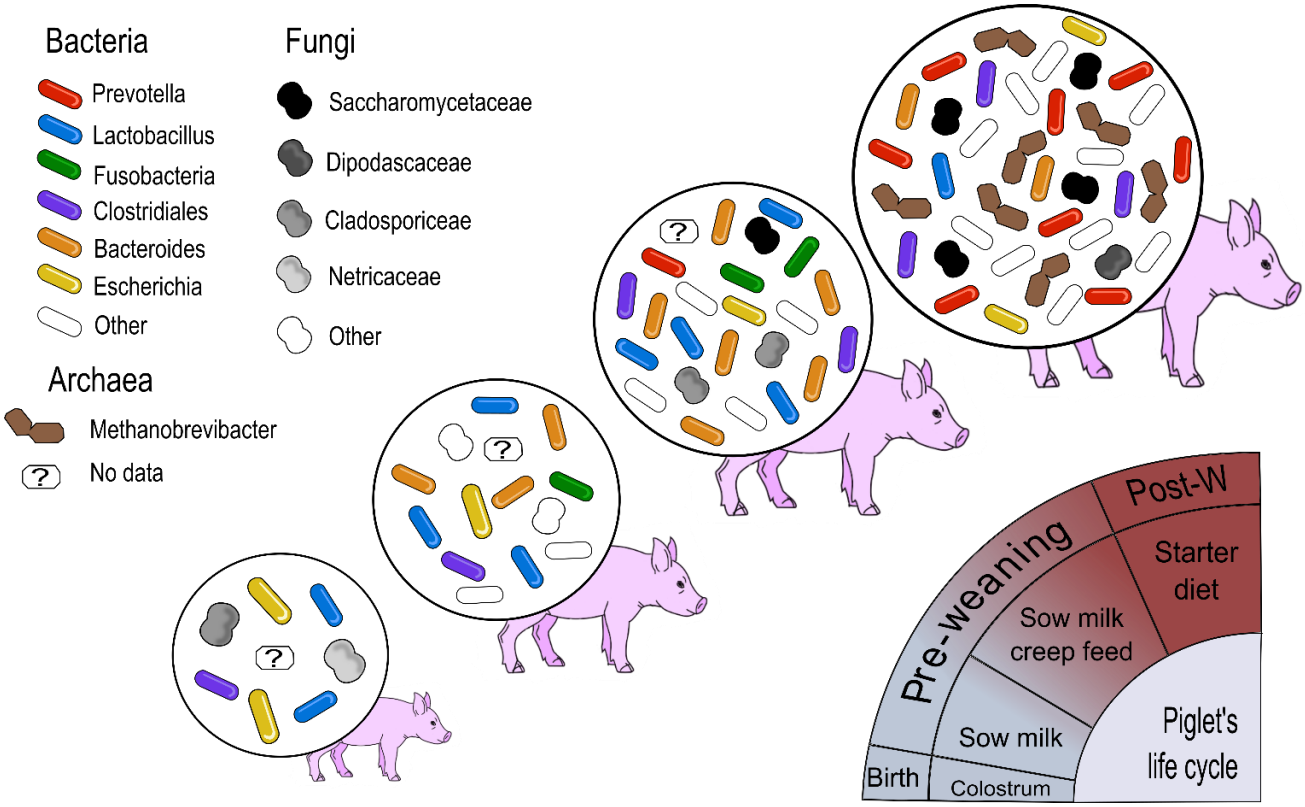


Figure 10. Schematic summary of healthy piglet fecal microbiota over time considering up to date knowledge (© Raphaële Gresse).

Table 5. Impact of weaning transition on the gut microbiota of healthy piglets (© Raphaële Gresse). (Only studies published after 2017 are reviewed, for literature published before 2017 see Gresse et al., 2017 in chapter 1).

Age of piglets at sampling	Diet and breeding conditions	Samples	Techniques	Qualitative modifications of gut microbiota	Composition of the gut microbiota		References
					Before weaning	After weaning	
14 day to 34 days	17 piglets were weaned at 3-week-old and housed in a commercial farm	Feces	Illumina MiSeq	Increase of alpha diversity Decrease of *Fusobacteria* and *Proteobacteria* Increase of *Bacteroidetes*	˜50 % of *Firmicutes* ˜29 % of *Bacteroidetes* ˜6 % of *Proteobacteria* ˜16 % of *Fusobacteria*	˜50 % of *Firmicutes* ˜40 % of *Bacteroidetes* ˜1.5 % of *Proteobacteria* ˜0 % of *Fusobacteria*	(Chen et al., 2017)
Birth to 35 days	16 commercial piglets were weaned at 28 days and fed with corn and soybean meal	Feces	454 pyrosequencing	Decrease of *Bacteroides genus* Increase of *Prevotella* genus Disappearance of *Fusobacterium*	4.9 % of *Bacteroides* 23 % of *Prevotella* 10.7% of *Fusobacteria*	1.4 % of *Bacteroides* 34% of *Prevotella* 0% of *Fusobacteria*	(Wang et al., 2017)
21 and 24 days	3 litters of 21d commercial suckling piglets were weaned at 21 days. 6 piglets were slaughtered before and 3 days after weaning	Colon	Illumina MiSeq	Decrease of *Bacteroides genus* Increase of *Prevotella* genus Other bacterial genera were increased after weaning such as *Lachnospiraceae*, *Negativicutes*, *Selenomonadales*, *Campylobacterales*	35 % of *Bacteroidetes* 51 % of *Firmicutes* 9% of *Proteobacteria* 20 % of *Prevotella* 20 % of *Lachnospiraceae* 10% of *Bacteroides*	56 % of *Bacteroidetes* 35 % of *Firmicutes* 3 % of *Proteobacteria* 37 % of *Prevotella* 7 % of *Lachnospiraceae* 4 % of *Bacteroides*	(Li et al., 2018)
28 and 42 days	Piglets were weaned at 28 day-old and housed litter-wise in controlled conditions	Small intestine Colon Feces	Illumina MiSeq	In the colon and feces, increase of *Firmicutes* and *Lactobacillus* Decrease of *Prevotella* in the colon but increase in the feces Disappearance of *Fusobacterium* in feces In the small intestine changes of unknown *Firmicutes* were observed	In the ileum: 98 % of *Firmicutes* 53% of *Lactobacillus* In the colon: 51 % of *Firmicutes* 38 % of *Bacteroidetes* 15 % of *Prevotella* 7 % of *Lactobacillus*	In the ileum: 99 % of *Firmicutes* 50 % of *Lactobacillus* In the colon: 72 % of *Firmicutes* 21 % of *Bacteroidetes* 8 % of *Prevotella* 27 % of *Lactobacillus*	(Poulsen et al., 2018)

10 and 33 days	392 piglets were weaned at 20 days and housed by groups of 6/7 piglets	All GIT segments	Illumina MiSeq	In all the GIT segments: Reduction of *Lactobacillus* Increased *Clostridia* and *Ruminococcaceae*	ND	ND	(De Rodas et al., 2018)
21 and 28 days	10 piglets were sampled at 21 days just prior to weaning and fed with a typical corn and soybean diet	Feces	Illumina MiSeq	Enrichment of *Prevotella, Lactobacillus* and *Lachnospiraceae* Decrease of *Treponema* and *Ruminococcaceae*	44 % of *Bacteroidetes* 41 % of *Firmicutes* 10 % of *Spirochaetes* 13 % of *Prevotella* 20 % of *Ruminococcaceae*	63 % of *Bacteroidetes* 34 % of *Firmicutes* 0 % of *Spirochaetes* 57 % of *Prevotella* 10 % of *Ruminococcaceae*	(Guevarra et al., 2018)
21 and 28 days	112 piglets were weaned at 21 days using a conventional diet	Feces	Illumina MiSeq	Increase of Family XIII *Clostridiaceae* and *Rickenellaceae* Decrease of *Bacteroidiaceae, and Enterococaceae*	˜ 2.5 % of Family XIII ˜15 % of *Bacteroidiaceae* ˜ 4 % of *Enterococaceae* ˜ 2 % of *Rickenellaceae*	˜ 7 % of Family XIII ˜0 % of *Bacteroidiaceae* ˜ 0 % of *Enterococaceae* ˜ 5 % of *Rickenellaceae*	(Summers et al., 2019)
7 and 27 days	From 10 piglets, 5 were euthanized at day 7 and 5 were weaned at 21 days and sacrificed at 27 days	Proximal jejunum (PJ) Distal jejunum (DJ) Colon (C)	Illumina MiSeq	Increase of alpha diversity Increase of *Bacteroidetes* Decrease of *Proteobacteria* Decrease of *Campylobacter, Helicobacter, Veillonella* Increase of *Clostridium, Streptococcus, Prevotella*	59% of *Firmicutes* 17 % of *Bacteroidetes* 13 % of *Proteobacteria* 43 % of *Prevotella* (PJ) 80 % of *Lactobacillus* (DJ) 43 % of *Lactobacillus* (C)	54% of *Firmicutes* 30 % of *Bacteroidetes* 0.3 % of *Proteobacteria* 38 % of *Prevotella* (C) 55 % of *Lactobacillus* (DJ) 50 % of *Lactobacillus* (PJ)	(Adhikari et al., 2019)

3. The piglet mucosal microbiota

3.1. Mucin-bacteria interactions

Although some microbial species are mainly found in the luminal part of the GIT, the mucus layer constitutes a competitive physical niche colonized by numbers of bacteria like mucin-binding and mucin-degrading bacteria. To our knowledge the specificity of mucin-bacteria interactions in piglets is not yet known, but these interactions have been reviewed recently in the human context for example by (Etienne-Mesmin et al., 2019) or Sicard et al., 2017. The mucus associated microbiota is more and more considered to greatly influence host health (Etienne-Mesmin et al., 2019; Sicard et al., 2017). Bacteria can bind to mucins via mucin-binding proteins, cell surface proteins, blood group binding adhesins, flagella or pili, degrade mucins using lipoproteins, proteases and glycoside hydrolases (figure 11) or modulate mucin composition by bacterial products such as SCFA (Burger-van Paassen et al., 2009); (Etienne-Mesmin et al., 2019; Sicard et al., 2017).

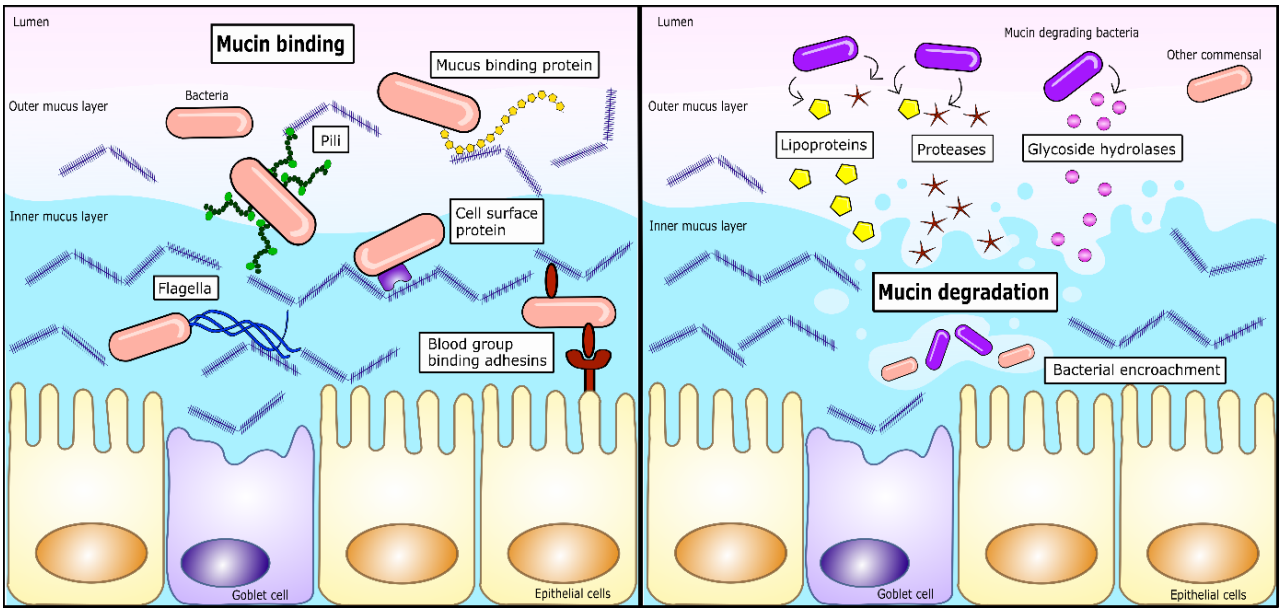


Figure 11. Interactions between mucins and the mucus associated microbiota. (Modified from Etienne-Mesmin et al. 2019; original figure realization: Raphaële Gresse)

The integrity of the mucus layer is of great importance for host health and for limiting access of invading pathogens to the intestinal epithelial cells (Donaldson et al., 2016; Etienne-Mesmin et al., 2019). Consequently, it can be hypothesized that the disruption of mucosa-associated microbiota, also called mucobiota, further impacts piglet health for example by triggering immune activation (Kelly et al., 2017; Metzler-Zebeli et al., 2018).

3.2. Composition of the piglet mucosal microbiota

Despite its significance, the piglet mucobiome just begins to be investigated. Up to date, few studies have investigated the mucobiome of piglets in different GIT segments (Adhikari et al., 2019; Argüello et al., 2018; Kelly et al., 2017; Mu et al., 2017; Schokker et al., 2018; J. Wang et al., 2019; Zwirzitz et al., 2019). During the early suckling period, mucosal microbiota is mainly composed of *Firmicutes* and *Bacteroidetes* with an emphasis of *Proteobacteria* in the small intestine (Adhikari et al., 2019; J. Wang et al., 2019). Mu et al. reported that, 2 weeks after weaning, the small intestinal mucobiota was mainly composed of *Proteobacteria* (~50 %), *Firmicutes* (~30 %) and a small proportion of *Tenericutes* (Mu et al., 2017). Kelly et al. highlighted in 28 day-old newly weaned piglets a very similar composition in the duodenum and jejunum mucosa, which both harboured around 80 % of *Proteobacteria* including around 45 % of *Campylobacteriaceae,* 30 % of *Helicobacteraceae* and 5 % of *Enterobacteriaceae* (Kelly et al., 2017). The major changes between duodenum/jejunum and ileum were an increase in *Enterobacteriaceae* and a decrease in *Campylobacteriaceae* and *Helicobacteraceae* in weaned piglets (Kelly et al., 2017). The mucus-associated colonic microbiota displayed higher relative abundance of *Prevotellaceae* and lower *Enterobacteriaceae* compared to that of the ileum. Finally, results of Kelly et al. showed that, compared to luminal cecum microbiota, mucosal cecum microbiota of weaned piglets harboured lower relative abundance of *Prevotellaceae, Lachnospiraceae, Ruminococcaceae* and *Veillonellaceae* whereas *Helicobacteraceae* and *Campylobacteriaceae* showed a greater abundance. Mucosal microbiota also seems to be shaped and more diversified across time, as mucosa ileal microbiota of 6-month-old pigs harboured, in comparison with that of newly weaned piglets, principally *Firmicutes* members such as *Clostridiaceae, Streptococcaceae, Lactobacillaceae, Lachnospiraceae* and *Veillonellaceae,* few members of *Bifidobacterium* and around 8% of

Proteobacteria (Zwirzitz et al., 2019). To our knowledge, no data have been published on the fungal and archaeal piglet mucosal microbiota.

To conclude on this chapter, the composition, diversity and functionality of the luminal microbiota have consequent impacts on piglet health. Due to its proximity with enterocytes, the mucobiota could further impact host physiology. In weaning piglets, this complex microbial ecosystem is still evolving and influenced by numbers of factors such as diet or age of weaning. Further investigations are needed to precisely understand the impact of microbiota changes at weaning.

Chapter 4. ETEC-mediated Post-Weaning Diarrhea in Swine Industry

1. ETEC infections in swine industry

E. coli is one of the most common pathogenic agents in humans and animals and is responsible for a wide range of intestinal and extra-intestinal diseases (Allocati et al., 2013; Gomes et al., 2016). Diarrheagenic *E. coli* (DEC) pathotypes are classified as enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), and enterotoxigenic *E. coli* (ETEC) depending on their colonization sites, virulence mechanisms, and associated symptoms (Croxen et al., 2013; Gomes et al., 2016). As reported in chapter 1, the ETEC pathotype is the most common in pigs but EPEC or STEC pathotypes can also induce diarrhea in piglets (Mandakini et al., 2015; Rhouma et al., 2017). ETEC can be carried without symptoms in 16.6% of healthy suckling piglets, 66 % of healthy weaned piglets and 17.3 % of healthy finisher pigs (Moredo et al., 2015). ETEC is responsible of colibacillosis occurring mainly in the neonatal period and post-weaning until 8 weeks of age (Rhouma et al., 2017). ETEC prevalence has been estimated to 60 to 80 % of piglets between day 1 and 4 post-weaning (Girard et al., 2018). The economic losses associated with ETEC infections in swine industry are estimated between 40 and 314 € per sow depending on the country and breeding conditions (Rhouma et al., 2017). However, despite the high prevalence of ETEC in piglets, post-weaning diarrhea is a multifactorial disease (Figure 12) with an exact etiology not yet clearly identified and which account for approximately 17% of death of piglets born in Europe (Lallès et al., 2007b).

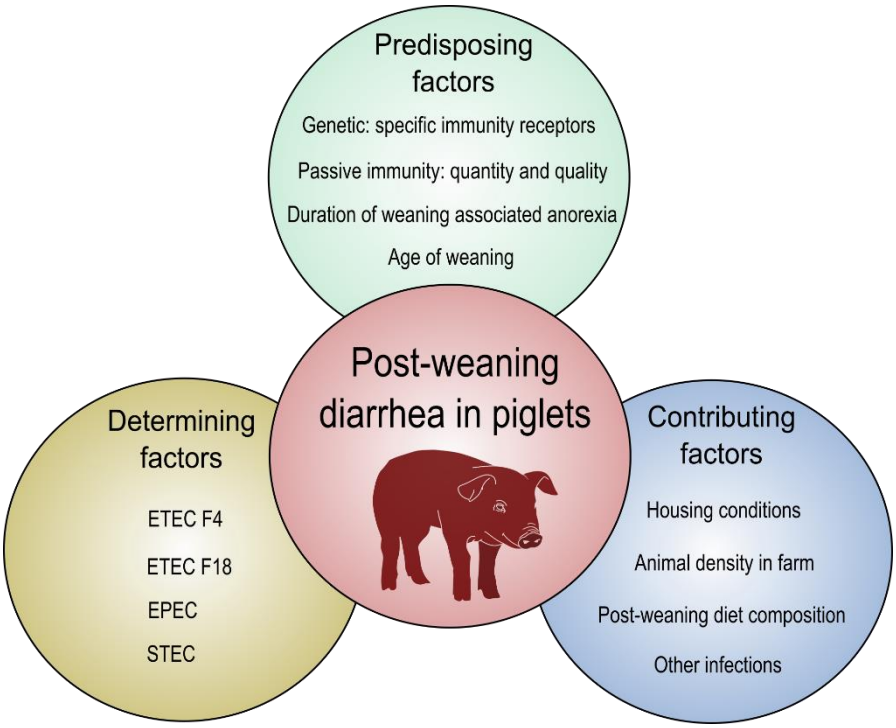


Figure 12. The multifactorial genesis of post-weaning diarrhea in piglets (modified from Rhouma et al., 2017).

2. Pathogenesis of ETEC mediated infections

ETEC include several virotypes characterized by different combinations of toxins and virulence factors (Fairbrother et al., 2005; Luppi, 2017). ETEC enter piglets by initial oral route and, in the presence of favourable conditions, colonize and proliferate in the small intestine by means of virulence factors (Luppi, 2017) which were extensively reviewed by Dubreuil et al. (Dubreuil et al., 2016). Once in the GIT, ETEC strains must access to the epithelium which is, under normal condition, covered by a mucus layer which, as seen in chapter 2, could be impaired after weaning due to disruptions of intestinal functions. Two mucinases, EatA, a serine protease autotransporter of *Enterobacteriaceae*, and YghJ, a mucin-binding metalloprotease highly conserved in ETEC, are able to degrade mucin proteins such as MUC2 and allow ETEC strains to temporary access to piglet enterocytes (Dubreuil et al., 2016). Most ETEC strains responsible for post-weaning diarrhea in piglets possess adhesins, surface proteins called *fimbriae*, predominantly F4 (also called K88) and F18, or non-fimbrial adhesins identified as adhesin involved in diffuse adherence (AIDA) (Dubreuil et al., 2016; Luppi, 2017; Luppi et al., 2016).

The role of the *fimbriae* is to allow the bacteria to adhere to specific receptors located on the brush boarder of piglet enterocytes (figure 13) (Dubreuil et al., 2016; Luppi, 2017). ETEC possessing *fimbriae* F4 colonize preferentially the whole length of jejunum and ileum while ETEC with fimbriae F5, F6, F41 mostly colonize the posterior jejunum and ileum (Fairbrother et al., 2005; Luppi, 2017). Susceptibility of piglets to ETEC virotypes changes with age. Neonatal piglets are mostly infected by F5, F6 and F41 while weaned piglets have increased susceptibility to F4 and F18 due to the presence of age-dependent receptors on piglet epithelial cells (Conway et al., 1990; Luppi, 2017; Luppi et al., 2016). The genetic background of piglets can also determine their susceptibility, for instance Flemish pigs have been diagnosed as highly susceptible to F4 and F18 ETEC strains (Nguyen et al., 2017). ETEC strains produce one or several enterotoxins, including heat stable enterotoxins (STa, STb), heat-labile enterotoxin LT and EAEC heat-stable enterotoxin (EAST1) (Dubreuil et al., 2016; Fairbrother et al., 2005; Gomes et al., 2016; Luppi et al., 2016). STa toxin binds to the extracellular domain of guanylyl cyclase (GC) and expresses its toxicity by stimulation of cyclic guanosine monophosphate (cGMP) production, a cyclic nucleotide, which induces electrolytes and fluid secretion by enterocytes and thus severe watery diarrhea (DUBREUIL, 2017; Dubreuil et al., 2016; Luppi, 2017). STb toxin binds to sulfatide (S), a glycosphingolipid widely distributed on the surface of epithelial cells which leads to assimilation of calcium into enterocytes inducing secretion of water, bicarbonate and electrolytes (DUBREUIL, 2017; Dubreuil et al., 2016; Luppi, 2017). LT delivery to host cells most probably occurs *via* vesicle after binding to its receptor, the glandioside GM1, which activates adenyl cyclase (AC) in enterocytes inducing cyclic adenosine monophosphate (cAMP) and thus, hypersecretion of water and electrolytes such chloride and bicarbonate into the lumen leading to dehydration (DUBREUIL, 2017; Dubreuil et al., 2016; Luppi, 2017). LT also shows to promote colonization of ETEC strains in the intestine of gnotobiotic piglets (Berberov et al., 2004) and the adherence of ETEC on the apical sites of porcine epithelial cells (Johnson et al., 2009). Very little is known about the role of EAST1, carried by many commensal and ETEC strains, in the etiology of diarrhea but, due to its 50% shared similarity with STa, EAST1 toxin is thought to possess the same pathogenicity (Dubreuil et al., 2016; Luppi, 2017). A recent study in ETEC-challenged piglets reported that ETEC could active the production of caspase-9, the extrinsic pathway of apoptosis in the jejunum (Xia et al., 2018). ETEC infections causes no important mucosal destruction or inflammation (DUBREUIL, 2017). However, ETEC secreted toxins and virulence factors disrupting enterocyte functions and facilitating bacterial colonization which induces pro- and anti-inflammatory responses that favour bacterial survival and growth (DUBREUIL, 2017).

The exact mechanism of ETEC-induced apoptosis remains however to be fully investigated. Figure 14 summarizes the pathogenesis of ETEC strains in weaned piglets.

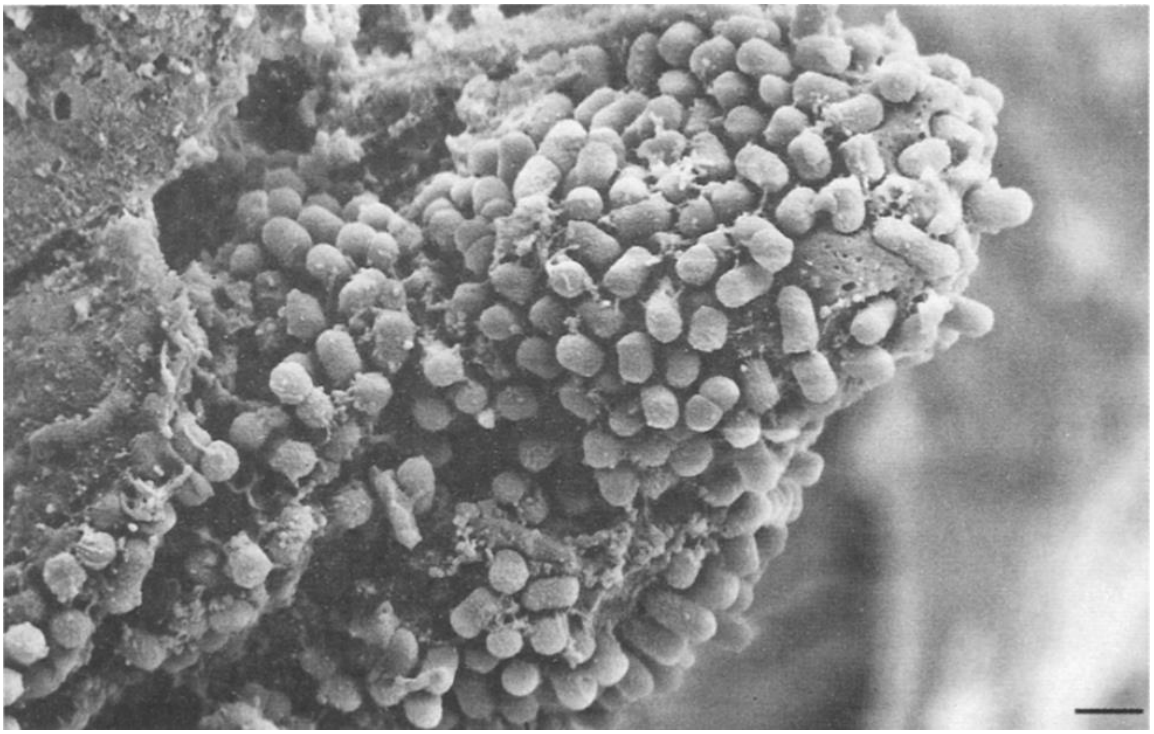


Figure 13. *E. coli* positive to F4 adhering to the apical face of an enterocyte located in the small intestine of weaned 45 days old piglets which died from post-weaning diarrhea (Wada et al., 1996).

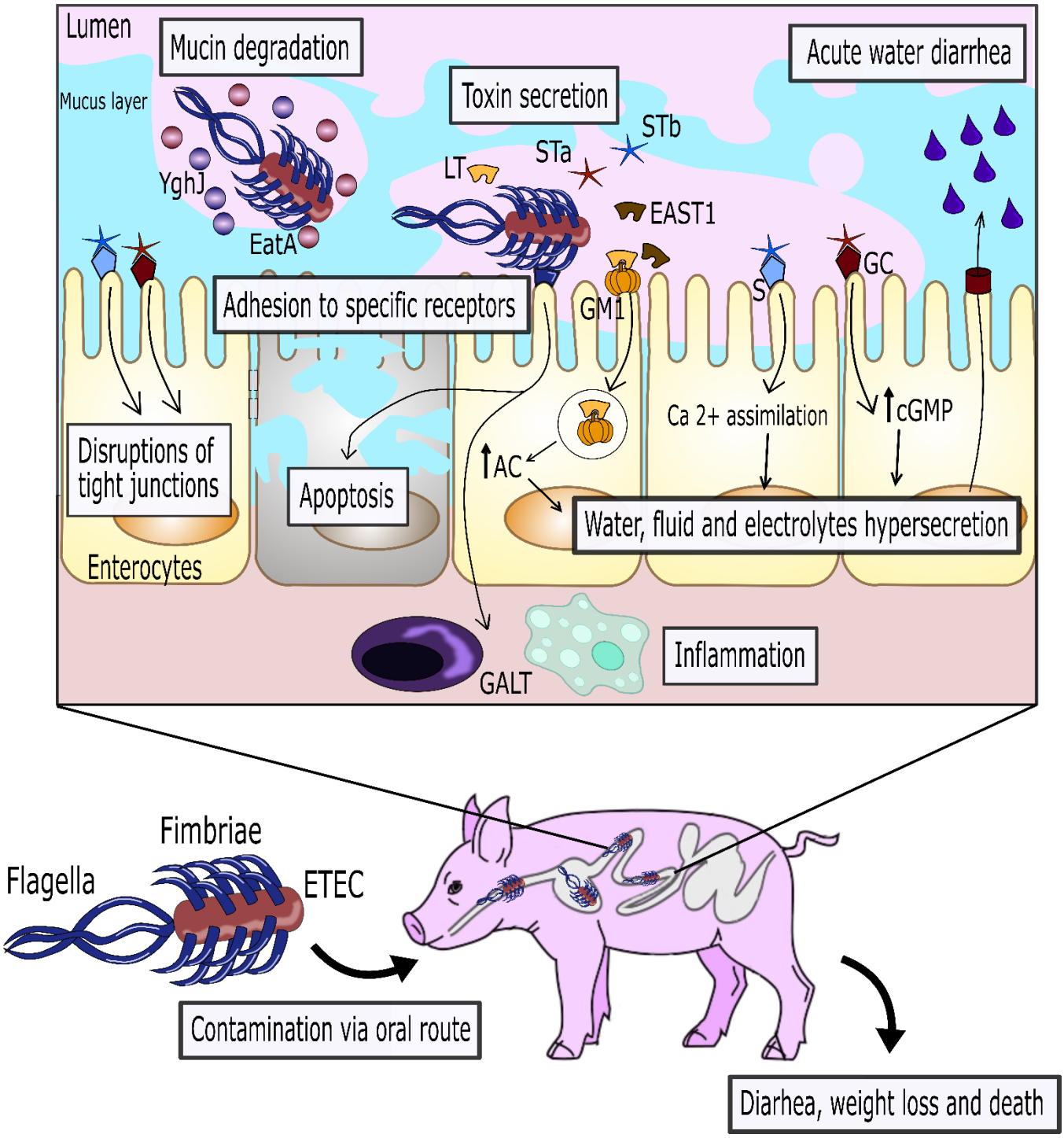


Figure 14. Overview of current knowledge about ETEC colonization and pathogenicity in newly weaned piglets (© Raphaële Gresse)**.**

3. Clinical symptoms and diagnosis of ETEC infections in piglets

The diagnosis of post-weaning diarrhea starts from the observation of clinical signs and gross lesions during necropsy prior to bacteriological investigations in order to determine the bacterial strain pathotype (Luppi, 2017). Post-weaning colibacillosis lasts around one week and is generally characterized by yellowish, grey or pink watery diarrhea with a characteristic smell (Luppi, 2017; Wada et al., 1996). Most of the infected piglets usually suffer from depression, reduced appetite and severe dehydration (DUBREUIL, 2017; Luppi, 2017). If the death of infected piglets is mostly due to severe dehydration, the increase in intestinal permeability due to disruptions of tight junctions during infections could also lead to death by septicaemia or endotemia (Fairbrother et al., 2005). At necropsy, ETEC infected piglets displayed dilated stomachs filled with clotted milk or dried feed and with severe hyperaemia, as well as dilated, oedematous and hyperaemic small intestine, and enlarged and hyperaemic mesenteric lymph nodes (Luppi, 2017; Wada et al., 1996). In piglets, ETEC infections are mostly diagnosed via the euthanasia of untreated diarrheic individuals or spontaneously dead animals which allow to collect samples and send them to a laboratory for pathogen analysis. Polymerase chain reaction (PCR) is used to identify virulence factors and confirm the presence of ETEC strains in intestinal piglet content (Luppi, 2017).

4. Dysbiotic microbiota and post-weaning infections

4.1. Microbiota driven susceptibility to infections

Such as highlighted in chapters 1 and 3, the weaning transition has been associated with a disrupted gut microbiota composition, also called dysbiosis, notably due to dietary changes and weaning stress factors like transient anorexia which could induce a vicious circle involving inflammation and leading to the production of oxygen and nitrate which could favour the growth of *E. coli* strains (figure 1). Studies investigating microbiota composition of diarrheic or ETEC challenged piglets published before 2017 were reported in Gresse *et al*. (chapter 1); the table 6 gives an update by presenting recent findings. Several studies related a decrease in *Proteobacteria, Enterobacteriaceae* or commensal *E. coli* species in the jejunal or fecal microbiota of diarrheic or ETEC challenged post-weaning piglets compared to healthy ones (Bin et al., 2018; Koo et al., 2020; Yang et al., 2019). *Lachnospiraceae, Ruminococcaceae* and

Prevotellaceae were increased while *Fusobacterium* and *Corynebacterium* were decreased in the fecal microbiota of 7 day-old piglets which didn't develop post-weaning diarrhea compared to weaned diarrheic piglets (Dou et al., 2017). Early-life microbiota composition could thus play a role in the susceptibility of piglets to develop post-weaning diarrhea or enteric infections (Dou et al., 2017). However, the process leading to dysbiosis and GI infections remains unclear and results between studies are sometimes inconsistent and highlighted the variation of susceptibility between individuals. Indeed, Yang et al. reported an increase in the relative abundance of *Lachnospiraceae* and *Ruminococcaceae* in the feces of healthy piglets compared to diarrheic piglets, they also highlighted, even in a non-totally consistent manner, that the *Prevotella* genus was higher in the faeces of diarrheic piglets compared to healthy piglets at 1, 3 and 5 weeks of age (Yang et al., 2019). Variability between studies can be due to host genetic factors which can impact the microbiota composition and the susceptibility to ETEC infections (Guevarra et al., 2019). Bian et al. notably highlighted a strong genetic effect on the fecal microbiota of suckling piglets from two pig lines, especially regarding the relative abundance of *Fusobacteriaceae*, *Erysipelotrichaceae* and *Bacteroides* (Bian et al., 2016) which could thus play a role in the future susceptibility to infections. Also, in a recent study, piglets from a susceptible phenotype to F18 ETEC infection harboured higher abundance of haemolytic bacteria and *Enterobacteriaceae* in their feces compared to resistant piglets (Riis Poulsen et al., 2018).

Table 6. Impact of post-weaning diarrhea or ETEC infections during weaning transition on the gut microbiota of piglets (© Raphaële Gresse). (Only studies published after 2017 are reviewed, for literature published before 2017 see Gresse et al, 2017 in chapter 1).

Age	Diet and breeding conditions	Samples	Techniques	Modifications of gut microbiota driven by infection or diarrhea	Composition of gut microbiota		References
					Healthy piglets	Diarrheic or infected piglets	
Piglets suffering from post-weaning diarrhea							
3 to 5 weeks	10 piglets were followed from birth and weaned at 4 weeks of age	Feces	16D rRNA gene sequencing qPCR	Decrease of diversity indices in diarrheic piglets Increased relative abundance (RA) of *Fusobacteriaceae, Prevotellaceae* in diarrheic piglets Increased RA of *Enterobacteriaceae, Ruminococcaceae* and *Lachnospiraceae* in healthy piglets Increased concentration of *Prevotella* and decreased concentration of *E. coli* in pre-diarrheic piglets	Non visible	Non visible	(Yang et al., 2019)
20 to 40 days	85 commercial piglets located in different regions of China early weaned at 21 days were followed from suckling to post-weaning	Feces	Illumina MiSeq	Increase of *Bacteroidiales* order and *Proteobacteria* in diarrheic weaned piglets Increase of *Prevotella* and *Escherichia-Shigella* RA in diarrheic weaned piglets	32% of *Bacteroidetes* 43% of *Firmicutes* 11% of *Proteobacteria*	16% of *Bacteroidetes* 42% of *Firmicutes* 33% of *Proteobacteria*	(Sun et al., 2019)
Piglets challenged with an ETEC strains							
36 days	30 MUC4-resistant piglets weaned at 21 days were orally challenged with 10^{10} CFU/day during 15 days	Colon	16S rRNA sequencing	ND	61% of *Bacteroidetes* 30% of *Firmicutes* 48% of *Prevotellaceae* 14% of *Ruminococcaceae*	53% of *Bacteroidetes* 32% of *Firmicutes* 36% of *Prevotellaceae* 17% of *Ruminococcaceae*	(Zhang et al., 2017)

18 to 23 days	41 piglets of 18 days of age received oral administration of 10^{10} CFU/ml of an ETEC K88 strain. 10 piglets received LB medium as a control	Jejunum	16S pyro-sequencing and rRNA sequencing	In the jejunum: Reduced *Proteobacteria*, *Escherichia coli* and diversity in diarrheic piglets Increased relative abundance of *Lactobacillus, Citrobacter, Klebsiella, Salmonella, Enterobacte,* in diarrheic piglets Reduction of *Bacteroidetes:Firmicutes* in diarrheic piglets	96% of *Proteobacteria* 2% of *Firmicutes* 88% of *Escherichia* 0.6% of *Lactobacillus* 0.3% of *Citrobacter* 0.8% of Klebsiella 1% of *Enterobacter*	81% of *Proteobacteria* 18% of *Firmicutes* 49% of *Escherichia* 10% of *Lactobacillus* 7.1% of *Citrobacter* 6.8% of Klebsiella 6.2% of *Enterobacter*	(Bin et al., 2018)
21 to 37 days	72 male piglets weaned at 21 days received 28 days oral dose of $5.8x10^9$CFU and $6.6x10^{10}$ CFU of ETEC F4+ strain	Colon	Illumina MiSeq	ND	ND	In the colon: 49.6% of *Firmicutes* 36% of *Bacteroidetes* 9.5% of *Proteobacteria* 9.8% of *Prevotellaceae* 16.6% of *Veillonellaceae*	(López-Colom et al., 2019)
21 to 44 days	37 piglets weaned at 21 days challenged with 8 mL of $6x10^9$ CFU/mL of ETEC K88 strain	Feces	qPCR	Reduction of *Bifidobacteria, Enterobacteriaceae* and *Clostridium* cluster IV in challenged piglets	ND	ND	(Koo et al., 2020)

4.2.Use of antibiotics against ETEC infections

As highlighted in chapter 1 in our published review, post-weaning diarrhea and enteric infections are associated with the increase of multi-resistant bacterial strains due to the massive use of antibiotics. To treat post-weaning ETEC infections, the antibiotic doses should rely on the adequate amount which should reach the small intestine, local history of previous treatments and the evaluation of the isolated pathogenic strains (Luppi, 2017). However, most of the cases require quick actions and are driven by local institutions or veterinary often leading to the use of non-adjusted doses. There are different classes of antibiotics used for treating ETEC infections in swine industry: β-lactam antibiotics (amoxicillin and the combination containing amoxicillin/clavulanic acid), cephalosporins (ceftiofur, cefquinome), aminoglucosides (apramycin, neomycin, gentamycin), aminocyclitols (spectinomycin) sulphonamide combined with trimethoprim (such as trimethoprim/sulphametoxazole), fluorochinolones (enrofloxacin, marbofloxacin and danofloxacin), quinolones (flumequine) and polymyxins (colistin sulphate) (Fairbrother et al., 2005; Luppi, 2017). *E. coli* of animal origin have the ability to acquire genes by horizontal transfer promoting the resistance against tetracyclines, phenicols, sulfamides, trimethoprim and fosfomycin conferring them broad spectrum resistance property (Poirel et al., 2018). In diarrheic Korean pigs, isolated *E. coli* displayed resistance to gentamicin (77%), trimethoprim-sulphametoxazole (75.7%), amoxicillin (75.7%), ampicillin (73%) and enrofloxacin (64.9%) (Lee et al., 2009). Colistin is the principal antibiotic used in agriculture and was estimated to reach 16,500 tonnes of demand by 2021 (Rhouma et al., 2017). However, since the increasing concerns about the wide spread of colistin resistance, colistin based treatments were considerably limited since 2013 in order to reduce the prevalence of antimicrobial resistance genes (https://ema.europa.eu). Several years after this limitation, a recent study however reported that in-feed antibiotic administration did not yet affect the abundance or diversity of antimicrobial resistance genes in the feces of sows and young pigs (Pollock et al., 2020). The prevalence of resistance gene was though reported to be different depending of the European country (Munk et al., 2018). Additionally, the effects of antimicrobial exposure on the levels of antimicrobial resistance genes in pigs are complex and different for each individual genes (Birkegård et al., 2017). These recent studies show that reducing antimicrobial resistance genes is a long and complex process which emphasize the need to limit even the use of antibiotics by finding and using non-pharmalogical alternatives.

To conclude, ETEC strains are the principal infectious agents responsible for post-weaning diarrhea which are associated with high mortality rates in piglets. Modifications of the gut microbiota, inflammation and host genetic factors play a role in the development of ETEC infections but their exact etiology remains far from clear. In parallel, antimicrobials represent the current principal strategy for treating ETEC infections in pig farms but the proportion of multi-resistant bacterial strains have considerably increased in the last decades. Regarding this pessimistic context, finding non antibiotic alternatives to maintain piglet health at the critical period of weaning, preserve the use of last-resort antimicrobials in human medicine and preserve public health becomes a real emergency.

Chapter 5. Yeast Probiotics in Piglets

1. Basics of probiotics

Probiotics are defined by the FAO/WHO as viable microorganisms that, when ingested in adequate amount, confer various beneficial effects to their host (Gibson et al., 2017; Hill et al., 2014). According to FAO/WHO guidelines, a probiotic strain needs to be strictly identified until the subspecies level when applicable and referenced with a numeric designation. These beneficial microorganisms can be bacteria or yeasts and should possess some obligate characteristics, such as the ability to survive to the host unfavourable conditions like digestive enzymes or low pH, to deliver their beneficial effects as well as a recommended adhesion to GIT epithelium (de Melo Pereira et al., 2018; Gibson et al., 2017; Hill et al., 2014). A wide variety of probiotic products are currently used and developed worldwide for a broad range of applications. Several recent reviews present the probiotics used in pig production and their proposed mode of action and effects (Barba-Vidal et al., 2017; Elghandour et al., 2020; Liao and Nyachoti, 2017; Ma et al., 2018; Wang and Gänzle, 2019). This chapter will focus on the use of yeasts to restore microbial balance and fight against diarrhoea and ETEC infections in piglets.

2. Yeasts as widely used feed additives and probiotics

2.1. General characteristics

Yeasts are facultative anaerobe unicellular eukaryotic microorganisms displaying round to ovoid shape, measuring between 5 to 10 µm of diameter and harbouring two layer walls and a complex internal cell structure (Salari and Salari, 2017; Shurson, 2018). Along their numerous properties, yeasts demonstrate high ability of growing under acidic pH and are viable up to 55°C (Salari and Salari, 2017; Sen and Mansell, 2020). Yeasts bring vitamins such as B6, thiamine, biotin or riboflavin, magnesium and zinc which make them nutritionally attractive for animals but also humans (Elghandour et al., 2020). Yeasts are additionally widely used worldwide for the production of fermented food and beverages and as medical product to treat

human gastrointestinal disorders, so they are generally considered as safe for humans and animals (MacKenzie et al., 2008; Moré and Swidsinski, 2015; Nash et al., 2017; Sen and Mansell, 2020).

2.2. Yeasts in swine industry

In livestock production, active dry yeasts, yeast derivatives or yeast fermented products have been used as feed additives in order to improve productivity, digestion, feed intake and animal health for more than 100 years (Elghandour et al., 2020; Shurson, 2018). The most common yeasts used in livestock production are *Saccharomyces cerevisiae* (SC) and *Saccharomyces cerevisiae* var. *boulardii* (SB) which differentiate to each other by various taxonomic, metabolic and genetic characteristics (Elghandour et al., 2020; Sen and Mansell, 2020). In animal feed, yeasts are generally delivered as active dry yeast containing about 95% of dry matter and 15 to 25 billion of live yeast cells per gram (Shurson, 2018; Stone, 2006). In swine industry, active dry yeasts are particularly used in gestating sows with the aim to improve diet digestibility, limit constipation and decrease stress, in lactating sows to improve colostrum and milk quality and quantity and increase litter size and piglet weight, and in fattening pigs to improve feed efficiency and meat quality (Chaucheyras-Durand and Durand, 2010). In young pigs, yeasts are also recommended to modulate the hindgut microbial populations and reduce diarrhea incidence (Chaucheyras-Durand and Durand, 2010).

3. Effects and potential mechanisms of yeasts against ETEC infections in piglets

One of the principal means of microbiota modulation by yeasts in piglets is to preserve or restore the balance between microbial communities prior to or following the critical period of weaning. Several recent studies investigated the potential beneficial effects of yeast on piglet microbiota (table 7). On healthy piglets, while some results are contradictory, the most reported effect was an increase in beneficial populations such as *Clostridiaceae*, *Ruminococcaceae* and *Actinobacteria* in the large intestine or fecal microbiota of post-weaning piglets (Brousseau et al., 2015; Kiros et al., 2019, 2018; Urubschurov et al., 2017). The modulation of beneficial commensals could strengthen the balance of intestinal microbiota which, by optimizing the occupation of ecological niches, reduce the colonization by intestinal pathogens (Gresse et al., 2017a). The mannan-oligosaccharides (MOS) located on the outer wall of yeasts displayed

some interesting characteristics as their administration to piglets showed to improve feed efficiency, weight gain, growth performance, enhance the functionality of macrophages, increase the number of lymphocytes in ileal Peyer's patches and increase resistance to colonization by Gram negative bacteria (Adewole et al., 2015; Davis et al., 2002; Kogan and Kocher, 2007; LeMieux et al., 2003; Pretus et al., 1991; Valpotić et al., 2016; Williams et al., 1996). In piglets, as previously described, the main challenge of the weaning period is the high prevalence and mortality from ETEC infections and post-weaning diarrhea. Several studies highlighted a reduction of ETEC or *Escherichia coli* shedding in feces of piglets which received daily an active dry yeast before and during an ETEC challenge, thus reducing the severity, the incidence and the duration or diarrheic episodes (Che et al., 2017; Trckova et al., 2014; Trevisi et al., 2017). Interestingly in younger piglets, a single dose of SB CNCM I-1079 could reduce the occurrence of neonatal diarrhea (Hancox et al., 2015). In addition to their effects on intestinal microbiota, SC was shown to stimulate host jejunal cells by increasing the number of mitotic cells and reducing apoptosis in weaned piglet challenged by ETEC F4 (Trevisi et al., 2017). SB CNCM I-1079 could reduce pathogen translocation to the lamina propria via the stimulation of the lymphocytes population and IgA secretion in weaned piglets challenged with ETEC F4 (Lessard et al., 2009).

Table 7. Effects of live yeasts on the microbiota of healthy or ETEC-challenged weaning piglets (© Raphaële Gresse).

Age of piglets when sampling	Diet and breeding conditions	Digestive samples	Techniques	Dose and strain	Modification of intestinal microbiota or colonization by pathogens	References
Healthy piglets						
10 to 37 days	Piglets receiving form birth probiotic treatment diluted in 2 mL of peptone. At weaning, continued to receive probiotic in a basal diet	Ileum Colon Feces	T-RFLP profiling	*Saccharomyces cerevisiae* var. *boulardii* Strain SB-CNCM I-1079 $2x10^9$ CFU/kg of feed	Increase in *Porphyromonadaceae* and *Ruminococcus* in the colon	(Brousseau et al., 2015)
32 to 60 days	48 piglets were at 27-28 days were assigned per group of 12 and received oral gavage of 5 mL of yeast 1, 2 or 3 times or sterile PBS during the time of experiment	Feces	454 pyro-sequencing	*Kazachstania slooffiae* (KS) $5.5x10^8$ CFU/5mL	Increase of alpha diversity indices, unclassified *Christensenellaceae* and *Ruminococcaceae* in piglets which received 2 or 3 doses of KS Increase of *Prevotella*, *Dialister* and *Acidaminococcus* in piglets which received 1 dose of KS	(Urubschurov et al., 2017)
1 to 32 days	A total 24 piglets from 4 sows received live yeast by oral gavage or sterile water since birth. After weaning at 27 days, piglets received a basal diet containing or not active dry yeast	Cecum Colon Feces	Illumina MiSeq	*Saccharomyces cerevisiae* CNCM I-4407 $2.5x10^{10}$ CFU/kg for oral gavage and 10^7 CFU/g of pelleted feed	Development of more homogenous microbial communities Increase of *Actinobacteria*, *Coriobacteriaceae*, *Ruminococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Peptococcaceae* Decrease of *Lactobacillus* and *Prevotella*	(Kiros et al., 2018)
21 to 70 days	360 piglets weaned at 21 days were fed with diet containing 3% of probiotic YL or SC	Jejunum Feces	Conventional culture	*Yarrowia lipolytica (YL)* *Saccharomyces cerevisiae (SC)* both at 3% of the diet (w/w)	No effects of yeasts on coliform and *Escherichia coli* numbers	(Czech et al., 2018)

28 days	32 piglets were treated since birth with a daily low or high dose of live yeast or water by oral gavage until weaning at 28 days of age	Cecum	454 pyro-sequencing	*Saccharomyces cerevisiae* CNCM I-4407 Low dose (LD): $5x10^9$ CFU per day per animal High dose (HD): $2.5x10^{10}$ CFU per day per animal	Decrease alpha diversity indices for both doses Higher proportions of *Actinobacteria, Collinsella* (LD) Decreased proportions of *Dorea, Prevotella, Unclassified Lachnospiraceae, Acidaminococcus and Unclassified Peptostreptococcaceae* (LD) and *Veillonella* (HD)	(Kiros et al., 2019)
Piglets challenged with an ETEC strain						
28 to 42 days	Live yeast were fed or not to sows from day 94 of gestation in their basal diet their piglets (n= 34) orally received yeast 3 times a week or not during the suckling period until weaning at 28 days, weaned piglets received basal diet containing yeast or not for 2 weeks. All piglets were orally challenged with $1.5x10^{11}$ CFU of ETEC K88 at day 30	Feces	Conventional culture	*Saccharomyces cerevisiae* CNCM I-4407 10^{10} CFU/kg of diet for sows and weaned piglets, and 10^{10} CFU/supplementation for suckling piglets	Lower shedding of ETEC from day 30 to day 36 Reduction of the number of days of fecal shedding of ETEC	(Trckova et al., 2014)
4 to 7 weeks	50 piglets weaned at 24 days challenged with $1.5x10^8$ CFU of ETEC F4	Feces	Conventional culture	*Saccharomyces cerevisiae* CNCM I-4407 $5x10^{10}$ CFU/kg	Reduction of ETEC excretion	(Trevisi et al., 2017)
29 days	36 piglets weaned at 21 days of age were fed with basal diet supplemented or not with live yeast and 36 piglets received 10^{11} CFU of ETEC K88 at 29 days	Colon	Conventional culture	*Saccharomyces cerevisiae* CNCM I-4407 10^{10} CFU/kg of diet	Increase in *Lactobacillus* counts Decrease in *Escherichia coli*	(Che et al., 2017)

4. Effects of yeasts on porcine intestinal epithelial cell lines

IPEC-J2, IPEC-1 and IPI-2I cell lines are the most used porcine intestinal cell lines to study interactions between epithelial cells, pathogens and the effects of probiotics or probiotic derived products. IPEC-J2 cells were isolated from neonatal piglet mid-jejunum while IPEC-1 were isolated from a mixture of ileum and jejunum from a neonatal piglet and IPI-2I were transformed with an SV-40 plasmid from the ileum of an adult boar (Brosnahan and Brown, 2012; Gonzalez-Vallina et al., 1996; Kaeffer et al., 1993). These three cell lines possess some functional differences such as differences in the level of some cytokines expression or the capacity to adhere specifically with some ETEC strains (Brosnahan and Brown, 2012).
Some *in vitro* studies performed on porcine intestinal cell lines reported interesting properties of *Saccharomyces* yeast in reducing the effects of ETEC infections. Although yeasts are thought to be unable to adhere to GIT epithelium, Kühle et al. highlighted that the SB CNCM I-1079 strain displayed 28% of adhesion to the porcine jejunal epithelial cell line IPEC-J2 (van der Aa Kühle et al., 2005). This property could help to reduce the virulence of ETEC strains by masking adherence sites as ETEC need first to adhere to enterocytes prior to secrete toxins (Luppi, 2017). Additionally, several studies reported that some SC and SB strains seem to possess immunomodulation properties by decreasing the gene expression of pro-inflammatory cytokines and chemokines such as TNFα, IL1α, IL8, CCL2 or CXCL2 in the porcine cell lines IPEC-J2 or IPI 2I during an ETEC F4 challenge (Badia et al., 2012b; van der Aa Kühle et al., 2005; Zanello et al., 2011). Co-incubation of ETEC F4 challenged IPEC-J2 cells and MOS was able to reduce ETEC adhesion and downregulate innate inflammatory response via the reduction of TNFα and several immunoglobulins gene expression (Hermes et al., 2011).

5. Additional modes of action

Other mechanisms of actions of yeasts against enteric pathogens have been reported from studies in other animal species or with other pathogens. For example, SB showed to secrete a large protease which inhibits the secretion of chloride induced by cAMP during LT toxins from *E. coli* pathogenesis in human intestinal cell lines (Czerucka et al., 1994; Czerucka and Rampal, 1999). Additionally, *SB* CNCM I-745 possesses a specific phosphatase that is able to dephosphorylate lipopolysaccharides (LPS), a molecule present on the surface of Gram-

negative bacteria such as *E. coli* and thus reduces LPS-induced inflammation in rat epithelial cells (Buts et al., 2006). Roussel *et al.* recently highlighted that a SC CNCM I-3856 strain could agglutinate with a human ETEC strain and reduce LT toxin expression during co-cultures (Roussel et al., 2018b). In other intestinal pathogens, *SB* CNCM I-745 demonstrated its ability to bind to cholera toxins of *Vibrio cholerae* and thus can reduce its action on enterocytes by avoiding binding of the toxin on its receptor (Czerucka and Rampal, 2019). Finally, a serine protease contained in *SB* CNCM I-745 culture supernatant can inhibit *Clostridium difficile* pathogenesis by both degrading the toxins and its receptor (Castagliuolo et al., 1996). Considering the previously reported *in vitro* and *in vivo* results, yeasts could indirectly or directly fight against intestinal pathogens, including ETEC strains, and could display various modes of action to reduce the pathogenesis of ETEC in piglets (figure 15). The other important point highlighted by those studies is that yeasts seem to show strain dependent effects.

To conclude, yeasts seem to display beneficial effects on piglet microbiota and intestinal epithelial cells which could help reducing the disruptions of microbiota observed at weaning and the pathogenicity of ETEC strains. However, if some mechanisms of action are already suggested, further investigations are needed to fully understand the complex yeast-ETEC-microbiota-host interactions.

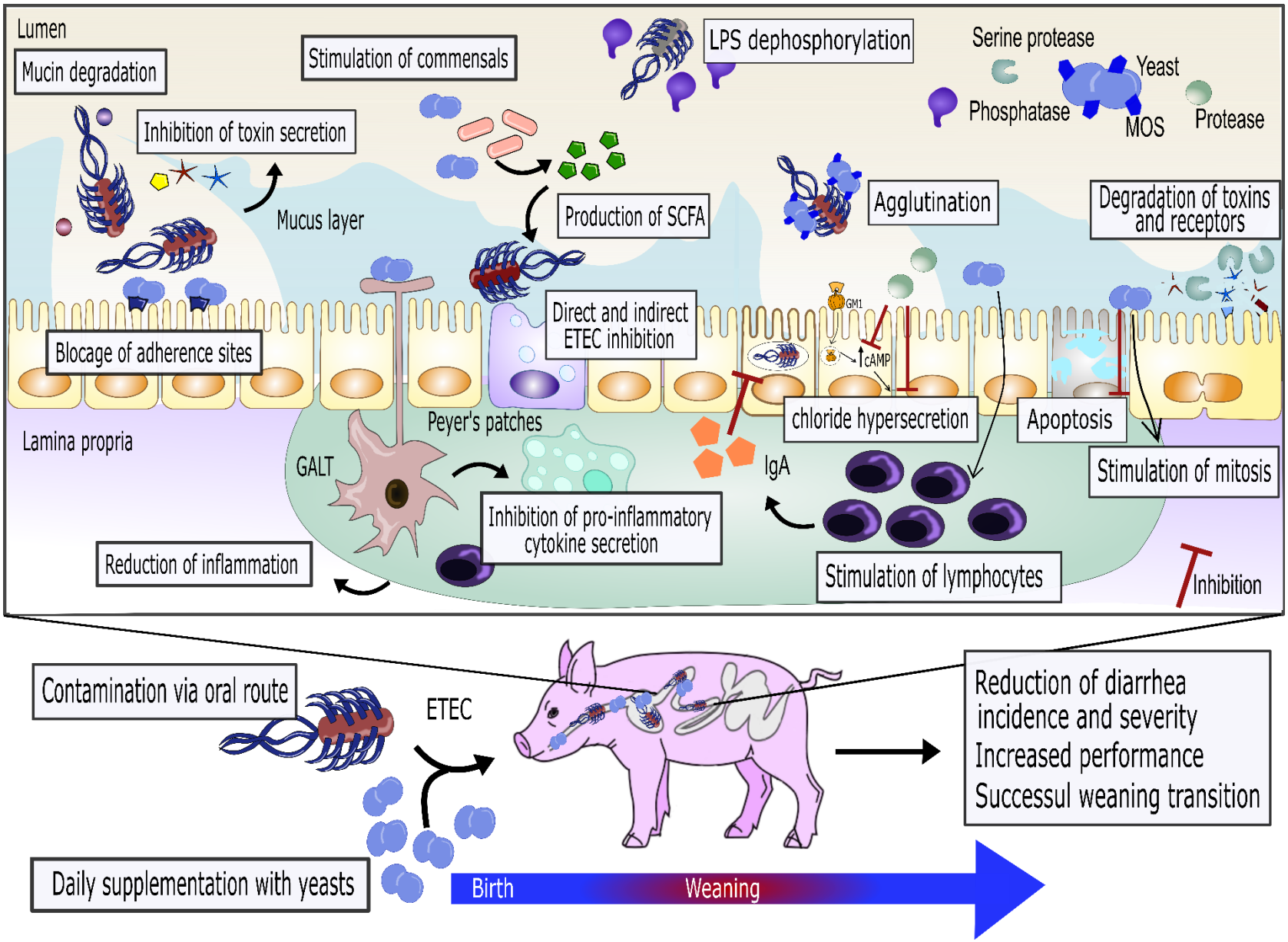


Figure 15. Possible mechanisms of actions of yeasts to fight against post-weaning ETEC infections in piglets (© Raphaële Gresse)

Chapter 6. *In Vitro* Simulation of the Pig Gut Ecosystem

Several *in vitro* models have been designed in the last decades, simulating the upper or lower parts of human or animal GIT and sometimes both. The great majority of *in vitro* models belong to batch, semi-continuous or continuous *in vitro* systems. Semi-continuous and continuous gut simulators, which allow long term experiments by providing nutrients and removal of fermentation product, have been adapted to various hosts, generally to humans. As seen in chapter 1, fermentation systems reproducing the colonic environment of pigs by cultivating fecal microbiota under controlled environmental conditions appear to be an effective solution to reduce the number of animal experiments (Gresse et al., 2017). In this chapter the focus will exclusively remain on semi-continuous or continuous *in vitro* model of the pig colon.

1. *In vitro* models of the adult pig colon

Cositec was the first semi-continuous *in vitro* model adapted to the pig colon (Pinloche et al., 2012). The Cositec model was however started from digestive content of the cecum and colon of finisher pigs instead of using fecal samples to remain non-invasive to the animals (Pinloche et al., 2012). The TNO Gastro-Intestinal model 2 (TIM-2), a dynamic multi-compartmental system simulating originally the human ascendant, transverse and descendant colon, was inoculated with pig feces by Martinez *et al.* (2013). Unlike ARCOL, this system is continuously flushed with nitrogen to ensure anaerobiosis. Despite the fact that pig colon doesn't possess the same anatomy than human colon, it should be underlined that the unique adaptation of the pig version of TIM-2 was the setup of a temperature at 39°C (Martinez et al., 2013). Finally, the *in vitro* model PolyFermS was used to simulate the colonic environment of pigs using several successive bioreactors and flushing with carbon dioxide to ensure anaerobiosis (Tanner et al., 2014a). The nutritive medium of this model was accurately set up to mimic the ileal chyme of pigs. The distinct features of this dynamic model is the use of an immobilized microbiota in a

polymer solution formed of gellan gum, xanthan and sodium citrate. This particularity allow a very good stability of the *in vitro* microbiota and thus long term experiments of 54 days (Tanner et al., 2014a).

2. *In vitro* simulation of the piglet gut

2.1. PigutIVM: *in vitro* model of the fattening piglet colon

The PigutIVM was previously adapted from the ARCOL to simulate the digestive environment of the colon of 8 week piglets (Fleury et al., 2017). In this *in vitro* model, several adaptations were made to stick with the gut environment such as a temperature set up to 39°C, a pH of 6.2 and a nutritive medium mimicking the ileal chyme of weaner piglets. The particular feature of this continuous model was the anaerobiosis maintained with the sole activity of the microbiota allowing measurement of gas composition and redox potential which would thus be solely due to microbiota activity (Fleury et al., 2017).

2.2. baby-SPIME: simulation of abrupt dietary change

Recently, the SHIME for Simulator of the Human Intestinal Microbial Ecosystem (Molly et al., 1993), was adapted to a baby-SPIME (baby Simulator of Pig Intestinal Ecosystem) (Dufourny et al., 2019). As shown in figure 16, the baby-SPIME attempts to simulate the ileum and proximal colon environments of piglets during the transition from a diet made exclusively of milk to a solid based diet in a semi-continuous conformation (Dufourny et al., 2019).

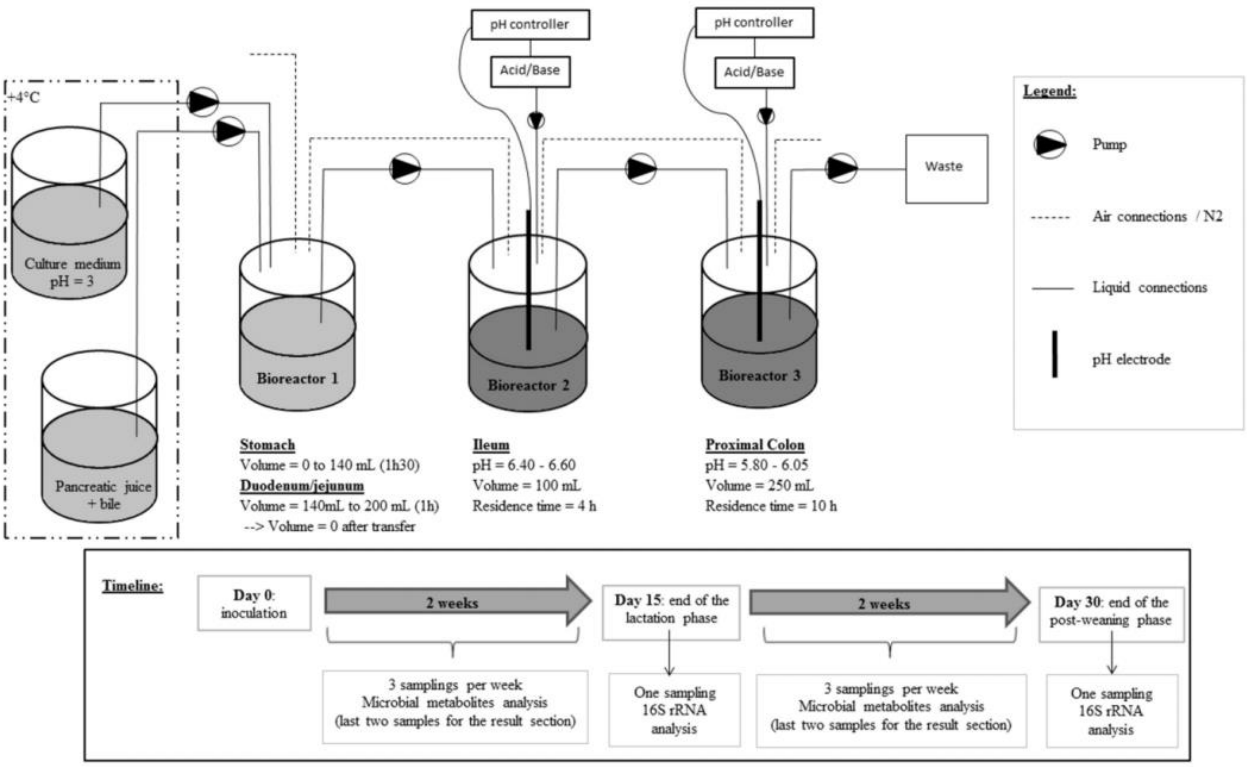


Figure 16. Schematic representation of the baby-SPIME model from Dufourny *et al.* 2019.

Briefly, the bioreactor 1, simulating the stomach, duodenum and jejunum received three times a day a culture media made of a commercial milk replacer for piglets principally composed of whey powder, vegetable oils and wheat flour as well as a medium simulating pig pancreatic juice and bile salts (Dufourny et al., 2019). These secretions have not been adapted to the piglet weaning conditions. The bioreactor 2 and 3 simulating respectively the ileum and proximal colon were inoculated simultaneously with frozen fecal samples from 4 suckling piglets of 27 day old and connected with the bioreactor 1 (Dufourny et al., 2019). After 15 days of fermentation, the culture media made of milk replacer was changed for a commercial post-weaning diet, though non-pre-digested. During the 4 fermentation runs, the pH and temperature were controlled and all the bioreactors were flushed once a day with nitrogen to maintain anaerobiosis (Dufourny et al., 2019). The microbiota of the colonic bioreactor 3 was overall composed of the genera *Mitsuokella, Prevotella, Bifidobacterium, Fusobacterium, Megasphaera* and *Bacteroides.* The principal effect of diet change in the baby-SPIME was a decrease of *Bacteroides* and an increase of *Prevotella.* The ileum bioreactor 2 actually acted like a pre-colon and failed to reproduce the ileal microbiota of piglets (Dufourny et al., 2019). The baby-SPIME was however not fully adapted to weaning conditions as important parameters such as pH, non-pre-digested diet, retention time and microbiota composition were not

compared with *in vivo* colonic data. Additionally, despite its complexity, the baby-SPIME however only aimed to simulate the luminal microbiota although several bacterial species colonize the gut dependently of their adhesion to mucus.

3. Possible optimisation of *in vitro* piglet gut models

3.1. With a mucin gel surface

Mucins are frequently added to nutritive media of *in vitro* models to provide specific sources of nutrients but the inclusion of mucin gel like structure to allow adhesion of a mucus-associated microbiota is less frequent and to our knowledge only used for simulation of human digestive environment (Gibson et al., 1988; null Macfarlane et al., 1998; Macfarlane et al., 2005; Van den Abbeele et al., 2013). The first inclusion of mucins as an attachment surface was under the form of mucin beads, from porcine gastric mucin and sodium alginate, packed into dialysis tubing inserted in the vessel of a dynamic model of the human colon (Probert and Gibson, 2004). These mucins beads were associated with a high level of *Escherichia coli* and overall very low level of obligate anaerobes compared to the fermentation medium (Probert and Gibson, 2004). Using the same dynamic human *in vitro* model, MacFarlane *et al.* (2005), included tubes containing a gel formed of porcine gastric mucin and agar replaced every 48 hours in a multi-compartment dynamic model of the human colon. This system was rapidly colonized by members of *Bacteroides, Enterobacteriaceae* and facultative anaerobes (Macfarlane et al., 2005).

Van den Addeele *et al.* (2013) developed an innovative system, the Mucosal-SHIME (M-SHIME), composed of plastic microcosms covered by porcine gastric mucins which were introduced into the SHIME bioreactors containing human colonic microbiota (figure 17). In several studies, the mucin-covered microcosms for the human proximal colon reactors showed to be particularly colonized by *Firmicutes* such as *Clostridia, Veillonellaceae, Ruminococcus, Roseburia* members or *Lactobacillus* species, while the distal colon microcosms were particularly colonized by *Pyramidobacter, Eubacterium, Enterobacteriaceae* and *Desulfovibrio* (De Paepe et al., 2018; Van den Abbeele et al., 2013, 2012). However, for the previously cited *in vitro* models, a constant flushing with nitrogen to maintain anaerobic

condition were mandatory in order to replace mucin gel surfaces continuously degraded and thus which need to be replaced every 48 hours while maintaining the anaerobic conditions.

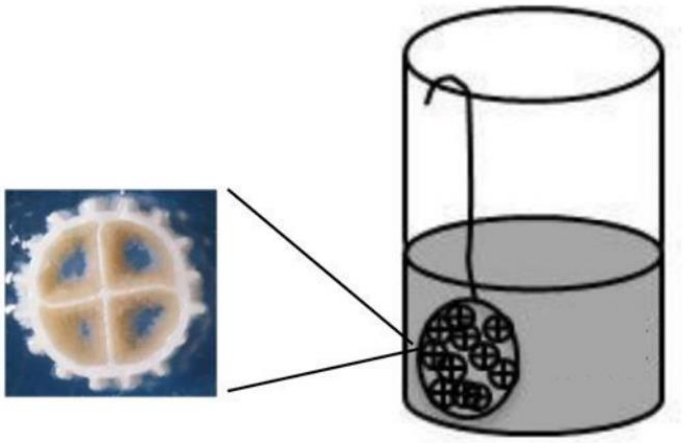


Figure 17. Mucin-covered plastic microcosms from the M-SHIME (Van den Abbeele et al., 2013)**.**

3.2. Coupling *in vitro* models with cell culture

Host-microbiota interactions can be studies using effluents from *in vitro* colon model coupled with *in vitro* cell culture of epithelial intestinal cell lines by investigating inflammatory responses, transepithelial resistance or expression of gene of interest (Bahrami et al., 2011; Defois et al., 2018; Marzorati et al., 2014). To our knowledge, effluents of *in vitro* model of the pig colon were never coupled with porcine epithelial cells but some studies did use this system for human investigations. For example, the effect of a probiotic supplementation on bacterial adhesion and cytokine expression was assessed by plating re-suspended pellet from effluents of a multi-stage *in vitro* model of the human colon on the human caco-2 cell line (Bahrami et al., 2011). Recently, supernatants from human colonic fermentors exposed to persistent organic pollutants were used to investigate cytokine production of the T7 human cell line (Defois et al., 2018).

Marzorati *et al.* (2014) also developed a more sophisticated system, the HMI device that can be combined with the SHIME model, to investigate host microbiota interactions (figure 18). The

HMI module contains both intestinal cells and a mucus layer separated by a semi permeable membrane allowing oxygen flow and the bi-directional transfer of bacterial fermentation metabolites or the withdraw of host defence molecules (Marzorati et al., 2014). The HMI module was used to study the *in vivo* functionality of adhering microbiota of the human GIT during the 48 hours of co-culture with enterocytes and the effects of a dried modified SC fermentation product (Marzorati et al., 2014).

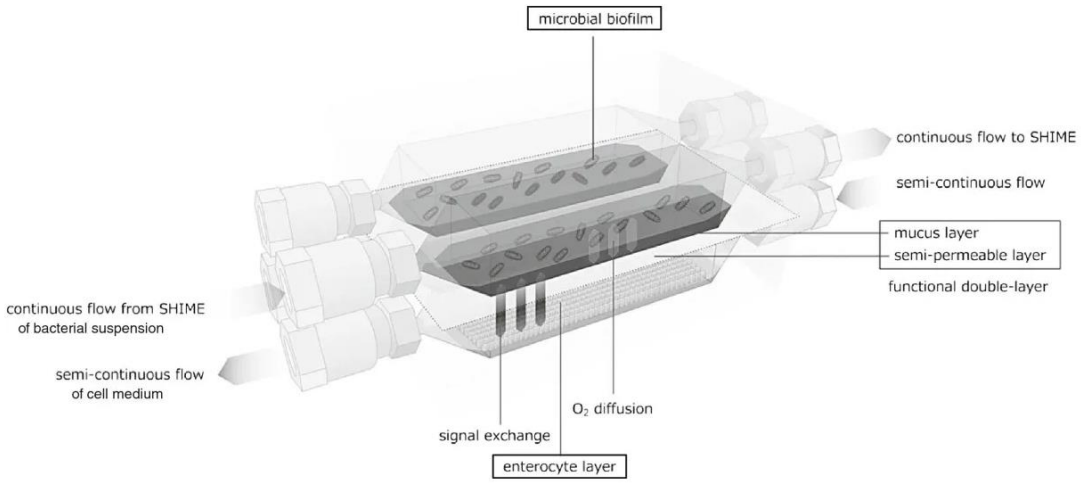


Figure 18. Organization of the HMI module (Marzorati et al., 2014)**.**

3. Pros and cons of *in vitro* gut models

In addition to the EU directive aiming to reduce, replace and refine the use of animals in research, the great majority of *in vitro* simulators of the digestive system possess the advantages to be used with standardized and reproducible procedures and globally cost less than an *in vivo* approach. Also, *in vitro* alternative assays require less time to be performed notably due to the absence of specific regulation or approval by an ethic committee. *In vitro* models are in that sense especially useful to investigate the effects of pathogens, toxic compounds, approved probiotics, pollutants or radioactive compounds on the microbiota where ethical approvals are further questioned. From a scientific point of view, the use of multicompartmental *in vitro* models allow the realisation of kinetics in a simulated GIT which are difficult to carry out in

animals due to the difficulty to follow the same individual over time, except by using cannulated animals, and the limited access to different digestive compartments.

However, the principal drawback of such techniques is the absence of absorptions, regulations, secretions or feedback from the host which are also actively shaping microbiota composition and activity. Generally, the mucus layer is not reproduced in *in vitro* models such as the microbiota of the upper part of digestive tract. Finally, when setting up *in vitro* models of specific species or under specific conditions, some important data can be missing in the literature such as normal redox potential, pH, characteristics of bile and digestive secretions, retentions (etc.) which lead to inaccurate estimations.

To conclude, the simulation of piglet colonic environment using *in vitro* models have a great potential to investigate the effects of components or diverse conditions on the gut microbiota by allowing the access to an *in vitro* colonic microbial communities over time. Several optimisation were recently carried out to mimic the mucobiota or investigate host-microbe interaction however not of piglet *in vitro* models. Existing models of the weaning piglets remains however incomplete by lacking of mucus layer, self-maintained anaerobiosis or adaptation of physiological parameters.

Chapter 7. Objectives and Outlines of the PhD

This literature review summarizes the up to date knowledge about the etiology of post-weaning diarrhea caused by ETEC infections in weaning piglets. Gut intestinal dysbiosis at weaning due to the encountered stressors such as, for instance dietary shift or transient anorexia, is thought to play a role in the etiology of post-weaning diarrhea and needs to be further investigated. Additionally, efficient alternatives to antimicrobial treatments in piglets urgently need to be found to counter the worrisome increasing resistance of bacteria to antimicrobials which are threatening animal and human health worldwide. In parallel, considering the EU directive aiming to reduce, refine and replace *in vivo* experiments by *in vitro* alternatives, the simulation of the piglet colonic environment including gut microbiota using bioreactors appears to be an ideal solution to conduct research on this problematic. Recently, optimisations of *in vitro* gut models were designed in order to, for example, simulate the mucus-adherent microbiota which is thought to play a consequent role in animal health. However, the current designed *in vitro* models of the piglet colon do not provide both a mucus surface, a self-maintained anaerobiosis and completely adapted parameters, which are important features to more closely mimic gut environment.

Therefore, the present PhD project aimed to:

- **Provide information about the GIT microbiota of commercial piglets to apprehend the most abundant microbial communities in fecal samples, luminal colonic digesta or mucosa, microbial activity and physico-chemical parameters such as pH that our *in vitro* model should be reproduced.**

- **Design and set up an *in vitro* model of the piglet colon containing mucin beads in a distinct compartment and to use this model to study the effect of feed deprivation and a preventive probiotic treatment on the composition and activity of the MPigut-IVM.**

- **Study the impact of the presence of an ETEC strain isolated from diarrheic piglets on the MPigut-IVM microbiota which were, such as during weaning transition, subjected to feed deprivation and dietary changes.**

- **Evaluate the potential protective effects of the active dry *Saccharomyces cerevisiae* (SB) CNCM I-1079 commercialised by Lallemand SAS in swine industry, on the MPigut-IVM microbiota which was subjected to feed deprivation, dietary changes and the presence of a pathogen.**

The following chapters corresponding to the above listed research questions will present the results of each of the experimental work presented in Figure 19.

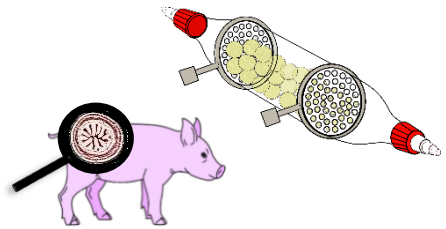
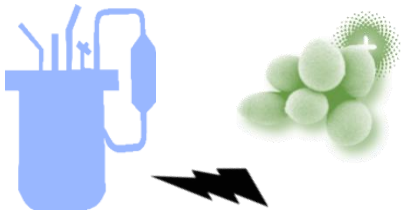
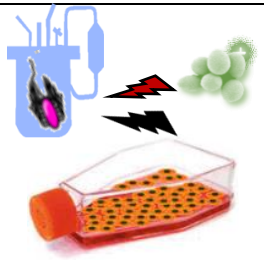
Dates & locations	01/2017	11/2017	03/2019	09/2019	11/2019
					
Not displayed in this manuscript	Set up of the mucin beads and their characteristics (colonization, degradation…)	Technical set up of the mucin bead module. Effects of SB CNCM I-1079 as a curative treatment after feed deprivation			
Chapter 1	Microbiota composition and functional profiling throughout the GIT of commercial weaning piglets				
Chapter 2		Development of the mucin containing piglet in vitro model (MPigut-IVM), effects of feed deprivation and SB CNCM I-1079 preventive daily dose			
Chapter 3			Impact of the presence of an ETEC F4 strain on the microbial communities of the MPigut-IVM and on porcine epithelial cells.		
Chapter 4			Effect of SB CNCM I-1079 and of the MPigut-IVM challenged by enterotoxigenic Escherichia coli (ETEC) on porcine cell lines		

Figure 19. Stage of the experimental work conducted during this PhD. The steps involving culture of intestinal porcine cells were conducted in the Department of Animal genetics at the University of Cordoba.

Experimental work

Chapter 8. Microbiota Composition and Functional Profiling Throughout the Gastrointestinal Tract of Commercial Weaning Piglets

An *in vivo* study investigating the gut microbiota of commercial piglets was needed in order to apprehend the most abundant microbial communities in fecal samples, luminal colonic digesta or mucosa, microbial activity and physico-chemical parameters such as pH that our colonic *in vitro* model should reproduce. In order to be fully consistent with the development of the *in vitro* model, this *in vivo* study was carried out in the commercial farm (SCEA Porc aubennes, Paulhaguet, Haute Loire, France) where the fecal inocula were collected for all our *in vitro* experiments. To complete the few existing literature at the beginning of this PhD project, the microbiota along the entire digestive tract of piglets was analysed by Illumina sequencing, quantitative PCR and functional predictions to get insight of potential functions associated to microbiota in each gut segment. Additionally, as very few data are available on the archaeal microbiota of pig and their distribution throughout the GIT remains unknown, both bacterial and archaeal microbiota were targeted in this study. Proportions and quantities of the main SCFAs were also evaluated at the whole GIT level to get information about fermentation activity. This study was performed on six male piglets of 28 days slaughtered on the weaning day to remain exactly in the same conditions of fecal collection used to inoculate the *in vitro* model.

The graphical abstract (Figure II) presented below summarizes the principal findings of this *in vivo* study. First, an important distinction in terms of bacterial and archaeal composition and activity was observed between small and large intestine segments. Indeed, the small intestine of piglet was free from archaeal community and contained a low concentration of SCFAs, quite weak bacterial diversity but some variability among piglets and functional predictions mainly focused on cellular processes. In comparison, the large intestinal segments exhibited a quantitatively important archaeal community, a diversified bacterial microbiota, high

concentrations of SCFAs and a larger proportion of functional predictions focused on metabolites production. At last, the presence of opportunistic pathogens such as *Escherichia Shigella* or *Campylobacter* was highlighted in the entire GIT of weaning piglets.

This chapter was valorised by a first authorship publication in the journal Microorganisms, published in September 2019.

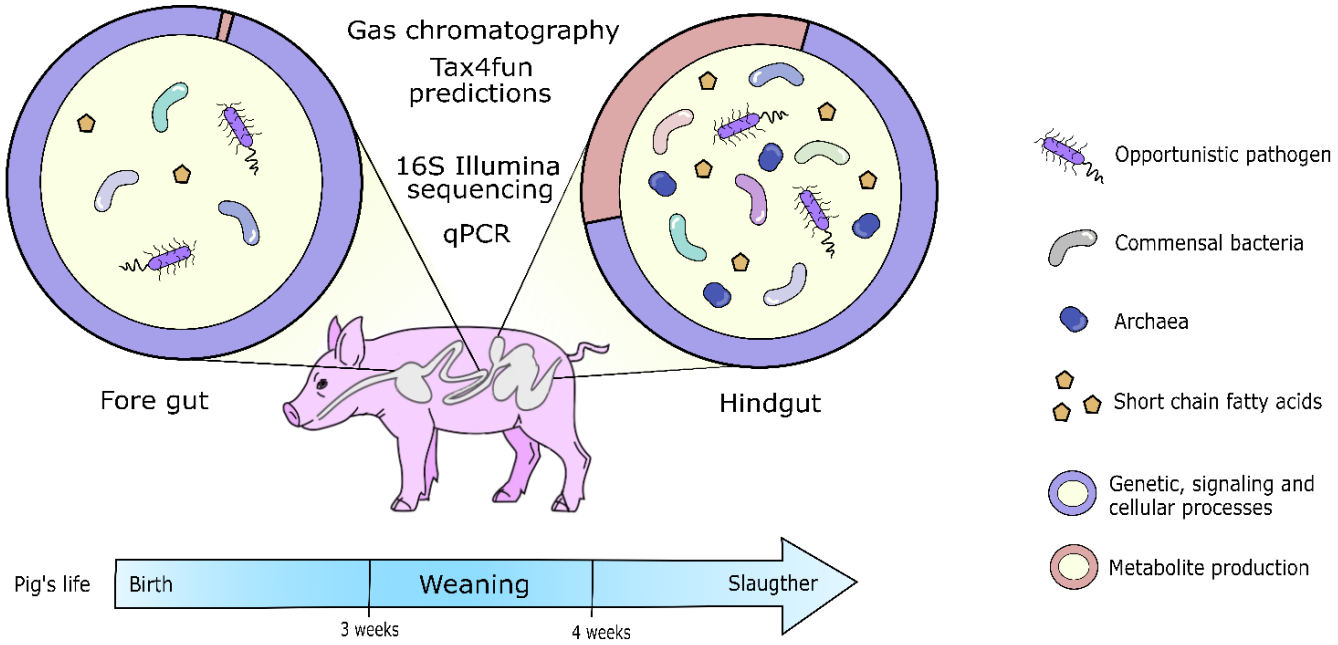


Figure II. Graphical abstract of Chapter 8.

microorganisms

MDPI

Article

Microbiota Composition and Functional Profiling Throughout the Gastrointestinal Tract of Commercial Weaning Piglets

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Abstract: Dietary, environmental, and social stresses induced by weaning transition in pig production are associated with alterations of gut microbiota, diarrhea, and enteric infections. With the boom of -omic technologies, numerous studies have investigated the dynamics of fecal bacterial communities of piglets throughout weaning but much less research has been focused on the composition and functional properties of microbial communities inhabiting other gastrointestinal segments. The objective of the present study was to bring additional information about the piglet bacterial and archaeal microbiota throughout the entire digestive tract, both at the structural level by using quantitative PCR and high-throughput sequencing, and on functionality by measurement of short-chain fatty acids and predictions using Tax4Fun tool. Our results highlighted strong structural and functional differences between microbial communities inhabiting the fore and the lower gut as well as a quantitatively important archaeal community in the hindgut. The presence of opportunistic pathogens was also noticed throughout the entire digestive tract and could trigger infection emergence. Understanding the role of the intestinal piglet microbiota at weaning could provide further information about the etiology of post-weaning infections and lead to the development of effective preventive solutions.

Keywords: weaning; piglets; microbiota; gastrointestinal tract

1. Introduction

In intensive swine production, early weaned piglets encounter social, environmental and dietary stresses generated by handling, transport, mixing litters, separation from the mother and the transition from a milk-based diet to a solid-based diet [1]. Dietary changes at weaning are associated with low feed and water intake, gastrointestinal (GI) changes and modifications of the intestinal piglet microbiota [2]. This huge microbial community has numerous roles benefiting the host including protection from pathogenic bacteria [3,4] and its alteration has already been linked with numerous diseases or infections [5]. Weaning transition in the piglet is associated with diarrhea and enteric infections which are the main causes of piglet death [6] and could be a direct consequence of microbial shifts observed at this critical period. Besides raising an economic burden in pig industry, weaning associated infections rear public health concerns due to the massive use of antimicrobials for therapeutic purposes [2,7].

Thus, there is a crucial need to gain further knowledge about weaning piglet microbiota functionality and composition to define non-pharmacological strategies to counteract post-weaning diarrhea. With the development of omics technologies, numerous of studies have characterized the

fecal microbiota composition of weaning piglets and its response towards weaning transition, antibiotic use, dietary changes, presence of pathogens or feed additives [8–14]. However, microbial interactions, activities and fermentations also occur in all the other segments of the pig gastrointestinal tract (GIT) which possess different physicochemical environments and, in consequence, different microbial communities [15]. Additionally, the small and large intestine are preferably used as colonization or infection sites by opportunistic pathogens and should be more largely studied to unravel the mechanisms inducing dysbiosis and infections. Only a few studies describe the microbial populations longitudinally harbored in the weaning piglet GIT using next-generation sequencing techniques and revealed distinct location-specific differences in microbial composition inside the different gut compartments [16–20]. Among these studies, four out of five used animals raised in a controlled laboratory environment [16–18]. However, as microbial population colonizing the intestine is impacted by the environment, including the use of commercial pigs in such research is also of great interest to be representative of swine industry conditions.

The microbial communities inhabiting the digestive tract have numerous functions such as participating to the digestion of organic compounds and fermentation of carbohydrates to make metabolites accessible for the host [3,4]. The differences of microbiota distribution throughout the entire GIT of piglets suggest that microbiota from different intestinal niches may have different roles [21]. Functions of weaning piglets GIT microbiota is yet to be explored. New bioinformatic tools such as PICRUSt [22] or Tax4Fun [23] were developed in the last decade and should be of a great help to predict the functionality of microbiome. Only one previous study investigated the functional predictions of piglet GIT microbiota, two weeks after weaning [21]. Functional capacities varied according to gut locations and from mucosal to luminal samples [21]. However, analyses of microbiome functions throughout the diverse GIT segments of weaning piglets remains to be performed. At least, if all piglet gut microbiome studies focus on bacterial composition, no omics investigations involve the archaeal microbiota yet.

The objective of the present study was to provide more information about the bacterial and archaeal GIT microbiota composition and functions of six male Landrace x Large White commercial piglets using 16S rDNA sequencing with Illumina MiSeq platform, quantitative PCR, gas chromatography for short-chain fatty acids (SCFA) quantification and the Tax4Fun pipeline. Sampling was performed on the commercial weaning day at 28 days of age on digesta from the stomach, duodenum/jejunum, ileum, cecum, proximal colon, distal colon, and feces. Mucosal scrapings from proximal colon, the most documented fermentative organ, were also collected to compare weaning piglet microbiota population and functions from the lumen to the mucosa.

2. Materials and Methods

2.1. Animal and Sample Collections

All experimental procedures were followed in accordance with the C2E2A Local Ethic Committee and the guidelines established by the European Community Concil under the Directive 2010/63/EU. The experiments were exempted from ethic evaluations because all animals were commercially raised and slaughtered on site under the supervision of the local veterinary. Animals were raised in a conventional pig farm located in the Haute-Loire area of the Auvergne-Rhône-Alpes region in France. All piglets remained with their mother and siblings during the suckling period. None of the selected piglets received antibiotic or had signs of enteric or metabolic disturbances from birth until slaughter. In addition to sow milk, piglets received water and pre-weaning diet (Supplementary Table S1) ad libitum. Six healthy male piglets, originating from a different mother, weighting between 10 and 12 kilos, (Landrace × Large White) at 28 days old, corresponding to the day of weaning, were randomly picked among the litters and slaughtered in order to collect their GIT. Immediately postmortem, the entire digestive tract was removed. The entire stomach; duodenum/jejunum, first part after the stomach's pyloric sphincter and the middle part of the small intestine; ileum, last part of the small

intestine before the ileocecal valve; cecum; proximal colon; first part of the large intestine after the ileocecal valve; distal colon, last part of the large intestine just before the rectum and rectum segments were carefully doubled clamped to avoid digesta leakage, kept at 4 °C and quickly transferred to the laboratory. Once reaching the laboratory facility, the GIT segments were separated by cutting between the double clamp of each section. Each segment of the digestive tract was weighted. pH was measured directly inside the organs using a pH1970 I pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) coupled with a LoT 406-M6-DKK-S7/25 probe (Mettler Toledo, Columbus, OH, USA). The pH probe was carefully rinsed with water after each pH measurement, and calibrated between each animal. The luminal contents of each intestinal compartment were entirely removed, pooled, mixed, aliquoted and immediately stored at −80 °C. The organs were washed using sterile 0.9% phosphate-buffer saline (PBS) and weighted again. For mucosal scrapings, the proximal colon was rinsed with sterile PBS to remove any digesta and its surface was scrapped using a sterile 76 × 25 mm Menzel-Gläser Superfrost (Thermo Scientific, Waltham, MA, USA) microscope slide. The scrapings were then stored at −80 °C.

The cecal, colonic and fecal concentrations of SCFAs were quantified by gas chromatography to determine the concentrations and proportions of acetate, propionate, isobutyrate, butyrate, caproate, isovalerate, and valerate. Approximately 500 µL of digestive contents were weighted, suspended with 500 µL of sterile water, mixed and centrifuged (14,000 g, 10 min, 4 °C). Eight hundred microliters of cell free supernatants were mixed with 500 µL of 0.4% (*w/v*) crotonic acid and 2% (*w/v*) metaphosphoric acid solutions. This mixture was centrifuged again, and the supernatant obtained was injected in a PerkinElmer Clarus 580 gas chromatograph (Waltham, Massachusetts, USA) for quantification of SCFA. The Mann and Whitney U test was used to compare the concentration of the main SCFA between GIT segments using the RStudio software version 1.0 (with R statistical package version 3.3.1, R Development Core Team, http://www.R-project.org).

2.2. DNA Extraction from Digestive Contents and Colon Mucosal Scrapings

Total DNA was extracted using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The quality of the eluted DNA was assessed by agarose gel electrophoresis. Extracts were quantified using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Samples were stored at −20 °C prior to use.

2.3. Quantification of Bacteria and Methanogenic Archaea Populations by QPCR

Two specific primer pairs were used for quantitative PCR (qPCR). The total bacteria were quantified using16SrDNA primers 338F 5'- ACTCCTACGGGAGGCAG-3' [24] and 530R 5'-GTATTACCGCGGCTGCTG-3' [25]. Methanogenic archaea were targeted using 16SrDNA primers 1174F 5'- GAG GAAGGAGTGGACGACGGTA-3' and 1389R 5'- ACGGGCGGTGTGTGCAAG-3' [26]. Real-time PCR assays were performed on a Rotor-Gene Q (Qiagen, Venlo, NL) in 20 µL reactions with QuantiFast SYBR GREEN master mix (Qiagen, Venlo, NL) with the additions of each primer at a concentration of 0.5 µM. The total bacteria 16S rDNA gene and 16S rDNA Archaea gene were respectively amplified using the following program: 2 min denaturation at 95 °C and 10 min denaturation at 95 °C; 40 and 45 cycles of 20 s at 95 °C and 60 s elongation and extension at 61 °C; and a melt curve step from 60 °C to 95 °C.

2.4. Standard Curve Assessments for Quantitative PCR Analyses

Conventional PCR for the amplification of the 16S ribosomal gene was carried out on genomic DNA from *Escherichia coli* (DSMZ N° 30083) and *Methanobrevibacter smithii* (DSMZ N° 861) (DSMZ, Braunschweig, Germany). The reaction was performed using the universal 16S primers 8F 5′-ACTCCTACGGGAGGCAG-3′ and 1492R 5′-GTATTACCGCGGCTGCTG-3′ and the Platinum™ *Taq* DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA). The PCR was carried out with a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: one cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s. The PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Venlo, NL) according to the manufacturer's instructions and were subjected before and after purification to a 1% agarose gel electrophoresis containing ethidium bromide and visualized for being approximately equal to 1484 bp using the ladder 500 bp Mol Ruler (Bio-Rad, Hercules, CA, USA). DNA concentration was measured via the Qubit dsDNA Broad Range Assay Kit (Invitrogen) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The 16S rDNA gene copy number was calculated using the formula: copy number/µL= $(C/X)*0.912.10^{12}$ with C: DNA concentration measured (ng/µL) and X: PCR fragment length (bp/copy) and diluted in 10-fold dilution series to be used as qPCR standards.

2.5. MiSeq 16S RDNA Sequencing and Bioinformatic Analysis

Prior to PCR amplification, the DNA concentration of all samples was measured using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and diluted to 2 ng/µL. The Bacterial V3-V4 region of 16S rDNA and the Archaeal 16S rDNA were respectively amplified with primers 357F 5′-CCTACGGGNGGCWGCAG-3′ and 805R 5′-GACTACHVGGGTATCTAATCC-3′ and primers 349F 5′-GYGCASCAGKCGMGAAW-3′ and 806R 5′-GGACTACVSGGGTATCTAAT -3′. Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system (Illumina, San Diego, CA, USA) performed at the Carver Biotechnology Center of the University of Illinois (Urbana, IL, USA). The demultiplexed paired end Illumina sequence reads in the FastQ format were uploaded into the Galaxy instance (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr) to be used in the FROGS (Find Rapidly OTU with Galaxy Solution) pipeline [27]. During the FROGS pre-process, sequences were depleted of barcode and the sequences with a non-appropriate length or containing ambiguous bases were removed. Next, reads were clustered into de novo operational taxonomic units (OTUs) using SWARM algorithm [28] with, at first, a denoising step to build very fine cluster using the minimal distance equal to 1 and, secondly, with an aggregation distance equal to 3. Chimeras were then detected and removed with VSEARCH [29]. Additionally, filters were applied to the OTUs in order to remove singletons [30,31]. The OTUs selected were taxonomically assigned using the Silva release 132 reference database [32].

2.6. Statistical Analysis of Sequencing Data

Statistical analysis was processed using the RStudio software version 1.0 (with R statistical package version 3.5.1, R Development Core Team, http://www.R-project.org). OTU structure and composition analyses were performed using the phyloseq R package [33]. Visualization of data was performed using the ggplot2 R package. Alpha diversity indices were calculated using a paired non-parametric t-test with the following indices: Inverse Simpson index, Chao 1 index, number of observed OTU phylogenetic diversity (PD) and Shannon index. Prior to beta diversity calculations, rarefaction using the transform counts methods was applied to the dataset. Statistical differences in Bray Curtis distance between GI locations were tested using a multi-analysis of variance (MANOVA) performed with ADONIS using the vegan R package [34] with 9999 permutations and represented by principal coordinate analysis (PCoA) plots. A non-metric multi-dimensional scaling (NMDS) on Bray Curtis distance was employed to visualize the differential abundance of main bacterial genera across the GIT

segments using heatmap representations. Statistical comparisons of mucosa versus digesta samples from proximal colon were performed using the Wald test of the DESeq2 R package at the genus level.

2.7. Function Prediction with Tax4Fun

Tax4Fun, an online tool hosted into Galaxy (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr), was used to predict functional genes of prokaryotic microorganisms across GIT sections [23]. Tax4Fun transforms OTUs picked up against the Silva 123 database into a taxonomic profile of KEGG (Kyoto Encyclopedia of Genes and Genomes) organisms and normalize these predictions by the 16S rDNA copy number. The most important functions of each intestinal segment were graphically represented using the Rstudio software version 1.0 (with R statistical package version 3.5.1, R Development Core Team, http://www.R-project.org).

3. Results

3.1. Physiological Parameters of the GIT of Piglets

The weight of organs and pH data collected while sampling is represented in Figure S1. As expected, the pH measured in the stomach was lower than that of the other anatomical regions of the GIT. Individual variability was remarkably reduced for the pH data of the cecum and proximal colon contents (Figure S1A). Segments from the foregut displayed higher weight but also greater inter-individual variability compared to hindgut anatomical regions (Figure S1B).

3.2. Concentration and Proportion of SCFAs Detected in the Hindgut

The SCFA concentrations varied in the different anatomical regions of GIT and was also quite variable among individuals. However, the total SCFA concentrations reached the highest values in the cecum followed by the proximal colon. Distal colon and rectum contents shared approximately the same total concentration of SCFAs. Acetic, propionic and butyric acids were the major SCFAs found in all compartments (Table 1). The concentrations of acetic acid were significantly higher in the cecum compared to proximal colon (p-value = 0.025) and in the proximal colon compared to distal colon (p-value = 0.008), but were not different between the distal colon and rectum contents (p-value > 0.05). Butyric and propionic acids were detected in significantly higher concentration in proximal colon compared to distal colon (p-value = 0.015). The relative abundance of acetate over total SCFAs tended to be lower in the proximal colon and very similar in the other segments, from 62.1 to 63.9% (Figure S2). The relative abundance of propionic acid was the highest in proximal colon and the lowest in rectum content. The relative abundance of butyric acid slightly increased from the cecum to the distal colon from 9.4% to 11%, with an intermediate proportion observed in the rectum.

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Table 1. Concentrations in mmol/L of SCFA measured in hindgut compartments of 28-day-old piglets by gas chromatography. (std= standard deviation).

Compartments	Concentration of Short-Chain Fatty Acid (mmol/L)															
	Acetate		Butyrate		Propionate		Valerate		Caproate		Iso Butyrate		Iso Valerate		Total SCFAs	
	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std	Individual Values	Mean ± std
Caecum	67.41	60.87 ± 15.75	8.03	9.06 ± 2.55	13.28	17.67 ± 4.68	3.12	3.17 ± 0.85	0.40	0.37 ± 0.17	2.28	2.28 ± 0.63	2.57	2.84 ± 0.73	97.09	96.24 ± 22.15
	54.52		8.68		17.61		2.93		0.06		2.53		3.58		89.92	
	78.89		10.11		15.83		3.82		0.49		2.89		3.13		115.17	
	33.90		4.72		13.18		1.63		0.34		1.09		1.50		56.36	
	71.05		10.96		25.15		3.58		0.35		2.66		3.26		117.00	
	59.43		11.84		20.95		3.92		0.55		2.23		2.99		101.91	
Proximal colon	33.18	39.00 ± 12.70	6.23	7.09 ± 2.37	12.88	14.03 ± 5.99	1.77	2.18 ± 0.83	0.08	0.23 ± 0.12	1.47	1.44 ± 0.55	2.59	2.23 ± 0.48	58.20	66.20 ± 21.59
	47.73		7.10		10.78		2.31		0.37		1.87		2.40		72.55	
	27.37		4.74		8.92		1.15		0.10		0.89		1.68		44.84	
	46.17		7.34		20.87		1.99		0.33		1.33		2.26		80.29	
	55.72		11.52		22.11		3.64		0.26		2.25		2.82		98.32	
	23.84		5.63		8.60		2.19		0.23		0.85		1.63		42.98	
Distal colon	12.61	17.46 ± 6.65	1.17	3.11 ± 2.21	3.75	5.03 ± 3.34	0.23	0.89 ± 0.83	0.00	0.11 ± 0.12	0.44	0.46 ± 0.18	1.06	1.05 ± 0.44	19.28	28.11 ± 12.86
	11.97		1.26		1.70		0.31		0.03		0.30		0.58		16.14	
	13.06		1.43		2.34		0.23		0.00		0.20		0.54		17.80	
	29.10		4.30		10.82		1.14		0.26		0.62		1.32		47.57	
	17.01		3.84		5.10		1.09		0.13		0.50		1.16		28.83	
	21.01		6.63		6.48		2.35		0.24		0.68		1.67		39.05	
Rectum	17.41	21.23 ± 7.47	4.51	3.51 ± 2.12	6.72	4.34 ± 2.60	0.56	1.03 ± 0.68	0.00	0.19 ± 0.21	1.25	0.90 ± 0.59	2.92	2.03 ± 1.18	33.37	33.22 ± 12.33
	9.41		1.83		1.49		0.56		0.10		0.28		0.70		14.37	
	29.08		1.13		5.27		0.64		0.00		0.52		1.26		37.90	
	29.18		6.69		7.71		2.22		0.53		1.88		3.87		52.07	
	21.50		4.64		2.88		1.49		0.17		0.89		2.04		33.60	
	20.82		2.24		1.96		0.72		0.31		0.56		1.40		28.02	

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3.3. QPCR Quantification of Total Bacteria and Methanogenic Archaea

Total bacteria were quantified in higher concentrations in the large intestine than in the stomach and small intestine regions, and were less variable between individuals in the lower parts of the gut (Figure 1A). The means of the log of total bacteria 16S copy numbers were increasing from the stomach (7.3 $\log_{10}$ copies/g) to the ileum (9.1 $\log_{10}$ copies/g), and were close to 10.5 in the lower gut segments. In the mucus collected in the proximal colon, the mean concentration (16S copy number) was 9.0 $\log_{10}$ copies/g. Methanogen archaea were not detected in samples from stomach and small intestine, but were quantified with an increasing gradient in digesta from the cecum (8.5 $\log_{10}$ copies/g) to the rectum (9.7 $\log_{10}$ copies/g) (Figure 1B). They were also detected in the mucus of the proximal colon, but in lower concentration (7.4 $\log_{10}$ copies/g).

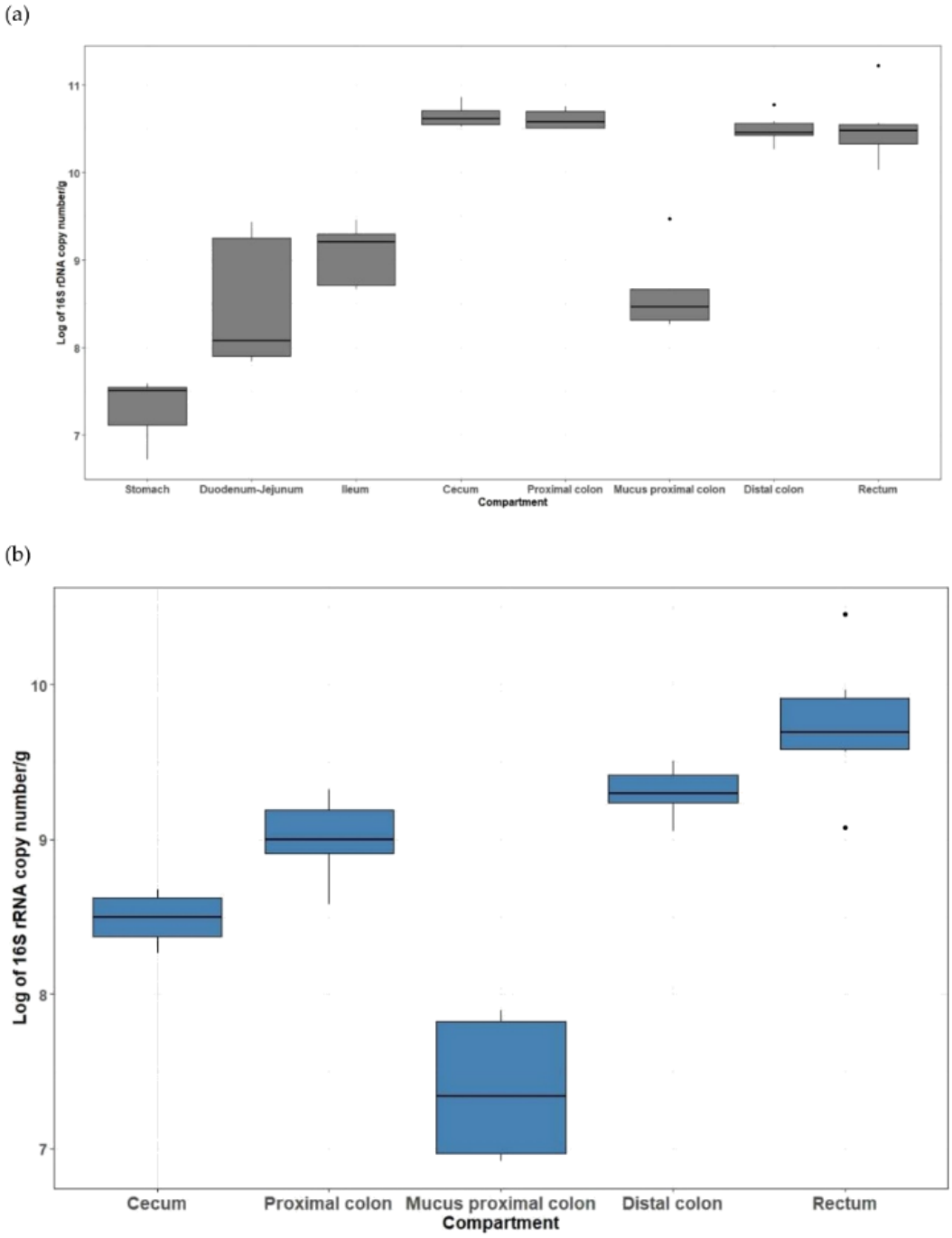

Figure 1. Quantification of total bacteria (**a**) and archaea (**b**) populations along the GIT of weaning piglet using qPCR on the 16S rRNA gene.

3.4. MiSeq Sequencing Data

A total number of 48 digestive contents and mucosal samples were collected during June to July 2017. The Illumina MiSeq run generated a total of 18,358,526 high quality sequences, lowered to 17,121,898 after removal of PhiX control reads. Primer sorted metrics total numbers of reads were equal to 5,364,263 and 1,882,030 respectively for V3-V4 16S primer set and the archaea primer set. Chimera removal, quality filtering and deletion of singletons led to a total of 12,970 identified taxa. The initial mean (± standard deviation) number of sequences per sample was 19,744 (± 6755) for the V3-V4 run and 15,467 (± 3086) for the cecum, proximal, and distal colon and rectum samples of the archaea run. After removal of chimeras and singletons, the mean (± standard deviation) number of sequences per sample was finally 12,361 (± 6374) for the V3-V4 run and 13,895 (± 2756) for the cecum, proximal, and distal colon and rectum samples of the archaea run. For the archaeal primer set, the mucosal samples collected at the proximal colon level displayed a lower mean (± standard deviation) number of sequences per sample equal to 2084 (± 1487) and 1643 (± 1384) after removal of chimeras and singletons. The very low number of sequences (<350 per samples) confirmed a poor detection of archaea in the upper part of piglet GIT.

3.5. Bacterial Communities All Along the GIT

3.5.1. At the Phylum Level

The most abundant phyla Firmicutes, Proteobacteria and Bacteroidetes displayed distinct profiles along the piglet GIT (Figure 2). The bacterial communities found in the duodenum and jejunum belonged predominantly to the Firmicutes phylum. The Proteobacteria phylum appeared particularly established in the gastric and ileal compartments, reaching ~ 25% of mean relative abundance. However, these upper GIT regions were submitted to high inter-individual variability (Figure S3). Despite a slight variability of the Firmicutes: Bacteroidetes ratio, the lower gut exhibited more diverse but more similar profiles between individuals (Figure 2 and Figure S3). Overall, the major phylum displayed in the lower gut segments was the Firmicutes followed by the Bacteroidetes, this latter being present with an increased proportion in colon areas compared with rectum content (Figure 2). The Proteobacteria and Epsilonbacteraeota phyla were better represented in the mucus samples. Supplementary Figure S3 also highlighted several particularities such as the presence of the phylum Synergistetes in one individual all along the lower part of the gut.

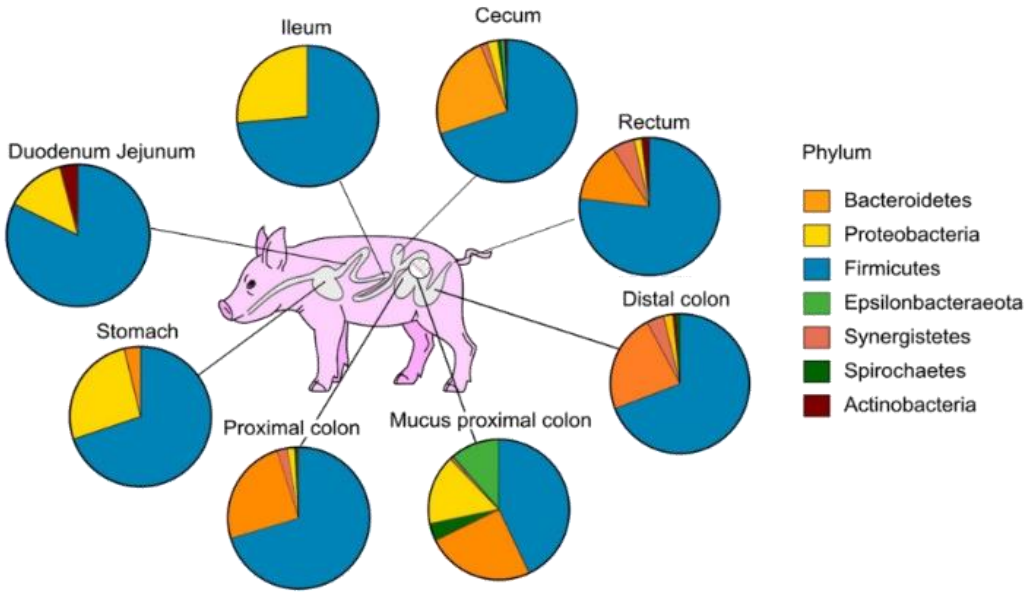

Figure 2. Mean relative abundance of the phylum-level microbiota across the weaning piglet GIT.

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3.5.2. At the Lower Taxonomic Level

The microbiota in the stomach and small intestine was inter-individually variable and principally composed of *Lactobacillaceae* and *Pasteurellaceae* families in the stomach, in addition to *Peptostreptococcaceae* and *Streptococcaceae* families in the small intestine (Figure 3). The *Enterobacteriaceae* family was also present in the small intestine, mainly in the ileum. The main families harbored in the hindgut were the *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, and the *Bacteroidaceae*. In the lower part of the gut, the variability across individuals was reduced. However, in the rectum, a reduced proportion of *Prevotellaceae* and *Bacteroidiaceae* was observed and members of *Enterobacteriaceae*, *Peptostreptococcaceae* or *Clostridiaceae* were found in variable abundances between individuals (Figure 3). The overall biolocalization of piglet bacterial community composition at the genus level is presented in Figure 4. The highest diversity was found in cecum, colon, and rectum segments which contain a great number of genera mainly consistent across samples. Many genera showed great specificity towards their location throughout the gut (Figure 4). For example, *Ruminococcaceae* and *Prevotellaceae* genera, *Rikenellaceae* RC9 gut group, *Lachnoclostridium*, *Parabacteroides*, and *Treponema* seemed to colonize almost exclusively the hindgut. Although *Staphylococcus* was mainly identified in the upper parts of the gut, no genus displayed absolute specificity toward the stomach or small intestine samples. (Figure S4) Some genera, such as *Campylobacter*, were not consistently present across individuals (Figure S4).

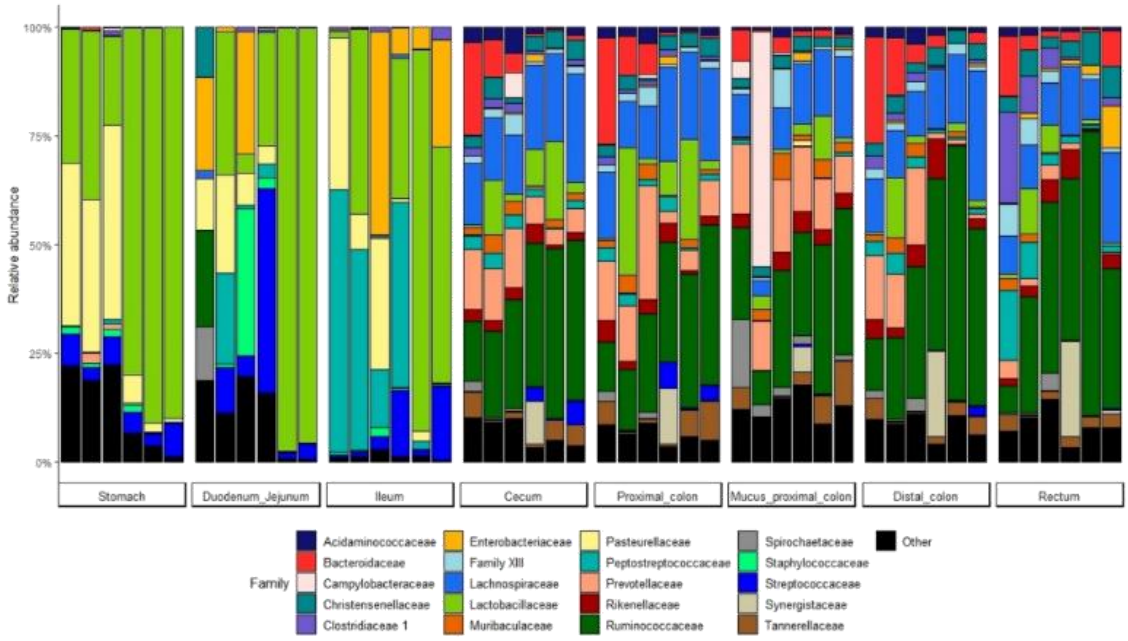

Figure 3. Relative abundance of the main bacterial families in the stomach and intestine segments of 6 weaning piglets.

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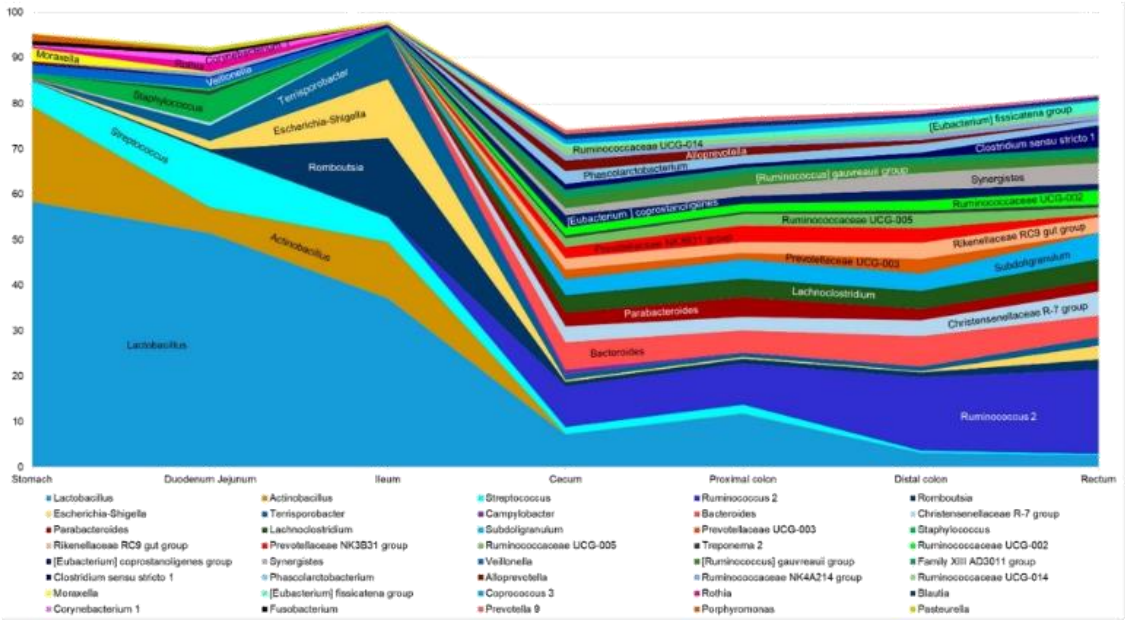

Figure 4. Mean relative abundance of the main bacterial genera along the GIT of weaning piglets.

3.6. Archaeal Microbiota Throughout the GIT

Archaeal community was exclusively composed of members belonging to the *Euryarchaeota* phylum (Figure S5). Despite some inter-individual variability across samples, the most represented genus was *Methanobrevibacter* belonging to the *Methanobacteriaceae* family reaching 98.1% of total sequences. Few other identified taxa were members of the *Methanomethylophilaceae* family belonging to the *Methanomassiliicoccales* order and representing 1.8% of total sequences (Figure 5).

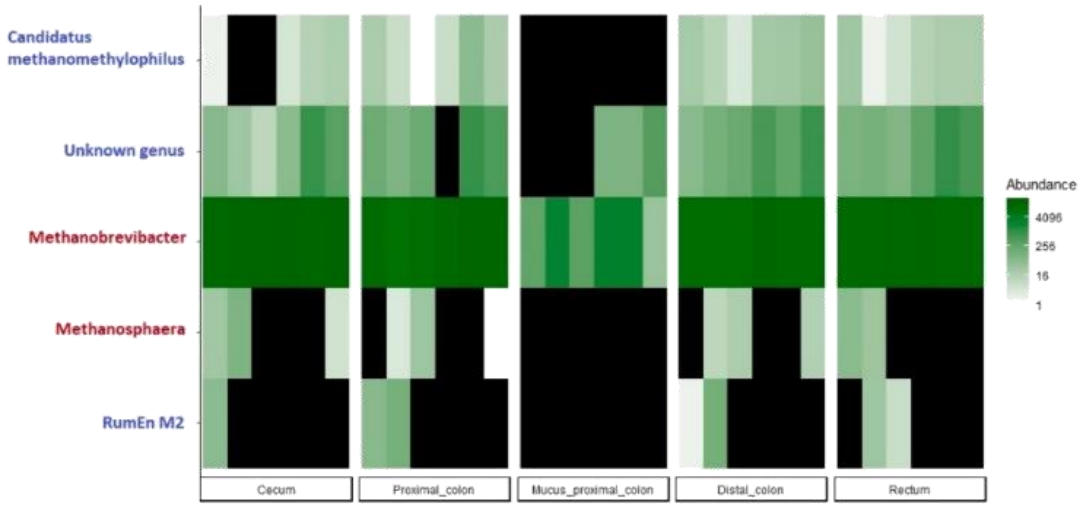

Figure 5. Top 20 of the most abundant archaeal OTUs along the GIT of weaning piglets (blue names correspond to *Methanomethylophilaceae* family and red names correspond to *Methanobacteriaceae* family, black color corresponds to an abundance of 0).

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3.7. Diversity and Richness Throughout the GIT Segments

3.7.1. Alpha Diversity and Species Richness

To further explore the microbiota structure among the different GI segments, bacterial and archaeal alpha diversity was evaluated according to the observed OTUs and the Shannon, Chao1 and inverse Simpson indices (Figure 6). Bacterial species richness and evenness, determined by the Shannon index, increased considerably from the small intestine to the hindgut (Figure 6A). The observed OTUs, chao1 index and inverse Simpson indices, reflecting respectively, the richness of species, the number of taxa observed in a sample and the species number and their abundance, displayed a similar profile confirming the increase of alpha diversity toward the cecum, colon, and rectum segments. Regarding the archaea, the hindgut digestive contents displayed similar alpha diversity indices (Figure 6B). For the mucosal colon samples, for both bacteria, and archaea, the observed OTUs and Shannon indices were numerically variable and lower than those found in the corresponding proximal colon lumen samples (Figure 6A,B).

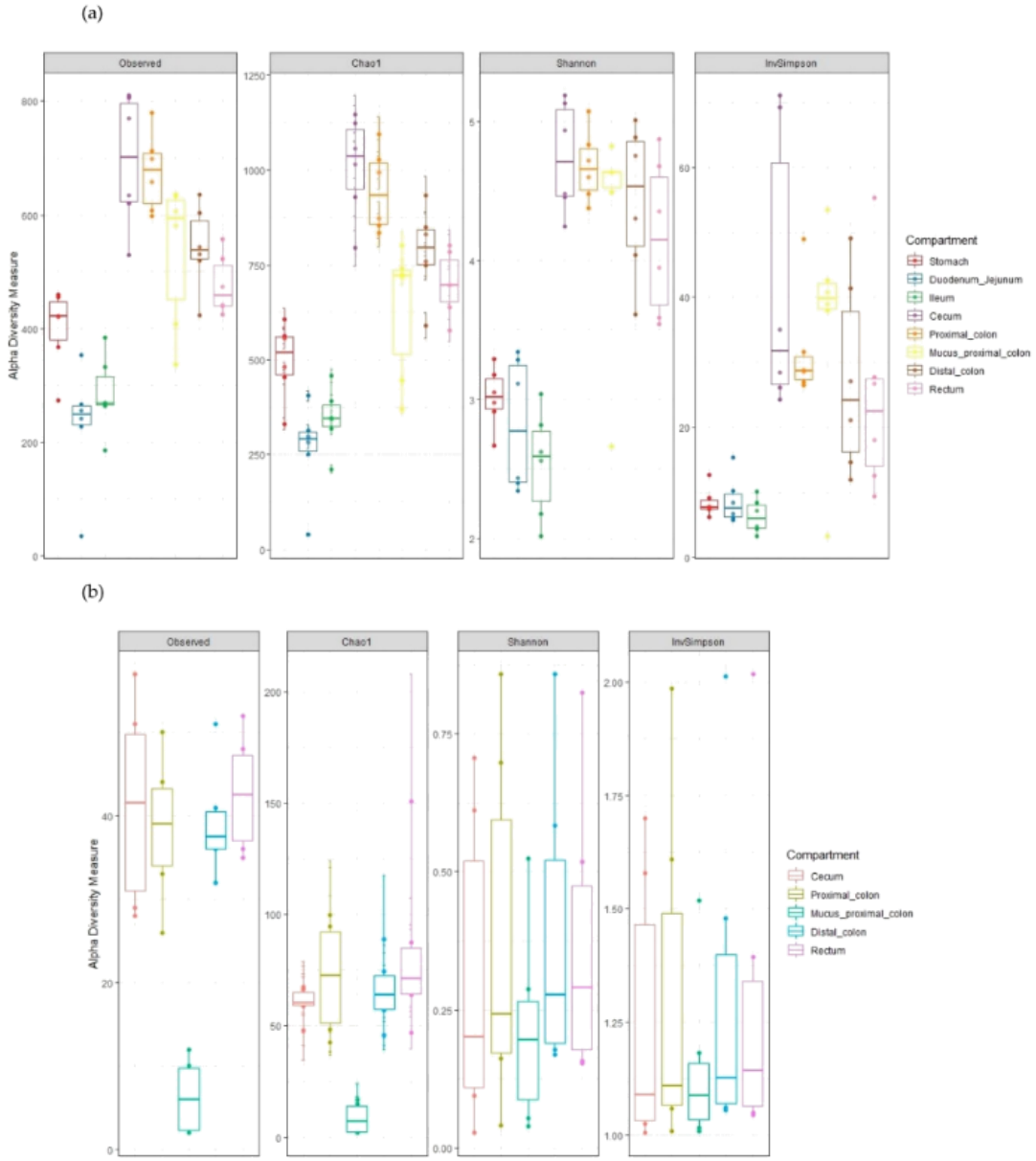

Figure 6. Alpha diversity measures on bacterial (**a**) and archaeal (**b**) OTUs across the GIT of weaning piglets.

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3.7.2. Microbial Community Analysis

Principal coordinate analysis was used to determine the similarities of microbial communities between different GI locations. Multi-dimensional scaling (MDS)/ principal coordinate analysis (PCoA) on Bray Curtis distance showed that stomach and small intestine samples formed a distinct cluster and could be separated from the samples collected in the hindgut. The mucus proximal samples could not be differentiated from the lumen proximal colon samples on the PCoA plot. Overall, the communities clustered by lower and upper position in the GIT (ADONIS: Bray Curtis, *P*-value = 1e-04, *R*-value = 0.266) (Figure 7).

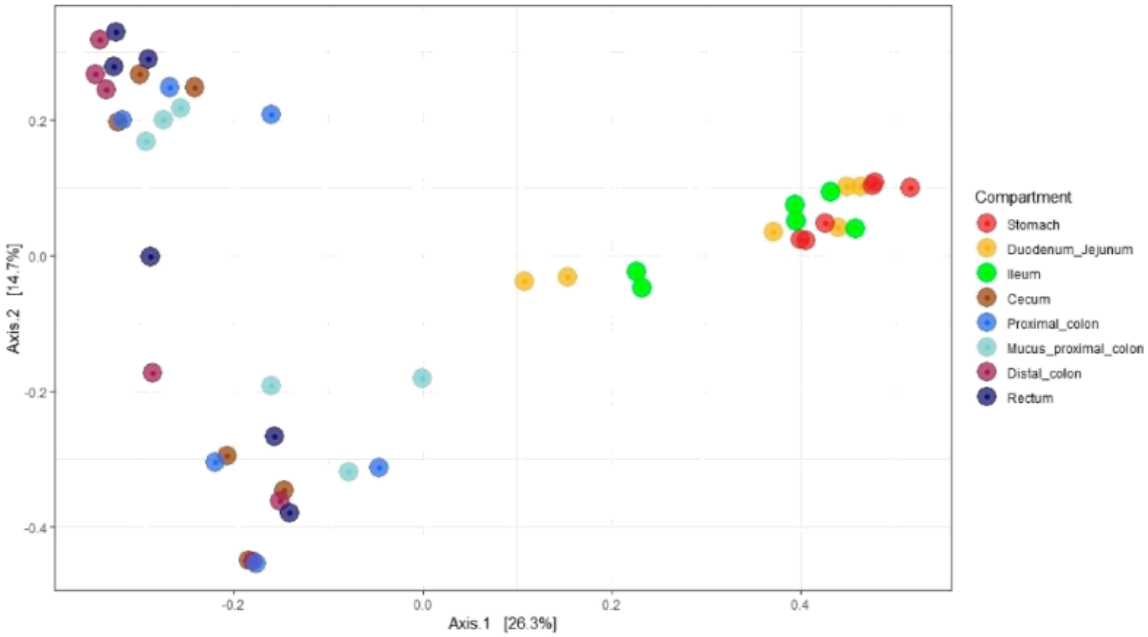

Figure 7. Bray Curtis multi-dimensional scaling / principal coordinate analysis of the bacterial communities across weaning piglet GI organs.

3.8. Differential Analysis of Mucosal Versus Luminal Proximal Colon Samples

Following the Wald test performed using the DESEQ2 R package, several OTUs were identified as significantly more abundant in mucosal scrapings compared with luminal digestive content (Figure 8). Among them, the genus *Mucispirillum* was found to be the most prevalent in mucus from proximal colon compared with luminal colonic content with a Log2FoldChange > 20, followed by the *Acinetobacter* and *Cerasicoccus* genera (7 > Log2FoldChange > 9) (Figure 8).

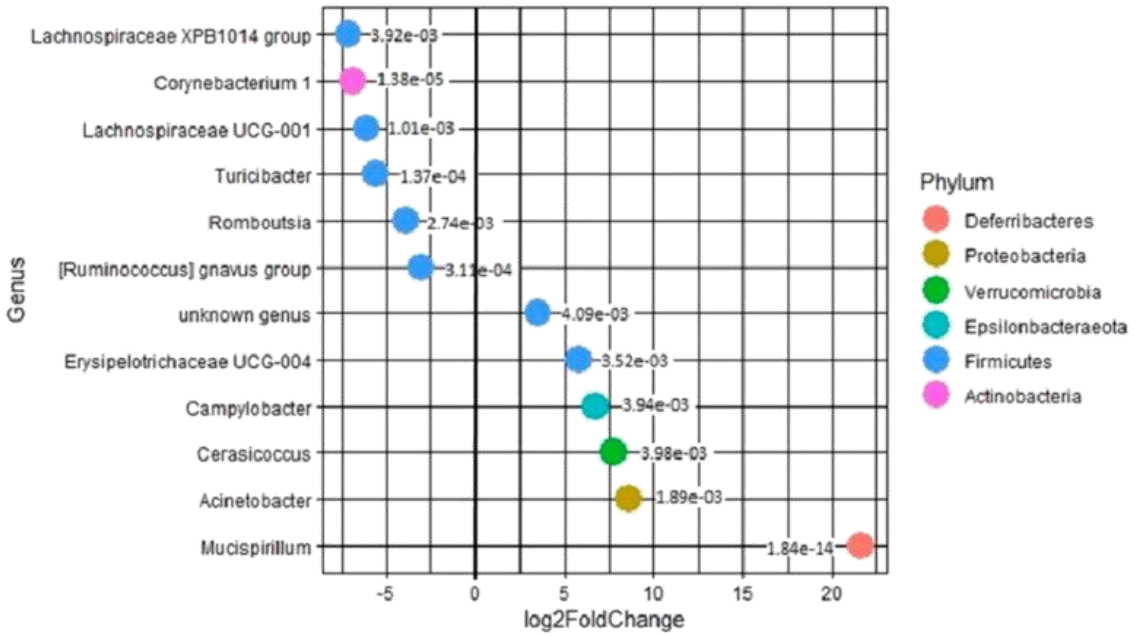

Figure 8. Differentially abundant genera between proximal colon mucosal scrapping and proximal colon digesta. Only the statistically significant genera are represented (*p* values are indicated on the figure).

3.9. Prediction of Microbiota Functional Capacity

Functional profiles were obtained through the Tax4Fun tools (Abhauer2015). Like for the microbiota composition, the stomach, duodenum, jejunum, and ileum displayed similar predicted functional profiles. In these compartments, the major identified pathways were DNA replication and repair, transport, genetic information processing and cellular community (Figure 9). Some functions were mostly predicted in these parts of the GIT such as infectious disease, prokaryotic defense system and endocrine system (Figure 9). The cecum and proximal colon (lumen and mucus) harbored very close profiles but rather different from those predicted in the upper part segments. Metabolic information related to signaling and cellular processes, signal transduction, energy metabolism, amino acid metabolism, and cell motility appear to be expanded in these areas. The metabolism of terpenoids and polyketides appears to be a specificity of the proximal colon mucus samples (Figure 9). In the distal colon segment, the relative abundances of several metabolic pathways increased compared to other colonic segments such as transport and catabolism, glycan synthesis, and carbohydrate metabolism. At last, the rectum content demonstrated a very distinctive profile. The functional capacity predicted in this area was mainly focused on carbohydrate, amino acids, lipid and energy metabolism and xenobiotics biodegradation and metabolism (Figure 9).

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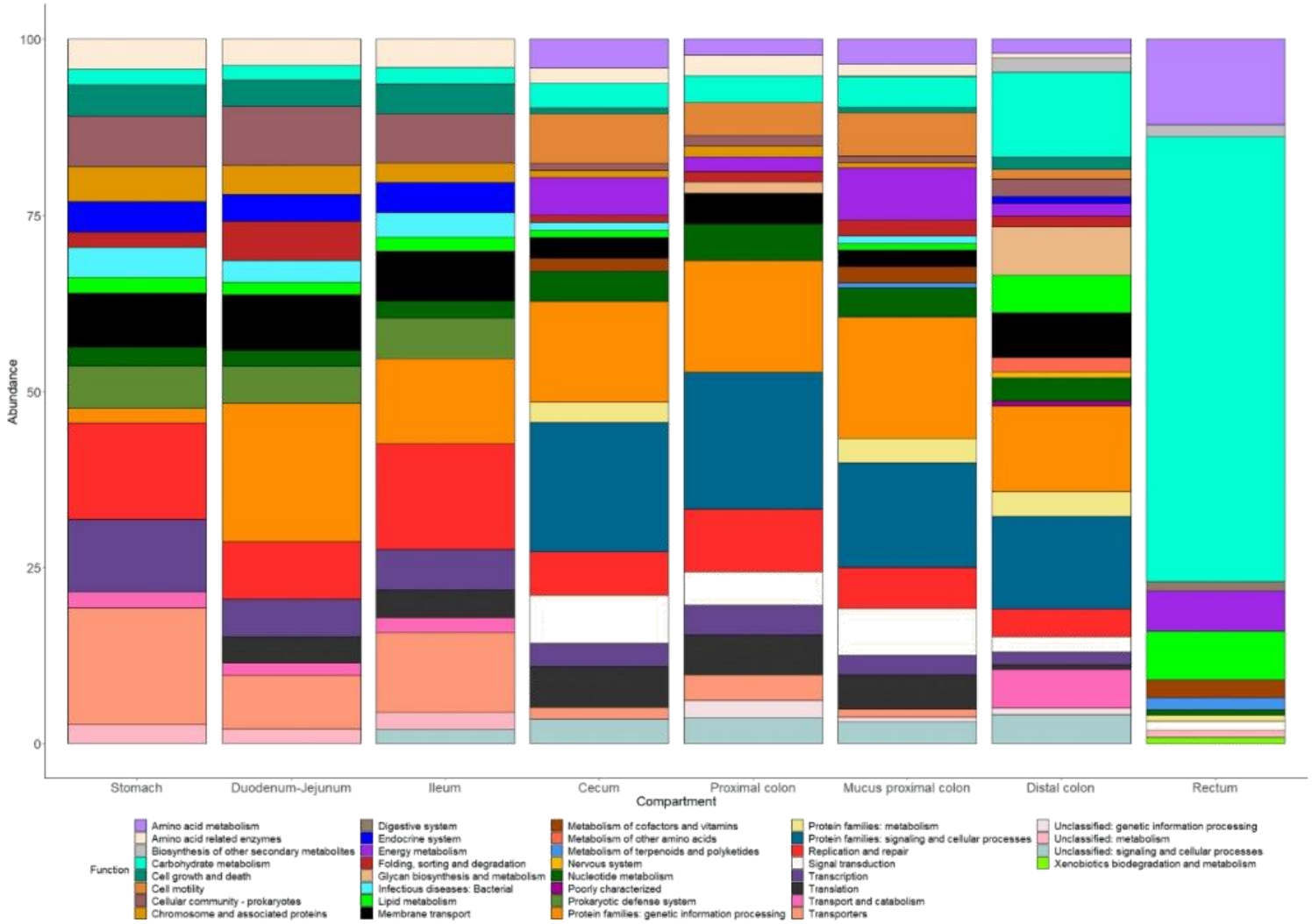

Figure 9. Prediction of functional capacity of the bacterial communities along the GIT of weaning piglets.

4. Discussion

Studies focusing on the weaning period raise special interests for swine production due to the high potential health concerns resulting from the increase of bacterial infections and post-weaning diarrhea. This study contributes to the growing knowledge of piglet microbiota structure and functions by combining compositional and structural sequencing and quantitative data. In this field, the -omics techniques considerably facilitated microbiome-oriented research in the past decade. Most of the metagenomic investigations in recent years described the shifts induced by weaning on piglet fecal bacterial communities [8–14]. However, the main feature reported in the present study was a clear separation between the upper part and the lower part of the piglet GIT, suggesting that characterization of fecal microbiota could be inadequate to investigate post-weaning infections etiology due to the fact numbers of pathogen infection or multiplication sites are located in the jejunum, ileum, or the colon segments. The segregation between the small and large intestine compartments was visible from the reported weight of organs to the microbial numbers and diversity, as assessed by qPCR and high-throughput sequencing. This could be due to shifts in the physicochemical conditions and differences of substrate availability between the superior and inferior gut segments as suggested elsewhere [15,17,35]. In our study, samples from small intestine were the most subjected to inter-individual variability in terms of composition but also bacterial diversity as reported in weaning piglets [19] but also in other animals [36,37]. The lack of stability of foregut bacterial communities could be due to the discontinuous influx of food depending on feed intake and diet type as a pre-weaning diet was offered to piglets in addition to sow milk. Indeed, several individuals harbored a great majority of *Lactobacillaceae* suggesting a preference for suckling behavior. It is also important to mention that the lower quantity of total bacteria detected in small intestine segments could impact the stability of bacterial composition. Genetics could also play a role in shaping the piglet intestine microbiota in this study as all animals were raised in different litters but in the same environment with the same diet at their disposal.

In the present study, the dominant phylum was attributed to the *Firmicutes* all along the GIT. Similar findings were described in fecal samples of 25- to 28-day-old weaning piglets and in all the GIT compartment of 42 day old weaned piglets from a close cross breed (Duroc × Landrace × Large White) [7,11,20]. The *Firmicutes* were also reported to be the most dominant in jejunum, ileum and colon of 28 day old German Large White x Piétrain weaning piglets [16]. Interestingly, the *Bacteroidetes* phylum was found to be the most represented in cecum content of 24 day old weaned piglets from the same cross breed as used in the present study (Landrace × Large White), suggesting a possible role of rearing environment or diet [38]. Despite the highest prevalence of *Firmicutes* across all the samples of our study, members of the *Bacteroidetes* phylum were found in higher relative abundance in cecum and colon mucosal scrapings and digestive contents. Across all GIT sites, OTUs classified as the most prevalent were *Lactobacillus, Actinobacillus, Romboutsia, Escherichia-Shigella, Terripsorobacter, and Campylobacter.* Up to now, the genus *Actinobacillus* has never been referenced as one of the most abundant in weaning piglet intestinal microbiota. Nonetheless, this taxon was identified to be predominant in piglet oropharynx which could explain its high abundance in stomach and small intestine samples of our study [39]. The *Lactobacillus* genus was already found to be predominant in the fecal microbiota of two weeks old suckling piglets [12]. In our study, the *Lactobacillus* genus was present in all the samples starting from 60% of relative abundance in stomach digesta and decreasing across the digestive tract to reach less than 5% of relative abundance in rectum content, suggesting the real dynamic of piglet microbiota across GIT segments. *Terripsorobacter* and *Romboutsia* were also detected throughout the whole GIT segments in our samples. These two genera belonging to *Clostridium* cluster XI, were previously characterized as dominant in the intestine of piglets regardless of their age [19]. Several species belonging to these genera are considered to be anaerobic pathogens [19,40,41].

Escherichia-Shigella species are facultative aerobes which are usually detected in higher proportion close to the intestinal mucosa due to the higher oxygen concentration released by the epithelial barrier in this area [17]. Even though members of this group cannot be distinguished by Illumina sequencing

techniques, the *Escherichia-Shigella* genus also hosts a variety of opportunistic pathogens such as enterotoxigenic *E. coli* (ETEC). ETEC is a very common cause of post-weaning diarrhea in piglets and is known to act mainly in the small intestine [12]. In our study, OTUs belonging to *Escherichia-Shigella* genus were detected in higher proportion in duodenum/jejunum and ileum samples, consistently with the potential site of action of ETEC strains. Additionally, *Escherichia-Shigella* group was detected in higher relative abundance in mucosal scrapings sampled from porcine colon confirming the affinity of this group for mucus secreting enterocytes [42]. A study from Bin et al. revealed that ETEC-induced diarrhea led to changes in the microbiota composition in the jejunum and feces of 18 day old piglets [6]. At genus level, diarrheal piglets had an increased percentage of *Lactococcus* in jejunum microbiota and *Escherichia* in feces, a lower abundance of *Prevotella* in feces and a lower abundance of *Escherichia* in jejunum [6]. Interestingly, piglets that recovered from diarrhea harbored a higher percentage of *Escherichia* genus in their jejunal microbiota suggesting that microbiota may play a resistant role to diarrhea after exposure to inducers [6]. The *Campylobacter* OTUs were also mainly found in mucosal scrapings of proximal colon, especially in one particular animal which was however devoid of intestinal lesions. *Campylobacter* species are known to adhere to the mucus surface, produce toxins and activate inflammation, leading to a reduction in nutrient use efficiency [19]. However, in pig gut, *Campylobacter* is considered to be a commensal bacterium [19,43]. *Campylobacter jejuni* and *Campylobacter coli* were even identified as the two most prevalent species in pigs at various ages [19,43]. A cecum mucus layer enriched in *Campylobacter* has been already observed, which supports the results of the present study, though it concerned only some individuals [44]. Other differences between mucosa and lumen were observed in our work using DESEQ2 analysis. The genus *Mucispirillum* belongs to the *Deferribacteres* phylum and is represented by a single species *Mucispirillum schaedleri*, which has a strong predilection for mucosal surfaces [13,45]. The latter information is consistent with our findings, as the *Mucispirillum* was 20 times more abundant in mucosal versus luminal proximal colon samples. *Mucispirillum* members were already detected in an increased amount in helminth infected pigs [46,47] but also in post-weaning piglet feces [11,13]. The *Acinetobacter* genus was also identified in a higher relative abundance in mucosal samples. *Acinetobacter* are strict anaerobes which were previously detected in high proportion in milk-fed piglet colon and were positively correlated with a metabolic pathways involved in the invasion of intestinal epithelial cells [48]. Interestingly, these bacteria are known to have heterotrophic nitrification and aerobic denitrification capabilities [49]. These data highlight the fact that gut compartments of healthy weaning piglets shelter a reservoir of opportunistic pathogens which could take advantage of any stress or dysbiosis related to weaning and thereby trigger pathologies.

This study contributes to increase our knowledge about the composition and the diversity of archaeal piglet microbiota all along the GIT. No data were previously published about the archaeal microbiota of piglets all along the GIT. Archaea are a separate domain of life inhabiting the GIT of animals initially studied regarding the high levels of methane initiated in livestock [50]. Methanogens diversity in piglets is yet poorly understood. One metagenomic study performed in growing pig mid colon revealed that archaeal species are influenced by diet composition [51]. Additionally, the unique study performed using PCR-DGGE analysis on the fecal archaeal microbiota of weaning piglets revealed a shift from the *Methanobrevibacter boviskoreani* to *M. smithii* species during weaning transition [52]. Our findings revealed that despite their poor diversity, archaea seem to be present in relatively high amounts in the hindgut, reaching 10^8 to 10^{10} 16S gene copy numbers per gram of digestive content from cecum to feces, where ecological conditions allow their establishment. All along the intestine, the most represented archaeal group was the genus *Methanobrevibacter* followed by an unknown genus from the *Methanomethylophilaceae* family belonging to the *Methanobrevibacter* order and *Thermoplasmata* class, both present in every sample from the hindgut including mucosal scraps of proximal colon. The genus *Methanobrevibacter* was already previously reported to be predominant in pig feces [52–54]. Su et al. 2014 [55] indicated the species *Methanobrevibacter smithii* was the most represented species in pre-weaning piglet fecal samples replaced by *M. boviskoreani* post-weaning. The genus *Candidatus methanomethylophilus* displayed a lower number of counts in our samples but seems to be present

in almost every segment of the luminal hindgut. The *Methanomassiliicoccales* order has been already identified in diverse anaerobic environments including the GIT of humans and animals [56], but to our knowledge, this is the first time this group is detected in pigs.

Very few data are available about functional properties of bacterial and archaeal GI microbiota of weaning piglet. This is important to increase our understanding of the potential contribution of microbiota to the physiology and metabolism of young piglets. Microbial fermentation products such as SCFAs represent the major source of carbon from non-digestible carbohydrates to the host and play numerous roles in host metabolic health [57]. Changes of SCFA luminal concentration and composition were previously reported in the large intestine of piglets after weaning [58]. In this study, the three main SCFAs: butyrate, acetate, and propionate, were detected in higher concentrations in cecum and proximal colon contents compared to distal colon and rectum samples. Indeed, the process of SCFA absorption remains not clear in weaning piglets, only 5% of the produced SCFAs in the hindgut is thought to be generally excreted in feces [58,59]. A study from Nakatani et al. (2018) [58] evaluated the SCFA concentrations in the cecum of 28 day old piglets and found similar concentration of butyrate and propionate, respectively 10 and 15 mmol/kg. However, the concentration of acetate described by Nakatani et al. was approximatively twice lower than the one detected in cecal samples of this study. This difference could be attributed to variabilities in piglet microbiota composition between the two studies. Acetate production is yet widely distributed among bacteria and cannot be related to specific bacterial groups [57]. The *Ruminococcaceae, Clostridiaceae* and *Lachnospiraceae* families known to produce butyric acid [60] were identified in our study with a higher relative abundance inside cecum and proximal colon segments consistently with the higher concentration of butyric acid detected in these compartments. Propionic acid is mainly provided by *Prevotellaceae*, *Bacteroidiaceae* families or the *Negativicutes* class [59]. In our samples, the lower concentration of propionate in distal colon and rectum digesta was in concordance with the low relative abundance of *Prevotellaceae, Bacteroidiaceae, Acidaminococcaceae, and Phascolarctobacterium.* Isovaleric acid has been previously positively correlated with *Christensenella* and *Methanobrevibacter* genera in human microbiota [61]. In our study, isovalerate shown to be twice more present in rectum content compared to other hindgut segments. Interestingly, our qPCR and metagenomic results highlighted a higher quantity of methanogenic archaea and a higher relative abundance of *Christensenellaceae* group in the rectum area.

The methanogenic archaeal community represent microbiota keystone species by transforming end-product from bacterial fermentation such as hydrogen, carbon dioxide or acetate into methane and potentially influencing the overall gut microbial populations [50,51]. In feces, the *Methanobrevibacter* genera performs hydrogenotrophic methanogenesis and transforms hydrogen and carbon dioxide derived from bacterial fermentations into methane and H_2O [52]. The use of H_2 and CO_2 as an energy source is thought to optimize fermentation and oxidation processes [62]. Like in other animal GIT, *Methanomassiliicoccales* probably carry out methylotrophic methanogenesis in the pig intestine [56]. These data suggest the main function of the archaeal microbiota of piglet would be CH_4 production and could potentially play an important role in piglet health.

With the recent rise of –omic technologies, the KEGG pathways obtained using Tax4Fun analysis offers the possibility to predict putative functions carried out by gut bacteria. In piglets, such analyses were already performed in fecal samples by Hu et al. (2016) [11] and Dou et al. (2017) [63] using the commonly used PICRUST tool, which is comparable to Tax4Fun in terms of robustness [64], but never all along the entire GIT as performed in our study. Within our sequencing results, two distinct types of profiles were generated by functional predictions in the piglet GIT segments. In stomach, duodenum/jejunum and ileum contents, most of the identified pathways belonged to microbial physiology such as DNA replication and repair, genetic information processing, membrane transport or pathways related to cellular community which might be explained by the adaptation of microorganisms to a challenging physicochemical environment. Also, in weaning piglets, gut organs are still maturing which probably induces a very tight microbe-host crosstalk and the expression of numerous pathways related to prokaryotic cellular machinery. In the cecum, proximal colon, proximal colon mucosal

scrapings, and distal colon, pathways related to energy, amino acids and carbohydrate metabolism were detected in higher abundance compared to foregut compartments probably due to the higher abundance of *Clostridium, Lachnospiraceae, and Prevotelleaceae* members. However, the most represented pathways in piglet hindgut belonged to prokaryotic signaling and cellular processes which may highlight the high communication level between microorganisms, their competition for persisting inside the same ecological niches and the progressive establishment of the still immature microbial intestinal ecosystem of weaning piglets. The metabolic pathways involving the metabolism of terpenoids and polyketides was only detected in proximal colon mucosal scrapings. Terpenoids and polyketides are large families of active natural compounds produced by a wide variety of living form including bacteria [65,66] suggesting that the emergence of this pathway in our mucosal samples could be due to differences in the relative abundances of other metabolic routes. In the distal colon segments, the relative abundance of pathways belonging to amino acid, carbohydrate, lipid and glycan metabolism and biosynthesis of secondary metabolites is increased compared to cecum and proximal colon area potentially due to lower level of host-microbiota crosstalk in this area. The rectum segments harbored a totally different profile. Indeed, contrary to the other GIT segments, no pathway was related to cellular processes or host-microbiota crosstalk. In the rectal area, carbohydrate metabolism was the most abundant followed by amino acid metabolism, glycan biosynthesis, fatty acid biosynthesis, and metabolism of cofactors and vitamins, consistent with the study of Hu et al. 2016 [11] focusing exclusively on piglet fecal samples. The distinct metabolic profile of rectum contents could be explained by an absence of, or much reduced exchanges between the microbiota and host epithelial cells. Even though the KEGG pathways obtained via Tax4Fun analysis stays predictions which must be considered carefully, the present results strongly emphasize the need for investigations about the activity of microbiota all along the small and large intestine of weaning piglets.

5. Conclusions

To conclude our findings, participate to the gain of knowledge about the composition and possible functional properties of microbial population inhabiting the total digestive tract of healthy commercial weaning piglets. Our study highlighted the strong differences in the shaping of weaning piglet maturing microbiota between GIT segments and the need to further explore the functional microbiota living inside the small and large intestines. Two of the main observations made throughout this study was the detection of an abundant archaeal microbiome in the large intestine as well as the presence of opportunistic pathogens inside the fore and lower piglet gut before weaning. This pathogen reservoir may trigger infection emergence during the critical weaning period leading to severe post-weaning diarrhea and massive antibiotic use. Therefore, understanding the role of the intestinal microbiota in preventing or eliciting the emergence of pathogens during the sensible weaning period in commercial piglets is of a great importance to find effective preventive actions to reduce the risk of post-weaning infections.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/9/343/s1, Figure S1: Physiological data on the GIT of weaning piglets: (a) pH values inside different locations of the GIT, (b): Weight of each segment of the intestine with (blue) or without the total content (red), Figure S2: Mean of relative abundance of SCFAs in cecum. proximal colon. distal colon and rectum of weaning piglets, Figure S3: Relative abundance of the main bacterial phyla in the stomach and intestine segments of 6 weaning piglets, Figure S4: Top 20 of the most abundant bacterial OTUs along the GIT of weaning piglets. OTUs' colors correspond to phyla assignation (Dark green = *Spirochaetes*. yellow = *Proteobacteria*. blue = *Firmicutes*. light green = *Epsilonbacteraeota*. orange = *Bacteroidetes*, black: no detection), Figure S5: Relative abundance of the main archaeal families in the low intestine segments of 6 weaning piglets, Table S1: Description of the commercial pre-weaning diet. The table contains the analytical composition of a pelleted diet that remains at the disposal of piglet from the second week of the nursing period. Ingredients: barley. wheat. extruded wheat. whey powder. soybean meal. wheat flour. oat flakes. protein isolates from whey powder. colza oil. soy protein isolate. potato protein. milk powder. vegetable oils. sugar.

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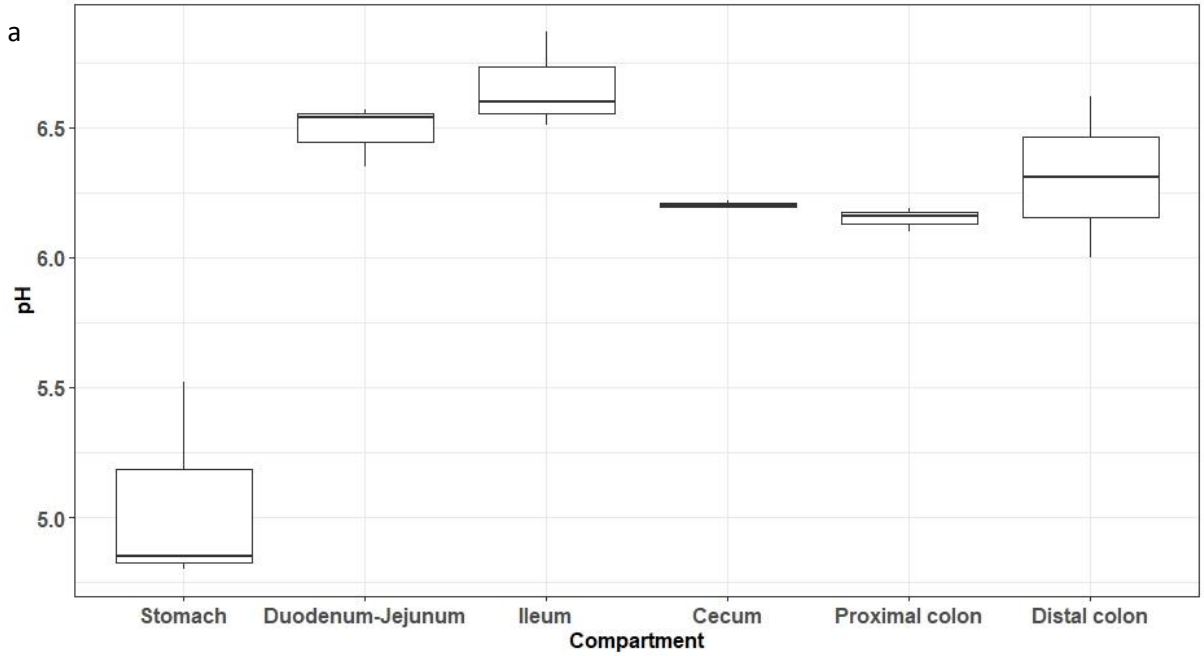
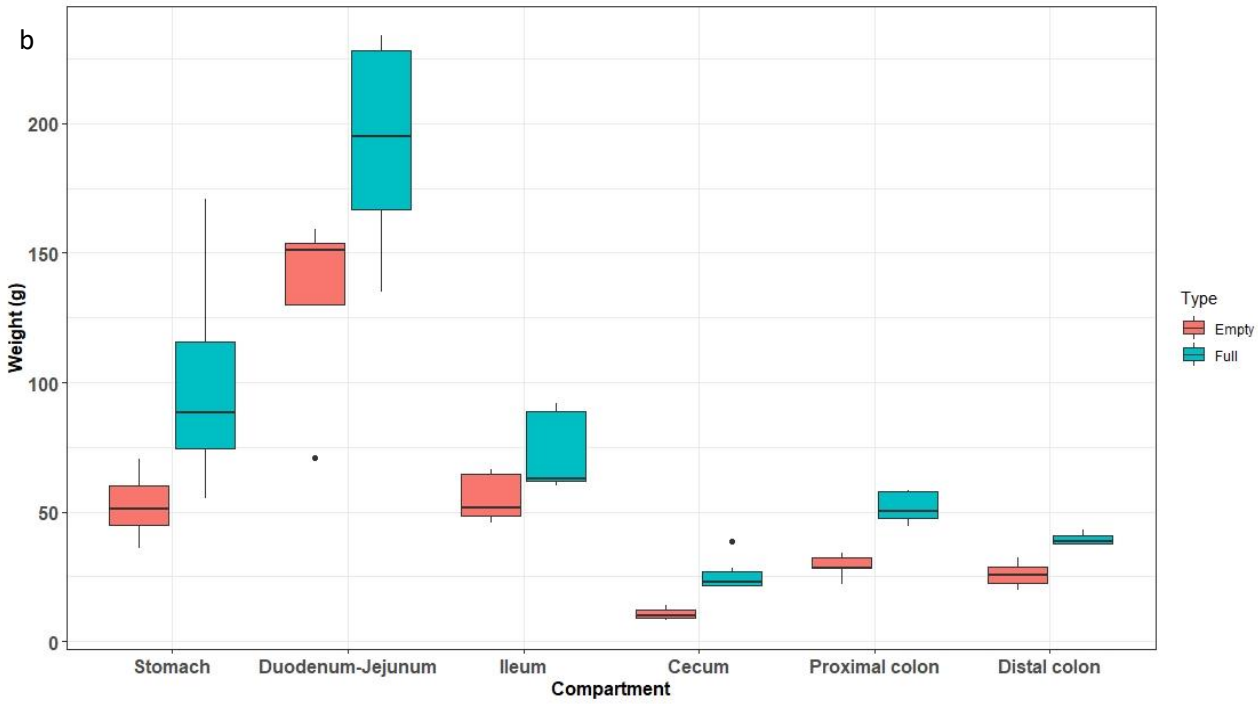
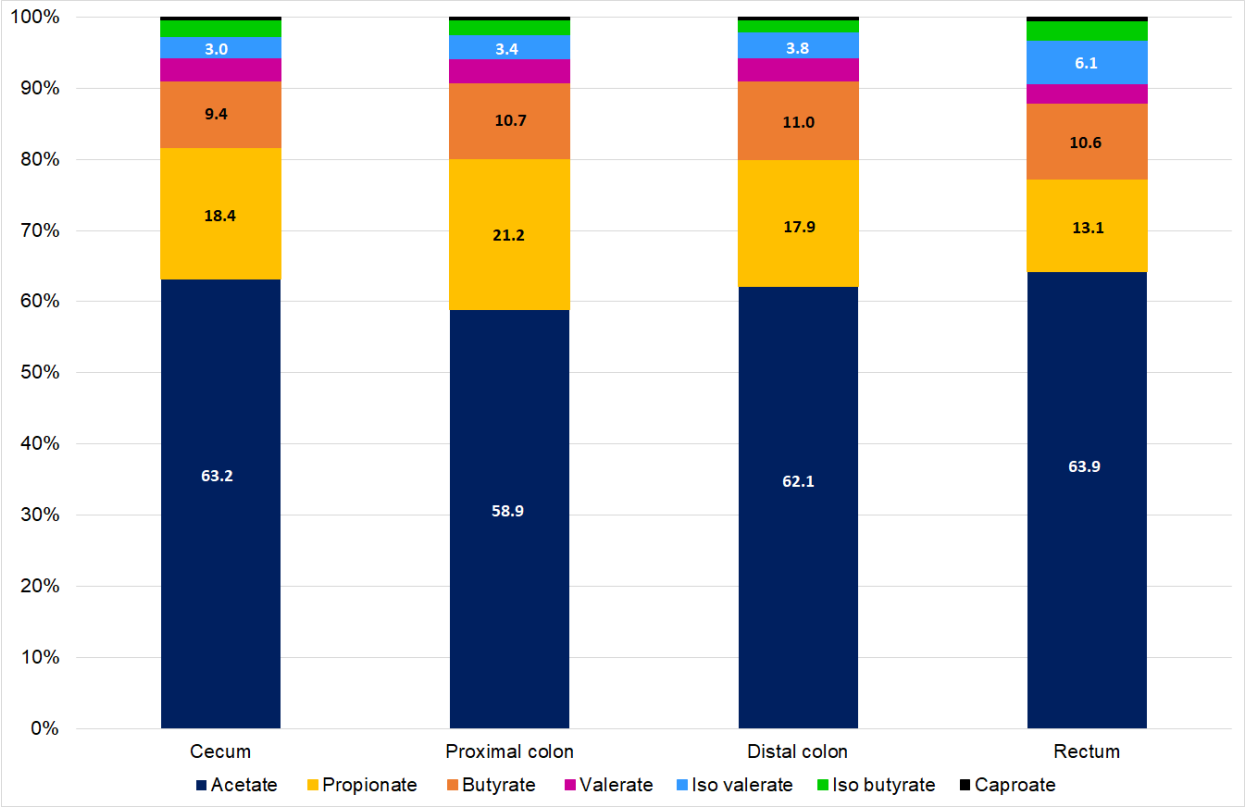
SUPPLEMENTARY MATERIAL

Supplementary figure 1: Physiological data on the GIT of weaning piglets: (a) pH values inside different locations of the GIT, (b): Weight of each segment of the intestine with (blue) or without the total content (red).

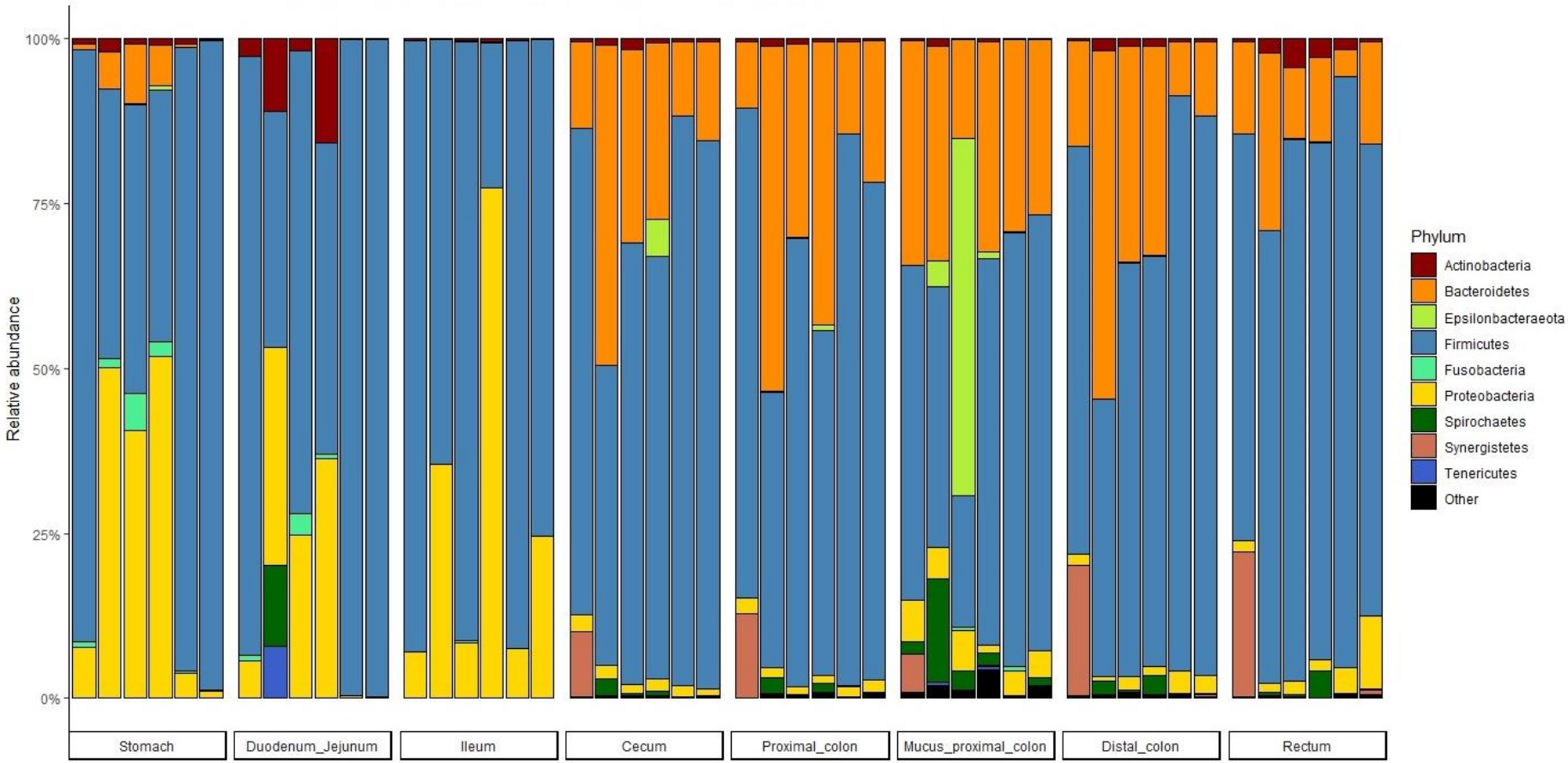




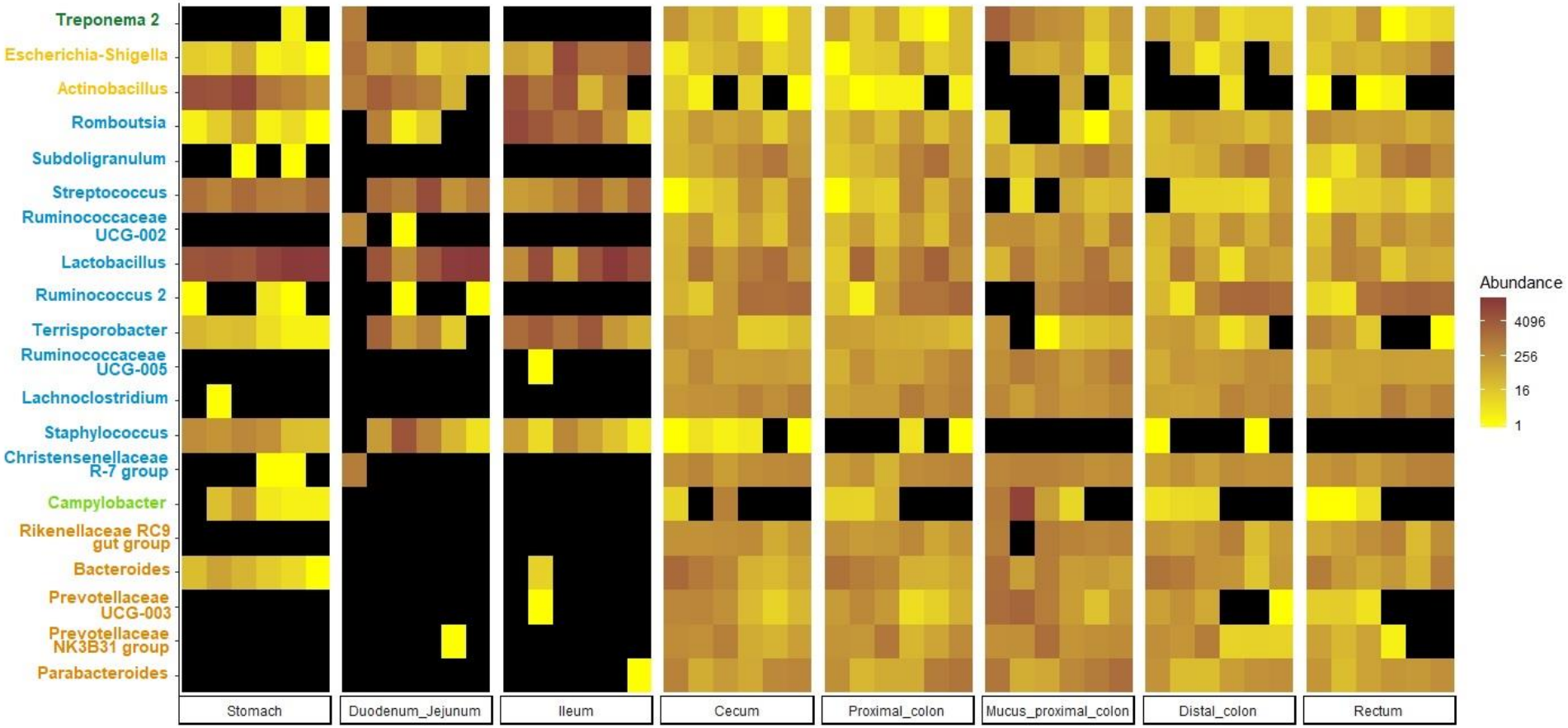
Supplementary figure 2: Mean of relative abundance of SCFAs in cecum, proximal colon, distal colon and rectum of weaning piglets.



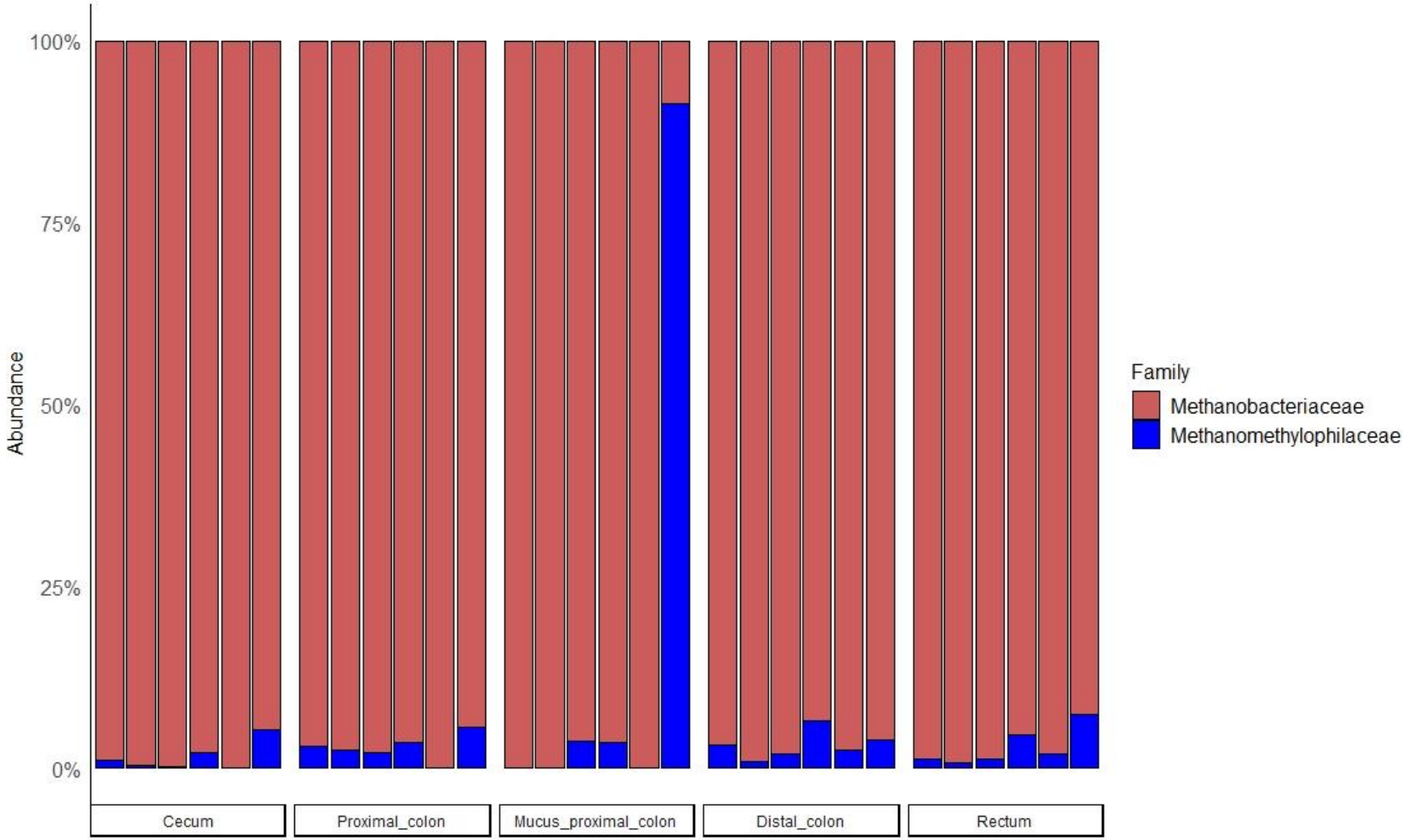
Supplementary figure 3: Relative abundance of the main bacterial phyla in the stomach and intestine segments of 6 weaning piglets.



Supplementary figure 4: Top 20 of the most abundant bacterial OTUs along the GIT of weaning piglets, OTUs' colors correspond to phyla assignation (Dark green = *Spirochaetes*, yellow = *Proteobacteria*, blue = *Firmicutes*, light green = *Epsilonbacteraeota*, orange = *Bacteroidetes*, black: no detection).



Supplementary figure 5: Relative abundance of the main archaeal families in the low intestine segments of 6 weaning piglets.



Supplementary table 1: Description of the commercial pre-weaning diet. The table contains the analytical composition of a pelleted diet that remains at the disposal of piglet from the second week of the nursing period. Ingredients: barley, wheat, extruded wheat, whey powder, soybean meal, wheat flour, oat flakes, protein isolates from whey powder, colza oil, soy protein isolate, potato protein, milk powder, vegetable oils, sugar.

Analytical constituents	
Crude proteins	18.00%
Crude cellulose	4.3%
Crude fat	7.0%
Minerals	5.3%
Lysine	1.62%
Methionine	0.63%
Calcium	0.61%
Sodium	0.25%
Phosphorus	0.55%
Trace elements (mg/kg)	
Iron	101
Iodine	1.5
Copper	151
Manganese	120
Zinc	131
Vitamins (IU/kg)	
A	16 000
D3	2000
E	150
C	100

Chapter 9. Development of a Novel Mucin Containing *In Vitro* Model of the Piglet Colon (MPigut-IVM). Application to the Study of Feed Deprivation and Probiotics

Once the colonic luminal and mucosa microbiota of our commercial piglets was characterized, the next step was to work on the development of the *in vitro* model using mini ARCOL-type bioreactors and tailor made mucin bead compartments in order to reproduce both the piglet colonic lumen and the mucin rich environment of the piglet colonic mucus layer. The development of the mucin phase was made in collaboration with Pr. Dr Tom van de Wiele from the CMET of Gent University. This step was performed with the aim of answering the research questions:

- Is our newly developed mucin beads system colonized by an adherent microbiota?
- Does our newly developed mucin beads system still allow the maintenance of anaerobiosis by the sole activity of the microbiota?
- Does the MPigut-IVM microbiota is stable during fermentations?
- Does the MPigut-IVM display reproducible results while maintain inter-inocula variability?
- Does the MPigut-IVM accurately reproduce the composition and activity of the colonic luminal and mucosal of piglet colon?

At weaning piglets encountered several stressful event such as separation with the mother, mixing litter or dietary change having detrimental consequences on their physiology and behaviour. Among the behavioural consequence induced by weaning, a transient anorexia period is often experienced by piglets which is thought to be one of biggest factors impacting intestinal morphology and function. Thus, once the *in vitro* model was developed the first application focused on evaluating the impact of feed deprivation on the MPigut-IVM microbiota composition and activity in order to reproduce the transient anorexia associated with weaning. The principal questions raised by this part of the experiment were:

- What is the effect of feed deprivation on the MPigut-IVM microbiota composition and activity?
- Does the feed deprivation impact differentially the bioreactor medium and he mucin beads?
- Are these changes lasting or transient in the MPigut-IVM?

The first part of this chapter focusing on the development of the model as well as the impact of feed deprivation on the *in vitro* piglet colonic microbiota have been submitted to the journal Gut Microbes. The Figure A summarizes the results obtained in Chapter 9.

Finally, an annexe of this chapter focuses on the effects of a daily supplementation with SB CNCM I-1079 on the microbiota composition and activity which was subjected to feed deprivation in the MPigut-IVM. The part is presented separately because it won't be submitted for publication.

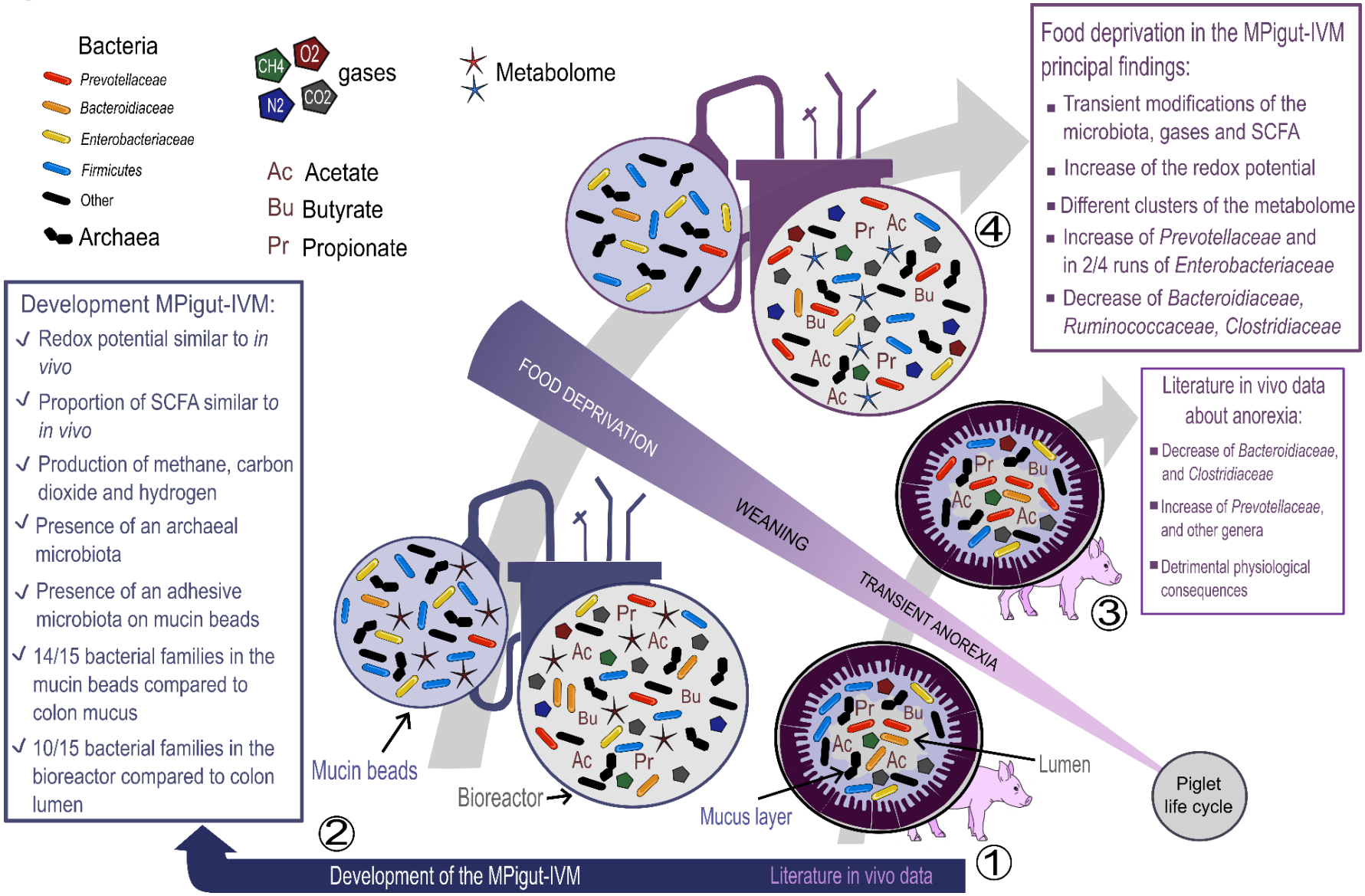


Figure III. Graphical abstract of Chapter 9.

Weaning-associated feed deprivation stress causes microbiome disruptions in a novel mucin containing *in vitro* model of the piglet colon (MPigut-IVM)

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ABSTRACT

Post-weaning infectious diarrhea in piglets, associated with high mortality and economic losses is a major problem in swine industry. Risks factors for the etiology of post-weaning diarrhea include the modifications of intestinal microbiota, also called dysbiosis, but remain to be fully elucidated. As animal experimental trials are increasingly questioned for cost and ethical reasons, appropriate *in vitro* gut models are representative first choice tools to address such complex questions. We developed a new *in vitro* model of the weaning piglet colon (MPigut-IVM) including a mucin bead compartment to reproduce the mucus surface from the gut to which gut microbes can adhere. The microbiota inhabiting the mucus layer is thought to be an important regulator of health due the proximity of host intestinal epithelium. Our results indicated that the MPigut-IVM is able to establish a representative piglet archaeal and bacterial colon microbiota in terms of taxonomic composition and function. The MPigut-IVM was consequently used to investigate the potential effects of feed deprivation, a common consequence of weaning in piglets, on the microbiota. The lack of nutrients in the MPigut-IVM led to an increased abundance of *Prevotellaceae* and *Escherichia-Shigella* and a decrease in *Bacteroidiaceae* and confirms previous *in vivo* findings. On top of a strong increase in redox potential, the feed deprivation stress induced modifications of microbial metabolite production such as a decrease in acetate and an increase in proportional valerate, isovalerate and isobutyrate production. Thus, weaning-associated feed deprivation in piglets provoke disruptions of MPigut-IVM microbiota composition and functionality and could be implicated in the onset of post-weaning dysbiosis in piglets.

BACKGROUND

In modern swine industry, piglet weaning occurring mostly between 3 to 4 weeks of age is the most critical event in pig life due to sudden social, environmental and dietary stresses (Lallès et al., 2007c). Weaning is frequently associated with changes in diet composition but also considerably impact the behavior of piglets resulting in vocalizations, fighting, and generally, weaning anorexia (Lallès et al., 2007c; Barbara A. McCracken et al., 1999; Sutherland et al., 2014). Post weaning-associated fasting periods affect growth performance and body weight (Campbell et al., 2013; Kats et al., 1992). Piglets approximately lose about 100–250 g body weight the first day after weaning which directly affects the total days to market (Kats et al., 1992). Reduced feed intake after weaning is variable from 24h to 4 days depending of individuals (Bruininx et al., 2002) and has been reported to be the most important factor compromising intestinal barrier function ahead of dietary change (Spreeuwenberg et al., 2001). The lack of nutrients in the intestine of piglets may contribute to intestinal inflammation and changes in intestinal morphology such as reduced villus height and increased intestinal permeability which facilitate the crossing of the mucus layer and the intestinal epithelial barrier by toxins and bacteria (Campbell et al., 2013; Barbara A. McCracken et al., 1999; van Nieuwamerongen et al., 2018). This mucus layer is a permeable gel overlying intestinal epithelial cells and separating them from gut luminal content including commensal bacteria and invading pathogens(Etienne-Mesmin et al., 2019). The mucus-associated microbiota, also referred to as mucobiome(Belzer et al., 2017), uses specific adhesins to bind to the polysaccharides composing mucins(Etienne-Mesmin et al., 2019). The mucobiome is thought to be an important regulator of host health. Such as in the lumen, a disproportion of mucus-associated microbial could, impact the balance between gut commensals and thus lead to the development of a disrupted microbiota(Etienne-Mesmin et al., 2019), also called dysbiosis. Regarding its numerous reported effects, the weaning-associated feed deprivation period could play a role in the etiology of post-weaning intestinal dysbiosis due to the lack of nutrients and the increase degradation of mucins and lead to a higher risk of developing enteric infection also called post-weaning diarrhea. To fight against post-weaning diarrhea, current strategies generally involve preventive and curative treatments with antibiotics which raise serious public health concerns due to the increasing antibiotic resistance prevalence (Barton, 2014; Gresse et al., 2017). Thus, there is a real need to find non-antibiotic solutions such as dietary compounds, prebiotics or probiotics and restore gut microbial balance at weaning.

Even though *in vivo* experiments remain the gold standard for evaluating the effects of non-pharmacological compounds on gut microbiota, pig *in vitro* digestion models constitute first-choice alternatives for ethical, technical, cost, and regulatory reasons. Continuous *in vitro* fermentation models can closely mimic pig colon physiology by simulating pH of colonic environment, consistent transit time, anaerobic habitat and supply of a nutrient medium close to the composition of ileal or cecal effluents, while maintaining a functional microbiota (Fleury et al., 2017; Messens et al., 2010; Pinloche et al., 2012; Ricca et al., 2010; Tanner et al., 2014b). Few semi-continuous or continuous *in vitro* models of the pig colon have been developed but the Pigut-IVM (Piglet Gut *In vitro* Model) and the BABY-SPIME (Baby Simulator of Pig Intestinal Microbial Ecosystem) are the only developed models aiming to reproduce the specific conditions encountered in the colon lumen of piglets (Dufourny et al., 2019; Fleury et al., 2017). Up to date, none of these models is able to mimic the gut mucosal surface. Incorporating mucin in fermentation models would make a surface available for microbial adhesion in order to get a more complete picture of the colonic ecosystem. The aim of this study was thus to develop, based on *in vivo* data, a continuous *in vitro* fermentation model including a mucin bead compartment in order to reproduce at best the colonic luminal and mucus microenvironments of 4-week old piglets. Once developed, this *in vitro* model was applied to evaluate the consequences of 48h feed deprivation on piglet microbiota composition and functionality, which was used to mimic a weaning feed deprivation stress.

MATERIALS AND METHODS

Samples collection and treatments

All animals were housed in a conventional pig farm located in the Haute-Loire area of the Auvergne-Rhône-Alpes region in France. Piglets remained with their mother and siblings during the suckling period. In addition to sow milk, piglets received water and pre-weaning diet (supplementary table 1, Chapter 8) *ad libitum* one week after birth. None of the piglets had signs of enteric or metabolic disturbances. The animals did not receive any antibiotic in the 27 days prior to sample collection day. As freezing process showed to affect bacterial abundances in pig feces (Metzler-Zebeli et al., 2016), fecal samples from 4-weeks old healthy male piglets destined to fermentation runs (Landrace x Large White) were collected using sterile bottles and immediately stored under anaerobiosis conditions using GENbag anaer gas pack systems (Biomerieux, Marcy L'Etoile, France) during transport to laboratory. In order to make a reliable comparison between our *in vitro* colonic model with *in vivo* colonic samples, luminal and mucosal proximal colon samples were collected from 5 healthy male piglets after slaughter on site. The experimental procedures were followed in accordance with the C2E2A Local Ethic Committee and the guidelines established by the European Community Council under the Directive 2010/63/EU. The experiments were exempted from ethic evaluations because all animals were commercially raised and slaughtered on site under the supervision of the local veterinary. Proximal colon mucosal and luminal samples were collected from the study of Gresse et *al* were procedures are further described (Gresse et al., 2019).

Description of the MPigut-IVM parameters

Five hundred mL MiniBio bioreactors (Applikon Biotechnology, Delft, The Netherlands) equipped with stirrers, ports and probes (figure 1) were used under continuous conditions to simulate the colonic environment based on *in vivo* data collected previously in the same commercial pig farm (Gresse et al., 2019). For the inoculation of one bioreactor, feces from 6 to 8 piglets, corresponding to 10g, were pooled and mixed under anaerobic conditions with 30mM sterile sodium phosphate buffer (pH 6.0) to reach a total volume of 50mL. The fecal inoculum was filtered through a 500 µm inox sieve. Fifty mL of the fecal suspension was added per bioreactor to 150 mL of nutritive medium (see below) while flushing with O_2-free N_2 gas, in order to ensure anaerobic conditions at the beginning of fermentations. Afterwards, during the fermentation course, the anaerobic conditions were maintained exclusively through the

activity of the resident microbiota and by ensuring the airtightness of the system. The temperature of the fermentation was set up to 39°C and maintained inside the main medium using an incorporated panel heater and inside the mucin bead compartment using a hot water bath. pH was recorded every 300 seconds in the bioreactor medium using a pH sensor (Mettler Toledo, Viroflay, France) and adjusted to a value of 6.0 with an automatic addition of 2M NaOH. Redox potential was constantly measured in the bioreactor medium using a redox sensor (Mettler Toledo, Viroflay, France).

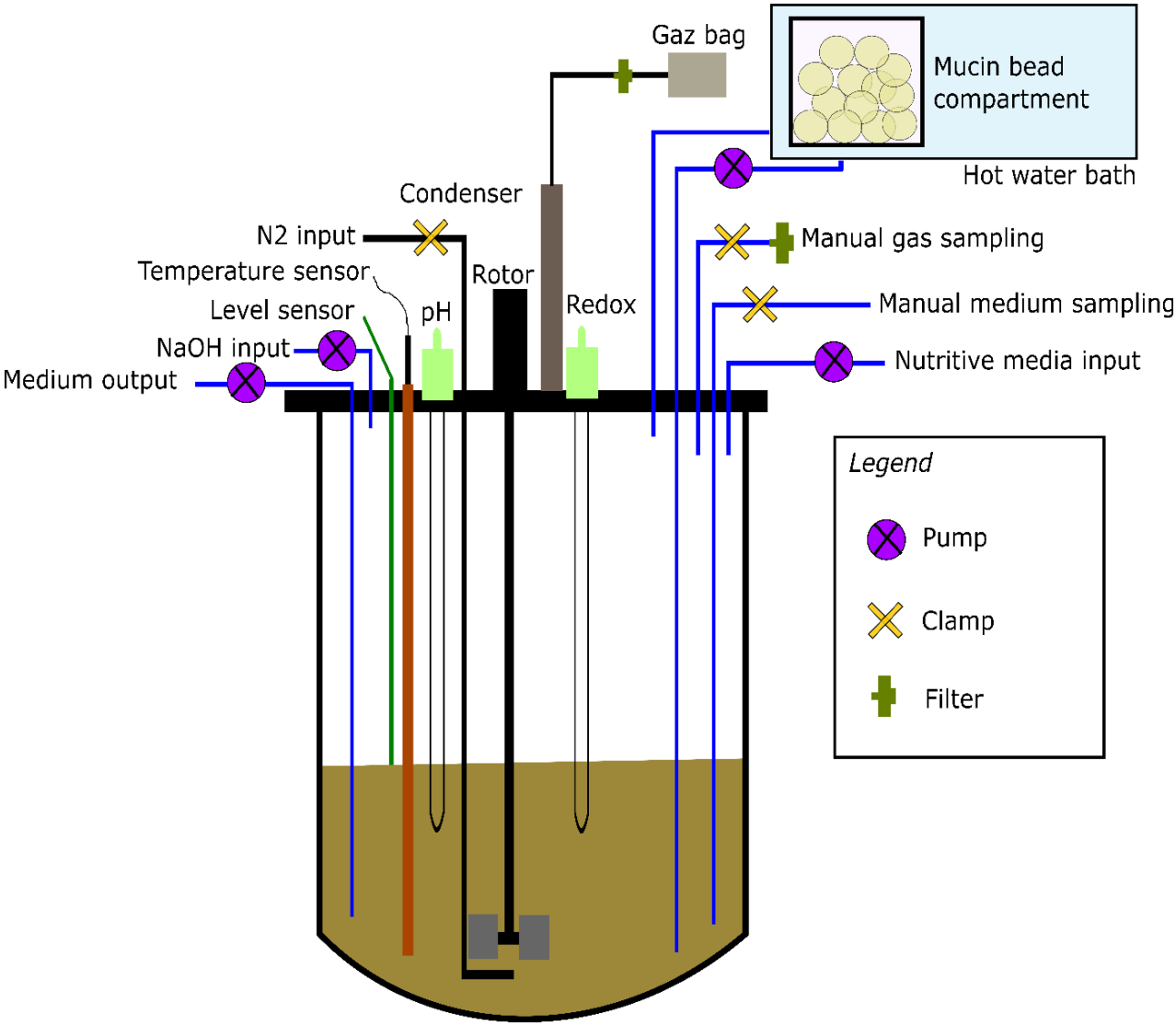


Figure 1. Schematic view of the new mucin implemented *in vitro* of the piglet colon (MPigut-IVM).

A nutritive medium formula containing various sources of carbohydrates, proteins, minerals and vitamins was set up using both the method used by McFarlane et *al.*(G. T. Macfarlane et al., 1998) et and on the basis of ingredients generally found in piglet feed at weaning, digestibility indices of ingredients, the estimated ileal chyme of piglet and the nutrients or growth factors necessary to maintain a viable microbiota in bioreactors (Supplementary Table 1). The fermentation medium was stirred at a constant speed of 300 rpm during the total duration of the experiment. The nutritive medium was continuously introduced into the bioreactors using a peristaltic pump at a speed of 0.17 mL/min. The volume of bioreactors was monitored using a level sensor and maintained at a constant value of 200 mL by automatic withdrawal of the fermentation medium. The constant arrival speed of the nutritive medium inside the bioreactors and the regular withdraw of the fermentation medium ensured a retention time of 18h to mimic the colonic retention time of 4-week old piglets(Wilson and Leibholz, 1981). Anaerobic conditions and gas composition were checked every day by analyzing O_2, CO_2, CH_4 and H_2 produced during the fermentation process in the atmospheric phase of the bioreactors using a HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled with a TCD detector (Agilent Technologies, Santa Clara, USA) and two series columns, Molecular Sieve 5A and Porapack Q (Aligent Technologies, Santa Clara, USA).

Mucin bead production and compartment

Mucin from porcine stomach type II (Sigma-Aldrich, Saint-Louis, Missouri, USA) and sodium alginate (Sigma-Aldrich, Saint-Louis, Missouri, USA) were diluted in sterile distilled water, at a concentration of 5% and 2%, respectively. Sterile sodium bicarbonate 1M was used to adjust the pH at 6.0. To produce mucin beads of 4 mm of diameter, the mucin solution was dropped using a peristaltic pump and a 2.06 x 0.85mm Tygon R3607 pipe (Saint Gobain, Courbevoie, France) at an approximate height of 40cm into a 0.2M solution of sterile $CaCl_2$ under agitation. At the beginning of fermentations, 350 ± 20 mucin beads were introduced in specific glass compartments allowing the circulation of the fermentation medium on its entire length and ensuring the contact of the resident microbiota with the mucin beads. Mucin beads were maintained in the compartments using plastic filters. One mucin bead compartment was connected to one mini-bioreactor medium using pipes and one peristaltic pump. The specific design of the mucin bead compartment is illustrated in figure 2 and an overview of the whole system is shown in figure 3. During the fermentation process, the mucin beads were totally replaced every 48h to ensure a continuous availability of mucin adherent surfaces. During the

time of bead replacement, the medium of the bead compartment was kept under CO_2 flushing to avoid oxygen entrance. The temperature of 39°C was maintained in the mucin bead compartment using a hot water bath.

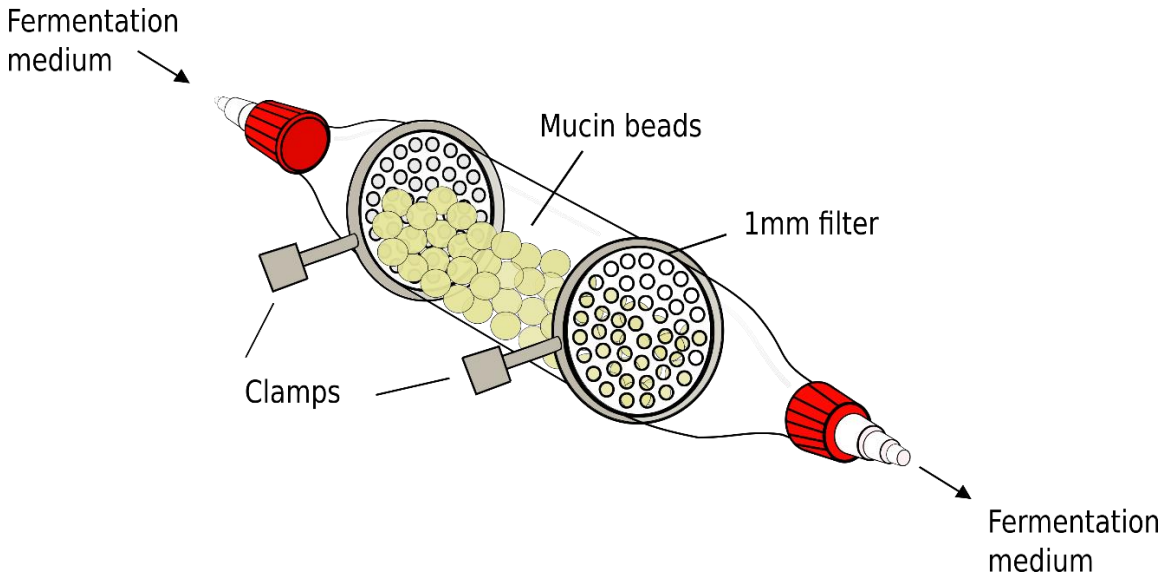


Figure 2. Schematic representation of the mucin bead compartment of the MPigut-IVM.

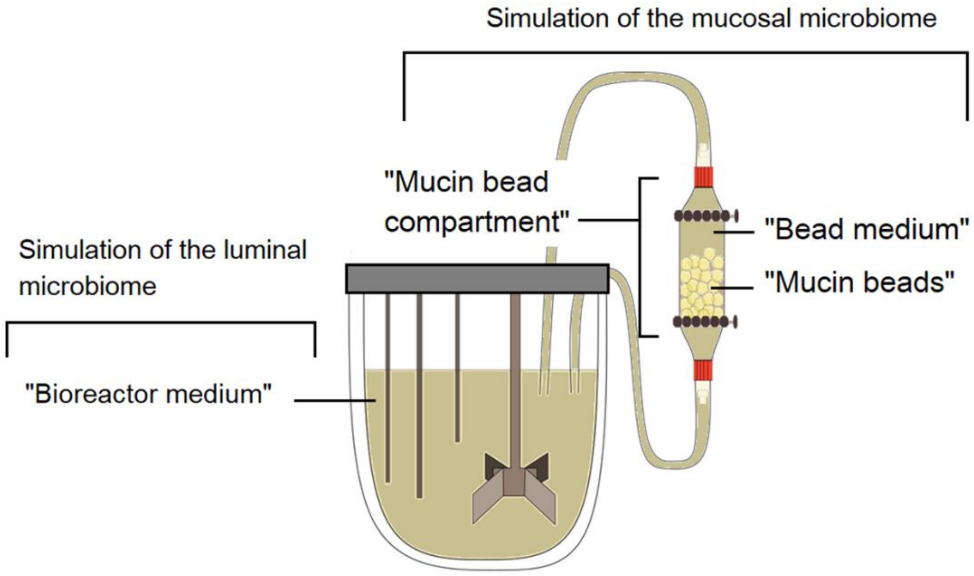


Figure 3. Denomination of the MPigut-IVM samples throughout this publication.

Experimental design of fermentations using the newly developed MPigut-IVM.

Control fermentations

The aim of these fermentation runs was to ensure that a viable and functional resident microbiota, comparable to the microbiota found *in vivo* in the colon of piglets, was maintained inside the fermentation vessels and on the mucin beads for a period of 15 days. As a strong control, both technical and biological replicates were performed without any treatment and to determine the number of days necessary to allow microbial populations to stabilize (figure 4). Control assays lasted 15 days as a compromise between a good stability of microbial communities and the focus on the first few days after weaning.

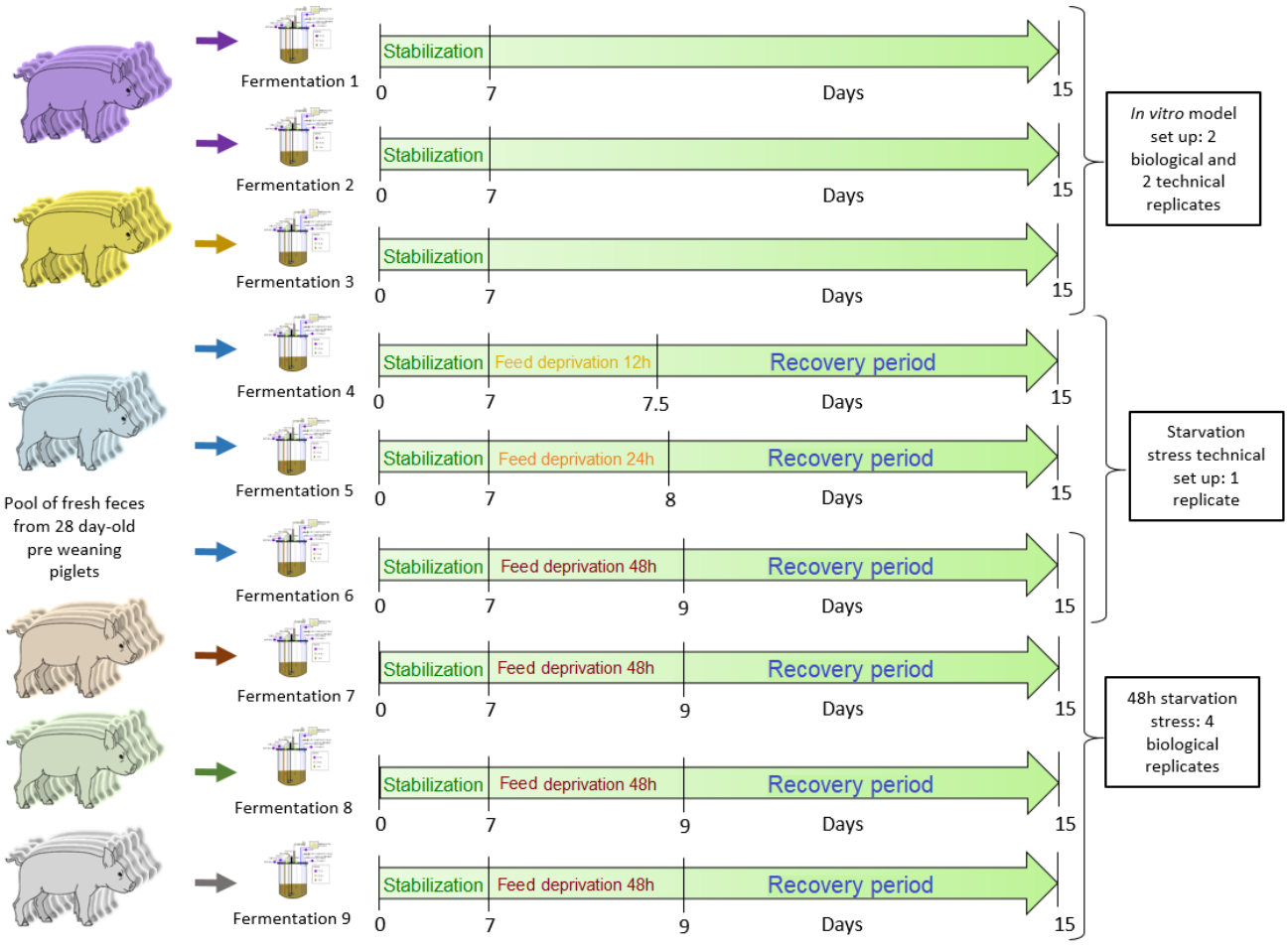


Figure 4. Experimental design of the fermentation experiments performed using the MPigut-IVM.

Application to the simulation of weaning stressors

The MPigut-IVM was then applied in a context of dietary stress, *i.e*. a feed deprivation period that piglets frequently encounter at weaning. To set up the most appropriate duration of the feed deprivation and evaluate its impact, the first experiment consisted in applying a feed deprivation period of 12, 24 and 48h in 3 bioreactors inoculated with the same fecal pooled sample. To simulate feed deprivation, the flow of nutritive medium was interrupted during 12, 24 or 48h seven days after starting the fermentation. The 48h feed deprivation experiment was in total repeated 4 times with 4 different fecal inocula and fermentations were numbered from 1 to 4 (figure 4).

Sampling of the MPigut-IVM

Samples from bioreactor mediums also referring to the luminal microbiota were collected every day. As mucin beads and bead medium were collected every 48h, no sampling was carried out during the feed deprivation periods but before (day 7) and after (day 9) (figure 4). Samples from the bioreactor medium and bead medium were centrifuged at 4°C, 10 000g and for 45 min. Pellets and supernatants were respectively stored at -20 and -80°C until analysis. After collection, mucin beads were gently washed 3 times in sterile 1X PBS and stored at -20°C.

Observation of the mucin beads by scanning electron microscopy (SEM)

To control that microorganisms were able to adhere to the bead surface during the fermentation process, some beads were sampled after a 48h stay in the mucin bead compartment during a control assay. Beads were gently washed 3 times in sterile PBS 1X. Then, they were fixated by immersion at 4°C overnight in a 0.2M sodium cacodylate (pH 7.4) buffer containing 2% glutaraldehyde. After fixation, mucin beads were dehydrated using successive 20 min incubations in ethanol 70, 95 and 100%. A final dehydration step included 20 min incubation in a mix of ethanol 100% and hexamethyldisilazane. Samples were dried overnight under a chamber and stained with gold-palladium deposition. SEM images were taken with a JEOL 6060 Low vacuum microscope (Jeol Europe SAS, Croissy sur Seine, France) at the Centre Imagerie Cellulaire Santé (CICS) from Clermont-Auvergne University.

DNA extraction from MPigut-IVM samples

Total DNA was extracted from all samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The quality of the eluted DNA was assessed by agarose gel electrophoresis. Extracts were quantified using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Samples were stored at −20 °C prior to analysis.

Quantification of bacterial groups and methanogenic archaea populations by qPCR

The list of primer pairs and their optimal conditions used for quantitative PCR are presented in Supplementary Table 2. Real-time PCR assays were performed on a Rotor-Gene Q (Qiagen, Venlo, NL) in 20 µL reactions with QuantiFast SYBR GREEN master mix (Qiagen, Venlo, NL) or Taqman Fast Advanced Master mix (Applied Biosystems, Foster City, California, USA) with the additions of each primer at their optimal concentration (Supplementary Table 2). The 16S rDNA genes were amplified using the following program: 2 min denaturation at 95°C and 10 min denaturation at 95°C; 40 and 45 cycles of 20s at 95°C and 60s elongation and extension at the optimum annealing temperature and, when performing SYBR GREEN based assay, a melt curve step from 60°C to 95°C. After log transformation of the data, a mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used to compare the number of 16S gene copy per g of samples between days of fermentation using the R packages lme4 and car. (Cordonnier, 2015; Huijsdens et al., 2002; Mohamed et al., 2004; Ohene-Adjei et al., 2008; Yu et al., 2005; Zwielehner et al., 2011)

MiSeq 16S rDNA sequencing and bioinformatic analysis

The DNA concentration of all samples was measured using the Qubit dsDNA High Sensivity Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and diluted to 2 ng/µL prior to PCR amplification. The Bacterial V3-V4 region of 16S rDNA and the Archaeal 16S rDNA were respectively amplified with primers 357F 5'-CCTACGGGNGGCWGCAG-3'(Yu et al., 2005) and 805R 5'-GACTACHVGGGTATCTAATCC-3'(Lane et al., 1985a) and primers 349F 5'-GYGCASCAGKCGMGAAW-3' and 806R 5'-GGACTACVSGGGTATCTAAT -3'(Ohene-

Adjei et al., 2008). Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system (Illumina, San Diego, CA, USA) performed at the Carver Biotechnology Center of the University of Illinois (Urbana, IL, USA). The demultiplexed paired end Illumina sequence reads in the FastQ format were uploaded into the Galaxy instance (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr) to be used in the FROGS (Find Rapidly OTU with Galaxy Solution) pipeline(Escudié et al., 2018). During the FROGS pre-process, sequences were depleted of barcode and the sequences with a non-appropriate length or containing ambiguous bases were removed. Next, reads were clustered into de novo operational taxonomic units (OTUs) using SWARM algorithm(Mahé et al., 2014) with, at first, a denoising step to build very fine cluster using the minimal distance equal to 1 and, secondly, with an aggregation distance equal to 3. Chimeras were then detected and removed with VSEARCH(Rognes et al., 2016). Additionally, filters were applied to the OTUs in order to remove singletons(Auer et al., 2017; Bokulich et al., 2013). The OTUs selected were taxonomically assigned using the Silva release 132 reference database(Quast et al., 2013).

Statistical analysis of sequencing data

Statistical analysis was processed using the RStudio software version 1.0 (with R software version 3.5.1, R Development Core Team, http://www.R-project.org). OTU structure and composition analyses were performed using the phyloseq R package(McMurdie and Holmes, 2013). Visualization of data was performed using the ggplot2 R package. Following log transformation of the data, univariate statistical analysis was performed on taxa relative abundance with the R packages lme4 and car. A mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used. Prior to alpha and beta diversity calculations, rarefaction using the transform counts methods was applied to the dataset. Alpha diversity indices were calculated using a Wilcoxon test with the following indices: Inverse Simpson index, Chao 1 index, number of observed OTU phylogenetic diversity (PD) and Shannon index. Statistical differences in Bray Curtis distance between the mucin beads and the bioreactor medium were tested using a multi-analysis of variance (MANOVA) performed with ADONIS using the vegan R package with 9999 permutations and represented by principal coordinate analysis (PCoA) plots. Statistical comparisons of samples from the stabilization and recovery phases of the fermentation 6, 7, 8 and 9 were performed using the Wald test of the DESeq2 R

package at the genus level. In all statistical analyses, only *p*-values below 0.05 were considered as significant.

Quantification of short chain fatty acids (SCFAs) by gas chromatography

The SCFAs were quantified in the bioreactor medium and bead medium by gas chromatography in order to determine concentrations of acetate, propionate, isobutyrate, butyrate, caproate, isovalerate and valerate. Eight hundred µL of fermentation supernatants were mixed with 500 µL of 0.4% (w:v) crotonic acid and 2% (w:v) metaphosphoric acid solutions. This mixture was centrifuged again and the supernatant obtained was injected in a PerkinElmer Clarus 580 gas chromatograph (Waltham, Massachusetts, USA) for quantification of SCFAs. A mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used to compare the concentration of the main SCFAs between days of fermentation using the R packages lme4 and car.

Metabolite analysis by ^{1}H nuclear magnetic resonance (NMR)

Supernatants of the bioreactor medium, bead medium of the fermentations #6, 7, 8 and 9 were used for metabolomic profiling using NMR spectroscopy. Fermentation supernatants were centrifuged twice (18 000g, 4°C, 10 min) to remove particles. Fifty µL were mixed with 600µL NMR buffer composed of sodium phosphate 0.2 M, pH 7.4, trimethylsilylpropanoic acid 1 mmol/L, 80% deuterated water, and 20% water. The samples were homogenized, centrifuged and 600 µL were transferred to 5-mm NMR tubes. All NMR spectra were obtained with an Avance III HD NMR spectrometer operating at 600.13 MHz for ^{1}H resonance frequency using a 5 mm inverse detection CryoProbe (Bruker Biospin, Rheinstetten, Germany) in the MetaboHUB-MetaToul-AXIOM metabolomics platform (Toulouse, France). ^{1}H NMR spectra were acquired at 300 K using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence with presaturation. Pre-processing of the spectra (group delay correction, solvent suppression, apodization with a line broadening of 0.3 Hz, Fourier transform, zero order phase correction, shift referencing on TSP, baseline correction, setting of negative values to zero) was performed in the galaxy tool Workflow4Metabolomics following guidelines(Giacomoni et al., 2015). After water region (4.5 – 5.1 ppm) exclusion, spectra (0.5 – 9 ppm) were bucketed (0.01 ppm bucket width) and normalized by total area in Workflow4Metabolomics. Representative

samples were characterized by 2D NMR experiments (1H-1H COSY and 13C-1H HSQC). For metabolite identification, 1D and 2D NMR spectra of pure compounds prepared in the same buffer and acquired with the same spectrometer were overlayed with samples spectra. Annotated representative spectra are presented in supplemental figure 1. For each identified metabolite, buckets non-overlapping with other metabolites were selected for the quantification (supplemental table 4).

Statistical analysis for NMR metabolomics were performed using the R software (version 3.5.1). P artial-least square discriminant analysis (PLS-DA) were performed with mixOmics package (Rohart et al., 2017). Metabolites relative concentrations was used as variable matrix (X). For PLS-DA, time points (days of fermentation) were used as predictors (Y) and time-repeated measurement were considered by using a multilevel approach. Univariate statistical analysis was also performed on each metabolite relative concentration with the R packages lme4 and car. A mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used. A post-hoc test was used to compare the mean relative concentrations with Tukey correction. P-values were corrected for multiple testing (false discovery rate). Spearman correlation was used to correlate the *in vitro* metabolome with the 20 most abundant families of the MPigut-IVM using the cor function of the R software. *P*values were corrected for multiple testing using the FDR method and the package Hsmic on the R sofltware.

RESULTS

Set-up and validation of the MPigut-IVM (fermentations #1, 2 and 3)

Microbial activity of the piglet colon is successfully maintained in the MPigut-IVM

Redox potential was recorded every 300 s during the 15 days of the fermentation process (fermentation #1, 2, 3). During the first 24 hours of fermentation, the redox potential quickly decreased from the starting value of approximately -150mV to the average value of -261± 7 mV.

Fermentations shown string anaerobic conditions and displayed mean percentages of H_2, O_2, CO_2, N_2 and CH_4 respectively equal to 1.6 ± 2, 0.8 ± 0.5, 61.5± 5.7, 24.5 ± 8.4 and 11.7 ± 3.7 for the fermentations #1, 2 and 3 (figure 5).

The general SCFA profile remained close to that measured in the fecal inoculum (Supplementary figure 2A) and displayed mean relative percentages of 57.9 ± 1.2, 26.2 ± 1.2 and 16 ± 0.3 respectively for acetate, propionate and butyrate (figure 6). The average total SCFA concentration from day 7 to day 15 was 259.35 ± 16.59 mmol/L for the fermentations #1, 2 and 3.

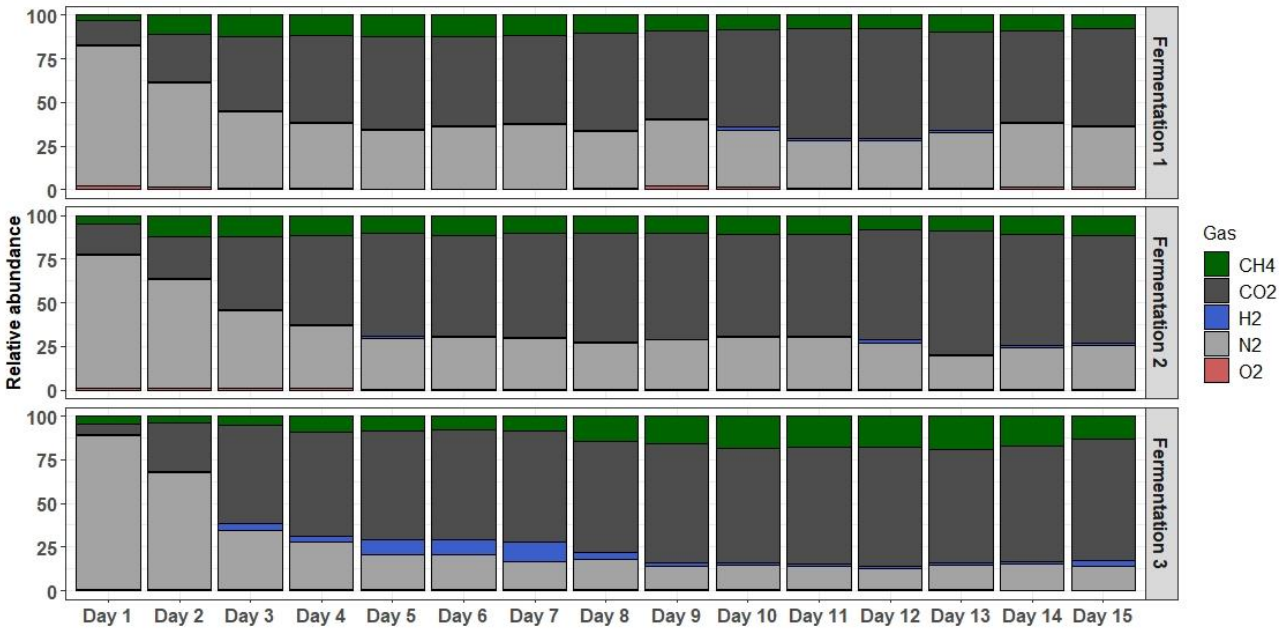


Figure 5. Relative abundance of gas produced by fermentation activity of the microbiota inhabiting the MPigut-IVM during control assays.

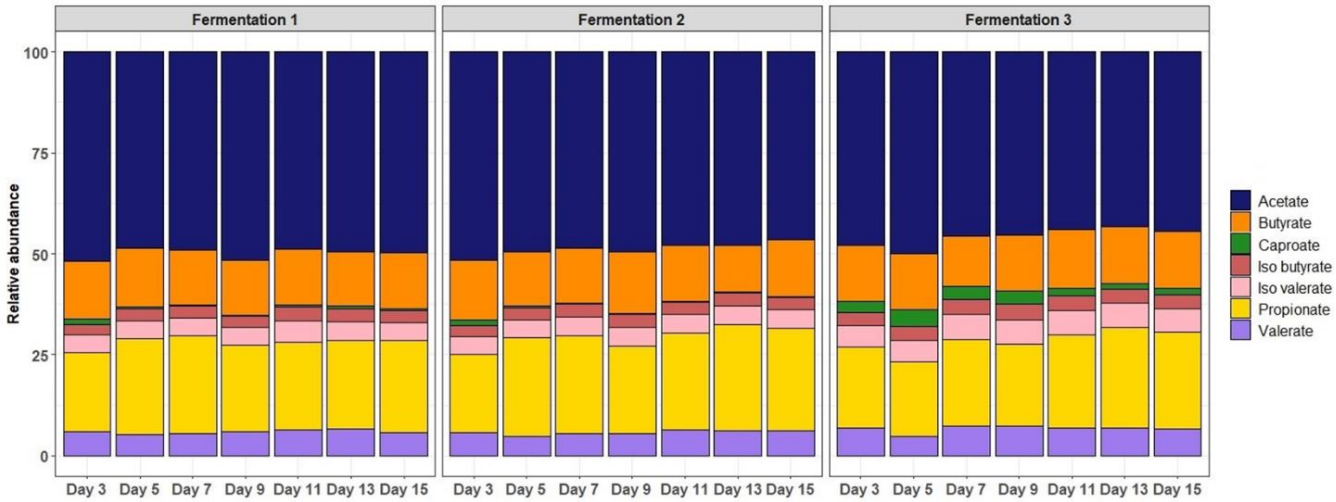


Figure 6. Short chain fatty acids (SCFA) relative abundance produced by fermentative activity of the microbiota inhabiting the MPigut-IVM during control assays in the bioreactor medium.

The MPigut-IVM harbors an abundant and taxonomically diversified microbiota

SEM observation of mucin beads incubated during 48h, corresponding to the time mucin beads stayed in the mucin bead compartment of the MPigut-IVM, confirmed the presence of an abundant adherent microbial population at the surface of the beads. A strong degradation of the bead surface was observed compared with non-incubated mucin beads (figure 7).

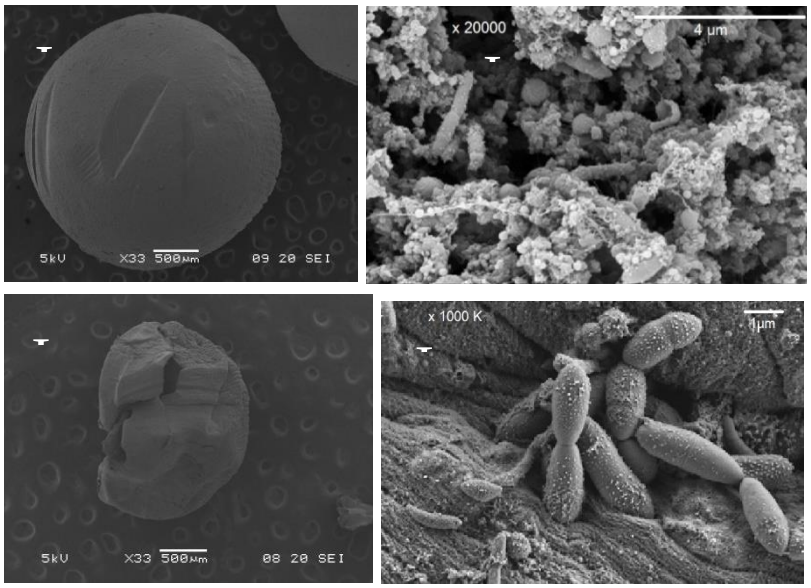


Figure 7. Evolution of the structure and colonization of a mucin bead: before (I) and after (II) 48h of incubation in the MPigut-IVM during the fermentation #1 and observation of the specific adherent microbiota at high magnitude (III & IV) by scanning electron microscopy.

QPCR results showed that compared to fecal inoculum *Bacteroidetes* level was increased in the bioreactor medium (Supplementary figure 2B). After day 7, *Firmicutes, Bacteroidetes, Escherichia/ Shigella* group, *Clostridium* cluster IV and *Clostridium cluster* XIVa populations were quantified at an average of 10.8 ± 0.4, 10.3 ± 0.4, 10.2 ± 0.4, 7.6 ± 1.3, 6 ± 0.7 and 10.2 ± 0.6 $\log_{10}$ copies of 16S rDNA / g of samples, respectively, in the bioreactor medium for the fermentations #1, 2 and 3. On mucin beads, after stabilization of populations, *Firmicutes, Bacteroidetes, Clostridium* cluster IV and *Clostridium cluster* XIVa groups mean abundances in mucin beads were of 9.2 ± 0.4, 8.2 ± 0.3, 8.7 ± 0.8 and 6.7 ± 1.2 $\log_{10}$ copies of 16S rDNA / g. *Clostridium cluster* XIVa population dramatically decreased across time. The level of total bacteria in the mucin beads was at the average of 9.4 ± 0.3 $\log_{10}$ copies of 16S rDNA / g, lower than the 10.8 ± 0.4 $\log_{10}$ copies of 16S rDNA / g of total bacteria observed in the bioreactor medium (Supplementary Figure 3).

The most abundant bacterial phyla in the bioreactor medium of MPigut-IVM were *Bacteroidetes* (51.3 ±11.2 %), *Firmicutes* (37.6 ± 9.5 %), and *Proteobacteria* (6.5 ±3.9%) (Supplementary figure 4A). On the mucin beads, the *Firmicutes* mean relative abundances was 72.1 ± 12.7, followed by *Proteobacteria* at 10.2 ± 6.7 %, followed by the *Bacteroidetes* and *Actinobacteria* phyla at respectively 7.7 ± 4.9, and 6.8 ± 4.6, from day 7 to day 15 (Supplementary figure 4B). At a lower taxonomic level, the MPigut-IVM was principally composed of *Bacteroidaceae, Lachnospiraceae, Prevotellaceae* and *Acidaminococcaceae* families in the bioreactor medium wheras some families predominant in the fecal inocula were exclusively found in the mucin beads such as *Clostridiaceae 1, Peptostreptococcaceae* and *Clostridium* Family XIII (figures 8). In the bioreactor medium, *Prevotellaceae* was found significantly lower on day 7 compared to all the other days. In the mucin beads, *Clostridium* Family XIII and *Coriobacteriaceae* were found significantly respectively higher and lower on day 7 compared to all the other days. No other significant differences were observed on other families as an evidence of the good stability of bacterial populations. Interestingly, an archaeal community was successfully maintained in the MPigut-IVM and was exclusively composed of members of the *Euryarchaeota* phylum including the families *Methanobacteriaceae* and *Methanomethylophilaceae* (supplementary figure 5). The fecal inocula were also exclusively composed of these two archaeal families (Supplementary figure 2).

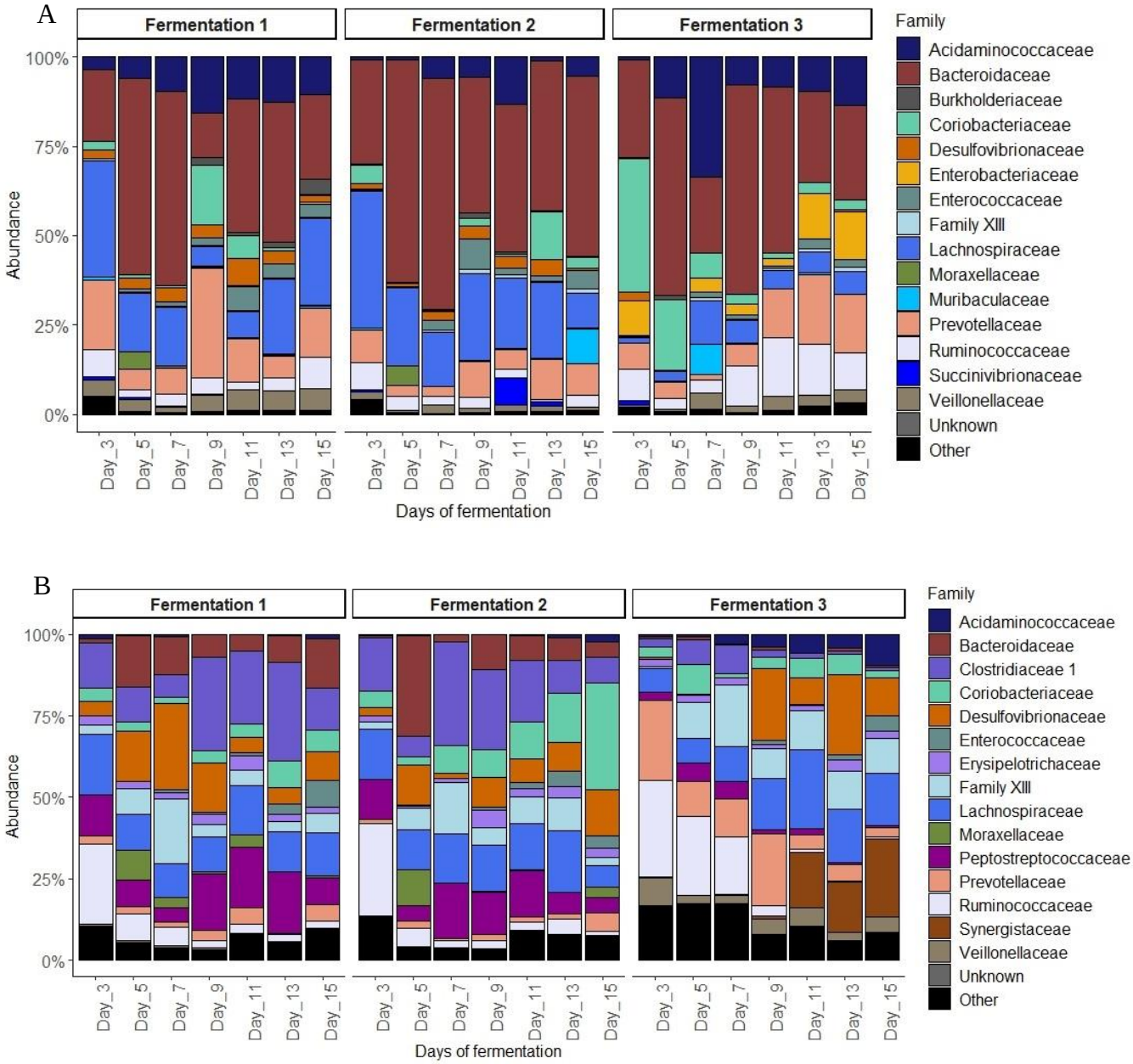


Figure 8. Relative abundance of the 15 principal bacterial families in the bioreactor medium (A) on the mucin beads (B) of the MPigut-IVM during control assays measured by 16S Illumina sequencing.

During the first 5 days, the Shannon diversity index, decreased from a mean value of 4 in the fecal inocula (Figure 9A) to mean values from 2.9 to 3.4 from day 7 until day 15 in the bioreactor medium. The observed number of OTUs were maintained at around 600 from day 7 to 15 in the bioreactor medium. On mucin beads, the observed number of OTUs and the Shannon index ranged respectively between 300 and 600 and between 2.4 and 3.5 from day 7

to the end of the fermentation runs (Figure 9B). Principal coordinate analysis (PCoA) on Bray Curtis distance showed that mucin beads and bioreactor medium as well as fecal inocula formed a distinct cluster (figure 10).

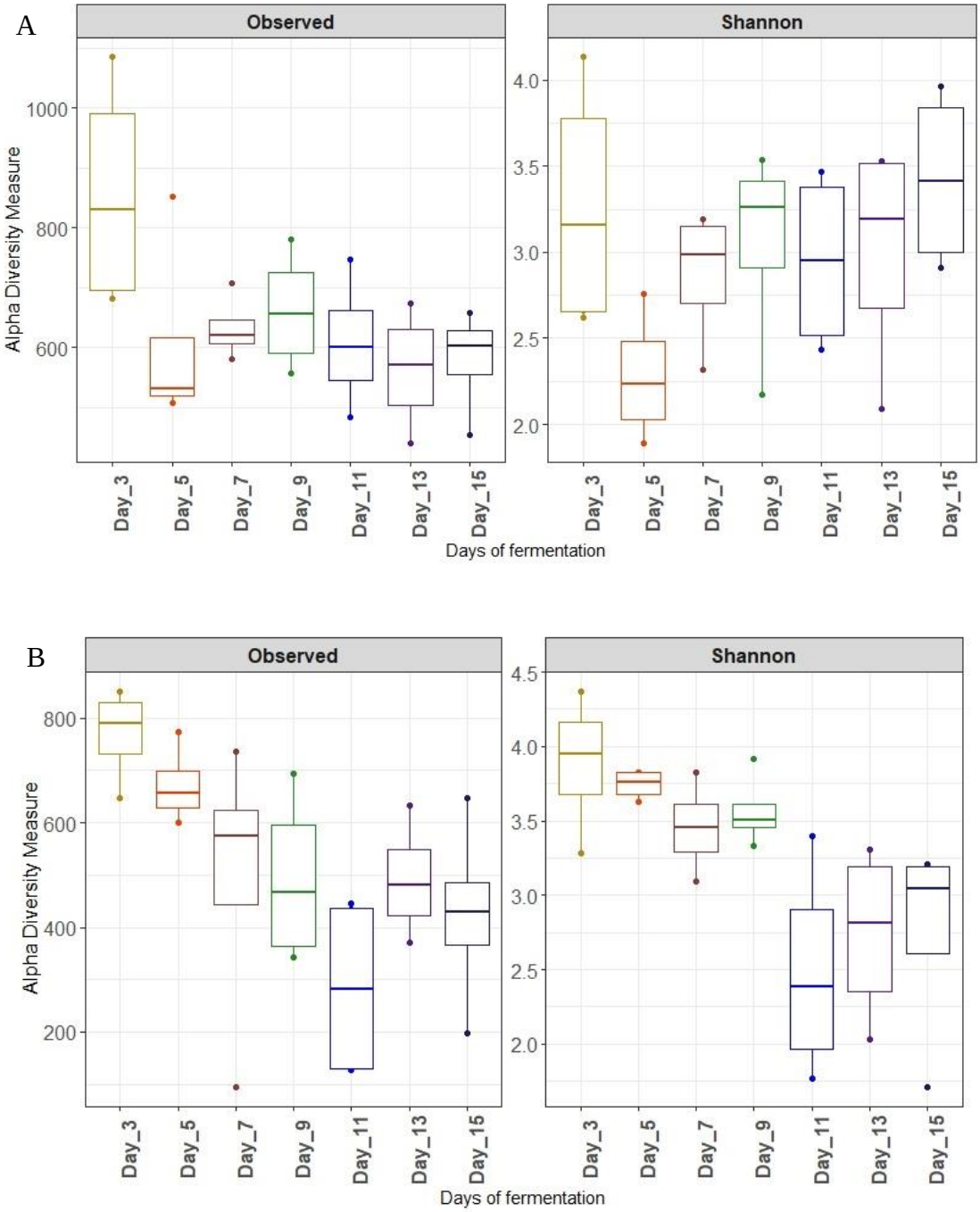


Figure 9. Alpha diversity indices based on bacterial OTUs in the bioreactor medium (A) on the mucin beads of the MPigut-IVM (B) during the fermentations #1, 2 and 3 (n = 3 for each time point).

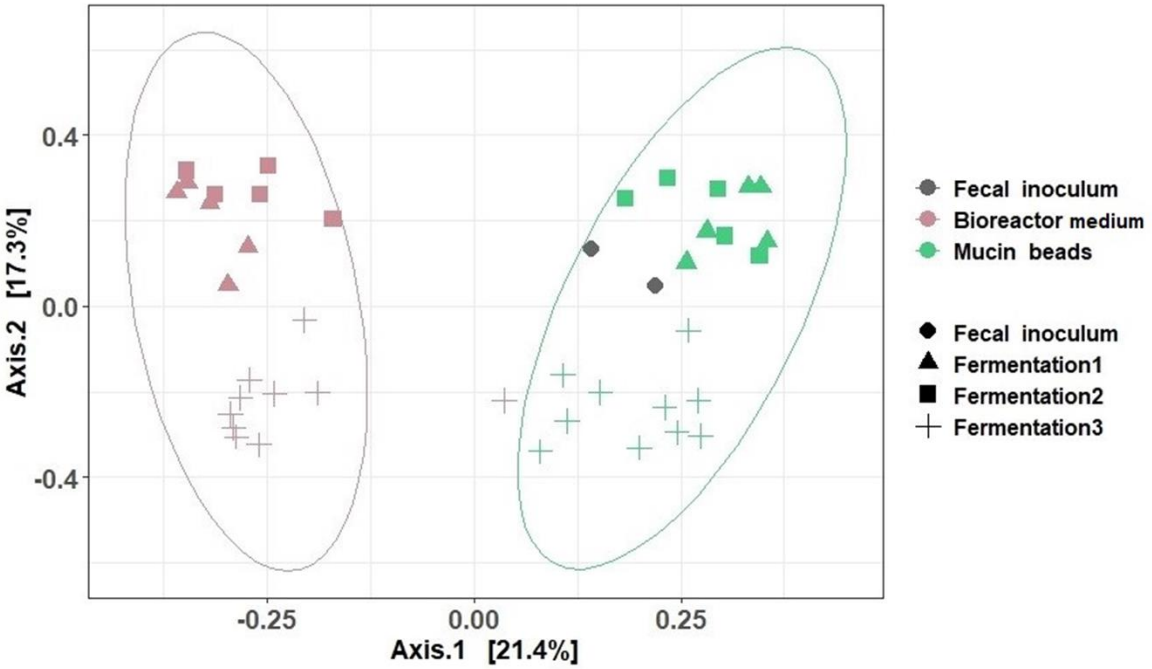


Figure 10. Principal component analysis (PCoA) plot with Bray-Curtis dissimilarity on the bacterial communities in the bioreactor medium and on the mucin beads of the MPigut-IVM from the end of stabilization phase (day 7) to the end of fermentation (day 15) of the fermentations #1, 2 and 3.

The stabilization phase is achieved after 7 days of fermentation

In *in vitro* continuous fermentation models, it is necessary to set up a stabilization period during which no treatments should be applied in order to make the transition from *in vivo* to *in vitro* conditions as well as the transition from a fecal to a colonic environment. Regarding the quantification of bacterial populations, the measurement of SCFA, gas composition, and the Illumina sequencing data of the fermentations #1, 2 and 3 stated above, a stabilization period of 7 days was the best compromise for the MPigut-IVM.

The microbiota of the MPigut-IVM is close to the in vivo microbiota of piglet proximal colon

The abundance of the 15 top bacterial families from all the bioreactor medium and mucin beads samples at day 7, were compared to *in vivo* colonic luminal and mucosal samples from 5 healthy 28-day-old piglets. In the bioreactor medium, the most abundant families retrieved, in particular *Veillonellaceae, Prevotellaceae, Muribaculaceae, Ruminococcaceae, Lachnospiraceae,*

Bacteroidiaceae and *Acidaminococaceae* were those also found in the *in vivo* colonic samples. *Tannerellaceae, Lactobacillaceae* and *Christensenellaceae* seemed to be overrepresented in the MPigut-IVM compared to *in vivo* (Figure 11). The *in vitro* microbiota communities of mucin beads showed a high similarity with *in vivo* samples from the proximal colon mucosa of piglets as visualized through Heatmaps (figure 12). Nevertheless, the *Atopobiaceae* and *Enterococcaceae* displayed a higher abundance, and *Campylobacteraceae* lower relative abundance, in *in vitro* mucin beads compared with *in vivo* mucobiome. Analysis of the *in vivo* microbiota also highlighted the variability between individuals which is kept in the MPigut-IVM.

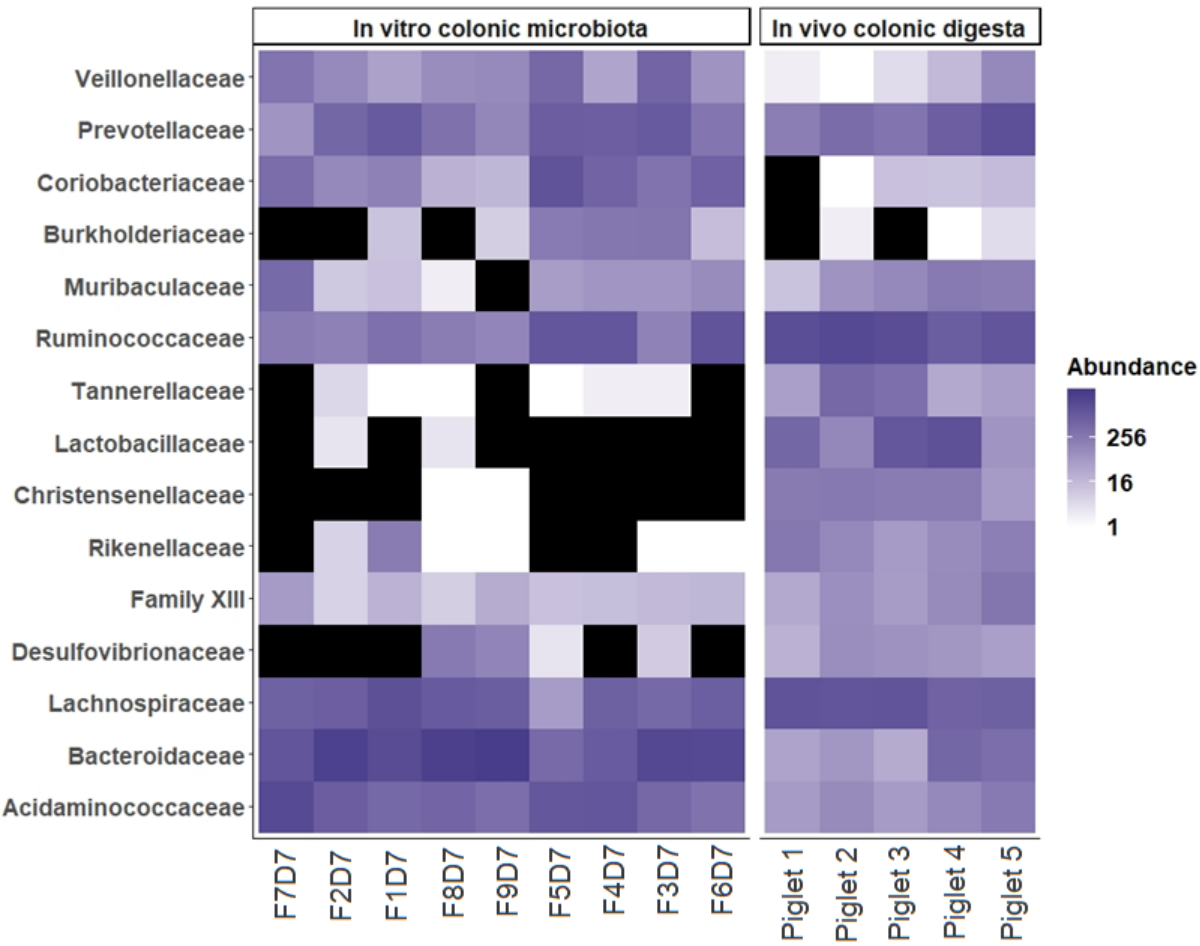


Figure 11. Comparison of the top 15 bacterial families between in vivo proximal colon luminal samples and in vitro fermentation samples from the bioreactor medium at day 7 corresponding to the end of the stabilization phase. The black colour corresponds to an abundance of 0. On the x axis: “F” = “Fermentation” and “D” = “Day”.

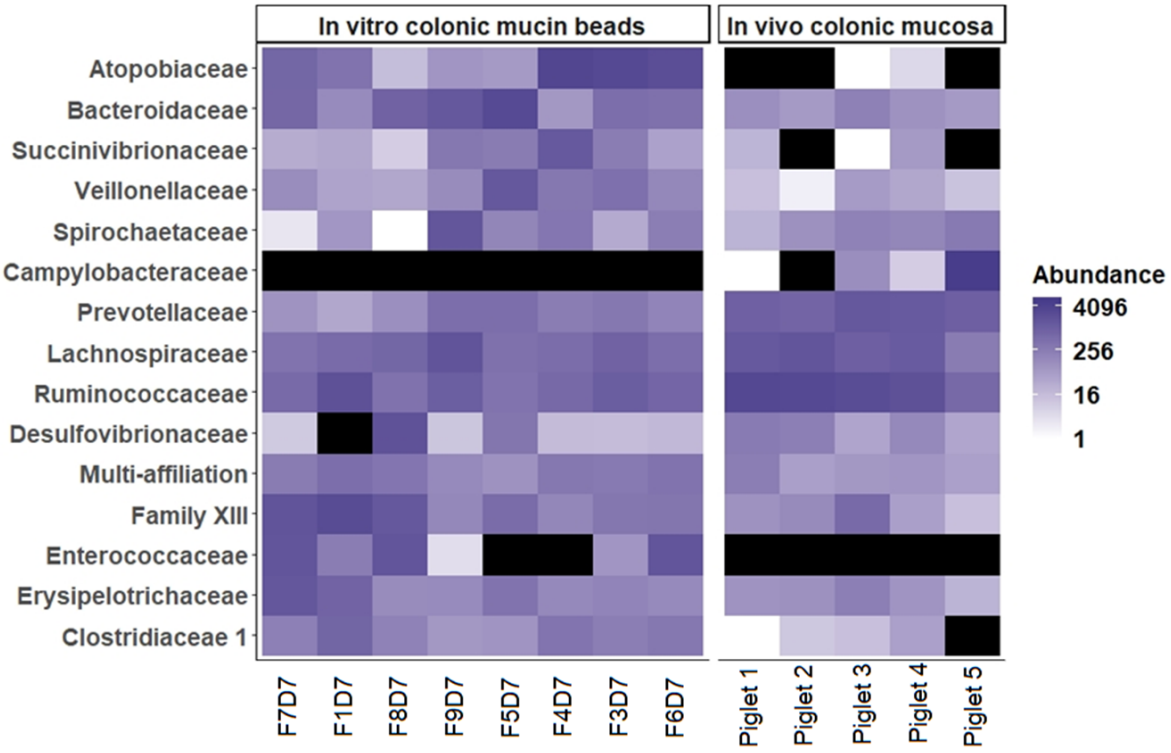


Figure 12. Comparison of the top 15 families between in vivo proximal colon mucosal samples and in vitro mucin beads at day 7 corresponding to the end of the stabilization phase. The black colour corresponds to an abundance of 0. On the x axis: "F" = "Fermentation" and "D" = "Day".

A feed deprivation stress triggers microbial dysbiosis in the MPigut-IVM

In order to better understand the etiology of microbial dysbiosis in post-weaning piglets, the effects of a feed deprivation stress were evaluated using the newly developed MPigut-IVM.

Effects of 12h, 24h and 48h feed deprivation stresses on the microbial composition

The fermentations #4, 5 and 6 were inoculated using the same fecal inoculum and were subjected to feed deprivation periods of 12, 24 or 48h, respectively. Due to the absence of replicates no statistics were performed on this section. Applications of gradual feed deprivation stress induces gradual modifications of the redox potential and bacterial composition. For instance, a gradual increase of *Enterobacteriaceae* was detected by qPCR and 16S sequencing both in the mucin bead and the bioreactor (supplementary figure 6 and figure 13).

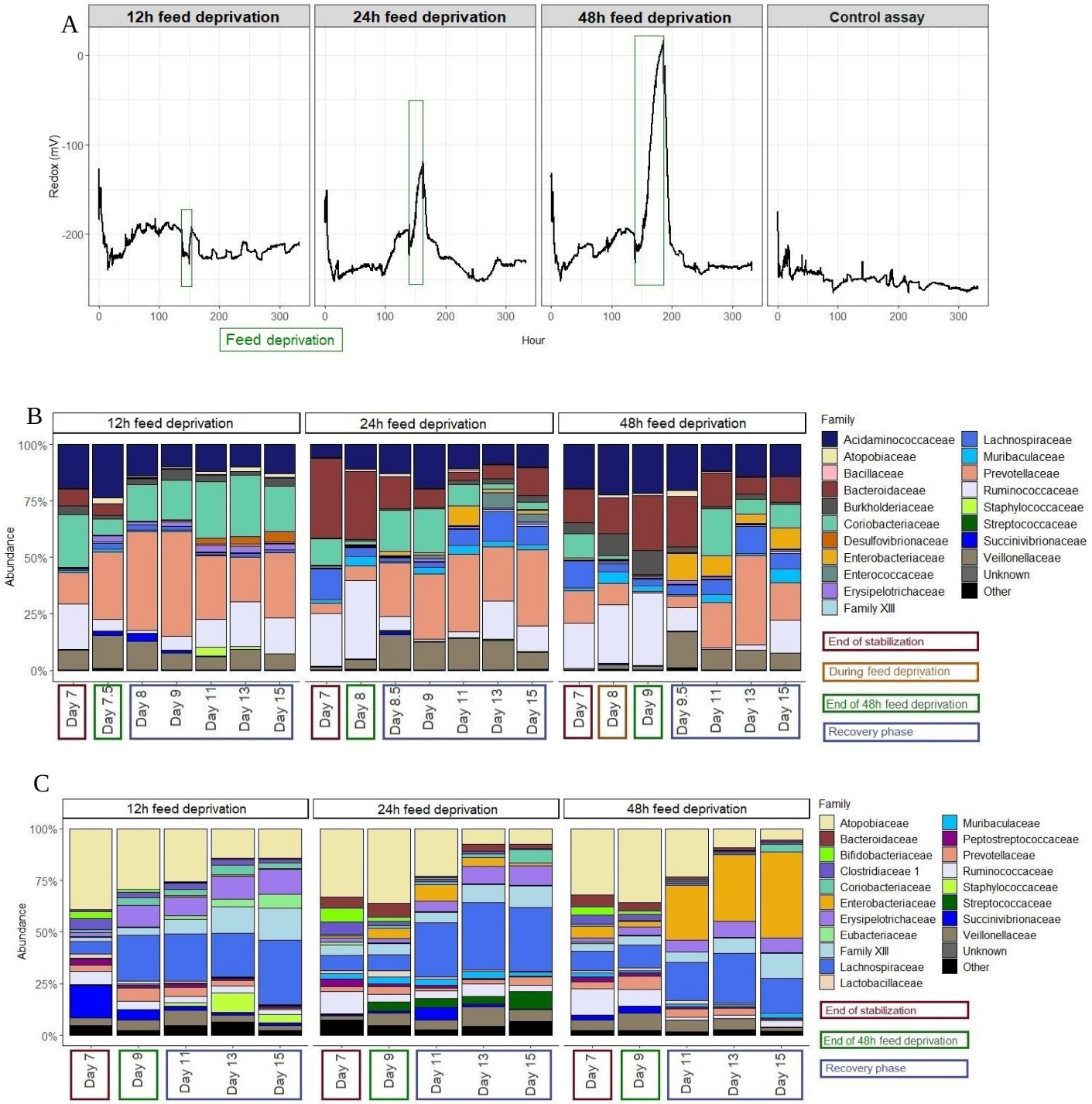


Figure 13. Effects of a feed deprivation stress of 12, 24, and 48h on the MPigut-IVM microbiota composition. On this figure, the "12h feed deprivation stress", "24h feed deprivation stress" and "48h feed deprivation stress" correspond to the fermentations #4, 5 and 6, respectively. (A) Evolution of the redox potential. Effect of a feed deprivation period of 12, 24 or 48 h on the relative abundance of the main bacterial families in the bioreactor medium (B) and on the mucin beads (C) of the MPigut-IVM as measured by 16S Illumina sequencing.

A 48h feed deprivation stress impacts the* in vitro *microbiota composition and activity

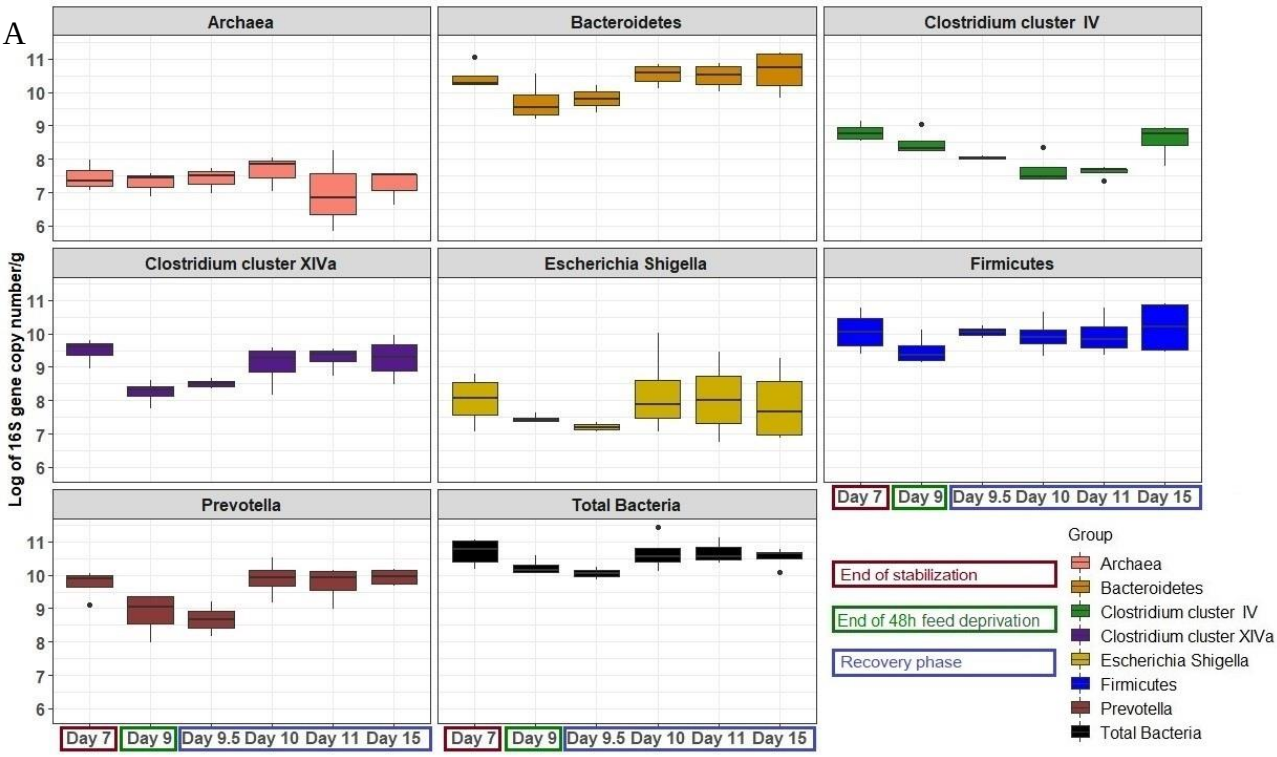
The fermentations #6, 7, 8 and 9 were inoculated with different fecal inocula (n=4) and were subjected to a 48h feed deprivation from day 7 to 9 of the fermentation runs.

QPCR quantification of bacterial and archaeal groups

In the bioreactor medium, compared to day 7, Prevotella, *Clostridium* cluster IV and *Firmicutes* groups were significantly reduced from day 9 to 11, *Clostridium* cluster IV and *Bacteroidetes*, were significantly reduced by from day 7 to day 15 (Figure 14A). The abundance of the Escherichia-*Shigella* group was quite variable between the four fermentation runs but a clear augmentation of the abundance of this group from 7.6 and 7.4 at day 9 to 9.3 and 8.3 at day 15 respectively in the fermentation # 6 and 7.

On mucin beads, compared to day 7, *Prevotella* group was significantly reduced at day 9 and *Clostridium* cluster IV displayed significantly lower values at day 11 and even at day 15. *Escherichia/Shigella* group was increased on the mucin beads at day 11, though no significantly (Figure 14B).

A



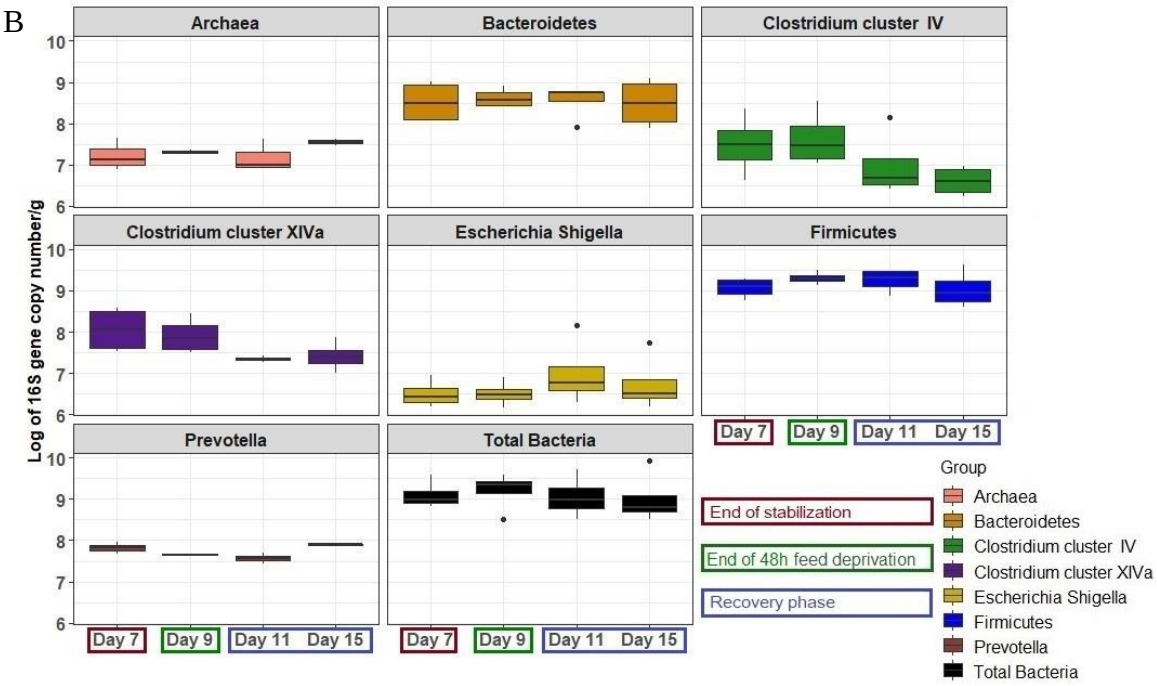


Figure 14. Quantification of bacterial and methanogen archaea in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during the fermentations #6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h (n = 4 for each time point).

Effects on the in vitro *microbiota using Illumina MiSeq sequencing technology*

Firmicutes relative abundance increased from 40.4 ± 9 % to 50.4 ± 10.4 % and *Bacteroidetes* relative abundance decreased significantly from 51.2 ± 14.2 % to 43.7 ± 14.5 % from day 7 to day 9. On the mucin beads, no significant differences were observed at the phylum level (Supplementary figure 7B). Compared to day 7, relative abundance of *Ruminococcaceae* and *Prevotellaceae* families were significantly reduced at the end of the feed deprivation period (*p*-value < 0.05) (day 9), *Prevotellaceae* family was significantly increased after the feed deprivation period (day 10), *Bacteroidiaceae* significantly increased at day 9 but significantly decreased at day 10 and 11, *Veillonellaceae* were significantly increased after the feed deprivation period until day 11, and *Coriobacteriaceae* and *Atopobiaceae* families were significantly more abundant at day 9. At day 15, the previously cited families recovered values similar to day 7 (figure 15A). The *Lachnospiraceae* family was significantly decreased from day 9 to day 15, compared to day 7 (figure 15A). On the mucin beads, the relative abundance of *Peptostreptococcaceae, Clostridiaceae 1, Ruminococcaceae* and *Veillonellaceae* families were lower from day 11 and day 15 compared to day 7 and day 9 (*p*-value < 0.05) (figure 4 D).

The *Enterobacteriaceae* family relative abundance showed a strong increase both on the mucin beads and in the bioreactor medium during the whole recovery period (day 9.5 to day 15) of the fermentations #6 and 7 (Figure 4 C & D). Interestingly, this family was also more abundant in the fecal inocula of the fermentations #6 and 7 compared to the fecal inocula of the fermentations #8 and 9 (see Supplementary figure 2D).

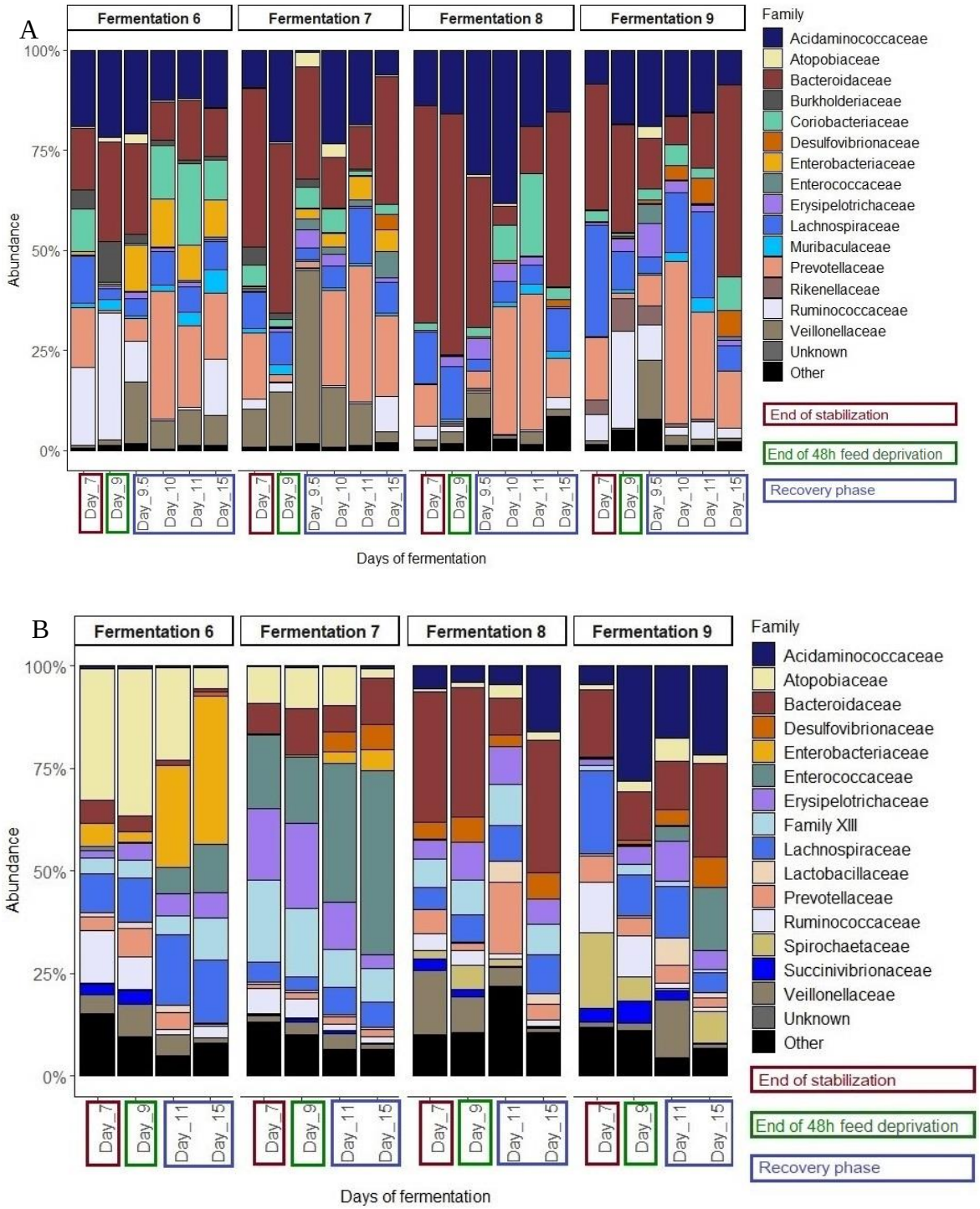


Figure 15. Relative abundance of the 15 main bacterial families in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during the fermentations

#6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h, as measured by 16S Illumina sequencing.

DESEQ2 analysis between day 7 and all the time points of the recovery period, showed that *Escherichia-Shigella* group was significantly more abundant after the feed deprivation stress along with *Pyramidobacter, Succiniclasticum, Bacillus, Mitsuokella* genera and several members of the *Lachnospiraceae* family, notably (figure 16).

Alpha diversity indices calculated for both the bioreactor medium and the mucin beads displayed variable values depending on the fermentation numbers but no statistical difference was observed (Supplementary figure 9).

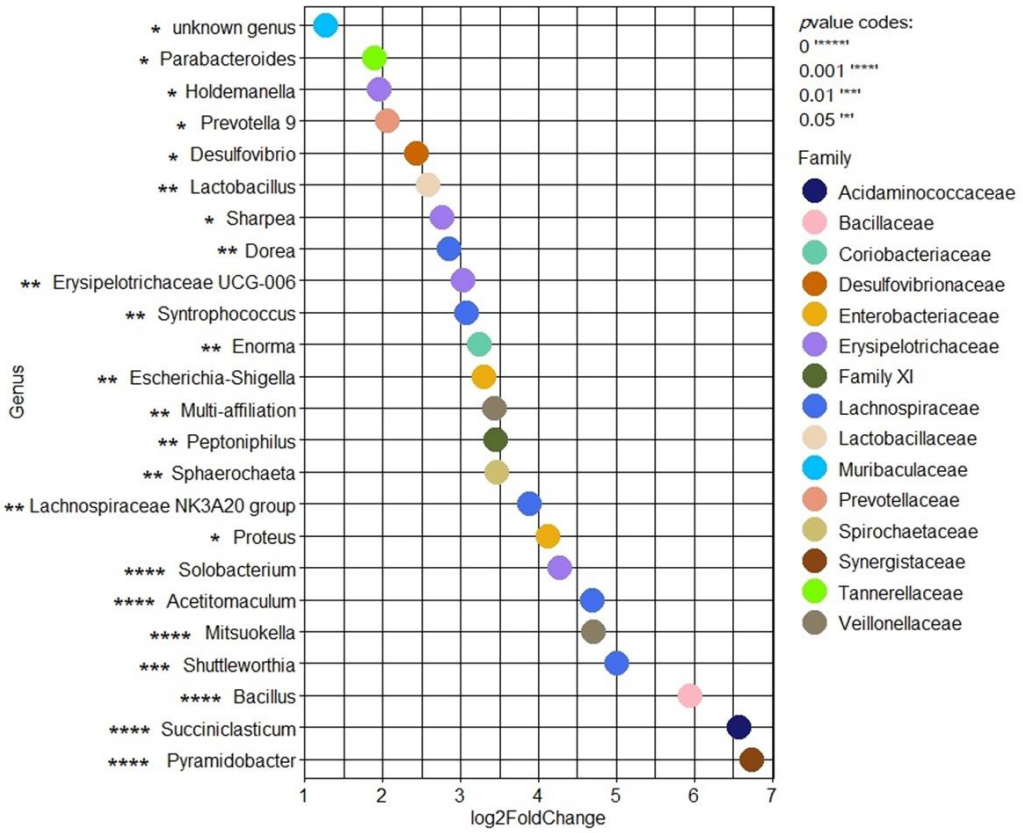


Figure 16. Differentially abundant genera between the end of the stabilization (day 7) and the recovery phase (day 9.5, 10. 11 and 15) in the bioreactor medium, mucin beads and bead medium of the fermentations #6, 7 8 and 9. Only significative Log2FoldChanges are represented on the figure. Pvalue codes are indicated on the figure.

Effects of feed deprivation stress on microbial activity

Measurement of redox potential, gases and SCFAs

A 48h feed deprivation stress induced a strong augmentation of the redox potential in the 4 fermentation runs to maximal values of +17, -21, -94 and -78 mV. In all bioreactors, the redox potential recovered a very low value (-250 mV) within ~20h after restarting of the feeding pump (Supplementary figure 10). The feed deprivation period of 48h in the MPigut-IVM led to modifications of gas composition with an slight augmentation of N_2 and O_2 percentages and a decrease of CO_2 percentage, which was not attributed to any leak of the system (Supplementary figure 11).

Compared to day 7, total SCFA concentration decreased, though not significantly, at day 9 (figure 17). ANOVA analyses revealed that, compared to day 7, acetate percentage was significantly reduced from day 9.5 to day 15, valerate was significantly increased at day 9 although propionate, isobutyrate and isovalerate were significantly reduced at day 9.5 (figure 18).

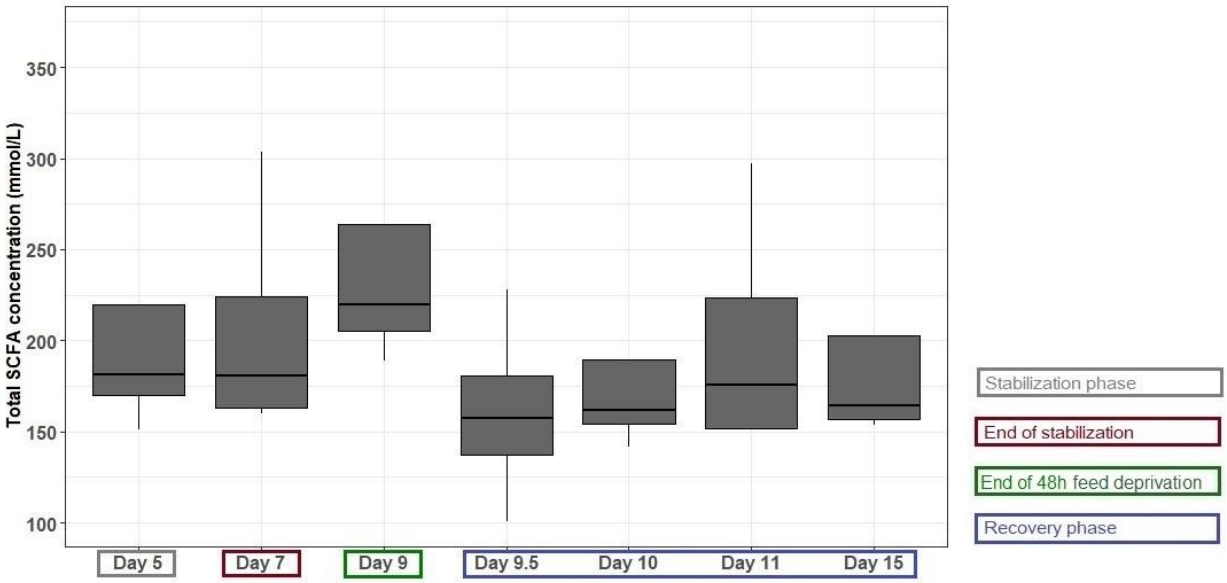


Figure 17. Evolution of the mean total concentration of SCFAs during the fermentation #6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h (n = 4 for each time point).

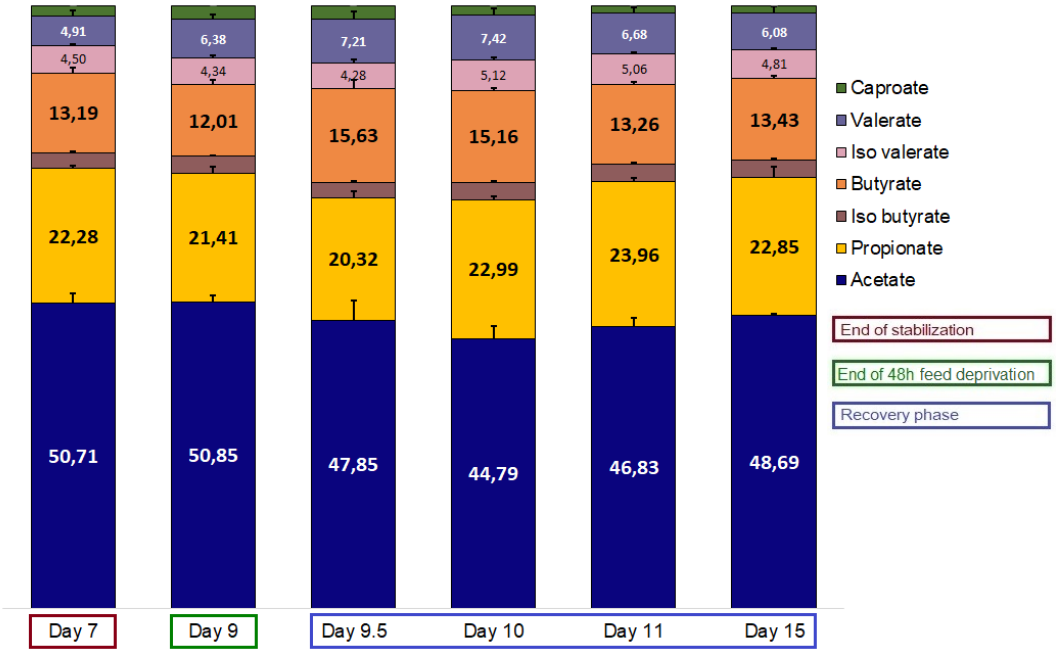


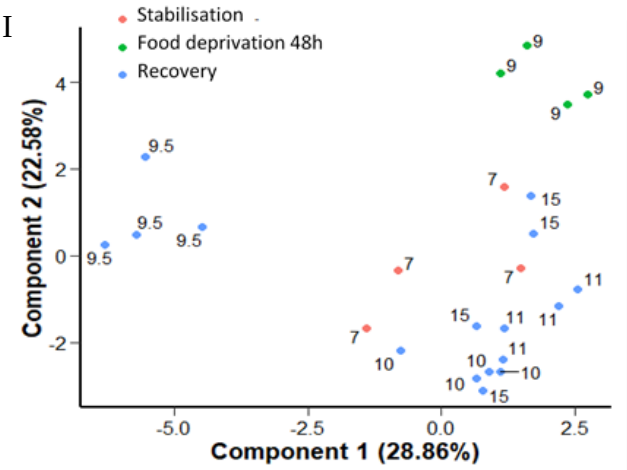
Figure 18. Evolution of the relative abundance of the main SCFAs during the fermentation 6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h (n = 4 for each time point).

Metabolome analysis using NMR

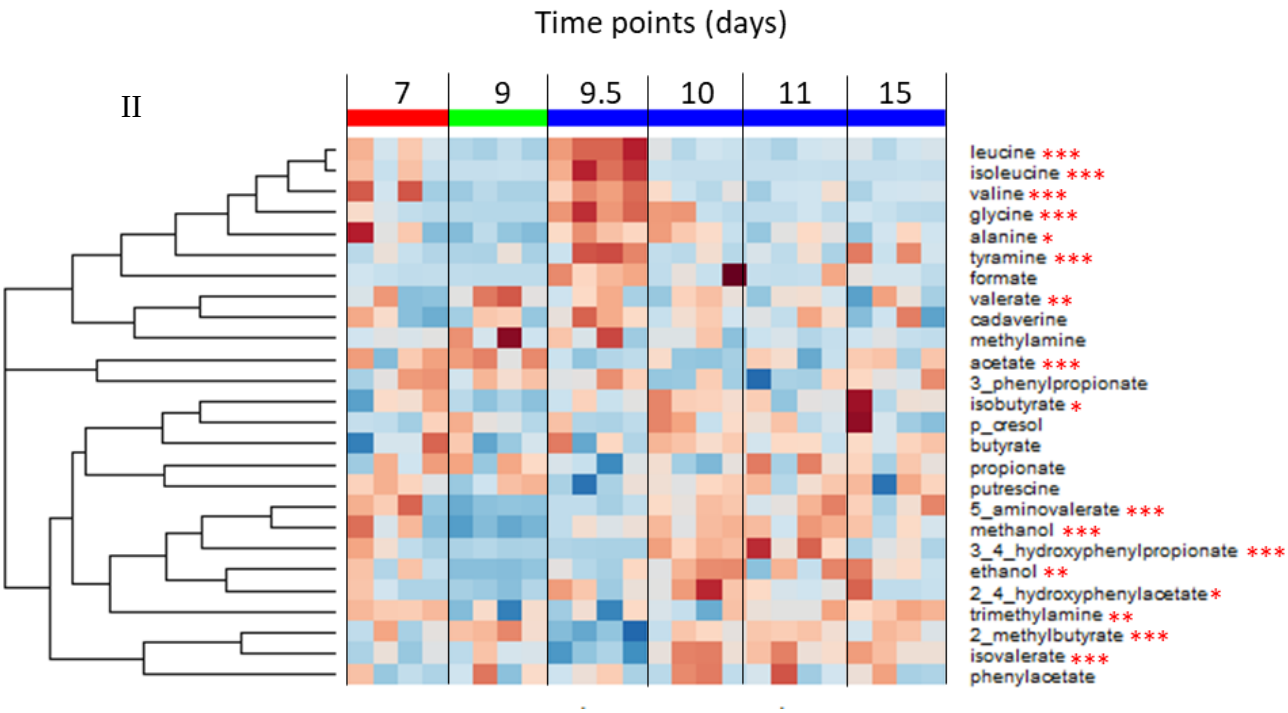
Overall, 26 metabolites could be identified (Supplementary table 3). PLS-DA revealed a dynamic remodeling of the metabolome in the bioreactor medium according to the stabilization, feed deprivation stress and recovery periods (figure 19 I and II). In the bioreactor medium, at day 9 compared to day 7, the concentration of methanol, trimethylamine, 5-aminovalerate and of the amino acids alanine, valine and leucine was decreased while valerate and 2-methylbutyrate concentrations increased. At day 9.5, a strong increase of the concentration of glycine, alanine, isoleucine, leucine, valine and tyramine and a decrease of isovalerate, acetate and 2-methylbutyrate was observed. At later time point (days 10 – 15), the concentration of amino acids decreased (glycine, isoleucine, leucine and valine) while the concentration of the bacterial metabolites 3-4-hydroxyphenylpropionate and isovalerate increased (figure 19 I and II, supplemental table 5).

In the bead medium, PLS-DA also revealed that the 48h feed deprivation decreased significantly the concentration of trimethylamine, valine and leucine. From day 9.5 to 15, the concentration of trimethylamine, isovalerate, 2-methylbutyrate, alanine and leucine gradually increased while methylamine and valerate decreased (figure 19 III and IV, supplemental table 5).

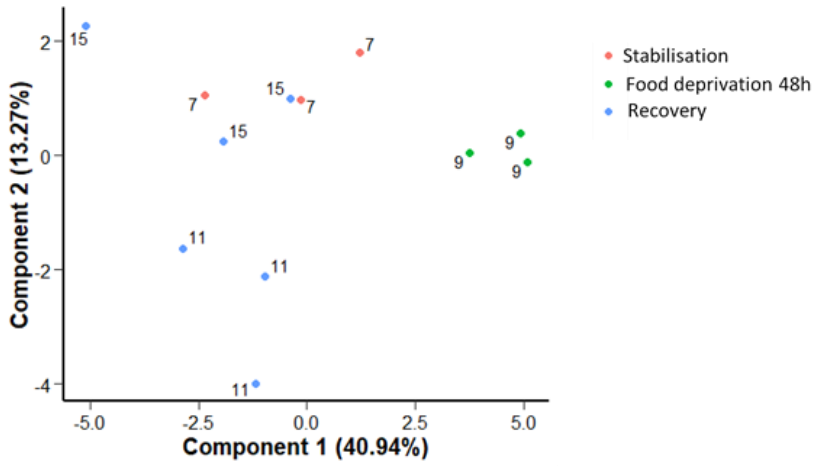
I


Stabilisation
Food deprivation 48h
Recovery
Component 2 (22.58%)
Component 1 (28.86%)

II


Time points (days)
7
9
9.5
10
11
15
leucine ***
isoleucine ***
valine ***
glycine ***
alanine *
tyramine ***
formate
valerate **
cadaverine
methylamine
acetate ***
3_phenylpropionate
isobutyrate *
p_cresol
butyrate
propionate
putrescine
5_aminovalerate ***
methanol ***
3_4_hydroxyphenylpropionate ***
ethanol **
2_4_hydroxyphenylacetate *
trimethylamine **
2_methylbutyrate ***
isovalerate ***
phenylacetate

III


Stabilisation
Food deprivation 48h
Recovery
Component 2 (13.27%)
Component 1 (40.94%)

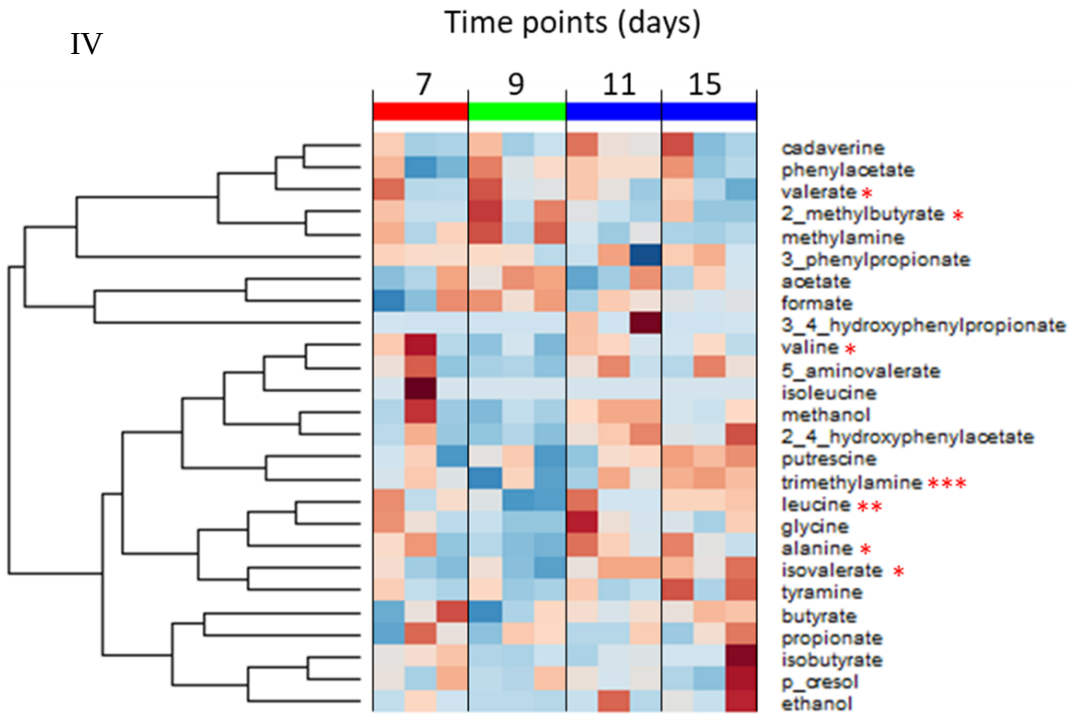


Figure 19. Analysis of the in vitro metabolome by nuclear magnetic resonance (NMR). Metabolomics analysis were performed by using NMR in the bioreactor medium (I and II) and bead medium (III and IV) compartments. Individual plots of partial least square-discriminant analysis using metabolites as variables and time points as predictors (I and III). Heatmap representing the relative concentrations of all identified metabolites (rows) in individual samples (columns). The color represents the Z-scores (row-scaled relative concentrations) from low (blue) to high (red) values. Metabolites (rows) were clustered by the average method. The mean relative abundances were analyzed by a mixed model and Anova.*: P<0.05, **: P<0.01, ***: P<0.001 (adjusted P-values by the false discovery rate method). The bead medium of the fermentation number 6 was not analyzed by NMR which explains the three replicates for the figure III.

Metabolome-microbiome Spearman's correlation

In the bioreactor medium (figure 20), the strongest positive spearman correlation (ρ (correlation coefficient) > 0.7, *p*values < 0.005) were between valerate and cadaverine and *Enterobacteriaceae*, between *Prevotellaceae* and hydroxyphenylpropionate, methanol, and aminovalerate, and between *Bacteroidiaceae* and phenylpropionate. Leucine was strongly negatively correlated with the *Muribaculaceae* family (ρ < -0.8, *p*value < 0.005).

In the bead medium (figure 21), valerate was positively correlated ($\rho > 0.8$, *p*val < 0.005) with *Eubacterium nodatum* group, *Clostridiaceae 1, Peptostreptococcaceae* and *Enterobacteriaceae* and negatively correlated ($\rho < -0.8$, *p*val < 0.005) with *Bacteroidaceae*, *Acidaminococcaceae* and *Prevotellaceae.* Butyrate and methyl butyrate were positively associated with *Acidaminococcaceae* and *Clostridiaceae* 1. Propionate and phenylacetate were respectively positively correlated ($\rho > 0.8$, *p*val < 0.005) with *Bacteroidaceae* / *Acidaminococcaceae* and *Atopobiaceae / Enterococcaceae* families. Biogenic amines, methylamine and trimethylamine, were positively correlated ($\rho > 0.8$, *p*val < 0.005) with *Bifidobacteriaceae*. At last, cadaverine could be positively correlated ($\rho > 0.8$, *p*val < 0.005) with *Atopobiaceae, Enterobacteriaceae* and *Enterococcaceae* and negatively correlated ($\rho < -0.8$, *p*val < 0.005) with notably *Bacteroidaceae.* Putrescine was positively correlated with *Desulfovibrionaceae* ($\rho > 0.8$, *p*val < 0.005) and negatively correlated ($\rho < -0.8$, *p*val < 0.05) with *Muribaculaceae, Peptostreptococcaceae* and *Ruminococcaceae.*

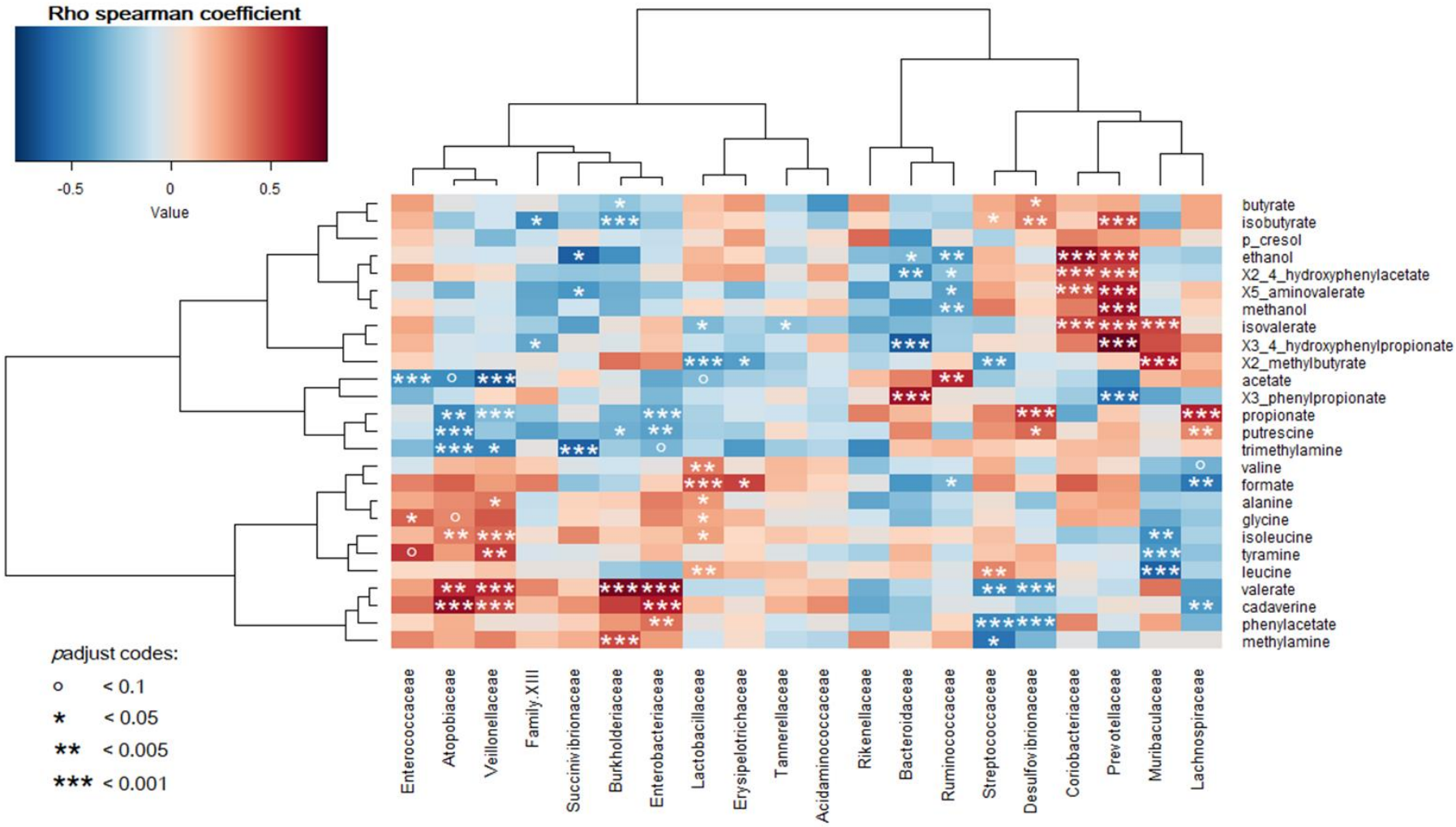


Figure 20. Spearman 's correlation between the relative abundance of the main bacterial families and the metabolites in the bioreactor medium of the MPigut-IVM during the fermentation 6, 7, 8 and 9 at day 7, 9, 9.5, 10, 11 and 15. Cells are colored based upon the Spearman correlation coefficient. Asterisks indicate significant pvalues (< 0.05).

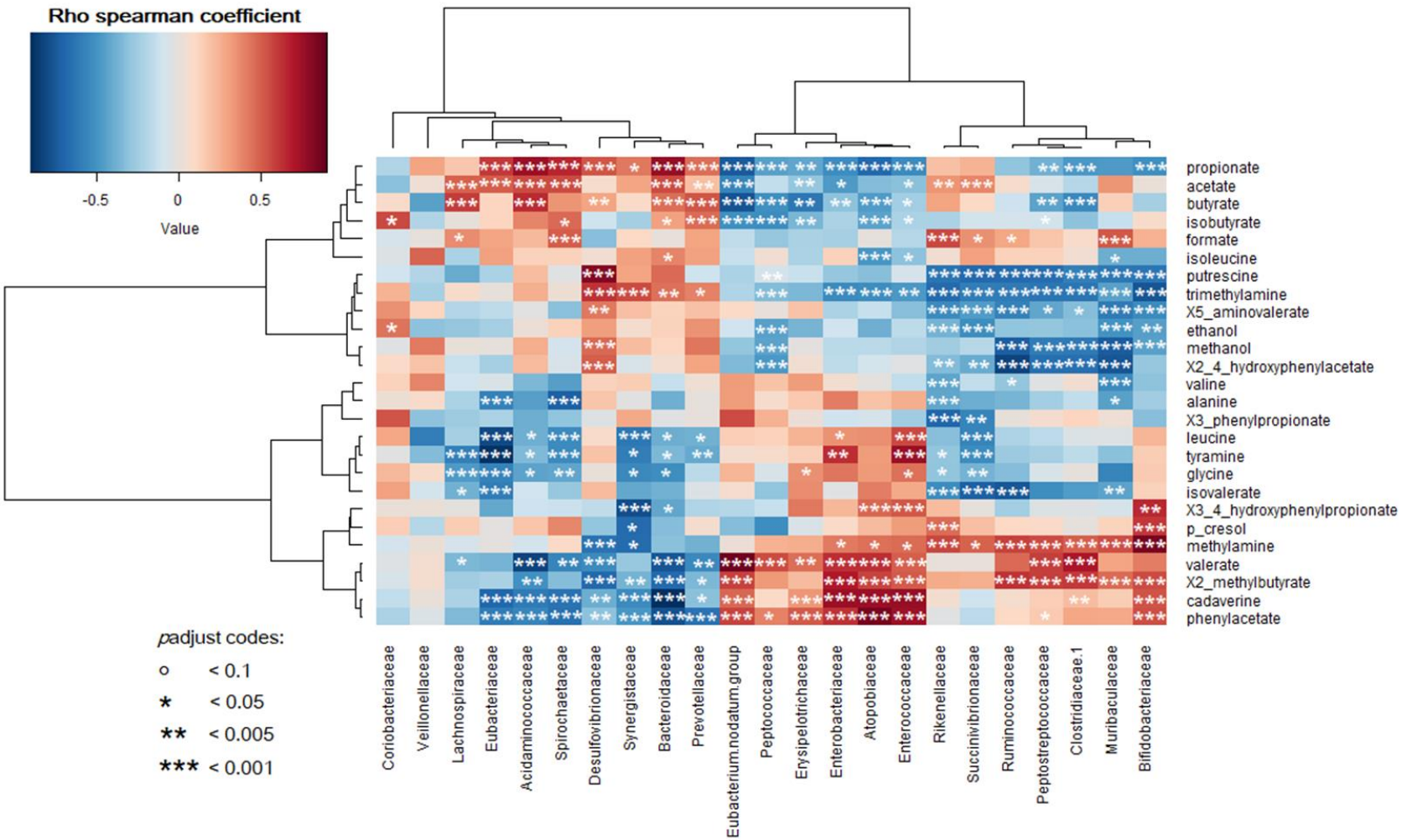


Figure 21. Spearman 's correlation between the relative abundance of the main bacterial families and the metabolites on the mucin beads of the MPigut-IVM during the fermentation 6, 7, 8 and 9 at day 7, 9, 11 and 15. Cells are colored based upon the Spearman correlation coefficient. Asterisks indicate significant pvalues (< 0.05).

DISCUSSION

We developed a novel *in vitro* technology to accurately mimic the piglet gut. As opposed to existing models (Dufourny et al., 2019; Fleury et al., 2017; Tran et al., 2016; Van den Abbeele et al., 2012), the MPigut-IVM possess a unique multiparametric set up by incorporating a mucus environment, an independently established anaerobic redox condition, a fine-tuned and representative piglet feed and a physiologically representative residence time (Wilson and Leibholz, 1981). Our newly developed MPigut-IVM shows high reproducibility between replicates while maintaining the precious inter-inocula specificities and displays consistent shifts from a fecal to a colon microbiota such as the increase of the *Bacteroidetes* phylum (Gresse et al., 2019). The augmentation of *Bacteroidetes* and *Proteobacteria* relative abundance is commonly observed in *in vitro* models due to initially higher oxygen concentration and reactor startup. Additionally, the MPigut-IVM was able to maintain an *archaeal* microbiota, composed of *Methanobacteriaceae* and *Methanomethylophilaceae* consistently with *in vivo* data (Gresse et al., 2019), which was hitherto not observed in *in vitro* model (Dufourny et al., 2019; Fleury et al., 2017; Tanner et al., 2014b).

In agreement with *in vivo* data (Gresse et al., 2019; Kelly et al., 2017), the microbiota composition of the MPigut-IVM mucin beads was clearly distinct from that of the bioreactor medium and, consistently with *in vivo* data, displayed a higher relative abundance of *Proteobacteria* and reduced *Bacteroidetes* compared to luminal microbiota, here simulated by the bioreactor medium (Gresse et al., 2019; Kelly et al., 2017). *Erysipelotrichaceae, Peptostreptococcaceae, Clostridiaceae* 1 and *Clostridium* Family XIII were more abundant in the mucin beads compared to the bioreactor medium, hypothetically due to their restore mucin-binding or mucin-degrading capacities. At last, the comparison between the fifteen most abundant families in mucin beads and our mucosal proximal colon samples clearly showed very similar profiles indicating a well-established and specific mucin-associated bacterial community in the MPigut-IVM.

Regarding microbial activity, methane was detected during the fermentation process indicating the presence of an active methanogenic archaea community, in agreement with *in vivo* observations in piglet (Jensen and Jørgensen, 1994; Robinson et al., 1989). In comparison with the proportion of acetate, propionate and butyrate in the fecal inoculum, the *in vitro* relative abundance of acetate and butyrate were respectively slightly decreased or increased after the stabilization period of 7 days which was in concordance with our previous *in vivo* proximal colon data (Gresse et al., 2019) and literature (Nakatani et al., 2018). Besides SCFA, NMR

metabolomics revealed that MPigut-IVM microbiota produced a wide diversity of metabolites such as biogenic amines (e.g. cadaverine, 5-aminovalerate, and putrescine) and aromatic metabolites (e.g. 3-phenylpropionate, phenylacetate, *p*-cresol). These results indicate that the functional capacity of the piglet gut microbiota is maintained *in vitro*.

Once characterized, the MPigut-IVM was applied to evaluate the impact of weaning feed deprivation period, the most critical factor involved in the intestinal barrier dysfunction and potentially affecting the microbiota composition and function(Spreeuwenberg et al., 2001). In the MPigut-IVM subjected to a feed deprivation stress, *Bacteroidiaceae* family was found significantly lower, while the *Prevotellaceae* increased in the bioreactor medium after the feed deprivation period compared to the end of the stabilization phase. Due to the selective distribution of the microbiota in the *in vitro* "luminal" and "mucus"-associated phases, the reduction of *Clostridiaceae*, was observed on the mucin beads. The *Clostridiaceae* family contains numbers of mucin-adherent butyrate and acetate producer and is associated with a healthy microbiome (Van den Abbeele et al., 2013). Up to date, very few researches have investigated the impact of weaning feed deprivation period on the intestinal microbiota and function of weaning piglets. Recent investigations have reported that deoxynivalenol (DON), the most common cereal crop mycotoxin, could induce anorexia and disruptions of the cecal and colon microbiota composition and SCFA concentration of 27 day old piglets (S. Wang et al., 2019). In this study, the DON-induced anorexia was characterized by a significant decrease in genera belonging to *Clostridiaceae* and *Bacteroidiaceae* and a significant increase in members of *Prevotellaceae* (S. Wang et al., 2019). *Pyramidobacter, Succiniclasticum, Bacillus* or *Shuttleworthia* genera, previously associated with production of acetate, iso valerate, lactate or propionate (Downes et al., 2009, 2002; Lan et al., 2002), were significantly increased from more than 4.5 log2 fold change in the MPigut-IVM after the feed deprivation period. DESEQ2 analysis also indicated that potential opportunistic pathogens could be favored by the feed deprivation period such as *Solobacterium* (Liu et al., 2019), *Sphaerochaeta*(Caro-Quintero et al., 2012), *Peptoniphilus* (Müller-Schulte et al., 2019) and two *Enterobacteriaceae* family members, *Proteus* (Schaffer and Pearson, 2015) and *Escherichia Shigella* genera (Dubreuil et al., 2016). The effect of the feed deprivation period in the MPigut-IVM was markedly observed in 2 out of the 4 fermentations which harbored the higher percentage of *Enterobacteriaceae* after the stabilization period. Interestingly, the fermentations #6 and 7 which displayed the higher increase of *Enterobacteriaceae* and *Escherichia coli* where also those which displayed the greatest impact of the feed deprivation stress on the redox potential. The reasons of the

increase in redox potential after the feed deprivation stress in the MPigut-IVM remain unclear but certainly due to reducing availability of nutrients and thus reduced fermentative activity. Redox potential reflects the reducing capacity of the gut environment which is influenced by several factors such as for instance diffusion of oxygen or release of reactive oxygen species or reactive nitrate species (Circu and Aw, 2011; Friedman et al., 2017; Pérez et al., 2017). The relationship between intestinal redox potential, diet and intestinal microbiota of piglets is not elucidated yet. In a study from Xu et *al.* (J. Xu et al., 2014), early weaning was associated to a decrease in antioxidant capacities, increases in colon hydroxyl radicals and H_2O_2 and increase in *Escherichia coli* counts. *Escherichia* species already proved to be tolerant to oxidative stress contrary to other species from the gut microbiota which possess a high sensitivity (Qiao et al., 2013). We could speculate that individuals possessing more oxidative stress-resistant bacterial species would be more competitive towards *Enterobacteriaceae* and would thus be less impacted by an increase of redox potential. These hypotheses could explain the variable effects of the feed deprivation stress on the *Enterobacteriaceae* family inside our MPigut-IVM.

In our *in vitro* model, the feed deprivation stress led to a decrease in total SCFA concentration in the bioreactor medium after the 48h stress period which was already observed in the cecum of suckling piglets after early exposure to antibiotic and could refer to a dysbiotic state (Yu et al., 2018). *In vivo*, modifications of SCFA profiles would have a direct impact on intestinal enterocytes but also on gut homeostasis (Tan et al., 2014). In addition to SCFA, metabolomics analysis showed that numbers of bacterial metabolites were significantly impacted by *in vitro*-simulated feed deprivation period. Indeed, biogenic amines and amino acids were decreased by the feed deprivation stress suggesting a reduction in proteolytic and peptidolytic bacterial activity. Amino acid components such as alanine, valine, leucine and isoleucine, were however increased both in the bioreactor medium and the mucin beads during the recovery period indicating strong resilience of the microbiota. Since microbial metabolites are considered as key molecular intermediates between the microbiota and host cells, a perspective of our work would be to treat pig intestinal epithelial cells (e.g. IPEC-J2 or organoids) with bacterial metabolites produced in the MPigut-IVM, alone or in combination. These experiments could improve our understanding of the molecular links between weaning-induced dysbiosis and gut barrier dysfunction in piglets.

Our study also reported strong correlations between some bacterial families and the *in vitro* metabolome detected by NMR which was for the first time performed in *in vitro* models. These findings highlighted the association of populations such as *Ruminococcaceae, Clostridiaceae,*

Prevotellaceae or *Bacteroidiaceae* with production of SCFAs(Tan et al., 2014) which confirmed that important functional groups are able to maintain their activity in the MPigut-IVM. Less known components such as polyamines, especially putrescine and cadaverine, were also detected in our samples and correlated with bacterial taxa. These molecules, absorbed by the host intestine, have a wide panel of biological functions like epigenetic regulations, stress resistance or cell proliferation (Igarashi and Kashiwagi, 2010) and could potentially have a role in the physiological consequences of the weaning period. Production and degradation of polyamines by the gut microbiota remain unclear but some bacterial taxa such as *Bifidobacterium* and *Escherichia coli* have been associated with putrescine production (Tofalo et al., 2019). Interestingly, in our *in vitro* model, *Bifidobacteriaceae* and *Enterobacteriaceae* families were positively correlated with a higher concentration of cadaverine. Further investigations would be needed to understand the metabolic role of this bacterial communities during weaning associated feed deprivation stress. Our metabolome and correlation data however highlighted that the MPigut-IVM was able to mimic the very complex colonic environment up to deciphering the production of a rich panel of microbial metabolites.

In vivo, weaning transient anorexia could lead to a more competitive environment for nutrient degradation inside the piglet colon and, such as observed in our *in vitro* model, disruptions of microbiome and metabolome leading to intestinal dysbiosis and post weaning infections. The few days following low feed intake at weaning seem to be a critical period during which high microbial competition occurs and the most robust species such as opportunistic pathogens could take the advantage to colonize released ecological niches. Further research using mucin associated *in vitro* model such as our MPigut-IVM including other weaning stress factors such as the presence of pathogens or a dietary change should benefit to a better understanding of the etiology of intestinal dysbiosis in post-weaning piglets. Ultimately, the MPigut-IVM could be used to evaluate the impact of potential non-pharmalogical therapeutic alternatives in order to limit the use of antibiotics to treat post-weaning diarrhea in piglets.

To conclude, our study reported the development of an innovative *in vitro* model of the piglet colon microbiota able to mimic gut luminal and mucus environments, while maintaining strict anaerobic conditions by the sole activity of the *in vitro* resident microbiota. We highlighted that weaning transient feed deprivation could lead to alterations of microbiota composition and metabolic profiles and might play a role in pathogen emergence. Despite the use of pooled fecal samples, some variability was

conserved which will be of a great use for testing the effect of stressors, feed additives or pharmaceutical products in a more robust and relevant manner.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Composition of the nutritive medium simulating the ileal chyme of 28 days old piglets.

Ingredients	g/L
Wheat starch	5
Pectin	2
Arabinogalactan	2
Cellulose	2
Soy protein isolate	10
Tryptone from casein	4.5
Mucin from porcine stomach type II	2
Whey powder	5
$FeSO_4$, $7H_2O$	0.005
L-cysteine HCl monohydrate	0.80
Bile extract porcine	0.40
KH_2PO_4	0.5
$NaHCO_3$	1.5
Hemin from porcine	0.005
NaCl	4.5
KCl	4.5
$MgSO_4$ anhy. (120.37 g/mol)	0.64
$CaCl_2$ $2H_2O$ (147.02 g/mol)	0.15
$MnCl_2$ $4H_2O$ (197.91 g/mol)	0.20

Supplementary Table 2. Primers and probes used for quantitative experiments on the MPigut-IVM in vitro gut microbiota**.**

Target	Type	Sequences	References	Concentration	Annealing temperature
***Escherichia coli/ Shigella* group**	Taqman	5'-CAT GCC GCG TGT ATG AAG AA-3' 5'-CGG GTA ACG TCA ATG AGC AAA-3' (6-FAM)-5'-TAT TAA CTT TAC TCC CTT CCT CCC CGC TGA A-3'(TAMRA)	(Huijsdens et al., 2002)	300 nM 300 nM 100 nM	61°C
Total Bacteria	SYBRGREEN	5'-ACT CCT ACG GGA GGC AG-3' 5'-GTA TTA CCG CGG CTG CTG-3'	(Yu et al., 2005)	500 nM 500 nM	61°C
Firmicutes	SYBRGREEN	5'-TGA AAC TYA AAG GAA TTG ACG-3' 5'-ACC ATG CAC CAC CTG TC-3'	(Cordonnier, 2015)	500 nM 500 nM	60°C
Bacteroidetes	SYBRGREEN	5'-CRA ACA GGA TTA GAT ACC CT-3' 5'-GGT AAG GTT CCT CGC GTA T-3'	(Cordonnier, 2015)	500 nM 500 nM	60°C
Prevotella	SYBRGREEN	5'-GGT TCT GAG AGG AAG GTC CCC-3' 5'-TCC TGC ACG CTA CTT GGC TG-3'	(Mohamed et al., 2004)	500 nM 500 nM	60°C
***Clostridium* cluster IV**	Taqman	5'-GCA CAA GCA GTG GAG T-3' 5'-CTT CCT CCG TTT TGT CAA-3' (6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	(Zwielehner et al., 2011)	500 nM 500 nM 300 nM	55°C
***Clostridium* cluster XIVa**	Taqman	5'-GCA GTG GGG AAT ATT GCA-3' 5'-CTT TGA GTT TCA TTC TTG CGA A-3' (6-FAM)-AAATGACGGTACCTGACTAA-(BHQ-1)	(Zwielehner et al., 2011)	500 nM 500 nM 300 nM	56°C
Methanogenic archaea	SYBRGREEN	5'-GAG GAA GGA GTG GAC GAC GGTA-3' 5'-ACG GGC GGT GTG TGC AAG-3'	(Ohene-Adjei et al., 2008)	500 nM 500 nM	61°C

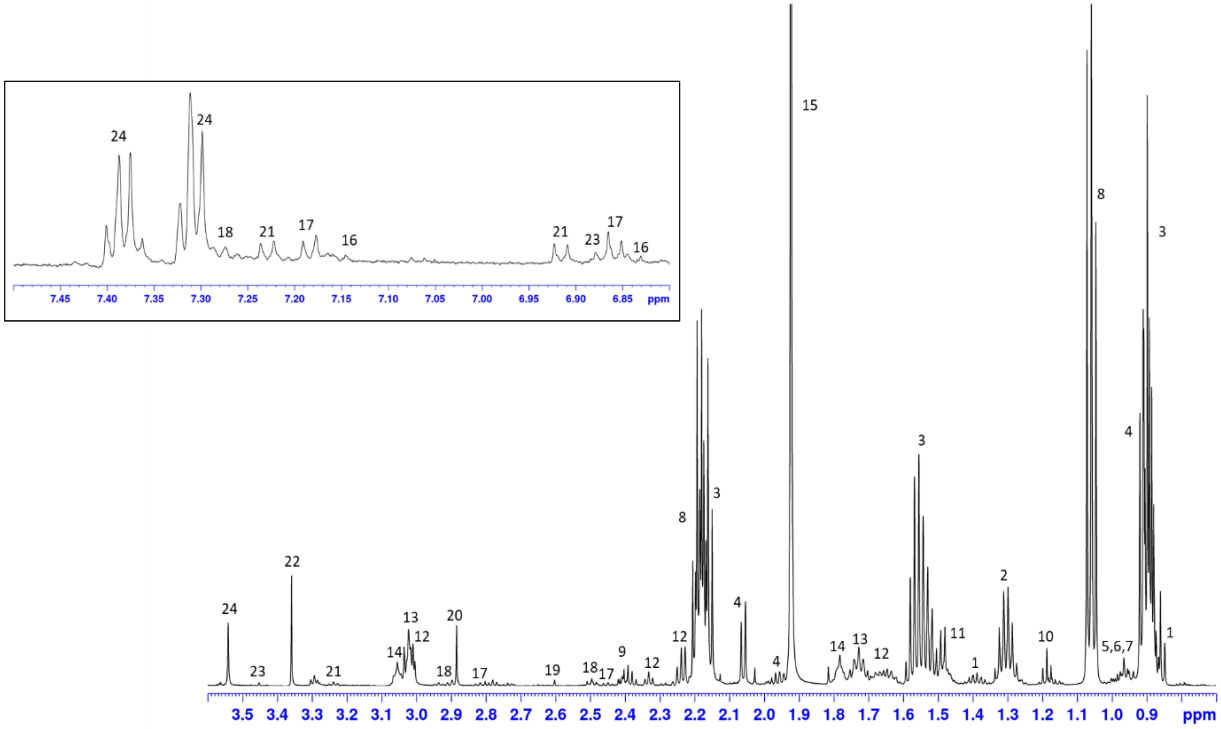
Supplementary Table 3: Identification of metabolites in NMR spectra. The numbers are reported in representative spectra in supplemental figure 1. s: singulet. d: doublet. t: triplet. q: quintuplet; m: multiplet. *: indicate the peak used for quantification.

	Metabolite	δ^1H (ppm)
1	2-methylbutyrate	0.86 (t). 1.39* (m)
2	Valerate	0.89 (t). 1.31* (m). 1.53 (m). 2.19 (t)
3	Butyrate	0.90* (t). 1.56 (m). 2.16 (t)
4	Isovalerate	0.91 (d). 1.96 (m). 2.06* (d)
5	Isoleucine	0.94 (t). 1.01* (d)
6	Leucine	0.97* (t)
7	Valine	1.00* (d). 1.05 (d). 3.62 (d)
8	Propionate	1.06* (t). 2.19 (m)
9	Isobutyrate	1.07 (d). 2.40* (m)
10	Ethanol	1.19* (t). 3.66 (m)
11	Alanine	1.48* (d). 3.79 (m)
12	5-aminovalerate	1.65 (m). 2.24* (t). 2.33 (t). 3.02 (t)
13	Cadaverine	1.73* (m). 3.02 (t)
14	Putrescine	1.78* (m). 3.05 (t)
15	Acetate	1.92* (s)
16	p-cresol	2.26 (s). 6.84* (d). 7.15 (d)
17	3-(4-hydroxyphenyl)propionate	2.45* (t). 2.82 (t). 6.86 (d). 7.18 (d)
18	3-phenylpropionate	2.50* (t). 2.89 (t). 7.27 (t). 7.32 (d). 7.37 (t)
19	Methylamine	2.60* (s)
20	Trimethylamine	2.88* (s)
21	Tyramine	2.94 (t). 3.24 (t). 6.92* (d). 7.23 (d)
22	Methanol	3.36* (s)
23	2-(4-hydroxyphenyl)acetate	3.45* (s). 6.87 (d). 7.17 (d)
24	Phenylacetate	3.54* (s). 7.31 (t). 7.39 (t)
25	Glycine	3.57* (s)
26	Formate	8.46* (s)

Supplementary Table 4: Statistical analysis of metabolites detected by NMR metabolomics in the bioreactor and bead medium. Means associated with a different letter are significantly different. P adj : adjusted p-values (FDR method).

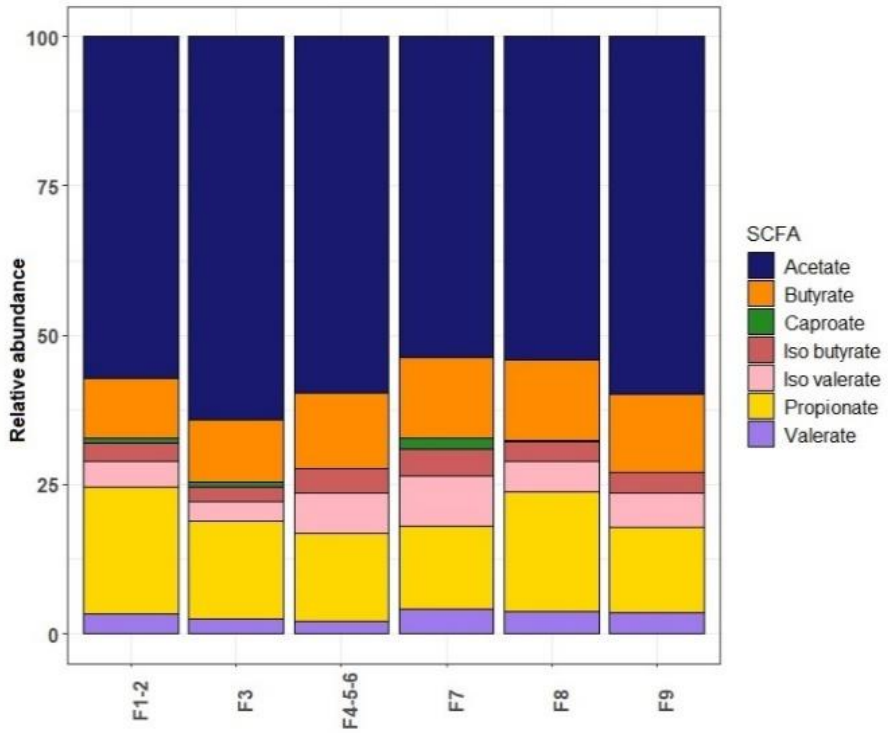
	Bioreactor medium								**Bead medium**					
	P values		*Time points*						*P values*		*Time points*			
	pval	**padj**	**7**	**9**	**9.5**	**10**	**11**	**15**	**pval**	**padj**	**7**	**9**	**11**	**15**
formate	0.038	0.055	a	a	a	a	a	a	0.287	0.355	a	a	a	a
tyramine	0.000	0.000	a	a	b	a	a	ab	0.020	0.052	ab	a	ab	b
p_cresol	0.334	0.362	a	a	a	a	a	a	0.929	0.929	a	a	a	a
glycine	0.000	0.000	ab	a	c	b	ab	ab	0.083	0.160	a	a	a	a
phenylacetate	0.701	0.701	a	a	a	a	a	a	0.018	0.051	a	b	ab	ab
2_4_hydroxyphenylacetate	0.021	0.034	ab	a	ab	b	ab	ab	0.077	0.160	a	a	a	a
methanol	0.000	0.000	b	a	ab	b	b	b	0.156	0.233	a	a	a	a
trimethylamine	0.001	0.002	b	a	a	ab	ab	b	0.000	0.001	b	a	ab	b
methylamine	0.070	0.091	a	a	a	a	a	a	0.003	0.023	ab	b	a	a
3_phenylpropionate	0.053	0.072	a	a	a	a	a	a	0.351	0.415	a	a	a	a
3_4_hydroxyphenylpropionate	0.000	0.000	ac	a	a	bc	c	ab	0.086	0.160	a	a	a	a
isobutyrate	0.022	0.034	ab	a	ab	b	ab	b	0.161	0.233	a	a	a	a
5_aminovalerate	0.000	0.000	b	a	ab	b	b	b	0.059	0.139	a	a	a	a
isovalerate	0.000	0.000	ab	bc	a	c	c	bc	0.010	0.041	ab	a	ab	b
acetate	0.000	0.000	bc	c	a	a	ab	ac	0.225	0.308	a	a	a	a
putrescine	0.084	0.095	a	a	a	a	a	a	0.119	0.194	a	a	a	a
cadaverine	0.081	0.095	a	a	a	a	a	a	0.092	0.160	a	a	a	a
alanine	0.008	0.013	b	a	b	ab	ab	ab	0.006	0.040	ab	a	b	b
2_methylbutyrate	0.000	0.000	b	c	a	bc	bc	bc	0.008	0.040	ab	b	a	a
valerate	0.004	0.008	a	b	ab	ab	a	a	0.015	0.049	ab	b	ab	a
ethanol	0.006	0.011	ab	a	ab	b	ab	b	0.516	0.559	a	a	a	a
propionate	0.074	0.091	a	a	a	a	a	a	0.886	0.922	a	a	a	a
isoleucine	0.000	0.000	a	a	b	a	a	a	0.391	0.442	a	a	a	a
valine	0.000	0.000	bc	a	c	ab	ab	ab	0.011	0.041	b	a	ab	ab
leucine	0.000	0.000	b	a	c	ab	ab	ab	0.000	0.005	b	a	b	b
butyrate	0.405	0.422	a	a	a	a	a	a	0.266	0.346	a	a	a	a

Supplementary Figure 1: Identification of metabolites in a representative NMR spectrum**.** Peaks are identified with a number corresponding to the metabolites described in supplementary Table 4. The inset shows the aromatic region (vertically expanded).

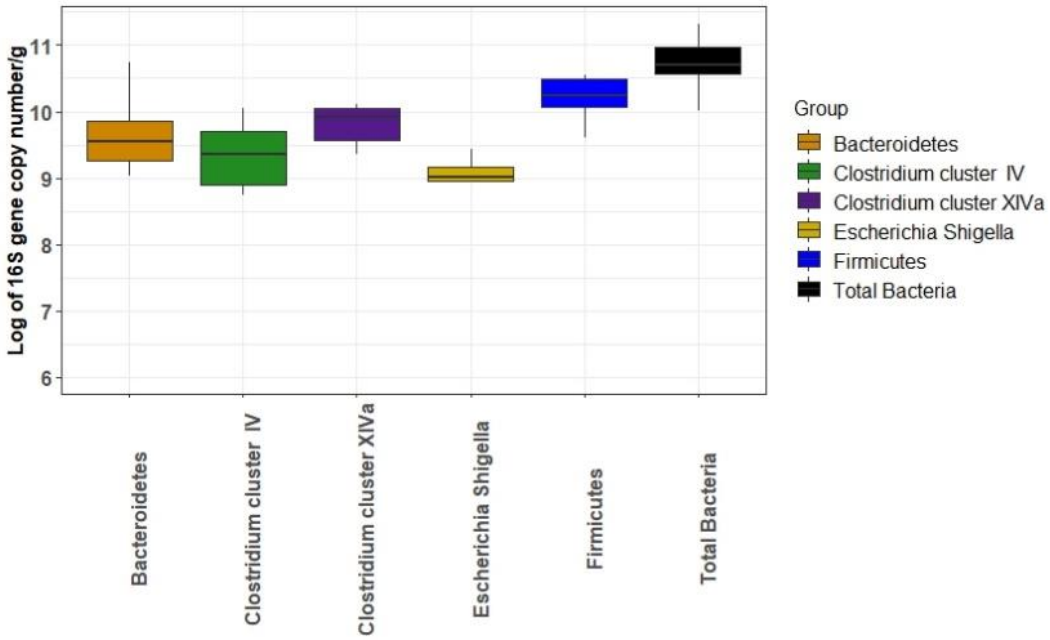


Supplementary Figure 2. Composition and metabolic activity of the microbiota from the fecal inocula in all fermentation runs**:** relative abundance of SCFA measured by gas chromatography (A), quantification of bacterial groups using QPCR (n = 6) (B) and relative abundance of the principal bacterial phyla (C), bacterial families (D), archaeal families (E) and alpha diversity based on Shannon index and the number of observed OTUs (F) measured by 16S Illumina sequencing. "F" = "Fermentation".

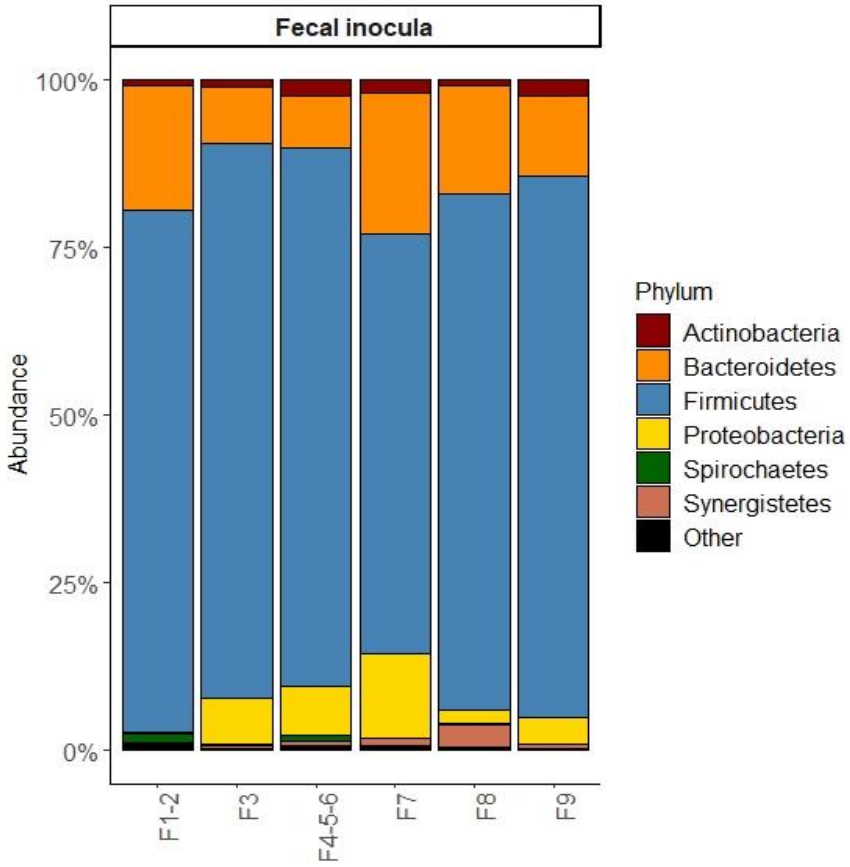
A



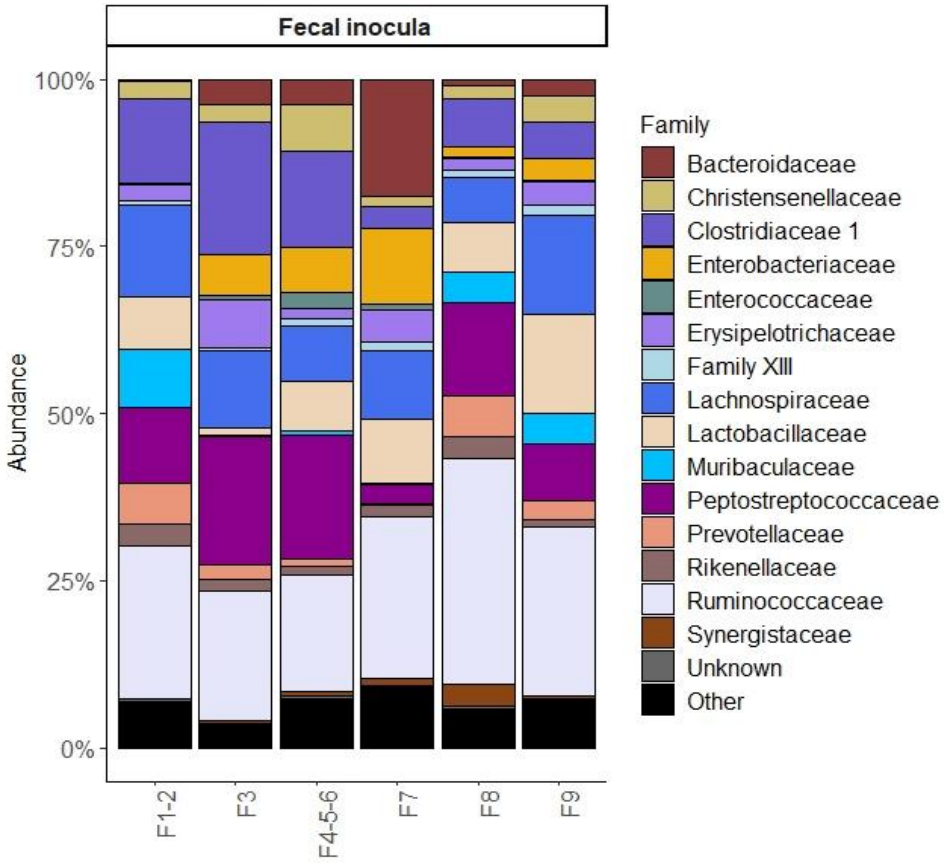
B



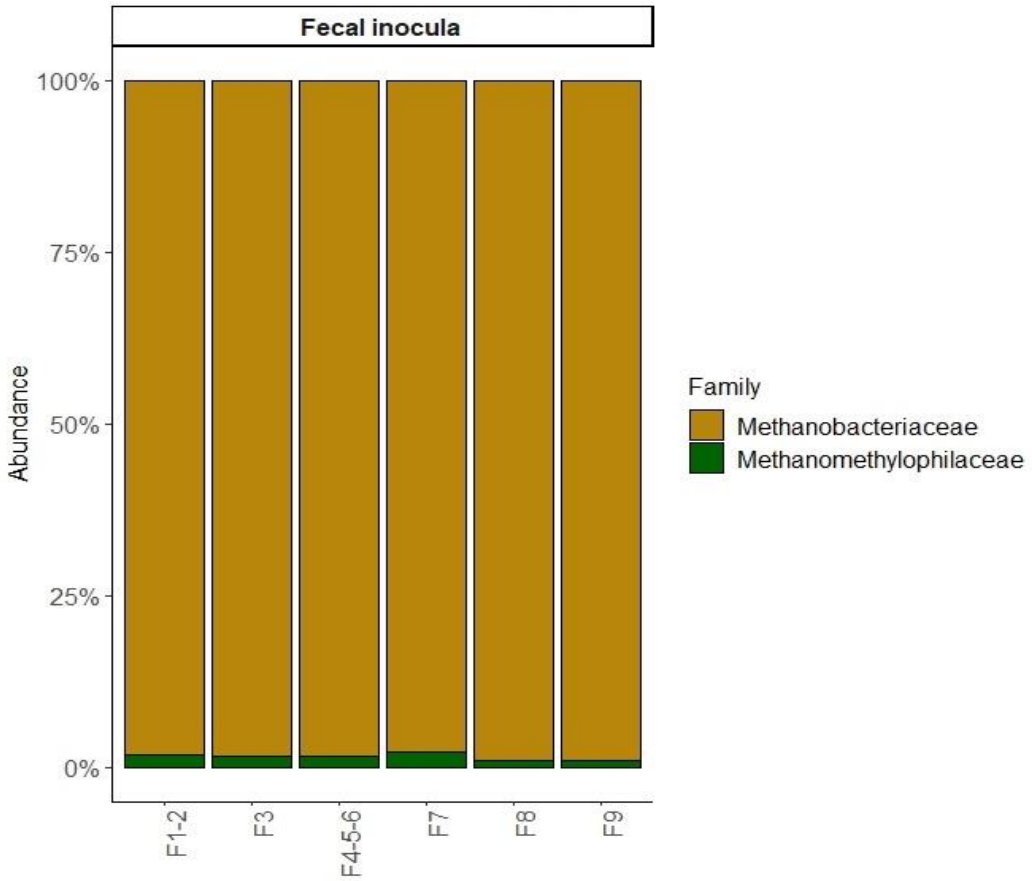
C


Fecal inocula
100%
75%
50%
25%
0%
Abundance
F1-2
F3
F4-5-6
F7
F8
F9
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Spirochaetes
Synergistetes
Other

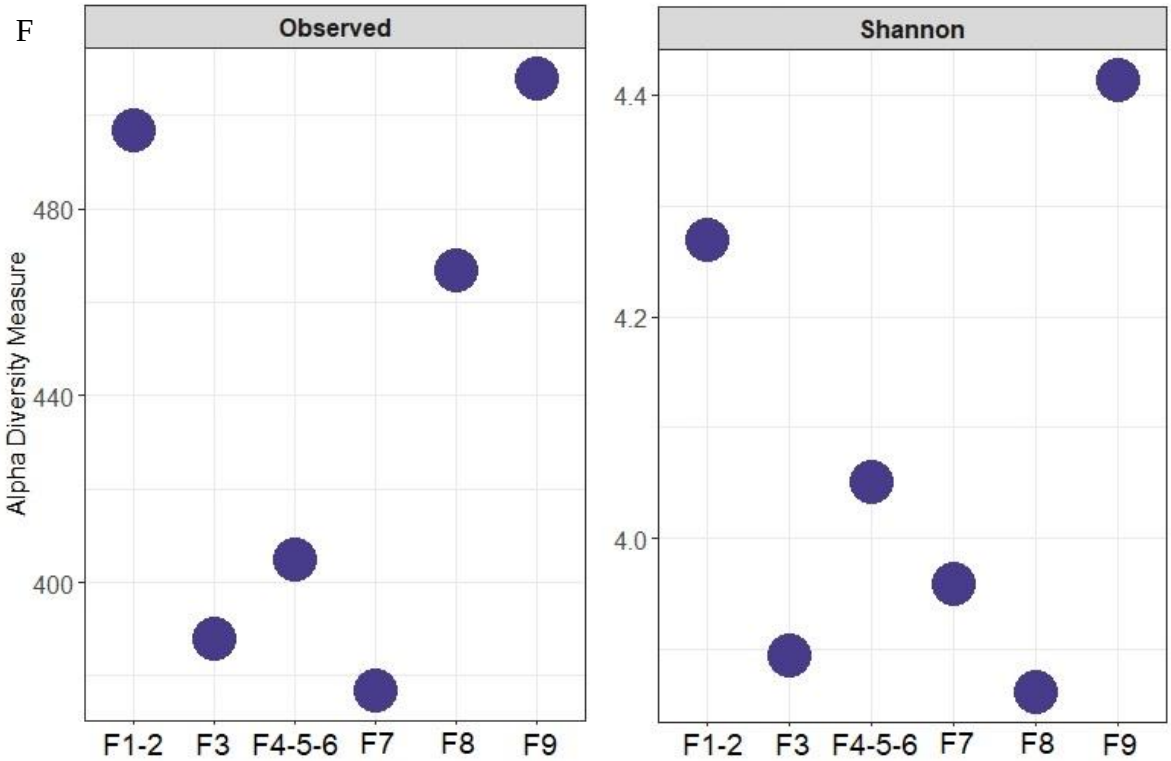
D


Fecal inocula
100%
75%
50%
25%
0%
Abundance
F1-2
F3
F4-5-6
F7
F8
F9
Family
Bacteroidaceae
Christensenellaceae
Clostridiaceae 1
Enterobacteriaceae
Enterococcaceae
Erysipelotrichaceae
Family XIII
Lachnospiraceae
Lactobacillaceae
Muribaculaceae
Peptostreptococcaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
Synergistaceae
Unknown
Other

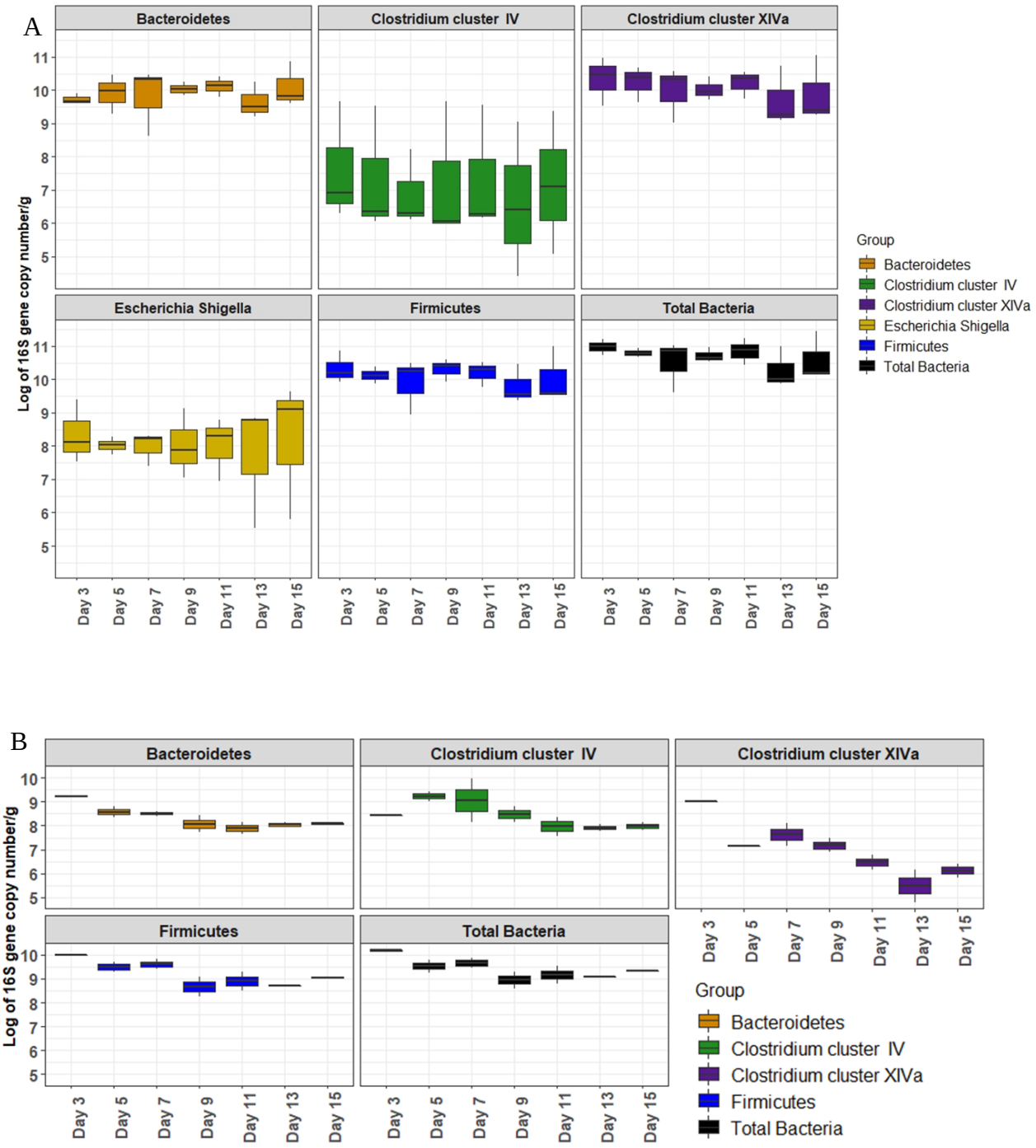
E


Fecal inocula
100%
75%
50%
25%
0%
Abundance
F1-2
F3
F4-5-6
F7
F8
F9
Family
Methanobacteriaceae
Methanomethylophilaceae

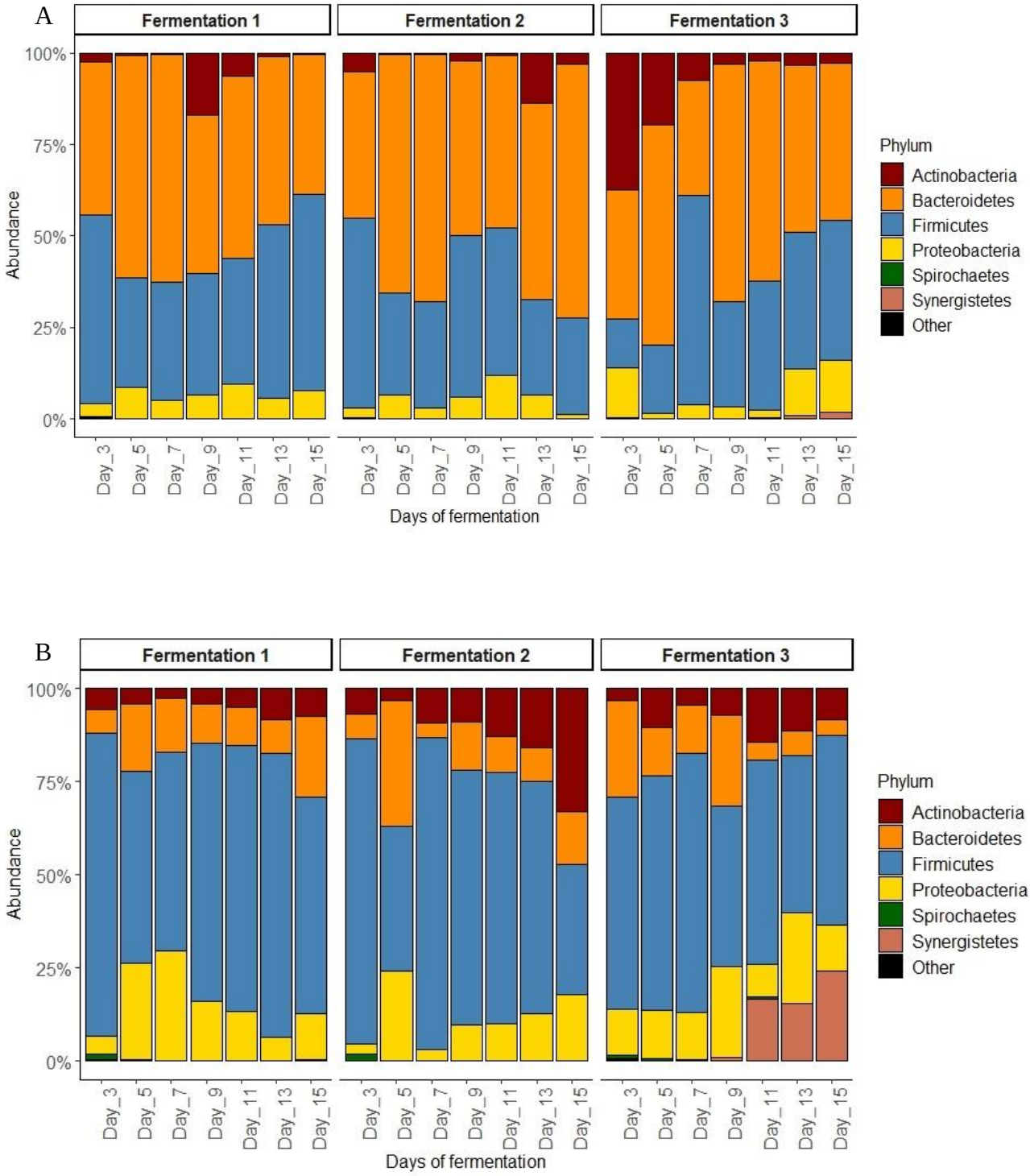
F


Observed
Shannon
Alpha Diversity Measure
480
440
400
4.4
4.2
4.0
F1-2
F3
F4-5-6
F7
F8
F9

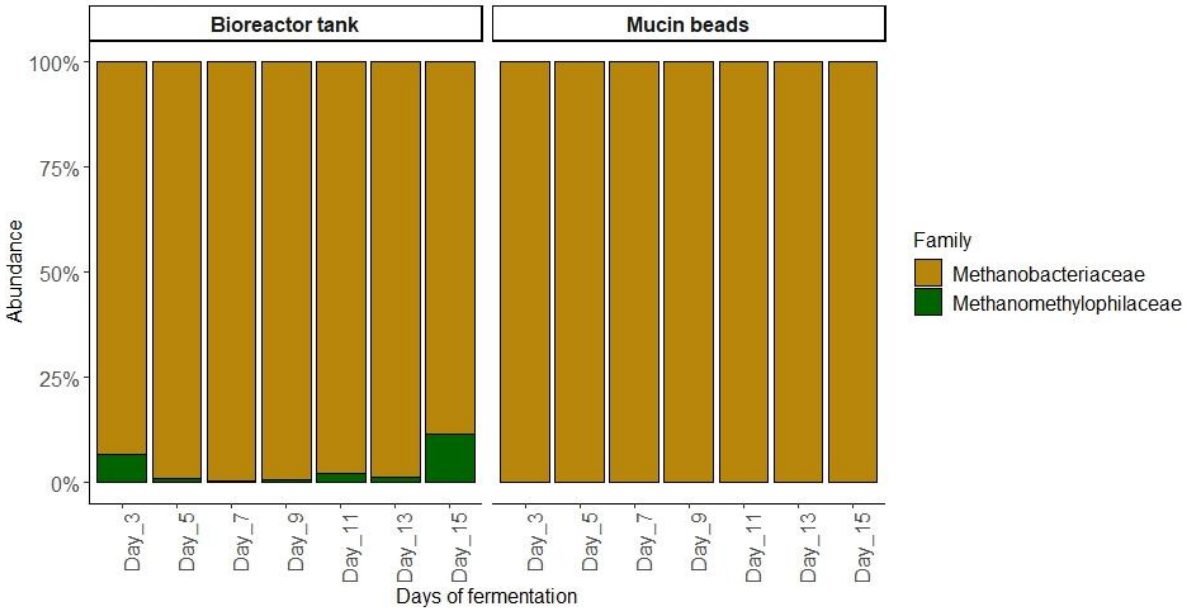
Supplementary Figure 3. *In vitro* microbiota composition inside the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during control assays (fermentations #1, 2 and 3), measured by QPCR (n = 3 for each time point).



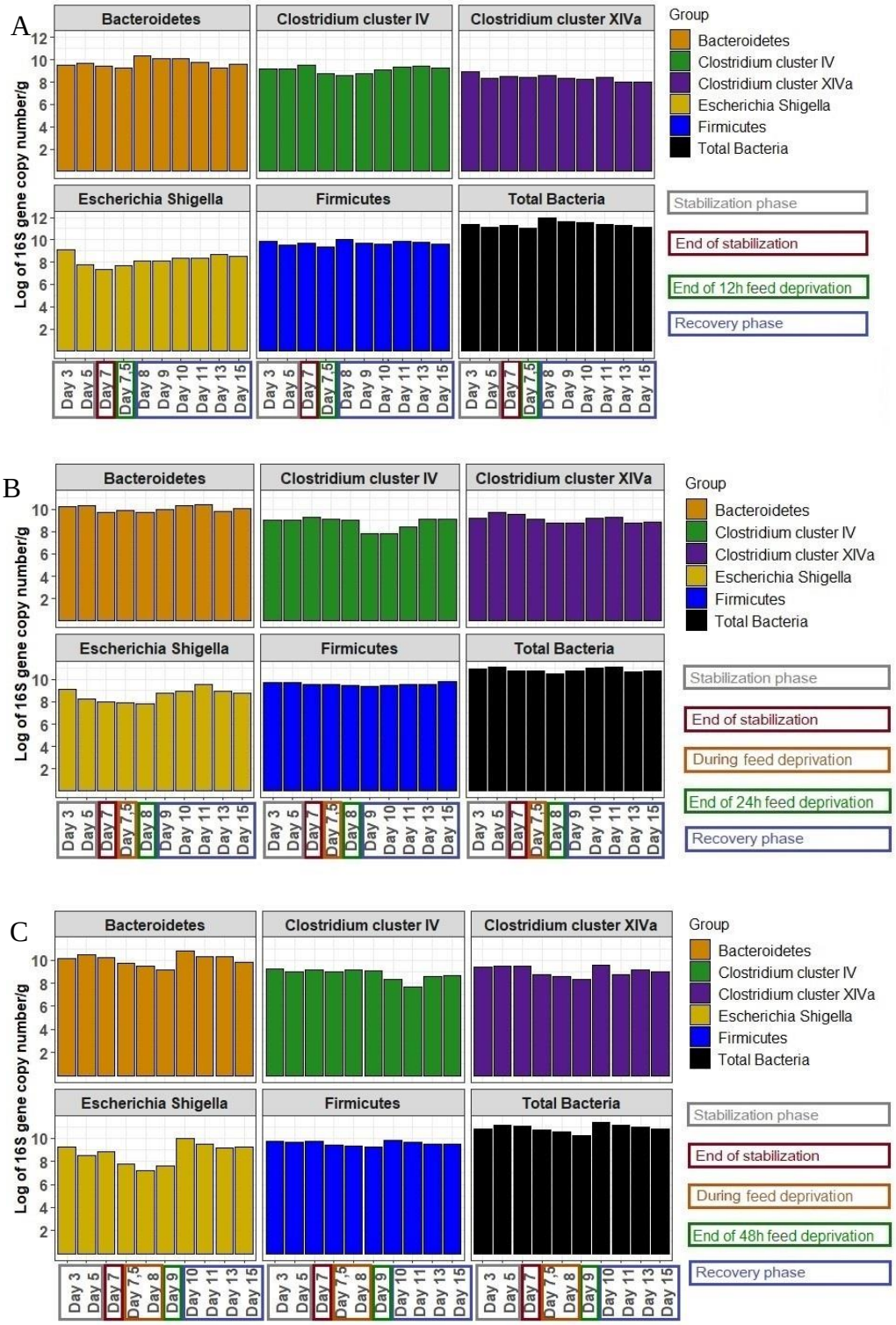
Supplementary Figure 4. Relative abundance of the main bacterial phyla in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during control assays (fermentations #1, 2 and 3), measured by 16S Illumina sequencing.



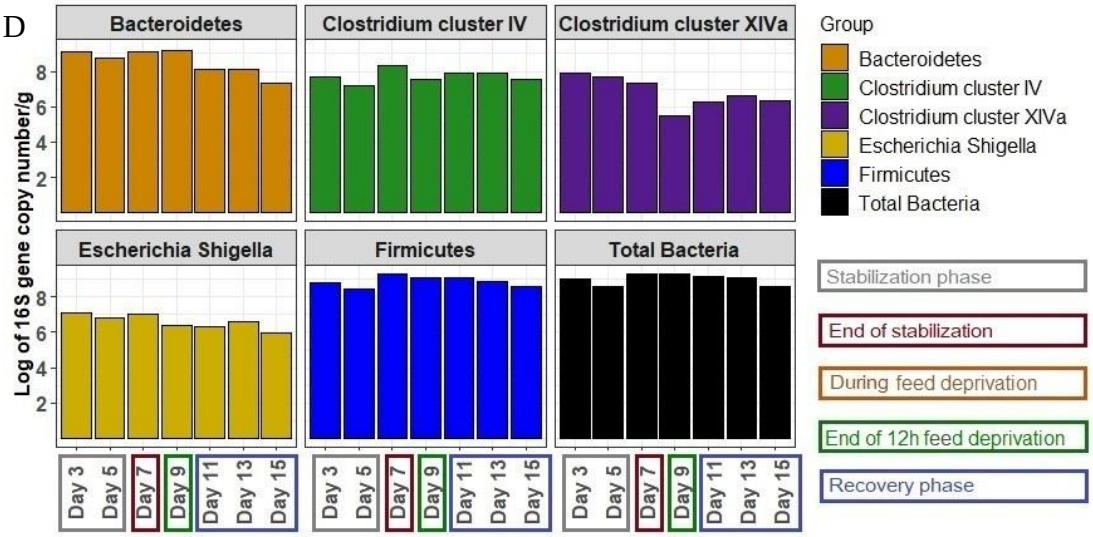
Supplementary Figure 5. Mean relative abundance of the archaeal families in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during control assays (fermentations #1, 2 and 3), measured by 16S Illumina sequencing (n = 3 for each time point).



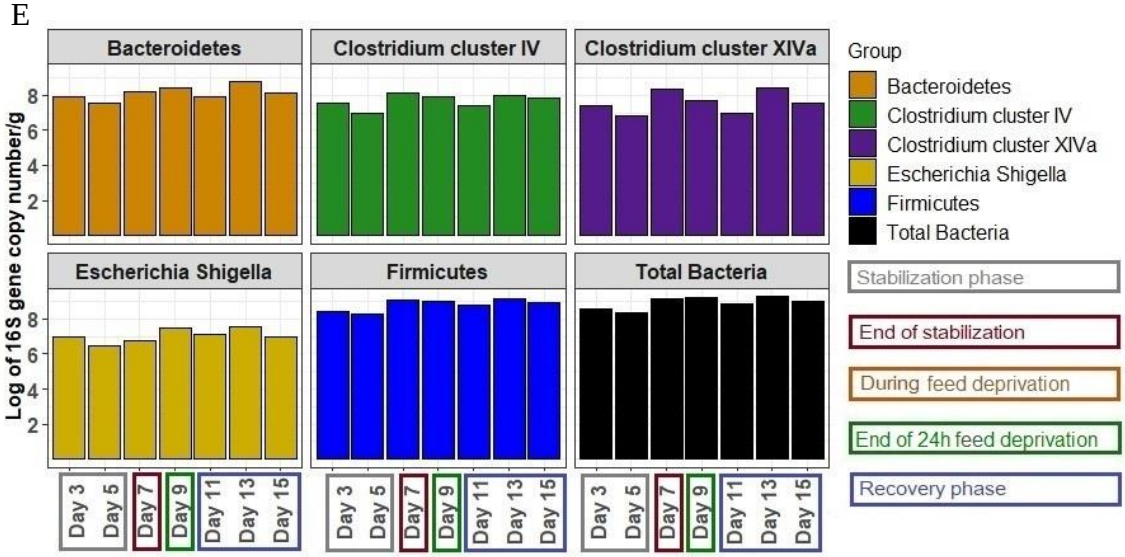
Supplementary Figure 6. *In vitro* microbiota composition in the bioreactor medium (A, B and C) and on the mucin beads (D, E and F) of the MPigut-IVM during the simulation of 12h (A and D), 24h (B and E) and 48h (c and f) feed deprivation stresses, measured by QPCR (n = 1 for each time point).



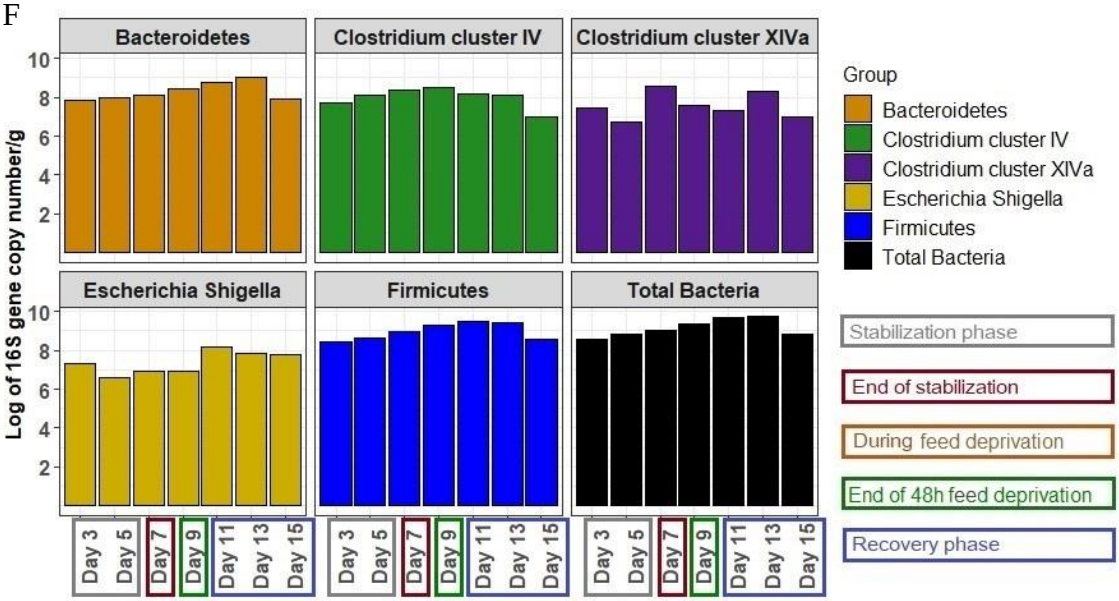
D


Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Log of 16S gene copy number/g
Group
Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Stabilization phase
End of stabilization
During feed deprivation
End of 12h feed deprivation
Recovery phase
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15

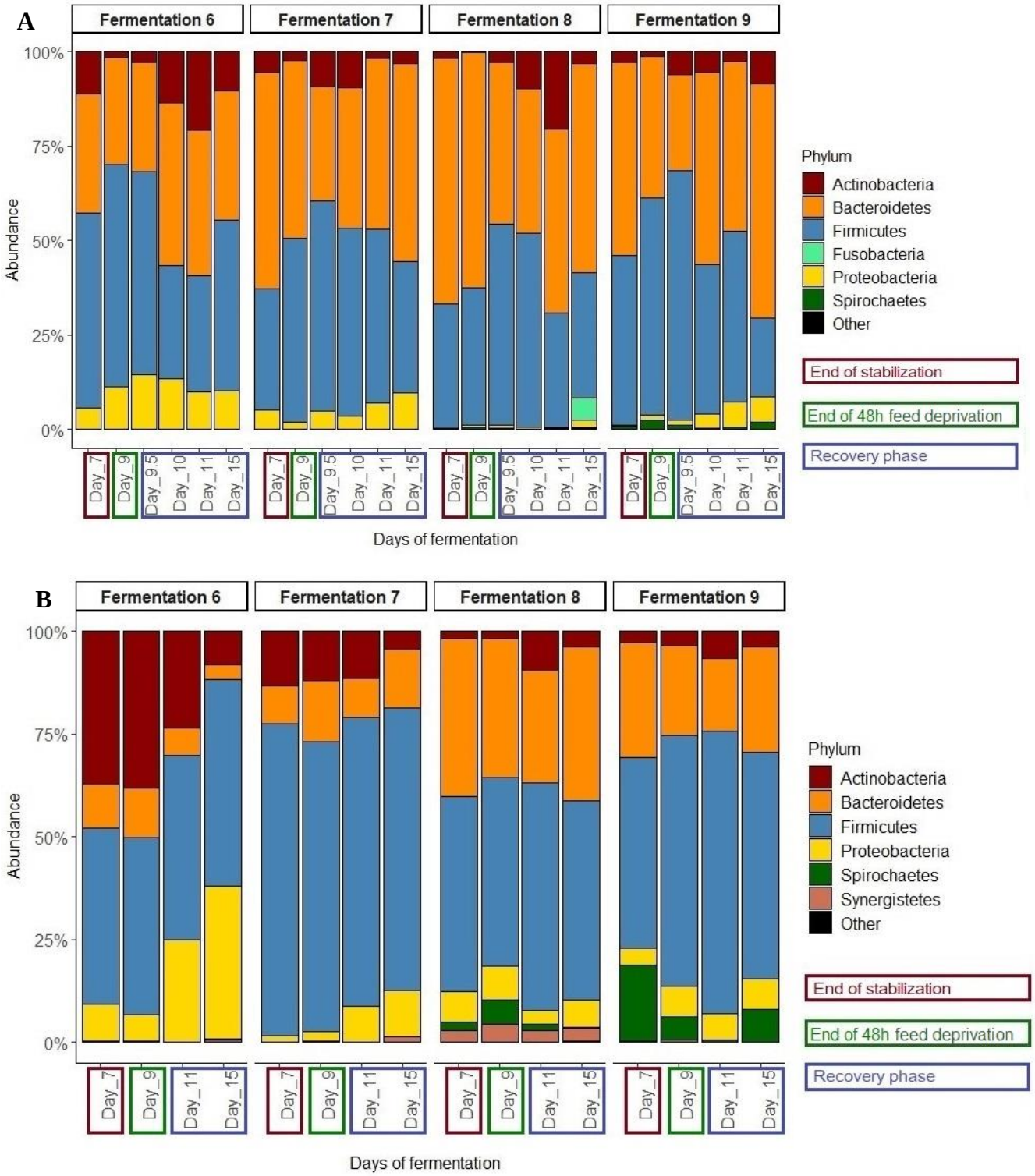
E


Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Log of 16S gene copy number/g
Group
Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Stabilization phase
End of stabilization
During feed deprivation
End of 24h feed deprivation
Recovery phase
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15

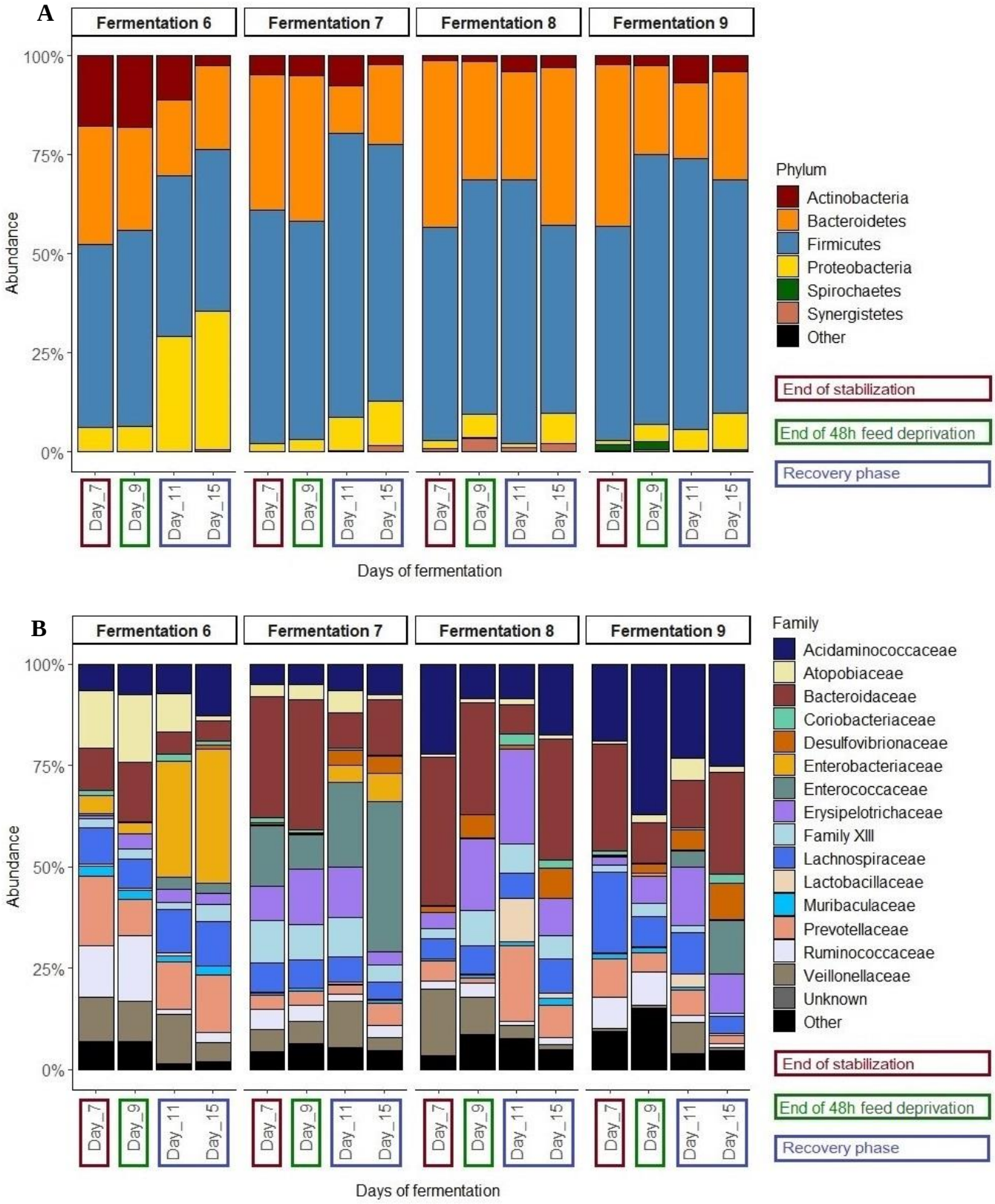
F


Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Log of 16S gene copy number/g
Group
Bacteroidetes
Clostridium cluster IV
Clostridium cluster XIVa
Escherichia Shigella
Firmicutes
Total Bacteria
Stabilization phase
End of stabilization
During feed deprivation
End of 48h feed deprivation
Recovery phase
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15

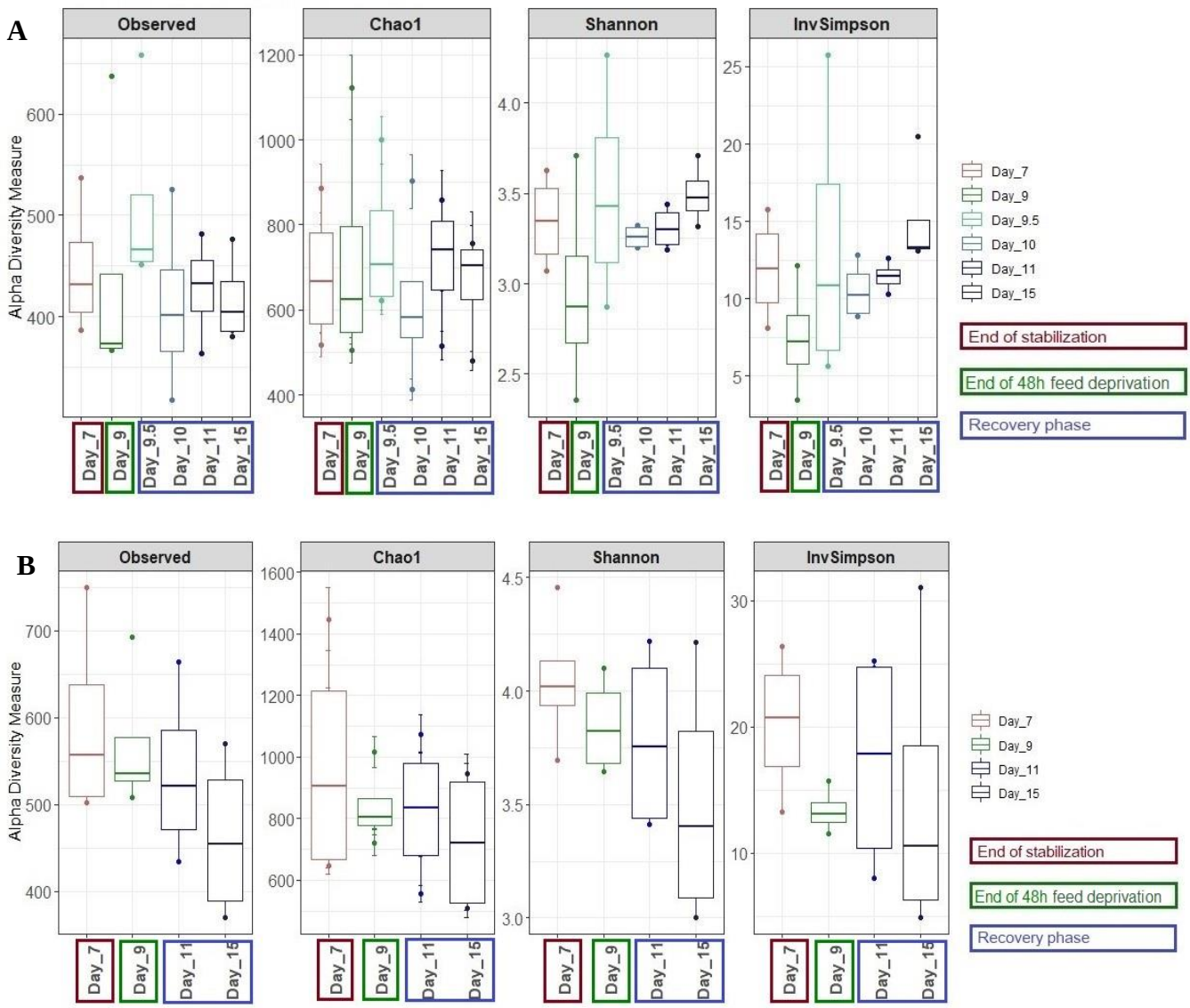
Supplementary Figure 7. Relative abundance of the main bacteria phyla in the bioreactor medium (A) and on the mucin beads (B) in the MPigut-IVM during the fermentations #6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h.



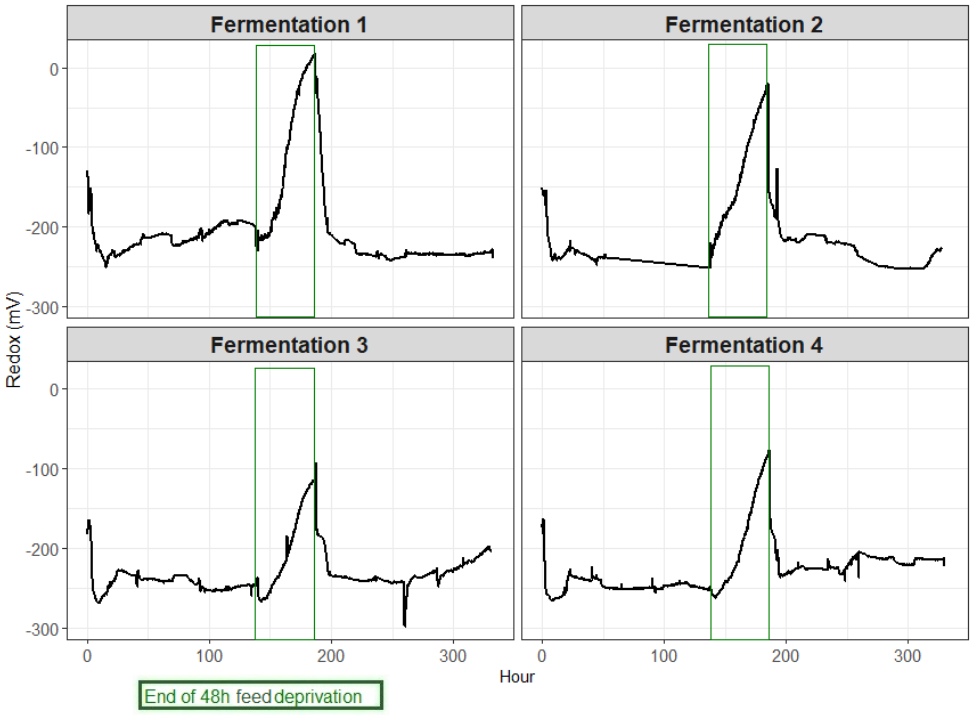
Supplementary Figure 8. Relative abundance of the main bacteria phyla (A) and families (B) in the bead medium of the MPigut-IVM during the fermentations #6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h.



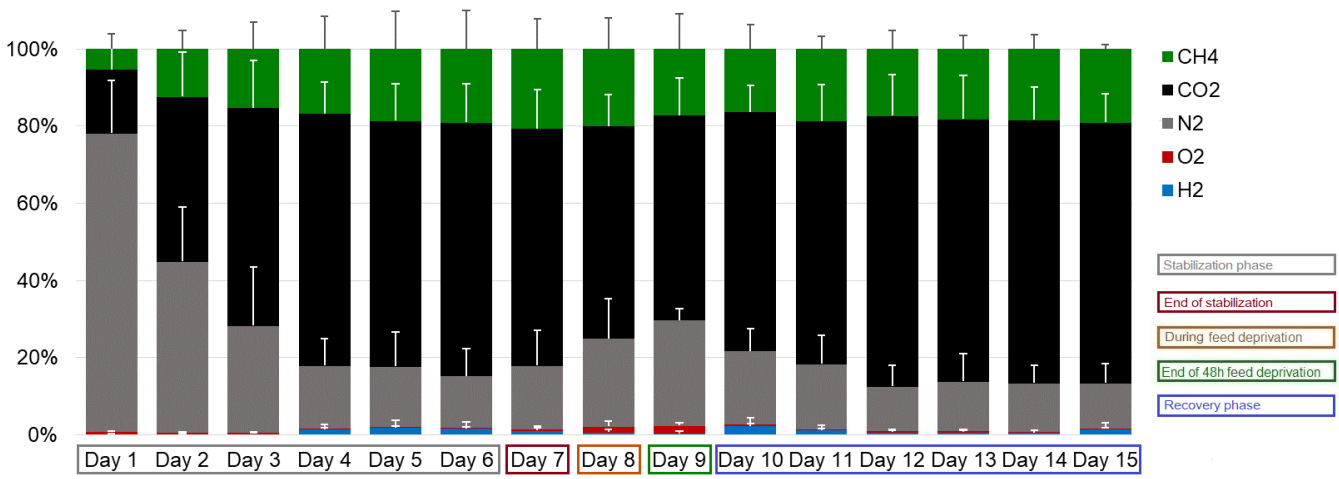
Supplementary Figure 9. Alpha diversity measures on bacterial OTUs in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during the fermentations #6, 7, 8 and 9 which were subjected to a feed deprivation stress of 48h (n = 4).



Supplementary Figure 10. Evolution of the redox potential inside the MPigut-IVM during the fermentations #6, 7, 8 and 9 which were subjected to a 48h feed deprivation stress.



Supplementary Figure 11. Effect of a feed deprivation stress of 48h on the gas composition inside the MPigut-IVM. This figure displays mean relative abundance values of gases and their error bars collected during the fermentations #6, 7, 8 and 9 (n = 4 for each time point).



Annexe

Saccharomyces cerevisiae var _Boulardii_ CNCM I-1079 influences bacterial communities in an _in vitro_ model of the weaning piglet colon (MPigut-IVM).

As seen in chapter 9 part 1, the simulated weaning-associated transient anorexia in the MPigut-IVM led to modifications of microbiota composition and functions consistent with *in vivo* findings. The few days following weaning transition is a very sensitive period, which, in case of microbiota disruption, could easily lead to the bloom of opportunistic pathogens. To prevent against the incidence of post-weaning gut dysbiosis and diarrhea, current investigations aim to find effective feed additives such as probiotics. In porcine industry, the probiotic strain *Saccharomyces cerevisiae* var *Boulardii* (SB) CNCM I-1079 is already commercialized as a feed additive under an active dry yeast form called Levucell SB (Lallemand, Blagnac, France) in order to improve gestating and lactating performance of sows, improve growth performance and feed efficiency in suckling, weaning and fattening pigs and reduce diarrhea (Chaucheyras-Durand and Durand, 2010). In *in vivo* studies, SB CNCM I-1079 daily supplementation was able to positively modulate the intestinal microbiota of piglets by increasing the relative abundance of beneficial SCFA-producing bacteria (Brousseau et al., 2015). Thus, SB CNCM I-1079 could be a candidate to fight against detrimental changes in microbiota composition at weaning. In this annex, we studied the impact of a daily realistic dose of SB CNCM I-1079 on the MPigut-IVM microbiota challenged with a feed deprivation stress of 48h, under experimental conditions similar to the ones described in chapter 9.

MATERIALS AND METHODS

The fermentation procedures and downstream analyses (DNA extraction, qPCR, quantification of SCFA, Illumina sequencing…) were performed as described in the first part on chapter 9 Part 1. The fermentations 7, 8 and 9 from chapter 9 were performed in technical replicates: a CTRL condition and a SB condition (Figure 22). For qPCR analysis, in addition to the primers used in Chapter 9 Part 1, the primers targeting *Saccharomyces cerevisiae* genus (F-5'-AGGAGTGCGGTTCTTTG -3' and R-5'- TACTTACCGAGGCAAGCTACA -3') were used at a concentration of 5 µM and at an annealing temperature 60°C (Chang et al., 2007).

SB CNCM I-1079 supplementation procedure

MPigut-IVM from the SB condition were supplemented with 1mL of SB CNCM I-1079 (Levucell SB, Lallemand, Blagnac, France) at a concentration of 10^6 CFU/mL, every day at fixed time, except during the feed deprivation period (day 8), while the CTRL condition received 1 mL of sterile 1X PBS. The choice of SB CNCM I-1079 dose concentration in the MPigut-IVM was established based on *in vivo* data from Lallemand reporting the number of live yeasts in the colon of piglets after an intake of $3x10^9$ CFU/day/piglet (Le Bon et al., 2016).

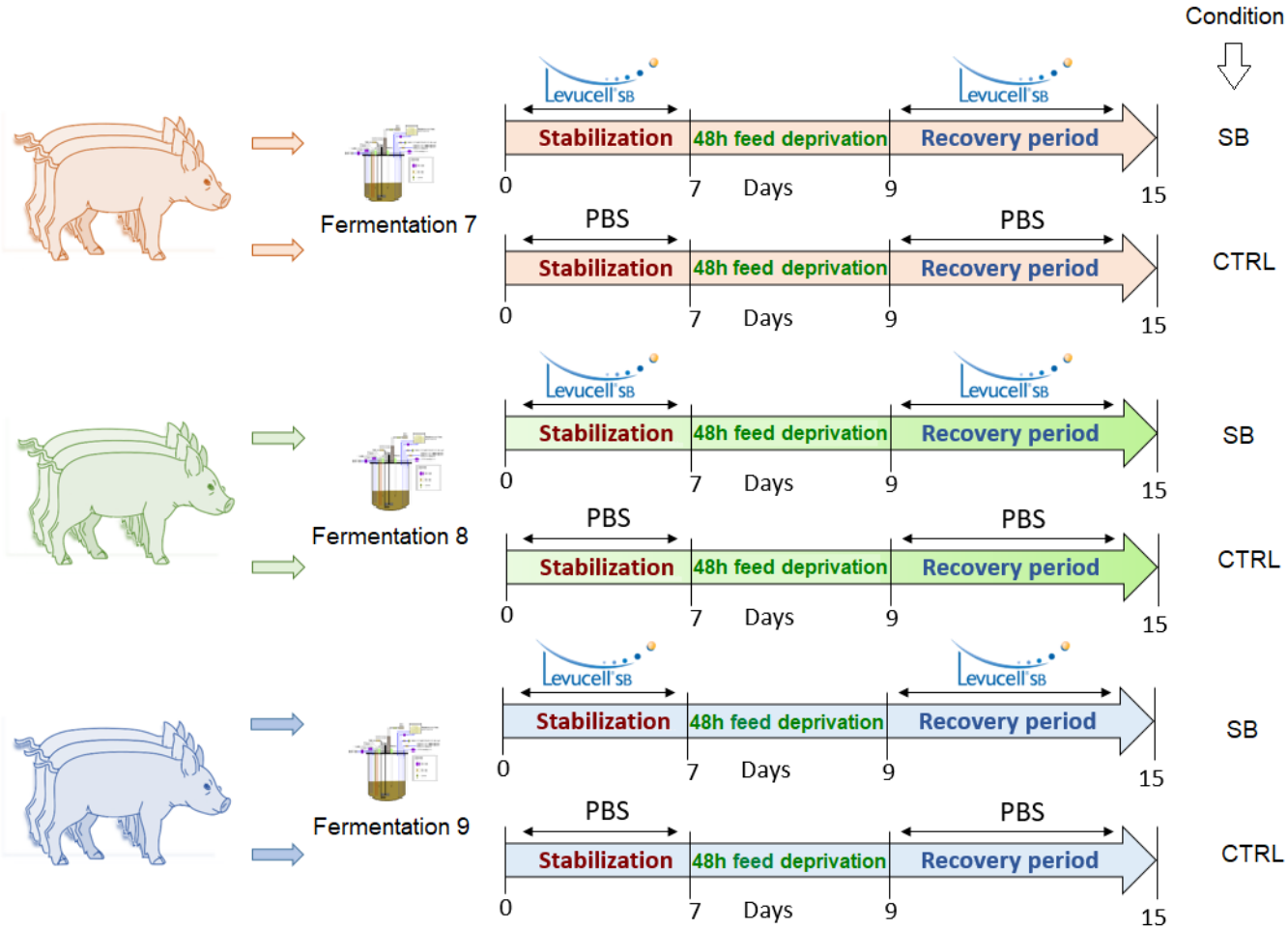


Figure 22. Experimental design of the MPigut-IVM runs presented in the annexe of the Chapter 9.

RESULTS

Impact of live SB CNCM I-1079 on microbiota composition and activity in the MPigut-IVM challenged with a feed deprivation stress

We have previously used the MPigut-IVM to simulate a feed deprivation stress associated with weaning and have reported the effects of an *in vitro* feed deprivation stress on the fermentor microbiota (see chapter 9). The same observations were obtained here in the control fermentations, and will thus not be detailed in the present report.

The daily supplementation of the MPigut-IVM with 10^6 CFU/mL of live SB CNCM I-1079 led to an expected significantly ($P<0.05$) higher concentration of *S. cerevisiae* in all samples from 0 to 6.1 ± 3.5 Log_{10} of target gene copy number/ mL of sample and to significantly higher concentration of *Escherichia /Shigella* group in the bioreactor medium from a mean of 7.5 ± 0.6 log of 16S gene copy / mL in the CTRL condition to a mean of 8.3 ± 0.9 log of 16S gene copy / mL in the SB condition (Figure 23). The total bacteria and archaea population levels were not impacted by the presence of SB. The Illumina MiSeq run revealed that the supplementation of the MPigut-IVM with SB CNCM I-1079 at a dose of 10^6 CFU/ mL had no impact on the microbiota at the phylum level both from the bioreactor medium and the mucin beads (Supplementary figure 12). On the mucin beads and at the family level, the SB daily supplementation significantly increased the relative abundance of the *Streptococcaceae* family from a mean relative abundance of 1.8 ± 2.8 % in the CTRL condition to 7.7 ± 8.2 % in SB condition and decreased the relative abundance of *Veillonellaceae* from a mean relative abundance of 4.9 ± 5.3 % in the CTRL condition to 3.0 ± 3.5 % in SB condition (Figure 24). In the bioreactor medium of the MPigut-IVM, SB supplementation had no impact on the bacterial microbiota (Supplementary figure 13) nor on archaeal community (data not shown). Alpha diversity indices did not display any difference between groups (data not shown).

SB treatment had no significant impact on gas composition (Supplementary figure 14), SCFA relative abundance (Supplementary Figure 15), total SCFA concentration (Supplementary figure 16) or the metabolome (data not shown) of the MPigut-IVM. However, the maximal redox potential values reached during the 48h feed deprivation period in CTRL conditions were significantly higher than in SB conditions (Figure 25).

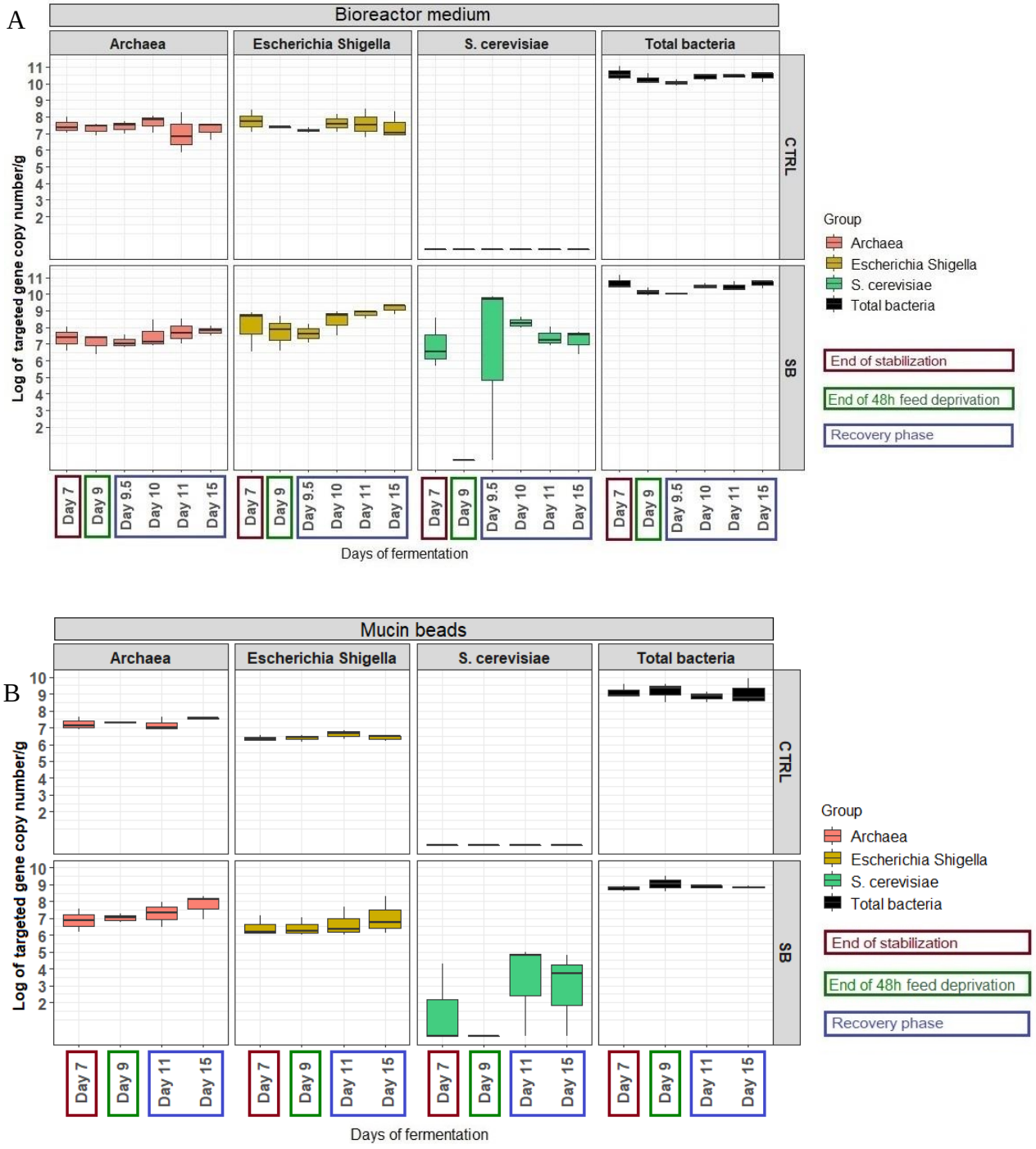


Figure 23. Quantification of total bacteria, *S. cerevisiae*, methanogenic archaea and *Escherichia/Shigella* in the bioreactor medium (A) and the mucin beads (B) of the MPigut-IVM during the CTRL and SB conditions.

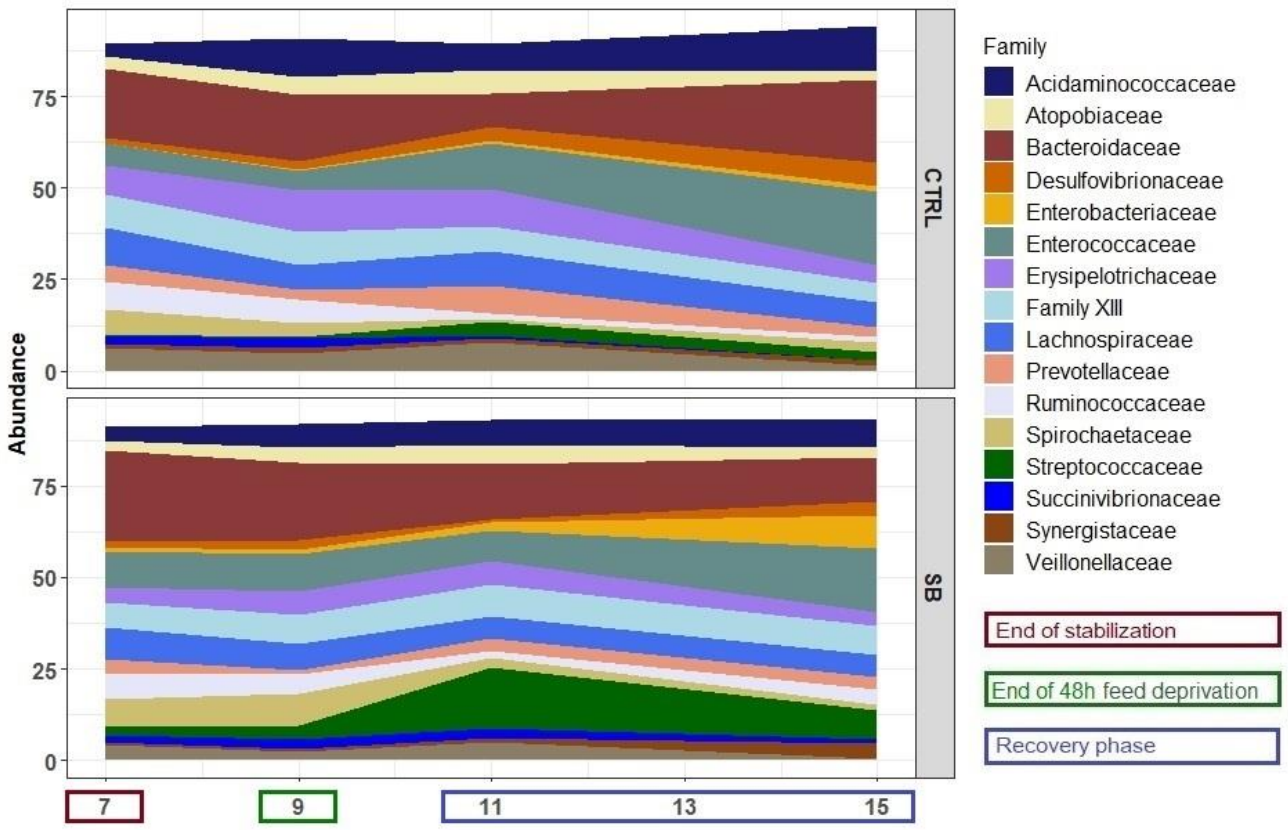


Figure 24. Relative abundance of the main bacterial families on the mucin beads of the MPigut-IVM for CTRL and SB conditions.

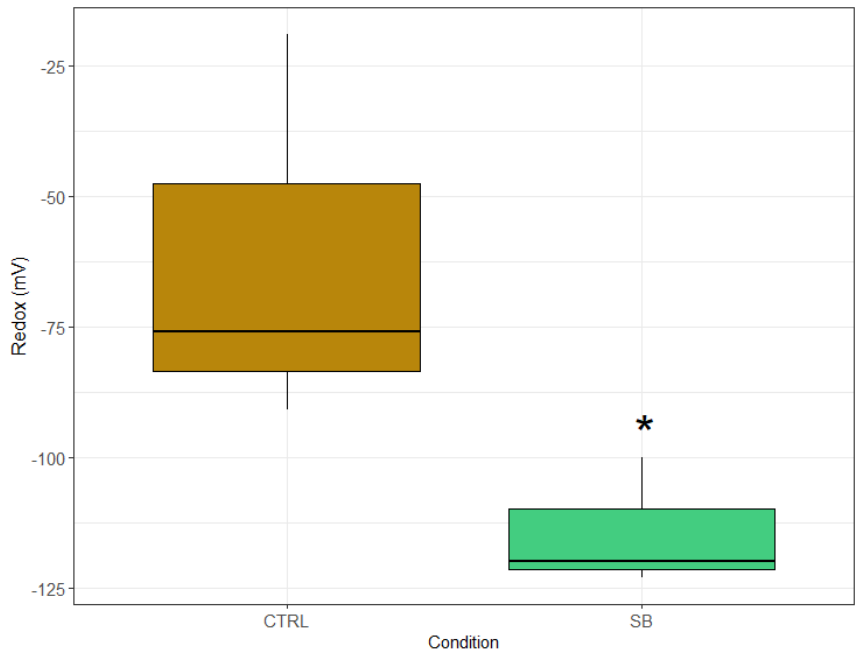


Figure 25. Maximal redox potential reached during the feed deprivation phase for CTRL and SB conditions. *Differences between the two conditions are significant with a *p*value < 0.05.

DISCUSSION

Rather few effects were provoked by the supplementation of a daily dose of SB in the MPigut-IVM. This can be explained by the choice of performing this experiment with realistic daily dose of 10^6 CFU/mL based on *in vivo* data (Le Bon et al., 2016), compared to normally higher dose of probiotics in *in vitro* models reaching most of the time 10^8 to 10^{10} CFU/mL (Fleury et al., 2017; Tanner et al., 2015). In a study involving human colonic *in vitro* models, Roussel *et al.* (2019) demonstrated a beneficial effect of another SB strain via an increase of SCFA and butyrate-producing bacteria such *Faecalibacterium* or *Roseburia.* However, in this study the daily dose of probiotics was reaching 10^8 CFU/mL (Roussel, 2019). Fleury *et al.* which studied the impact of a twice daily dose of SB CNCM I-1079 of $5x10^6$ CFU/mL corresponding to 10^7 CFU/day, observe no modifications of SCFA productions but could demonstrated an decrease of *E. coli* counts (Fleury et al., 2017). However, this last study consisted in only one biological repetitions and, as seen in our results, effects of SB CNCM I-1079 on *Escherichia Shigella* seems to be inoculum dependent. A noticeable effect of SB in the MPigut-IVM was the positive, although limited, impact on the maximum redox potential during the feed deprivation period. A lower redox potential reflects a better reducing capacity and thus a more active microbiota (Circu and Aw, 2011; Friedman et al., 2017). If no direct effects on SCFA or other bacterial metabolites detected by NMR were highlighted in MPigut-IVM samples in this study, SB could positively affect microbiota activity, which by maintaining few fermentation mechanisms, could help to reduce oxidative stress. An *in vivo* study reported that, besides reducing diarrhea rates, dietary *Saccharomyces cerevisiae* cell wall extracts could alleviate oxidative stress in weaned piglets via the increase of antioxidant enzyme concentration in the blood (Liu et al., 2017). Additionally, a study using a combined probiotic approach with *Clostridium butyricum* and a SB strain in neonatal piglets which received a daily dose of probiotic during the first 3 days after birth concluded that the early life probiotic dose induced lower oxidative stress after weaning by measuring antioxidant capacities in the blood (Xiang et al., 2020). However, no accurate functional explanations are yet available in the literature.

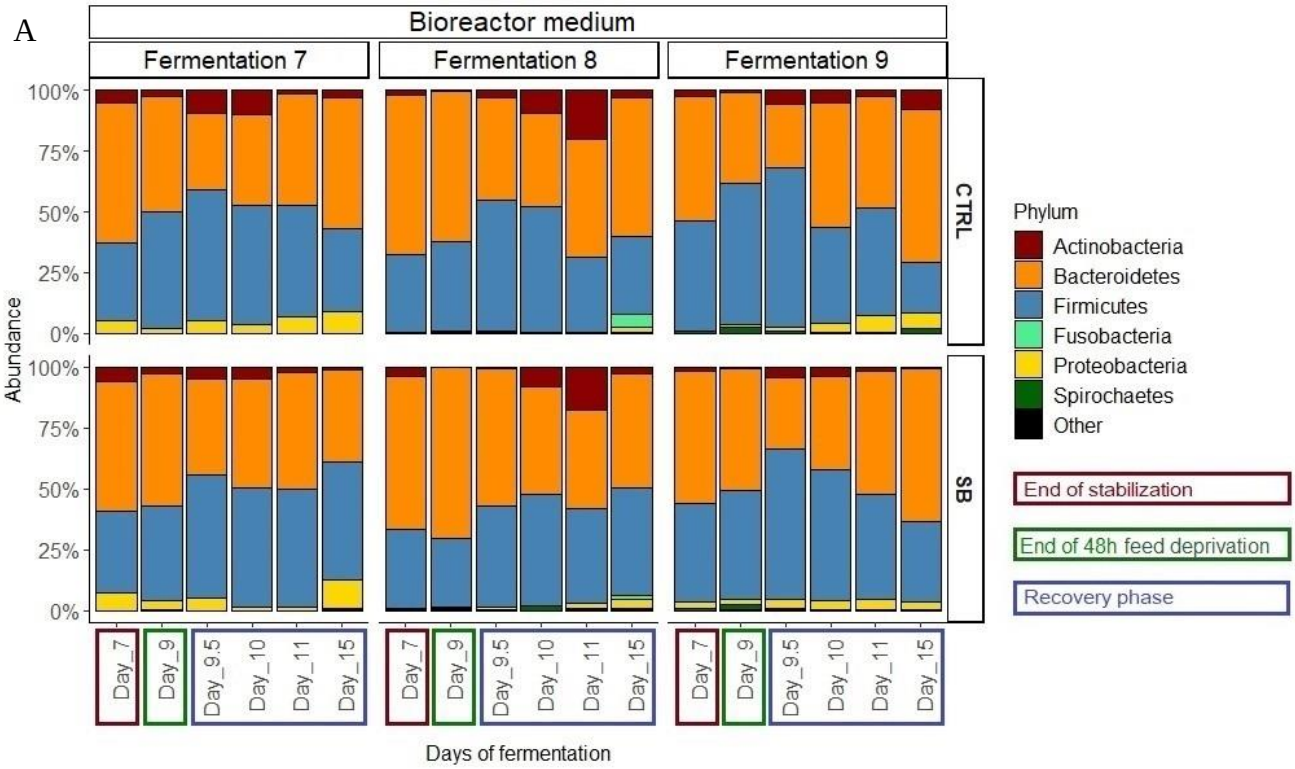
In the MPigut-IVM, the *SB* CNCM I-1079, supplemented at of 10^6 CFU/mL, seemed to favour the *Escherichia/Shigella* group in the bioreactor medium for which a 0.8 Log_{10} 16S copy number/mL was measured in the SB supplemented fermenters compared to controls. If the *Escherichia/Shigella* genus contains some opportunistic pathogens, such as ETEC, most of its members are recognized as commensals of mammalian GIT. Interestingly, Bin et *al.* highlighted that the relative abundance of *E. coli* was reduced from 86% to 49% in the jejunum

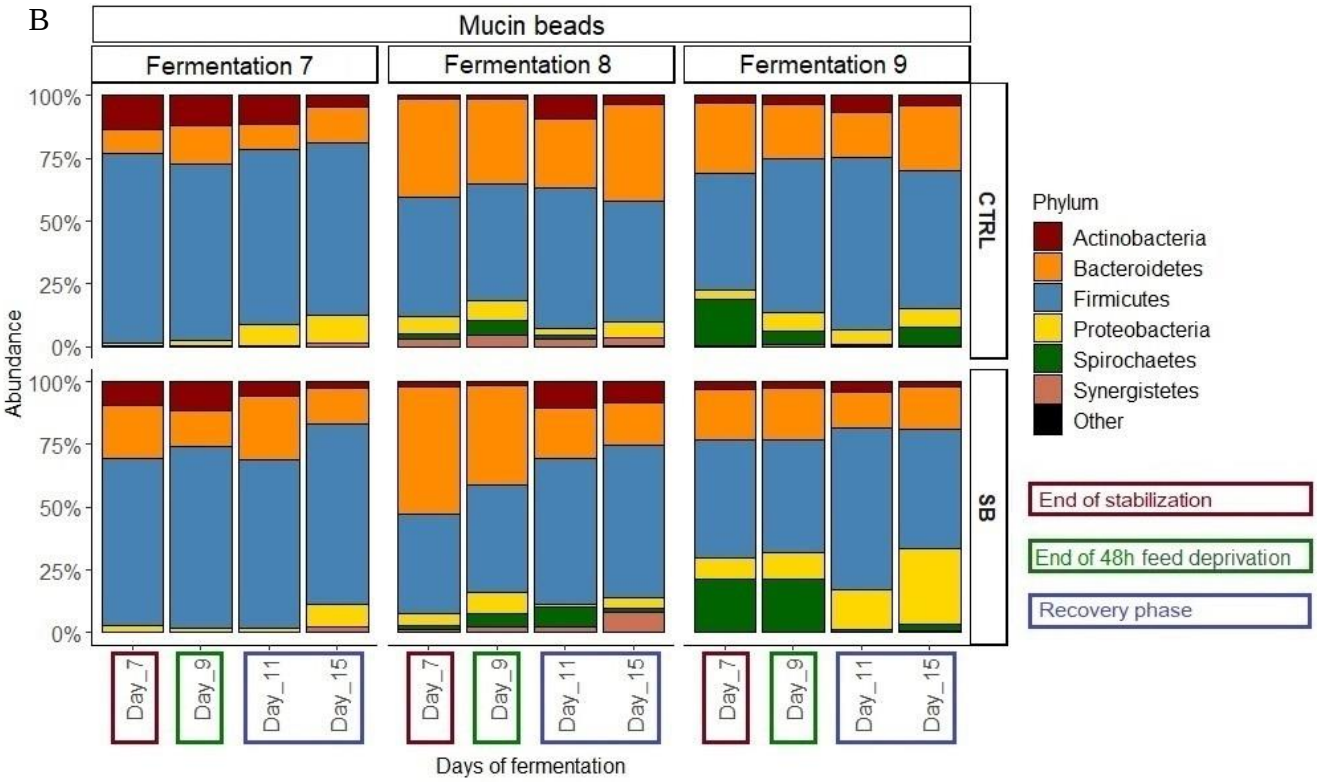
of ETEC-challenged diarrheic piglets compared to healthy individuals (Bin et al., 2018). This finding was later confirmed by Yang et *al.* who reported that *E. coli* was decreased in the fecal microbiota of pre-weaned diarrheic piglets (H. Yang et al., 2017). Besides, the probiotic strain *E. coli* Nissle 1917 has shown efficient preventive properties against ETEC-induced diarrhea in an infection model of pre-weaned piglets (Schroeder et al., 2006) and could suppress clinical signs, histopathological changes and induction of inflammatory cytokines associated with *Salmonella typhimurium* infections in germ-free piglets (Splichal et al., 2019). Further research is needed to confirm these data and provide insights about the implicated mechanisms, but commensal *E. coli* present in a sufficient amount in the GIT of pre-weaned piglets could provide an efficient protection against the onset of PWD as they would occupy the ecological niche of *E.coli* pathogenic strains. SB CNCM I-1079 daily dose also favoured the *Streptococcaceae* family in the mucin beads of the MPigut-IVM. *Streptococaceae* are normal commensals of piglet microbiota which were found significantly reduced in fecal microbiota of diarrheic weaning piglets compared to healthy piglets (Q. Yang et al., 2017).

To conclude, the present report indicated that the potential mode of action of probiotic yeast could be indeed the modulation of intestinal microbiota composition and activity. Further research is needed to better identify the microorganisms and their metabolites implicated that could help piglets to resist against intestinal dysbiosis and infections. Also, the dose effect of probiotics supplementation should be investigated.

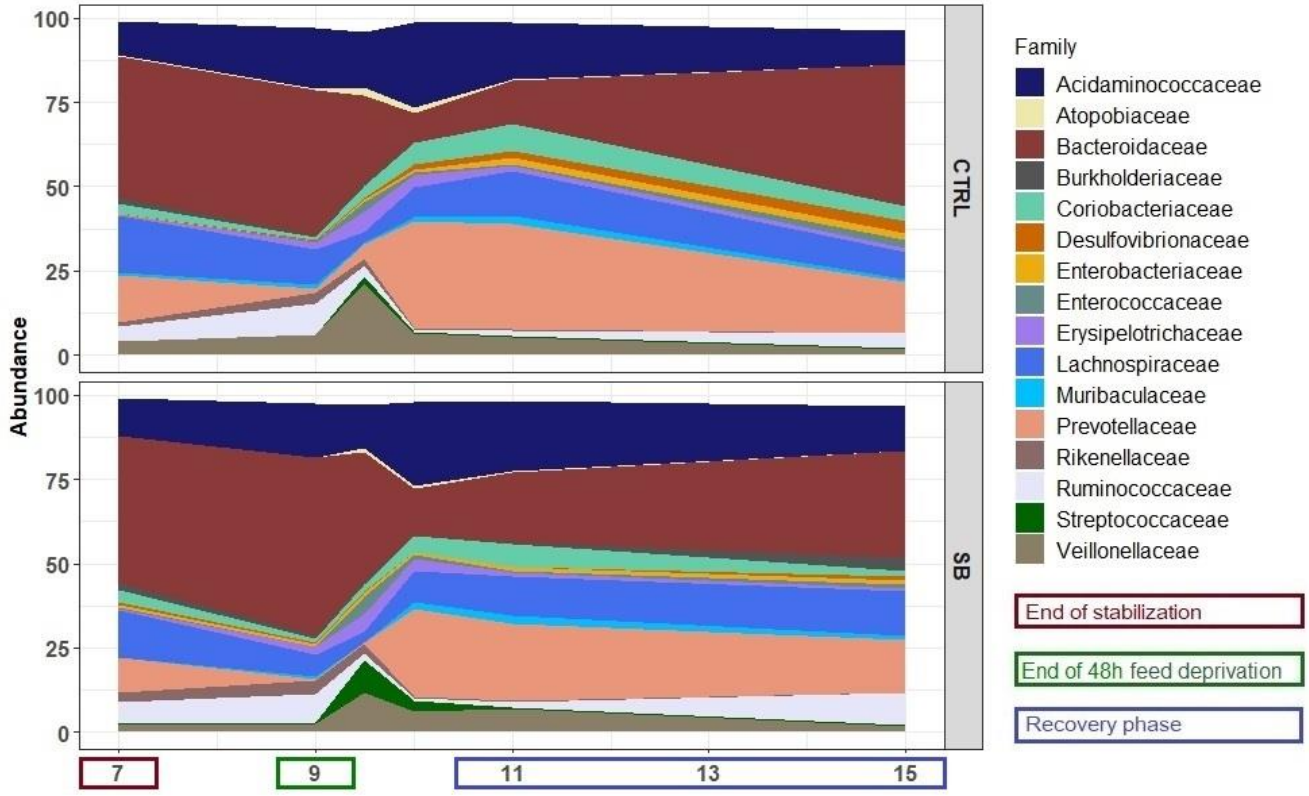
SUPPLEMENTARY MATERIAL

Supplementary figure 12. Relative abundance of the main bacterial phyla in the bioreactor medium (A) and the mucin beads (B) of the MPigut-IVM during the CTRL and SB conditions.

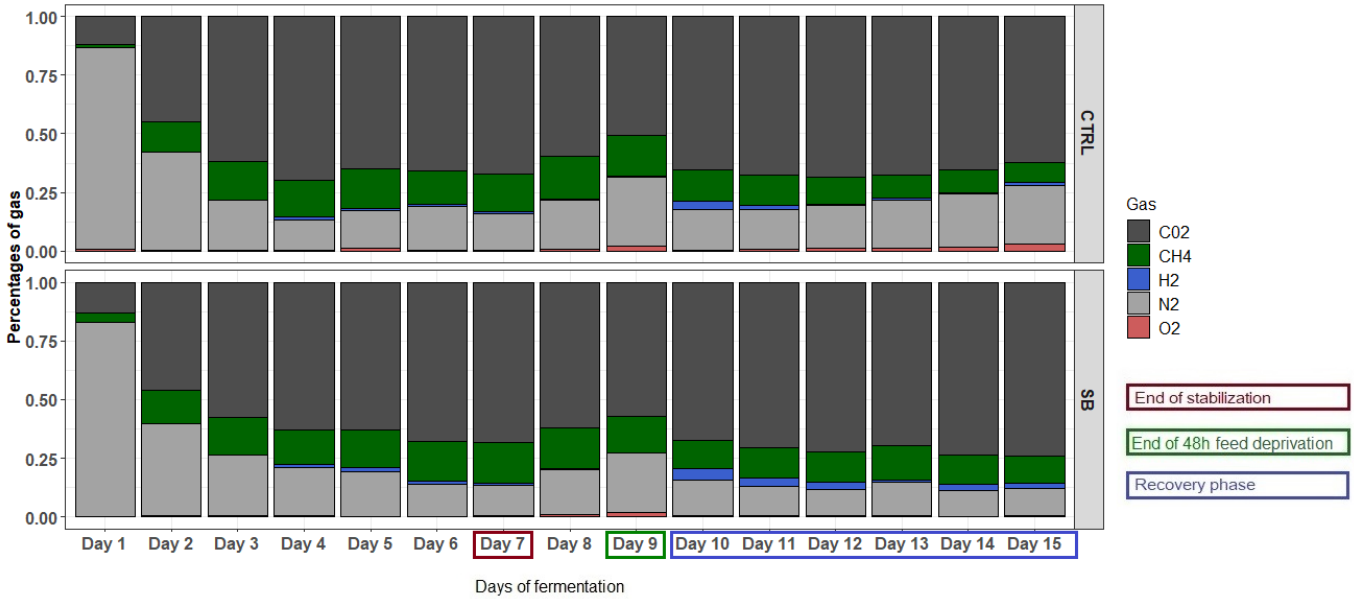




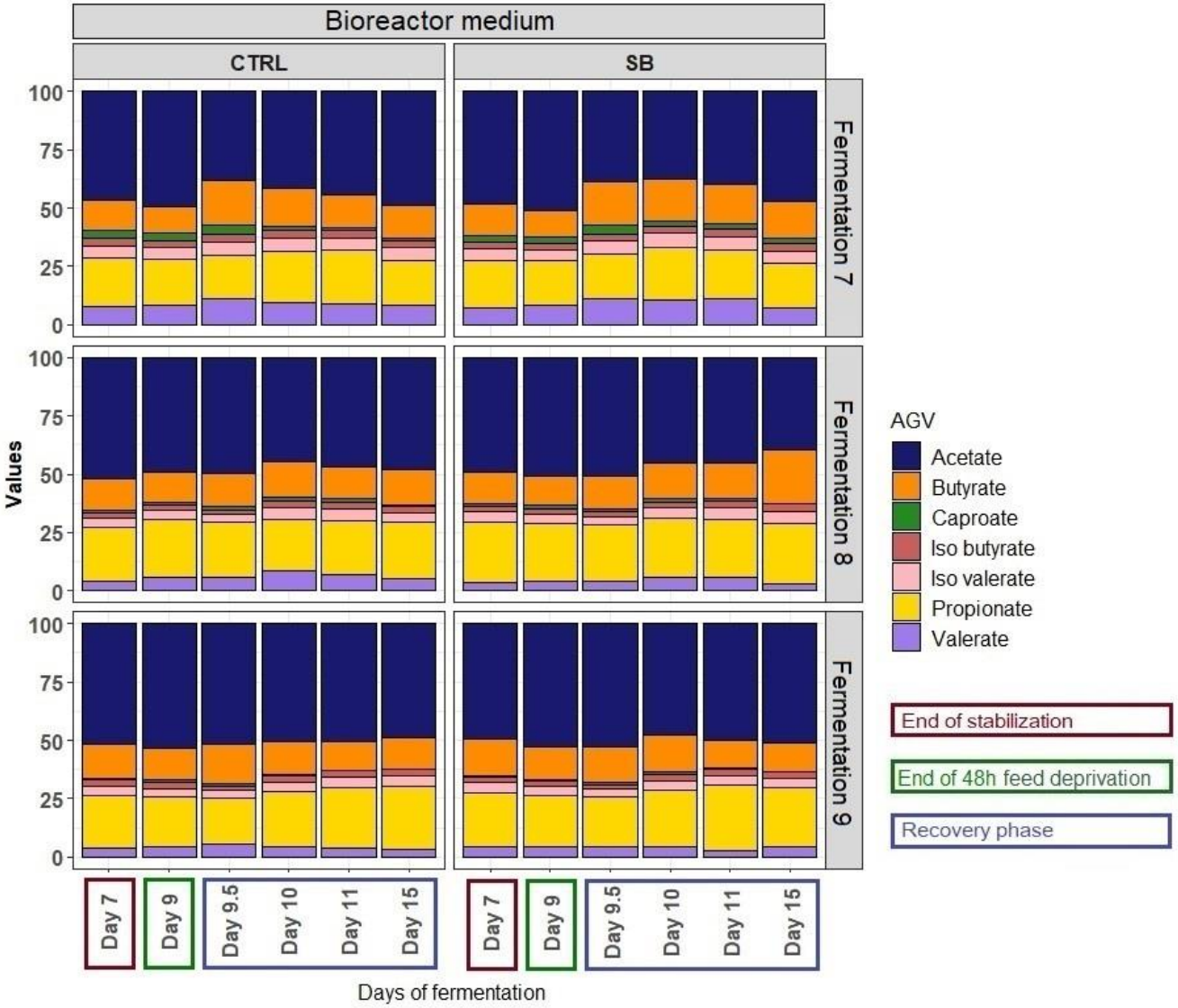
Supplementary figure 13. Mean relative abundance of the main bacterial families in the bioreactor of the MPigut-IVM during the SB and CTRL conditions.



Supplementary figure 14. Relative abundance of gas produced in the bioreactor medium of the MPigut-IVM during the CTRL and SB conditions.



Supplementary figure 15. Relative abundance of the SCFA in the bioreactor medium of the MPigut-IVM during the CTRL and SB conditions.



Chapter 10. Impact of Pathogen Exposure and Dietary Stress at Weaning: Application to a Mucin Associated *In Vitro* Model of the Piglet Colon (MPigut-IVM)

As seen in Chapter 9, the MPigut-IVM displayed a good reproducibility, stability and consistency toward *in vivo* microbiota while keeping enough variability with biological replicates to closely mimic individual heterogeneity. The simulation of the weaning associated transient anorexia with a feed deprivation 48h-period in the MPigut-IVM led to transient changes in microbiota composition and activity, which, *in vivo,* could impact host physiology. In order to bring a more complete picture of the potential effects of weaning transition on the microbiota and the host, a dietary change was introduced in the MPigut-IVM just after the feed deprivation period. This dietary change consisted in the introduction of new sources of plant protein, higher total amount of proteins and fibers and lower amount of milk derived products to simulate to the principal characteristics of dietary change in swine industry. Additionally, as often observed at weaning transition, the effects of a pathogen exposure on the MPigut-IVM was tested after the simulated weaning transition (feed deprivation + dietary change). This experimentation was performed using an ETEC strain isolated from a diarrheic Spanish piglet kindly provided from Pr. Dr. Juan J. Garrido in order to be consistent with *in vivo* conditions. In total, 3 biological replicates corresponding to fermentations initiated with 3 different fecal inocula (pools of feces) were carried out with or without pathogen exposure. This bunch of experiments was performed to answer the following questions:

- Does a dietary shift impact further the effect of feed deprivation on the MPigut-IVM microbiota composition and activity?
- Is the ETEC strain able to colonise and maintain in the MPigut-IVM and the mucin beads?

- Does the presence of an ETEC strain impact the MPigut-IVM microbiota composition and activity in the absence of the host?
- What is the effect of a dysbiotic microbiota on the survival and virulence of an ETEC strain?

Additionally, supernatants of the MPigut-IVM contents were coupled with porcine intestinal cell lines to study host responses towards microbiota changes and pathogen exposure. This part of the work was performed during a PhD secondment at the Department of Animal Genetics of Cordoba University under the supervision of Pr. Dr. Juan J. Garrido. Prior to this experiment preliminary assays were performed to establish optimal incubation conditions. A set of genes involved in immune response, tight junctions, and mucin production was selected to evaluate the responses of porcine epithelial cells to MPigut-IVM samples of the bead medium which had been subjected to the simulated weaning transition and ETEC challenge.

The graphical abstract presented hereafter summarizes the principal findings of this Chapter (figure IV). Both dietary change and pathogen inoculation had consequences on the MPigut-IVM microbiota composition and activity such as modifications of the *Prevotella/ Bacteroides* ratio. Additionally, effluents collected from the MPigut-IVM after introduction of the ETEC strain were able to modulate gene expression of some targeted genes by porcine epithelial cells.

This Chapter corresponds to an article in preparation for the Journal Applied and Environmental Microbiology.

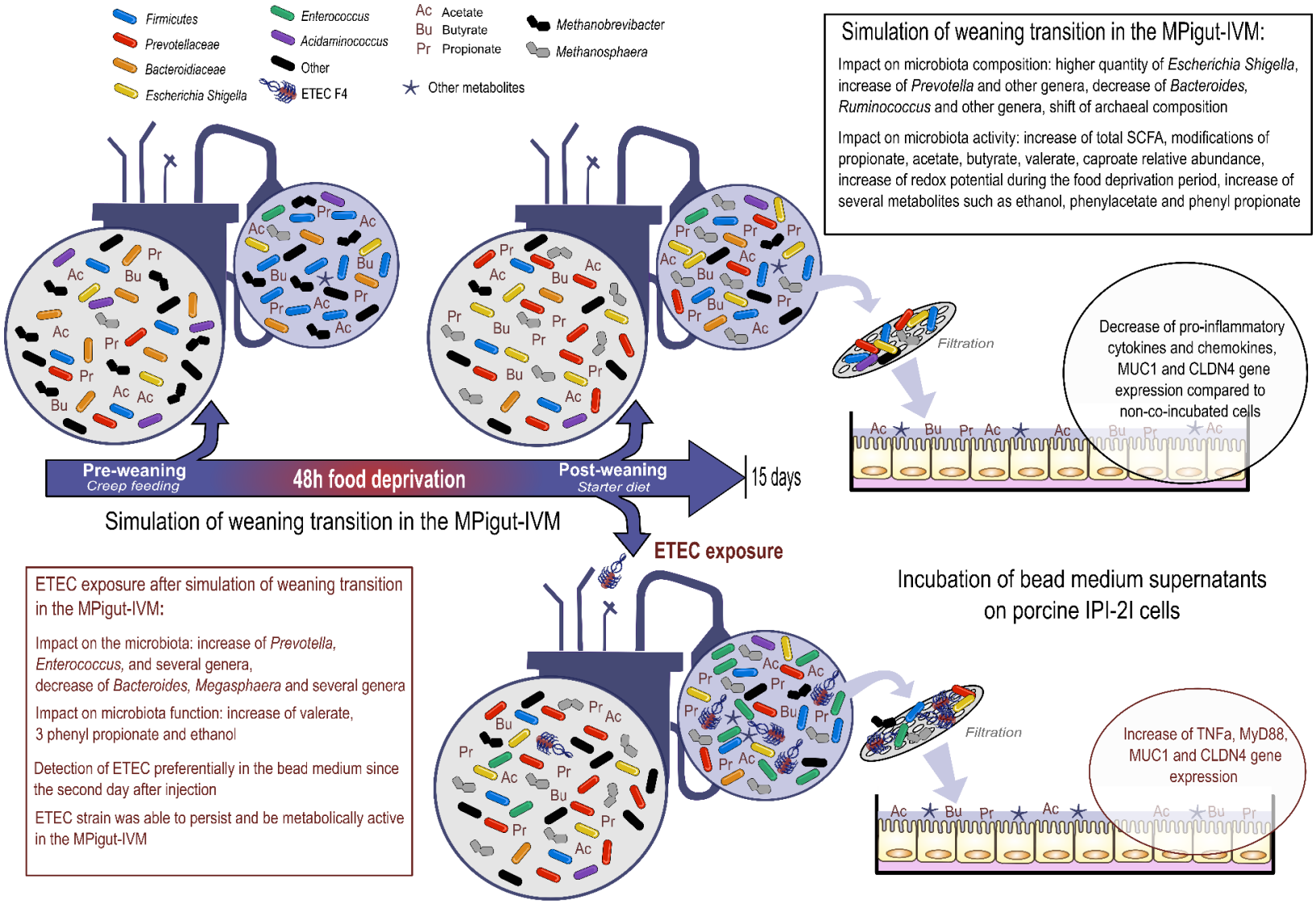


Figure IV. Graphical abstract of the Chapter 10.

Impact of pathogen exposure and dietary stress at weaning: application to a mucin associated *in vitro* model of the piglet colon (MPigut-IVM)

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IMPORTANCE

Weaning is the most critical event in swine industry due to the high amounts of stress encountered by animals at social, dietary or environmental levels. Physiological consequences of weaning stressors are profuse and lead to high incidence of post-weaning diarrhea and enteric infections in piglets. Mortality and morbidity associated with these infections induce substantial economic losses and the overuse use of antibiotics as therapeutics. The massive use of antibiotics considerably increases the spreading of antibiotic resistant genes in bacteria and represent a real threat for animal and human health. However, little is known about the etiology of post-weaning infections and it is needed to find non-pharmalogical effective alternatives to antibiotics in weaning piglets. By using an *in vitro* gut model mimicking colon microbial ecosystem and avoiding the use of animals, our research participates to the gain of knowledge about the onset of post-weaning diarrhea and infection in piglets.

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is the principal pathogen responsible for post-weaning diarrhea (PWD) in newly weaned piglets. Expansion of ETEC at weaning is thought to be the consequence of weaning stress factors such as dietary change, transient anorexia or increase in intestinal inflammation and permeability, but the exact mechanisms remain to be elucidated. As the use of animal experiments raise more and more ethical concerns, we used a recently developed *in vitro* model of piglet colonic microbiome and mucobiome, the MPigut-IVM, to evaluate the effects of feed deprivation stress, dietary change and pathogen exposure at weaning. Our data suggested that the tested factors impacted the composition and functionality of the MPigut-IVM microbiota. For example, the simulation of weaning led to an increase in relative abundance of the *Prevotellaceae* family which was further promoted by the presence of ETEC. Several beneficial families such as *Bacteroidiaceae* or *Ruminococcaceae* and beneficial short chain fatty acids like butyrate or acetate were, in contrary, reduced after weaning simulation. Finally, the incubation of MPigut-IVM supernatants with porcine intestinal cell line cultures showed that the presence of ETEC in the *in vitro* model led to an increase in the expression of proinflammatory genes by porcine cells. This study provides insights about the etiology of a dysbiosis microbiota in post-weaning piglets.

BACKGROUND

The early life shaping of the gut microbiota is influenced by several internal and external factors including diet (Frese et al., 2015). At weaning, piglets are exposed to social, environmental and dietary stresses engendering disruptions of the balance between intestinal microbial communities, also called dysbiosis (Gresse et al., 2017). Gut dysbiosis in post-weaning piglets is presumably associated with a higher risk of developing infectious post weaning diarrhea (PWD) (Gresse et al., 2017) which raises a big economic burden in swine industry because of the reduced growth performance or even high mortality of infected animals (Amezcua et al., 2002; Fairbrother et al., 2005; Luppi et al., 2016). Additionally, the massive use of antibiotics as preventive and curative treatment increases public health concerns due to the expansion of bacteria resistance against antibiotics (Gresse et al., 2017). The major pathogenic agent responsible for PWD is Enterotoxigenic *Escherichia coli* (ETEC) (Amezcua et al., 2002; Dubreuil et al., 2016; Fairbrother et al., 2005; Luppi et al., 2016; Rhouma et al., 2017). This pathotype is characterized by both the presence of fimbrial adhesins inducing the cell

attachment to porcine intestinal epithelial cells and the secretion of enterotoxins which impact intestinal homeostasis (Dubreuil et al., 2016; Luppi et al., 2016). The most prevalent ETEC strains found in 45.1% of diarrheic post-weaning piglets harbour the fimbriae F4 (also designated K88) and secrete heat-labile toxin (LT) and heat-stable toxins (St a or b) (Dubreuil et al., 2016; Fairbrother et al., 2005; Luppi et al., 2016). If contributing factors to the development of ETEC infections such as housing conditions, early weaning, feed management and genetic predispositions were previously identified (Laine et al., 2008; Madec et al., 1998; Main et al., 2004; Rhouma et al., 2016), the exact etiology of PWD and ETEC infections remains far from understood. One hypothesis incriminates the reduced feed intake encountered by piglets at weaning which causes feed deprivation periods of variable duration, contributes to intestinal inflammation and morphology disruptions and strongly correlates with the risk of developing enteric diseases (Le Dividich and Sève, 2000; Main et al., 2004; B. A. McCracken et al., 1999; Rhouma et al., 2017). In particular, the disturbance of the mucosa intestinal environment and its associated microbiota could promote the expansion of opportunistic pathogens such as *Enterobacteriaceae* and increase the permeability towards bacteria and their toxins (Gresse et al., 2017). As such mechanisms are very complex and probably caused by both host and microbe factors, understanding the origin of PWD is very challenging. *In vitro* models of the piglet intestine including gut microbiota are adequate tools to remove host influence and thus exclusively evaluate factors impacting or influenced by commensal microbes. Especially, the use of such *in vitro* techniques offers advantageous conditions when pathogenic strains are involved due to more standardized conditions, good reproducibility and ethical reasons (Payne et al., 2012). Hitherto, the PigutIVM (Piglet Gut In vitro Model) and the BABY-SPIME (Baby Simulator of Pig Intestinal Microbial Ecosystem) are the only developed models mimicking the specific physicochemical and microbial conditions encountered in the colon of piglets (Dufourny et al., 2019; Fleury et al., 2017). However, the recently designed MPigut-IVM (Mucin associated Piglet Gut In vitro Model) (Chapter 9) brought the unique feature of reproducing the mucus-associated microbiota of piglet colon using specifically developed mucin beads. In a previous study, the MPigut-IVM was used to evaluate the impact of a 48h feed deprivation on the colonic luminal and mucus associated piglet microbiota (Chapter 9).

In this study, we used the MPigut-IVM to evaluate the impact of a dietary change on the gut microbiota of 4 week old piglets after a 48h feed deprivation period. The MPigut-IVM was then exposed to an ETEC strain isolated from diarrheic piglets to study the interactions between

the pathogen and the microbiota of our model. Finally, to unravel the consequences of microbiota perturbation on the host, effluents of control and ETEC-inoculated MPigut-IVM were incubated with a porcine cell line.

MATERIAL AND METHODS

Sample collection and treatments

All animals were housed in a conventional pig farm located in the Haute-Loire area of the Auvergne-Rhône-Alpes region in France. Piglets remained with their mother and siblings during the suckling period. In addition to sow milk, piglets received water and pre-weaning diet (Supplementary Table 1 Chapter 8) ad libitum. None of the piglets had signs of enteric or metabolic disturbances. The animals didn't receive any antibiotic in the 27 days prior to fecal collection day. As freezing process showed to affect bacterial abundances in pig feces (Metzler-Zebeli et al., 2016a), fecal samples from 4-weeks old healthy male piglets destined to fermentation runs (Landrace x Large White) were collected using sterile bottles and immediately stored under anaerobiosis conditions using GENbag anaer gas pack systems (Biomerieux, Marcy L'Etoile, France) during transport to laboratory.

MPigut-IVM parameters

Five hundred mL MiniBio bioreactors (Applikon Biotechnology, Delft, The Netherlands) equipped with stirrers, ports and probes and inoculated with fecal samples from piglets were prepared as previously described (Chapter 9). To ensure anaerobic conditions at the beginning of fermentations, the same volume of fecal suspension was added per bioreactor to 150 mL of nutritive medium mimicking the composition of piglet ileal effluents (see in the next paragraph), while flushing with O_2-free N_2 gas. Afterwards, during the fermentation course, the anaerobic conditions were maintained exclusively by the activity of the resident microbiota and by ensuring the airtightness of the system. The temperature of the fermentation was set up to 39°C and maintained inside the bioreactor medium using an incorporated panel heater and inside the mucin bead compartment using a hot water bath. pH was recorded every 300 seconds using pH sensor (Mettler Toledo, Viroflay, France) and adjusted to a physiological value of 6.0

with an automatic addition of 2M NaOH. Redox potential was constantly measured using a redox sensor (Mettler Toledo, Viroflay, France) and corrected into an Eh value by adding 197mV to the E recorded values, according to the manufacturer's instructions. The fermentation medium was stirred at a constant speed of 300 rpm during the total duration of the experiment. The volume of bioreactors was monitored using a level sensor and maintained at a constant value of 200 mL by automatic withdrawal of the fermentation medium. Anaerobic conditions and gas composition were checked every day by analyzing O_2, CO_2, CH_4 and H_2 produced during the fermentation process in the atmospheric phase of the bioreactors using a HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled with a TCD detector (Agilent Technologies, Santa Clara, USA) and two series columns, Molecular Sieve 5A and Porapack Q (Aligent Technologies, Santa Clara, USA).

Composition of MPigut-IVM nutritive medium

A nutritive medium was continuously introduced into the bioreactors using peristaltic pumps at a speed of 0.17 mL/min. The constant arrival speed of the nutritive medium inside the bioreactors and the regular withdraw of the fermentation medium ensured a retention time of 18h to mimic the colonic retention time of 4-week old piglets (Wilson and Leibholz, 1981). The nutritive medium formula was prepared as previously described (Chapter 9) and considered as a digested pre-weaning diet (Supplementary Table 1). This pre-weaning diet was given during the first seven days of fermentation (Figure 1). Following the 48h feed deprivation period, which was simulated by stopping the nutrient supply, corn meal, potato protein and a higher concentration of soy proteins were included in the diet whereas milk derived proteins and products were reduced to simulate the more diverse post-weaning diet fed to piglets (Supplementary Table 1).

Mucin bead production and compartment

Mucin beads were prepared as previously described (Chapter 9). At the beginning of fermentations, 350 ± 20 mucin beads were introduced into their specific glass compartments. Mucin beads were totally replaced every 48h to ensure a continuous availability of mucin adherent surfaces. During the time of bead replacement, the medium of the bead compartment was kept under CO_2 flushing to avoid oxygen entrance.

In vitro fermentation procedures and sampling

The first seven days of the fermentation procedures represented the stabilization period and, as previously described, correspond to the appropriate time to observe a stabilization of microbiota composition, diversity and activity inside the MPigut-IVM (Chapter 9). At day 7, the flow of nutritive medium was interrupted during 48h to simulate feed deprivation observed at weaning. Experiments were designed as presented on Figure 1. Samples from the bioreactor medium were collected on day 7 before the start of the feed deprivation period, on day 9 right before the recovery period, and days 10, 10.5, 11, 12 and 15. Mucin beads and bead medium from the bead compartment were collected on day 7 before the start of the feed deprivation period, on day 9 right before the recovery period, and on days 11 and 15. Samples from the bioreactor medium and bead medium were centrifuged (4°C, 10000 g, 45 min). Pellets and supernatants were stored until analysis at -20 and -80°C, respectively. After collection, mucin beads were gently washed 3 times in sterile 1X PBS and stored at -20°C.

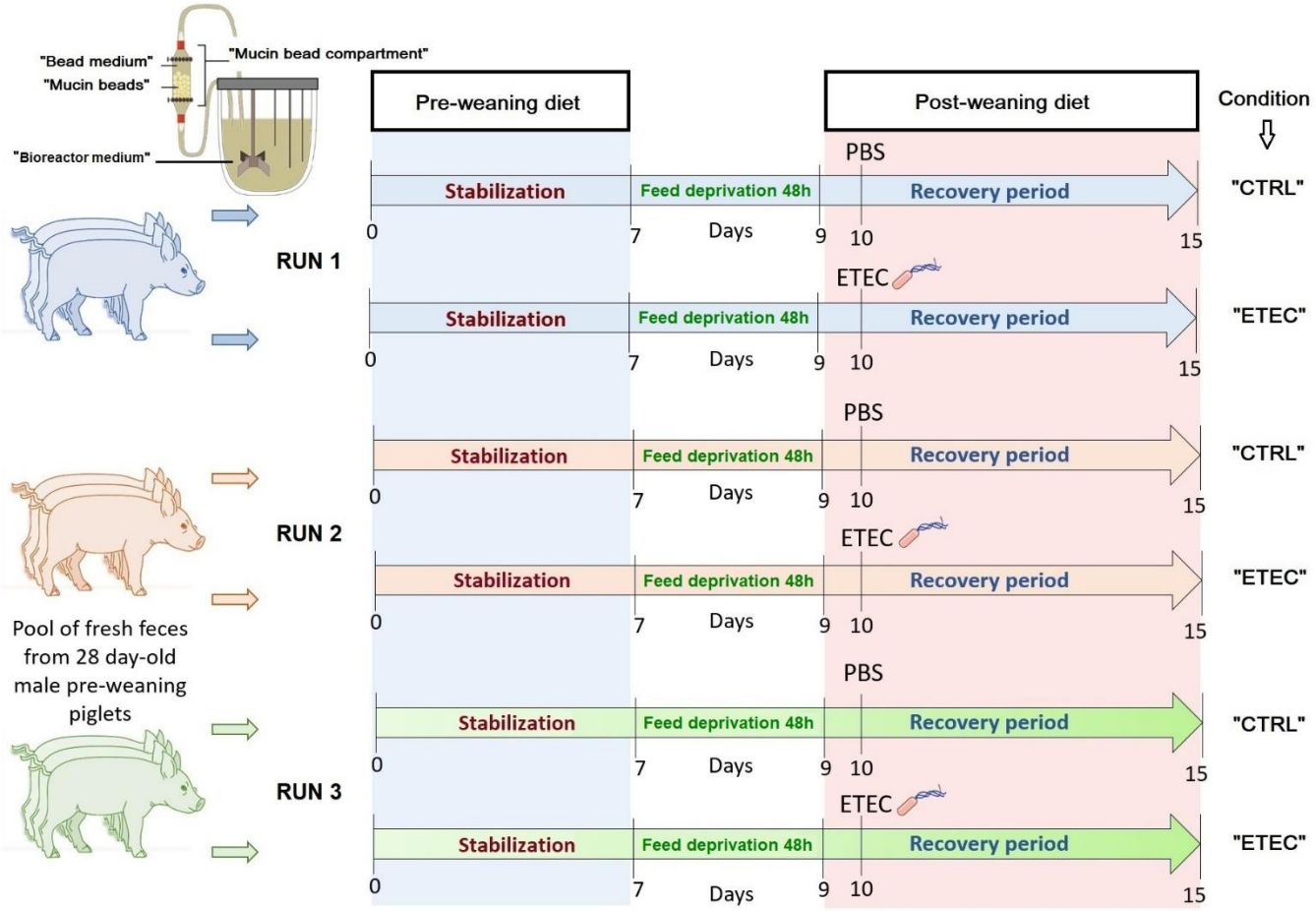


Figure 1. Experimental design of the *in vitro* fermentations and denomination applied to the MPigut-IVM in the Chapter 10.

ETEC strains, culture conditions and challenge of the MPigut-IVM

The ETEC Ec105 strain (F4, Stb+, East1+, LT+) was isolated from diarrheic piglets (Dr J.J. Garrido, Department of Animal Genetics, University of Cordoba, Spain). Prior to ETEC challenge in the *in vitro* model, bacteria were grown until OD_{600nm}= 0.6 in Luria Bertani (LB) broth (BD Difco, New Jersey, USA), at 39°C to be consistent with the temperature of the MPigut-IVM. The bacterial culture was then centrifuged (4°C, 10,000 g, 15 min) and the pellet was rinsed using sterile PBS 1X, resuspended in 1 mL of sterile PBS and injected inside the bioreactor medium of the "ETEC" condition at the day 10 of the fermentation (final concentration of 10^7 CFU/ mL in the fermentation medium). The control condition, referred as "CTRL", received 1 mL of sterile PBS 1x at the day 10 of the fermentation runs.

PMA treatments for qPCR

To avoid quantification of DNA after cell death, nucleic acid intercaling dyes, such as propidium monoazide (PMA) can be used as a treatment right after sample collection. Samples from the bioreactor medium and bead medium were collected in duplicate at all time points. The duplicates were stained with 50 µM PMAxx (Interchim, Montluçon, France) as described by Roussel et *al.*(Roussel et al., 2018a). The stained samples were incubated 5 min in the dark at room temperature, under agitation of 100 rpm. After incubation, samples were exposed to the blue light PMA-Lite LED Photolysis (Interchim, Montluçon, France) during 15 min to activate the PMAxx dye. Samples were then centrifuged (4,400 g, 4°C, 45 min). Pellets were washed twice with milli-Q water and stored at -20°C until DNA extraction.

DNA extraction from MPigut-IVM samples

Total DNA was extracted from all samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The quality of the eluted DNA was assessed by agarose gel electrophoresis. Extracts were quantified using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Samples were stored at −20 °C prior to analyses.

Quantification by qPCR

The list of primer pairs and their optimal conditions used for quantitative PCR of total bacteria, Methanogenic archaea and *Escherichia* / *Shigella* group are presented in Supplementary Table 2 (references (Huijsdens et al., 2002; Ohene-Adjei et al., 2008; Yu et al., 2005)). Real-time PCR assays were performed on a Rotor-Gene Q (Qiagen, Venlo, NL) in 20 µL reactions with QuantiFast SYBR GREEN master mix (Qiagen, Venlo, NL) or Taqman Fast Advanced Master mix (Applied Biosystems, Foster City, California, USA) with the addition of each primer at their optimal concentration (Supplementary Table 2). The 16S rDNA genes were amplified using the following program: 2 min denaturation at 95°C and 10 min denaturation at 95°C; 40 and 45 cycles of 20s at 95°C and 60s elongation and extension at the optimum annealing temperature and, when performing SYBR GREEN based assay, a melt curve step from 60°C to 95°C. Each reaction was run in duplicates. The melting curves of PCR amplicons from SYBR GREEN based assays were checked to ensure primer specificity. Ten-fold dilutions series of DNA from an ETEC Ec105 pure culture stained or not with PMA were used to control the reliability of the PMA treatment. A sample from ETEC Ec105 pure culture was subjected to a lethal treatment (95°C, 15min) and stained or not with PMA and used as a negative control for PMA-qPCR. The survival of the ETEC strain was monitored from day 10, the time of injection, to day 15 in the bioreactor medium, bead medium and on mucin beads *via* the quantification of the labile enterotoxin (LT) gene (Supplementary Table 3, references (Madoroba et al., 2009; Ngeleka et al., 2003; Nicklasson et al., 2012; Rahman et al., 2006; Roussel, 2019)). After log transformation of the data, a mixed-model one-way Anova (lmer and Anova functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used to compare the number of 16S gene copy per g of samples between days of fermentation using the R packages lme4 and car.

MiSeq 16S rDNA sequencing and bioinformatic analysis

The DNA concentration of all samples was measured using the Qubit dsDNA High Sensivity Assay Kit (Invitrogen, Carlsbad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and diluted to 2 ng/µl prior to PCR amplification. The Bacterial V3-V4 region of 16S rDNA and the Archaeal 16S rDNA were respectively amplified with primers 357F 5'-CCTACGGGNGGCWGCAG-3’(Yu et al., 2005) and 805R 5'-GACTACHVGGGTATCTAATCC-3’(Lane et al., 1985b) and primers 349F 5'-GYGCASCAGKCGMGAAW-3’ and 806R 5'-GGACTACVSGGGTATCTAAT -3’(Ohene-Adjei et al., 2008). Amplicons were generated using a Fluidigm Access Array followed by high-

throughput sequencing on an Illumina MiSeq system (Illumina, San Diego, CA, USA) performed at the Carver Biotechnology Center of the University of Illinois (Urbana, IL, USA). The demultiplexed paired end Illumina sequence reads in the FastQ format were uploaded into the Galaxy instance (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr) to be used in the FROGS (Find Rapidly OTU with Galaxy Solution) pipeline (Escudié et al., 2018). During the FROGS pre-process, sequences were depleted of barcode and the sequences with a non-appropriate length or containing ambiguous bases were removed. Next, reads were clustered into *de novo* operational taxonomic units (OTUs) using SWARM algorithm (Mahé et al., 2014) with, at first, a denoising step to build very fine cluster using the minimal distance equal to 1 and, secondly, with an aggregation distance equal to 3. Chimeras were then detected and removed with VSEARCH (Rognes et al., 2016). Additionally, filters were applied to the OTUs in order to remove singletons (Auer et al., 2017; Bokulich et al., 2013). The OTUs selected were taxonomically assigned using the Silva release 132 reference database (Quast et al., 2013).

Statistical analysis of sequencing data

Statistical analysis was processed using the RStudio software version 1.0 (with R software version 3.5.1, R Development Core Team, http://www.R-project.org). OTU structure and composition analyses were performed using the phyloseq R package(McMurdie and Holmes, 2013). Visualization of data was performed using the ggplot2 R package. Following log transformation of the data, univariate statistical analysis was performed on taxa relative abundance with the R packages lme4 and car. A mixed-model one-way Anova (lmer and Anova functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used. Prior to alpha and beta diversity calculations, rarefaction using the transform count methods was applied to the dataset. Alpha diversity indices were calculated using a Wilcoxon test with the number of observed OTU phylogenetic diversity (PD) and Shannon indices. Statistical differences in Bray Curtis distance between the mucin beads and the bioreactor medium were tested using a multi-analysis of variance (MANOVA) performed with ADONIS using the vegan R package with 9999 permutations and represented by principal coordinate analysis (PCoA) plots. Statistical comparisons of samples were performed using the Wald test of the DESeq2 R package at the genus level. In all statistical analyses, only *p*value < 0.05 were considered as significant.

Function Prediction with Tax4Fun

Tax4Fun(Aßhauer et al., 2015), an online tool hosted into Galaxy (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr), was used to predict functional genes of prokaryotic microorganisms in the MPigut-IVM. Tax4Fun transforms OTUs picked up against the Silva 123 database into a taxonomic profile of KEGG (Kyoto Encyclopedia of Genes and Genomes) organisms and normalize these predictions by the 16S rDNA copy number. The most important functions of CTRL and ETEC conditions were graphically represented using the RStudio software version 1.0 (with R statistical package version 3.5.1, R Development Core Team, http://www.R-project.org).

RNA isolation of MPigut-IVM samples

Total RNAs from bioreactor medium, mucin beads and bead medium were extracted using Trizol reagent (Invitrogen, Thermo Fisher, Waltham, USA) as described by Comtet-Marre et *al.* (Comtet-Marre et al., 2017). DNAse treatment with the rDNAse Set (Macherey-Nagel, Hoerdt, France) was performed to remove any contamination of genomic DNA according to the manufacturer's instructions. The integrity of few samples representative from the whole set was assessed using the Agilent 2100 Bioanalyzer using RNA Nano Chip (Agilent Technologies, Santa Clara, USA) to ensure sufficient quality for RT-qPCR. Quantity and purity of RNAs were measured using the Nanodrop One (Thermo Fisher Scientific, Waltham, USA) and RNAs were stored at -80°C until cDNA synthesis.

RT-qPCR

First, 1µg of RNA per sample was first reverse transcribed into complementary DNA (cDNA) with the SuperScript IV Reverse Transcriptase kit (Invitrogen, Thermo Fisher, Waltham, USA) in conformity with the manufacturer's instructions. QPCR was performed on the cDNAs as outlined in the section above. Primers and conditions used for qPCR on cDNAs are listed in Supplementary Table 3. CDNAs and DNA samples from RNA extraction of the ETEC Ec105 pure culture and from the MPigut-IVM ETEC challenge were used as a positive control. The comparative $E^{-\Delta\Delta Ct}$ method was applied to calculate the relative fold changes in ETEC virulence genes in the samples from the MPigut-IVM. BestKeeper excel-based tool(Pfaffl et al., 2004) was used to determine the geometric means of the three quantified reference genes, *arcA*, *gapA*

and *rpos* considered for normalization. Primer efficiency was determined using 10-fold dilution series of a set of samples representative from mucin beads, bead medium and bioreactor medium. The efficiency was calculated from the slope of the standard curves using the following equation $E = 10^{(-1/slope)}$, where E corresponds to high/acceptable amplification efficiency equals to 90-110 %.

Quantification of short chain fatty acids (SCFAs) by gas chromatography

The SCFAs were quantified in the bioreactor medium and bead medium by gas chromatography. Eight hundred µL of supernatants from bioreactor medium and bead medium were mixed with 500 µL of 0.4% (w:v) crotonic acid and 2% (w:v) metaphosphoric acid solutions. This mixture was centrifuged and the supernatant obtained was injected into a PerkinElmer Clarus 580 gas chromatograph (Waltham, Massachusetts, USA) for quantification of SCFAs. A mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation experiment as a random effect was used to compare the concentration of the main SCFAs between days of fermentation using the R packages lme4 and car.

Metabolite analysis by ^{1}H nuclear magnetic resonance (NMR)

Supernatants of bead medium collected on days 7 and 11 were used for metabolomic profiling using NMR spectroscopy. Samples preparation, acquisition of spectra and metabolite identification was described previously (Chapter 9). All NMR spectra were obtained with an Avance III HD NMR spectrometer operating at 600.13 MHz for ^{1}H resonance frequency using a 5 mm inverse detection CryoProbe (Bruker Biospin, Rheinstetten, Germany) in the MetaboHUB-MetaToul-AXIOM metabolomics platform (Toulouse, France).

Statistical analysis for NMR metabolomics were performed using the R software (version 3.5.1). Principal component analysis (PCA) and partial-least square discriminant analysis (PLS-DA) were performed with mixOmics package (Rohart et al., 2017). Metabolite relative concentration was used as variable matrix (X). For PLS-DA, time points (days of fermentation) were used as predictors (Y) and time-repeated measurement were considered by using a multilevel approach. Univariate statistical analysis was also performed on each metabolite relative concentration with the R packages lme4 and car. A mixed-model one-way ANOVA

(lmer and ANOVA functions) with time point (days of fermentation) or the ETEC and CTRL conditions as a fixed effect and fermentation experiment as a random effect was used. A post-hoc test was used to compare the mean relative concentrations with Tukey correction. P-values were corrected for multiple testing (false discovery rate). Spearman correlation was used to correlate the *in vitro* metabolome with the most abundant families of the MPigut-IVM using the cor function of the R software.

Incubation of MPigut-IVM supernatants on the IPI porcine cell line

IPI-2I cell culture

The IPI-2I cell line is derived from the ileum of an adult male pig and was immortalized by transfection with an SV40 plasmid (pSV3-neo)(Kaeffer et al., 1993). IPI-2I cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) medium (InvitrogenTM Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS, PAA Laboratories GmbH, Austria) and 4 mM L-glutamine (Sigma, St. Louis, MO, USA). Cells were seeded onto 48-well tissue culture plates at 25,000 cell/well in a volume of 200 µL and grown 24h in an atmosphere of 5% CO_2 at 37°C to allow for confluency for the day of experiment.

Exposure of IPI-2I cells to MPigutIVM supernatants

Supernatants from fecal inocula and bead medium at days 7, 9, 11, 13 and 15 were filtered using 0.2 µm sterile Minisart syringe filters (Sartorius, Göttingen, Germany) and 30 times diluted with DMEM (10% Fetal Calf Serum and 4mM L-glutamine). A thirty-fold dilution of each sample was established as the best compromise following preliminary tests estimating the survival of IPI-2I cells exposed to with dilutions series of MPigut-IVM supernatants (data not shown). The diluted samples were added in duplicate to confluent monolayers of IPI-2I cells in 48-well plates, as described above. Plates were incubated during 2h at 37 °C, 5% CO_2. At the end of the incubation, the supernatants were removed and IPI-2I cells were lysed by addition of 500 µL of NucleoZOL (Machery-Nagel, Hoerdt, France). Cell lysates were stored at -80°C prior to RNA isolation.

RNA isolation from IPI-2I lysates

Total cellular RNAs were extracted from IPI-2I lysed cells following the guideline provided by the NucleoZOL user manual (Machery-Nagel, Hoerdt, France). The TURBO DNA-free™ kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions to prevent DNA contamination. Purity and quality of the RNA extracts were controlled on 1% agarose gels. RNAs were then quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) using an optical density of 260 nm.

RT-qPCR on IPI-2I RNA extracts

Reverse transcription was performed using the qScript cDNA Synthesis Kit (Quantabio, Beverly, MA, USA). Briefly, 350 ng of RNA per sample was added to 5 µL of sScript Reaction Mix (5x) and 1 µL of qScript Reverse Transcriptase in a final volume of 15 µL. The reverse transcription mix was successively incubated 5 min at 22°C, 30 min at 42°C and 5 min at 85°C. The synthetized cDNAs were stored at -20°C until used. The targeted genes are listed in Supplementary Table 4 (Mariani et al., 2009). Quantifications were carried out in triplicates for each cDNA using a QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The cyclophilin A and β-actin genes were used as reference genes. PCR reactions were carried out in 96 well plates using 3 µL of 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), 0.4 µL of forward and reverse primer, 9.2 µL of milliQ water and 2 µL of cDNA. Tenfold dilution series of each primer pair were used as standard curves to determine primer efficiencies. Real time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. The appropriate reference gene and the Log2 fold change of each gene, compared with the IPI-2I cells which had not been exposed to bead medium supernatants, were determined by GenEx software (http://genex.gene-quantifcation.info/). A mixed-model one-way ANOVA (lmer and ANOVA functions) with time point (days of fermentation) as a fixed effect and fermentation number as a random effect was used to compare the significance between gene expression profile of the IPI-2I cells which were subjected to bead medium supernatants containing or not the ETEC Ec105 strain using the R packages lme4 and car.

RESULTS

Fecal inocula display few variability in their microbiota and SCFA proportions

Analyses on the fecal inocula used in the fermentations are presented in Figure 2. The fecal inocula used for the run #3 displayed higher proportions of *Proteobacteria* and *Enterobacteriaceae* compared with the two other fecal inocula. A higher proportion of acetate was measured in the fecal sample used as an inoculum for run #3 compared to the others. The relative abundance of *Clostridiaceae* 1 family was higher in the fecal inoculum of the run #2.

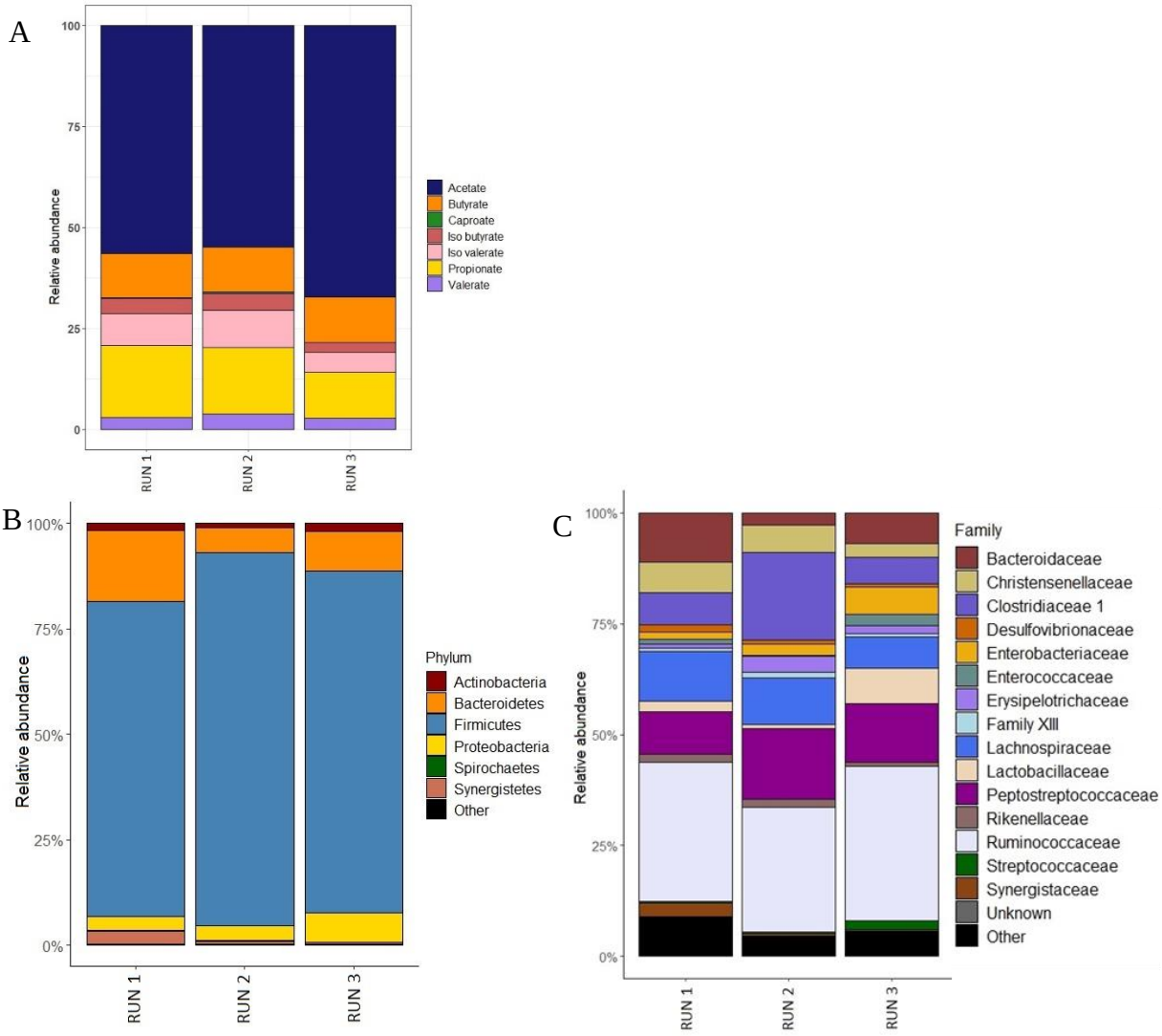


Figure 2. Analyses of the fecal inocula used for runs #1, 2 and 3: relative abundance of SCFAs measured by gas chromatography (A), relative abundances of the principal phyla (B) and families (C) measured by 16S Illumina sequencing.

A feed deprivation stress followed by a dietary shift and a challenge with a pathogenic strain affect the functions of the MPigut-IVM microbiota

Redox potential and gas composition

The 48h feed deprivation stress in the MPigut-IVM induced a reproducible and marked increase in redox potential in 5 out of the 6 fermentations but was followed by a decrease right after the beginning of the recovery phase when the feeding of the bioreactor restarted. In the Control fermentation of run #1, the increase in redox potential was weaker during the 48h feed deprivation period but continued during the recovery period (Figure 3).

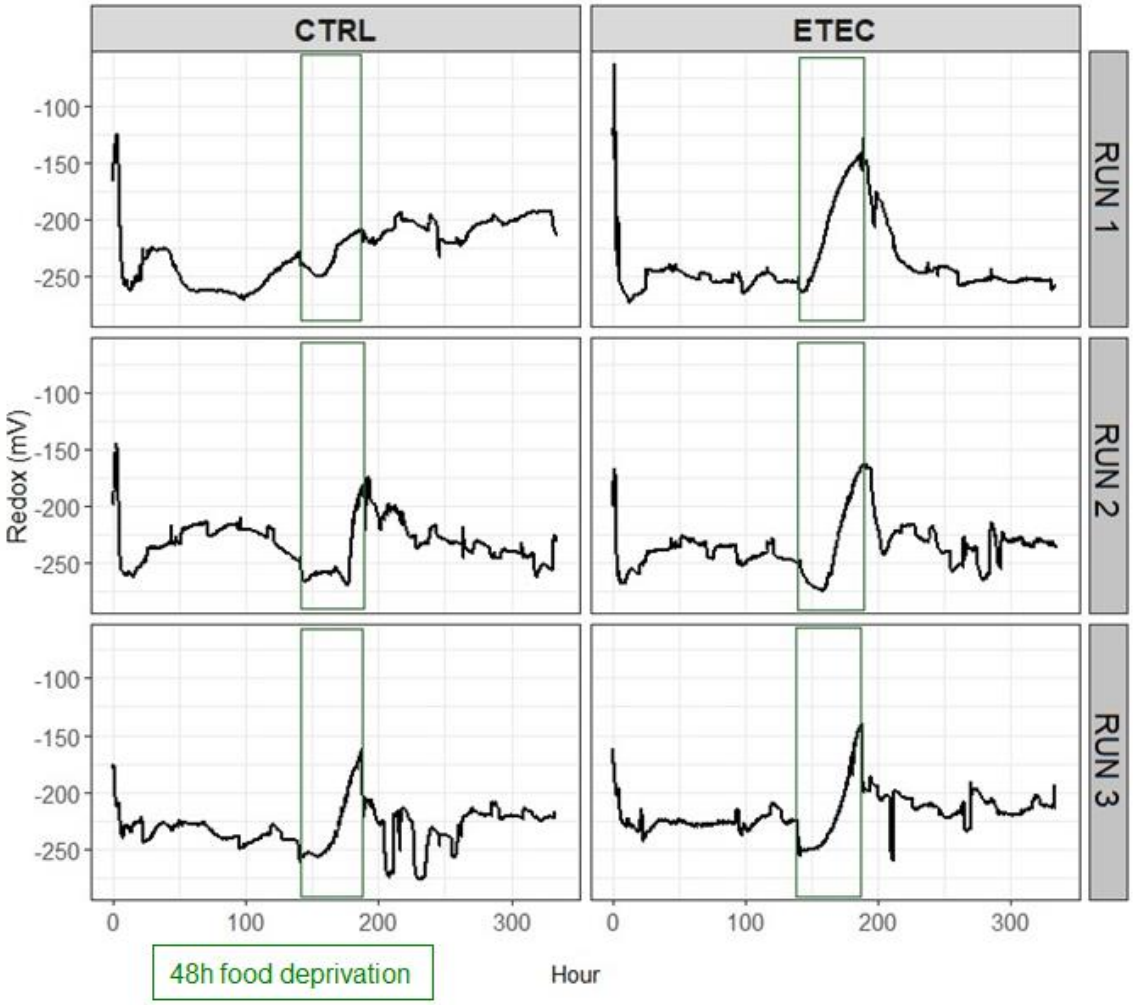


Figure 3. Evolution of the redox potential in the MPigut-IVM for the runs #1, 2 and 3 which were subjected to a 48h feed deprivation stress, a dietary shift and challenged or not with the ETEC Ec105 strain.

At the end of the stabilization period, the mean relative proportions of H_2, O_2, CO_2, N_2 and CH_4 during the fermentations A, B, C, D, E and F were respectively 2.1 ± 1.2, 0.3 ± 0.1, 73.7 ± 0.4, 5.9 ± 0.8 and 6.8 ± 1.1 % (Supplementary Figure 1). However, the feed deprivation period led to modifications in gas composition with an augmentation of N_2, CH_4 and O_2 percentages and a slight decrease of CO_2 and H_2 percentages, which were not attributed to any leak of the system. Indeed, during the feed deprivation period, a CO_2 bag was connected to the bioreactor medium using a one-way clamp to avoid a too strong depression of the system and incoming air. At the end of the feed deprivation stress, the mean relative proportions of H_2, O_2, CO_2, N_2 and CH_4 during the fermentations A, B, C, D, E and F were respectively 0.8 ± 0.6, 1 ± 0.4, 65.4 ± 2.0, 15.3 ± 2.8 and 9.1 ± 0.2 % (Supplementary Figure 1).

Effects of a feed deprivation stress followed by a dietary change on microbiota functional characteristics

The 48h feed deprivation stress followed by a dietary change led to significant (*p*value < 0.05) modifications in the relative abundance of SCFA during the recovery period compared to the stabilization period. In the bioreactor medium, ANOVA analyses revealed a significant increase in propionate and caproate proportions while acetate, isovalerate and butyrate proportions were decreased from day 10 to 15 compared to day 7. In the bead medium, the proportions of propionate, isovalerate and valerate increased while the proportions of acetate and butyrate decreased from day 10 to 15 compared to day 7 (Figure 4). The total concentration of SCFA was significantly increased from day 10 to 15 compared to day 7 both in the bioreactor medium and the bead medium (Figure 5). NMR allowed the identification of 26 metabolites in the bead medium of the MPigut-IVM. Among these metabolites, 3 phenylpropionate, phenylacetate, iso valerate, valerate, 2 methyl butyrate and ethanol concentrations were significantly increased by the feed deprivation period and diet change in the bead medium (Figure 6I). PLS-DA revealed a spatial distribution between the samples of day 7 receiving pre-weaning diet and samples from day 11 receiving post-weaning diet (Figure 6II).

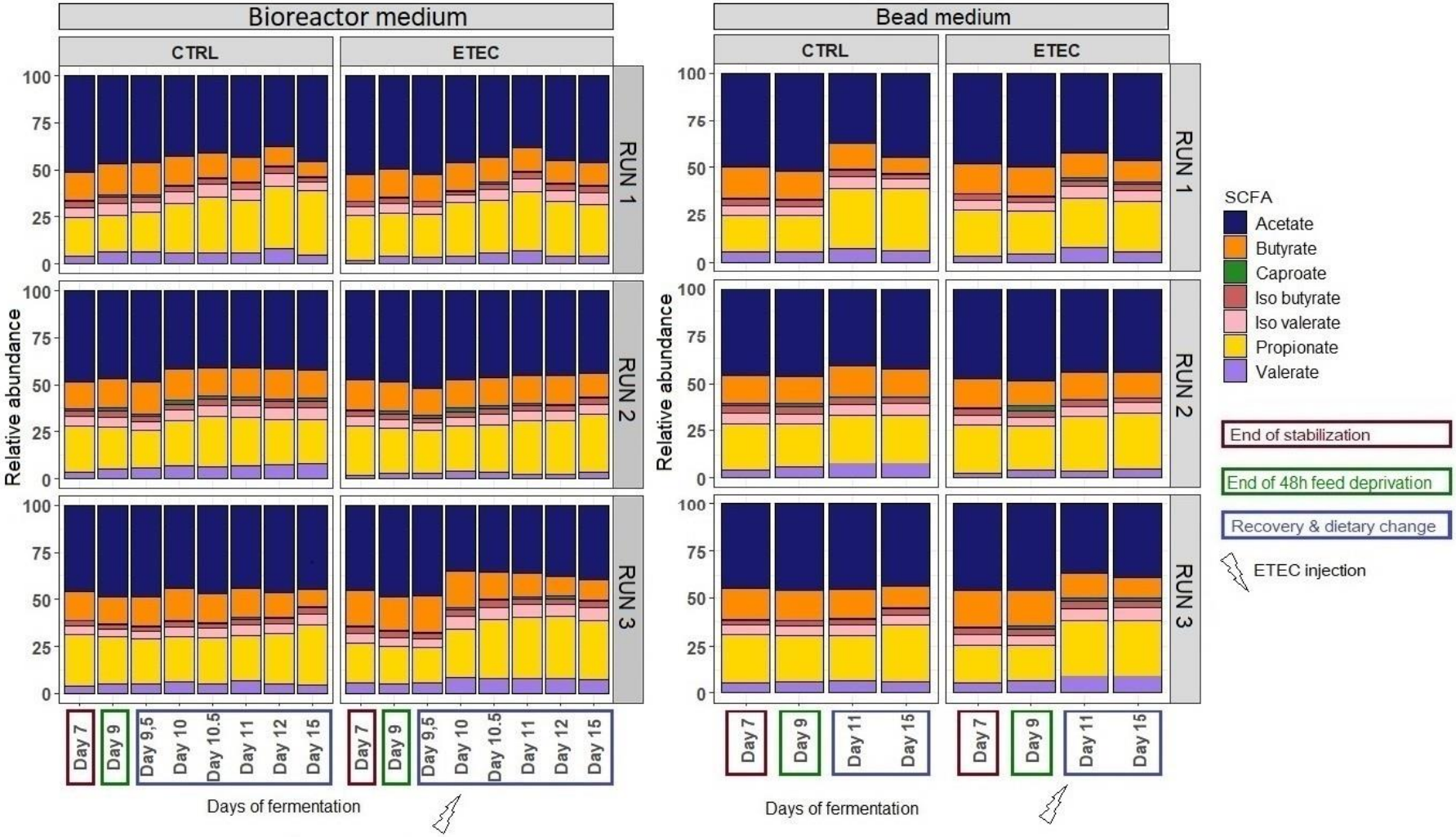


Figure 4. Short chain fatty acids (SCFA) relative abundances produced by fermentation activity of the microbiota inhabiting the MPigut-IVM challenged with a feed deprivation stress, a dietary shift and an ETEC strain.

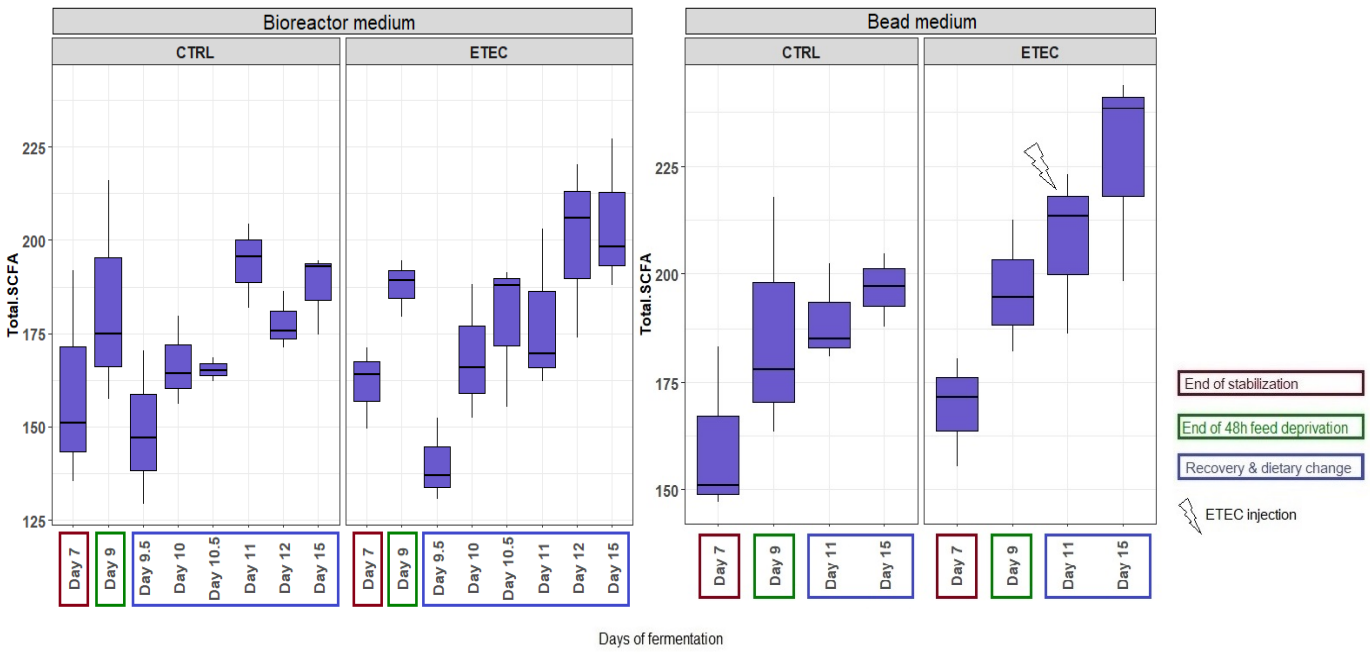


Figure 5. Evolution of the mean total concentration of SCFAs for the runs #1, 2 and 3 in the bioreactor medium and the mucin beads medium of the MPigut-IVM.

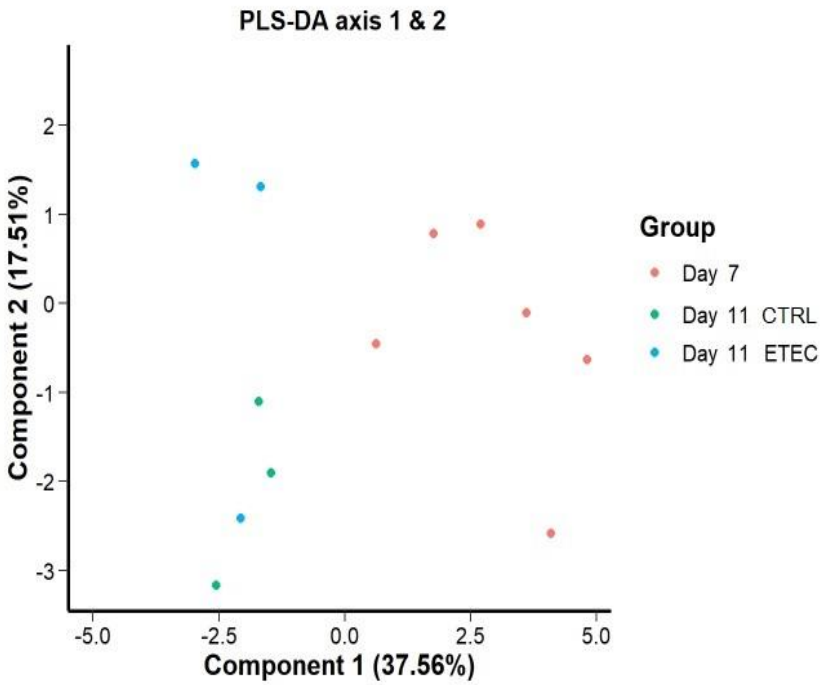


Figure 6I. Relative abundance of significantly different metabolites between MPigut-IVM challenged or not with the ETEC Ec105 strain in the bead medium of the day 7 and 11 analyzed by NMR.

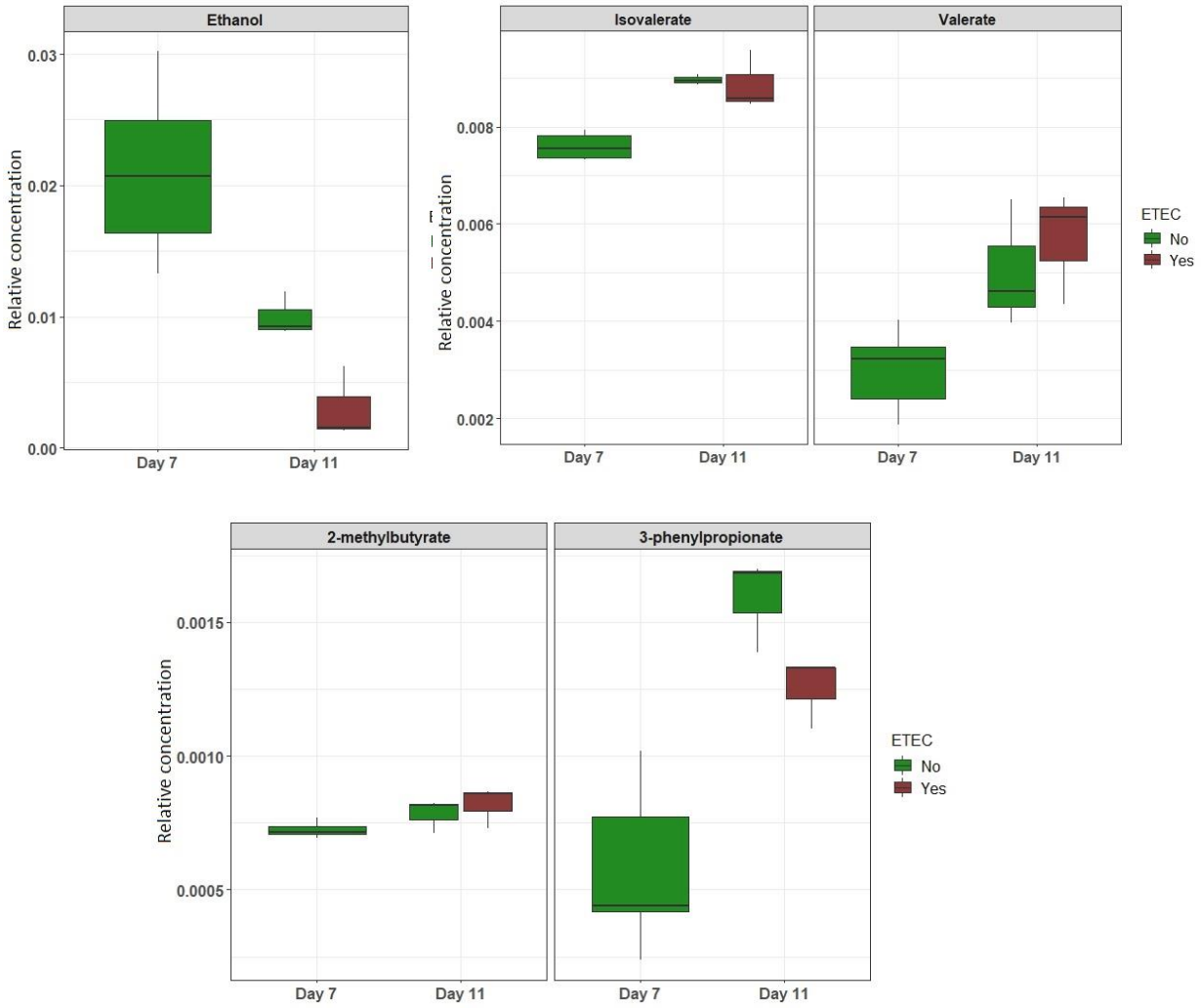


Figure 6II. Individual plots of partial least square-discriminant analysis using metabolites as variables and time points as predictors in the bead medium of the day 7 and 11 analyzed by NMR.

Effects of presence of the ETEC Ec105 strain in the MPigut-IVM

Relative abundances of SCFA were compared between the conditions ETEC and CTRL during the recovery period from day 10 to 15 only. The proportion of caproate and propionate tended ($0.05<p\text{value} < 0.1$) to be higher in the ETEC condition in the bead medium and bioreactor medium, respectively (Figure 4). Analysis of MPigut-IVM bead medium by NMR revealed that the presence of the ETEC Ec105 strain significantly decreased 3 phenyl propionate and ethanol

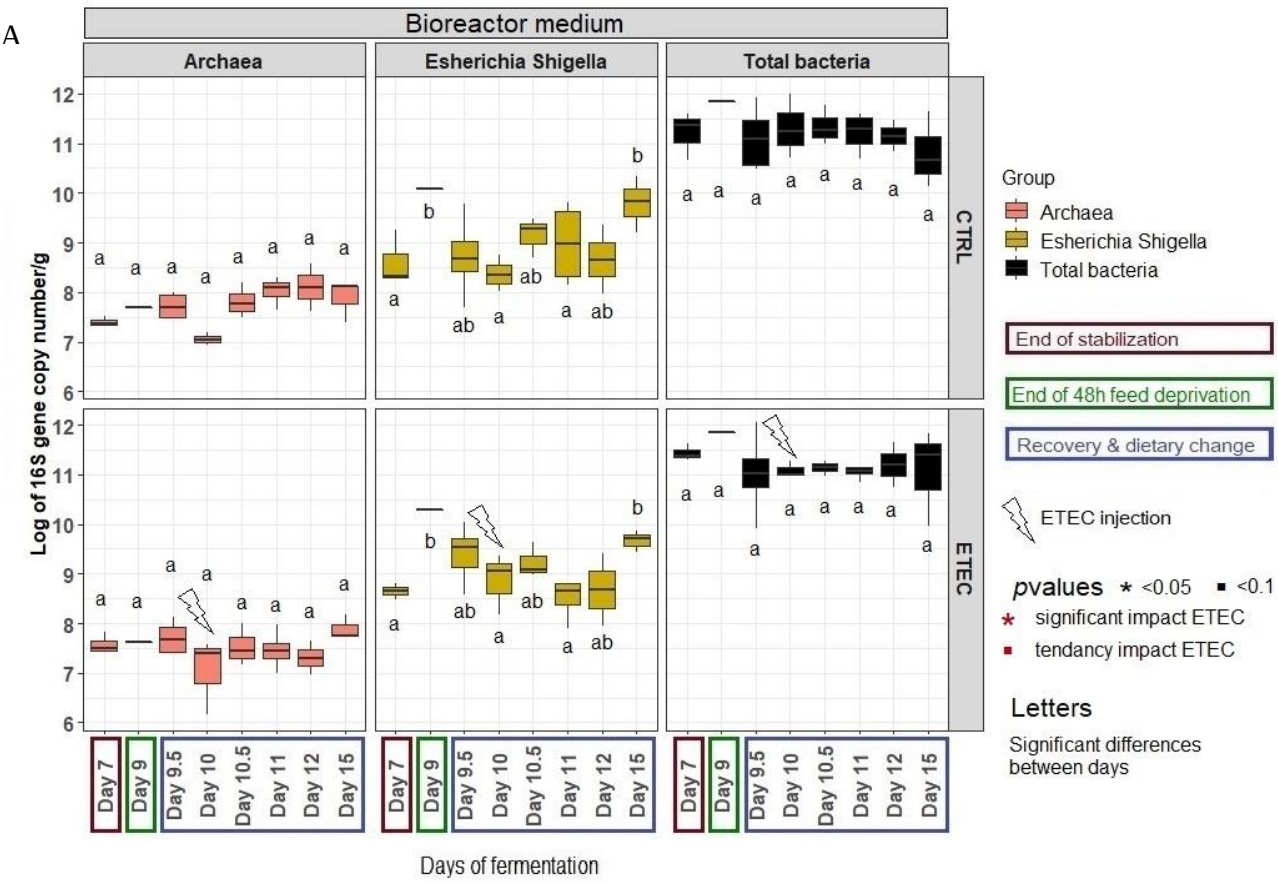
concentrations while the valerate concentration was significantly increased compared to the CTRL condition at day 11 (Figure 6I).

Feed deprivation stress, dietary change and the presence of the pathogen trigger microbiota composition disruptions in the MPigut-IVM

Effects of a feed deprivation stress followed by a dietary change on microbiota composition

Q-PCR quantifications of targeted bacterial groups showed that total bacteria were not affected by the 48h feed deprivation stress and the diet change (Figure 7A). However, *Escherichia/Shigella* group was significantly increased at day 9 and 15 in the bioreactor medium of both CTRL and ETEC conditions. In the mucin beads, both the archaea and *Escherichia/ Shigella* group were significantly increased at day 15 compared to all the other days for both the ETEC and CTRL conditions (Figure 7B). PMA-qPCR confirmed that bacteria from *Escherichia/Shigella* group were viable across time for the whole fermentation runs in all samples of the MPigut-IVM (data not shown).

A



B

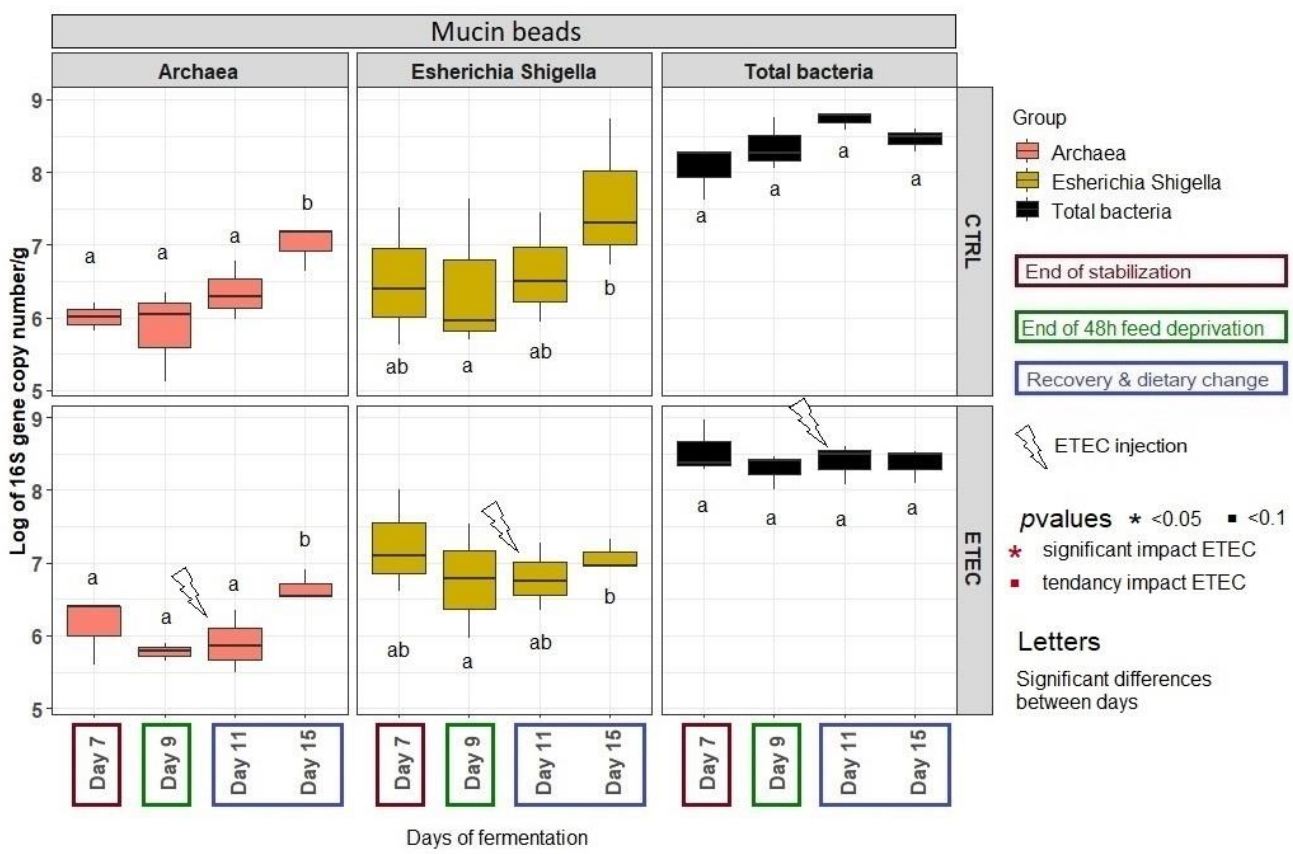


Figure 7. Quantification of total bacteria, *Escherichia coli* and methanogenic archaea populations in the bioreactor medium (A) and the mucin beads (B) of the MPigut-IVM. The days sharing the same letters are not significantly different from each other.

The Illumina MiSeq run generated a total of 8,107,484 and 1,467,731 high quality sequences respectively for the V3-V4 and archaeal sets of primers. Removal of PhiX control reads, removal of chimeras and filtering of singletons lead to a number of 51,703 ± 13,520 sequences for V3-V4 primers and 5,377 ± 4,645 sequences for archaeal primers per samples. To avoid any bias, samples containing less than 500 sequences after abundance filtering were removed from the dataset. A certain variability of microbiota composition was noticed between the run 1, 2 and 3. However, some populations responded in a similar manner to the different treatments and were significantly impacted by the feed deprivation period and dietary change in both CTRL and ETEC conditions. On the mucin beads, *Spirochaetes* significantly decreased from a relative abundance of 6.8 ± 5.7% at day 7 to 0.3 ± 0.3% of mean relative abundance from day 10 to 15 (Supplementary Figure 2). In the bioreactor, the *Proteobacteria* significantly increased from a relative abundance of 4.0 ± 2.5% at day 7 to 17.1 ± 4.1 % at day 9 and to 7.9 ± 5.6% from day 10 to 15 (Supplementary Figure 2). Looking at lower taxonomy level, some families

were also significantly impacted at the end of the feed deprivation stress or during the recovery period compared to the stabilization phase (Figure 8). In the bioreactor medium, the *Coriobacteriaceae* significantly decreased from a mean relative abundance of 11.9 ± 6.6 % at day 7 to a mean relative abundance of 2.2 ± 2 % from day 9 to day 15. In contrary, the *Enterobacteriaceae* and *Erysipelotrichaceae* families significantly increased from a mean relative abundance of respectively 0.9 ± 0.7 % and 1.5 ± 2 % at day 7 to mean relative abundances of 6.2 ± 5.4 % and 4.7 ± 4.1 % from day 9 to day 15 (Figure 8A). Finally, the *Prevotellaceae* significantly decreased from 6.5 ± 6.3% at day 7 to 4 ± 2.7 % at day 9 prior to significantly increase from day 10 to day 15 at a mean relative abundance of 27.1 ± 20 %. On the mucin beads, the *Bacteroidiaceae* and *Coriobacteriaceae* were significantly lower from respective mean relative abundances of 24.9 ± 9% and 5 ± 2.7 % from day 7 to 9 to mean relative abundances of respectively 10.3 ± 8.8% and 1.3 ± 0.9% from day 11 to 15 (Figure 8B). In contrary, the *Prevotellaceae* and *Atopobiaceae* were significantly higher from r mean relative abundances of 3.1 ± 2.4% and 2.4 ± 1.5 %, respectively, from day 7 to 9 to mean relative abundances of 9.7 ± 7% and 6.8 ± 4%, respectively, from day 11 to 15 (Figure 8B). The feed deprivation stress and dietary shift also led to modifications in relative proportions of several genera, both in bioreactor medium and mucin beads (Figure 9).

The archaeal microbiota was influenced by the feed deprivation period and the dietary change. In the bioreactor medium and the mucin beads of the control fermentations, the *Methanosphaera* genus was found in significantly higher mean relative abundance during the recovery period compared to the end of the stabilization period (Figure 10), and feed deprivation stress also affected *Methanobrevibacter* relative abundance which was significantly lower after stress but only on mucin beads (Figure 10B) .

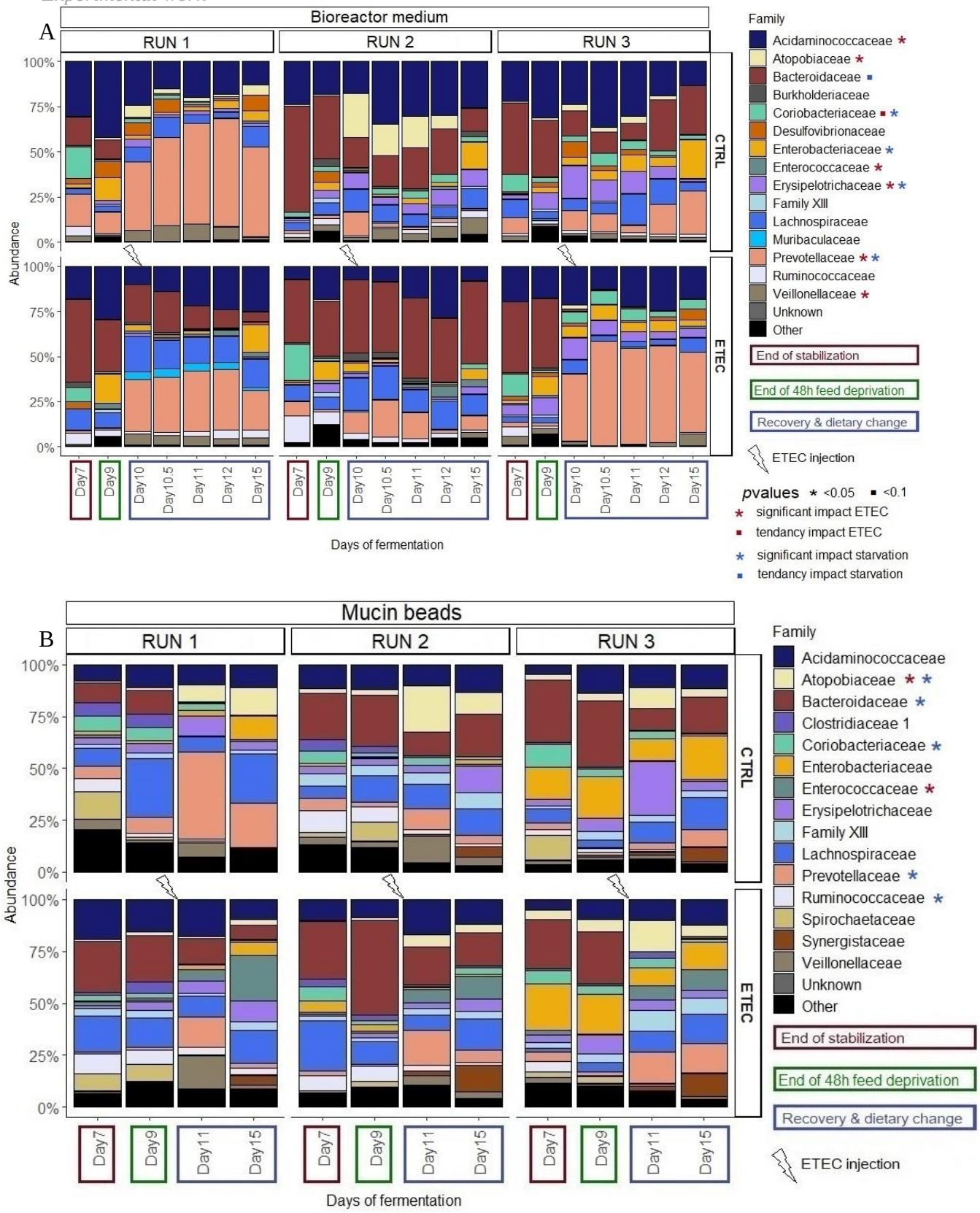
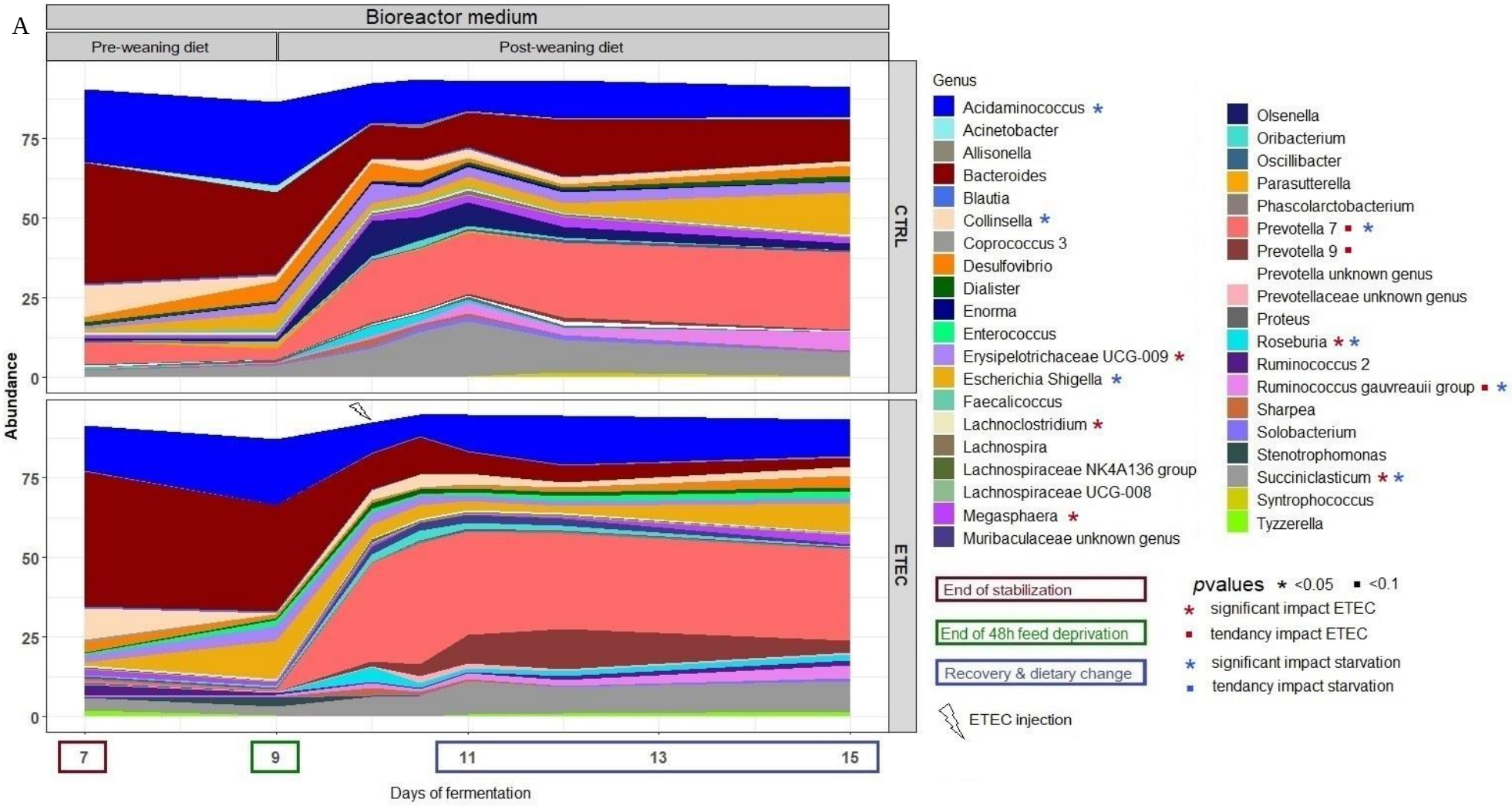


Figure 8. Relative abundances of the main bacterial families in the bioreactor medium (A) and the mucin beads (B) in MPigut-IVM during the runs #1, 2 and 3 which were subjected to a 48h feed deprivation stress, a dietary change and challenged or not with the ETEC Ec105 strain.


A
Bioreactor medium
Pre-weaning diet
Post-weaning diet
CTRL
ETEC
Abundance
0
25
50
75
7
9
11
13
15
Days of fermentation
Genus
Acidaminococcus *
Acinetobacter
Allisonella
Bacteroides
Blautia
Collinsella *
Coprococcus 3
Desulfovibrio
Dialister
Enorma
Enterococcus
Erysipelotrichaceae UCG-009 *
Escherichia Shigella *
Faecalicoccus
Lachnoclostridium *
Lachnospira
Lachnospiraceae NK4A136 group
Lachnospiraceae UCG-008
Megasphaera *
Muribaculaceae unknown genus
Olsenella
Oribacterium
Oscillibacter
Parasutterella
Phascolarctobacterium
Prevotella 7 ▪ *
Prevotella 9 ▪
Prevotella unknown genus
Prevotellaceae unknown genus
Proteus
Roseburia * *
Ruminococcus 2
Ruminococcus gauvreauii group ▪ *
Sharpea
Solobacterium
Stenotrophomonas
Succiniclasticum * *
Syntrophococcus
Tyzzerella
End of stabilization
End of 48h feed deprivation
Recovery & dietary change
ETEC injection
pvalues * <0.05 ▪ <0.1
* significant impact ETEC
▪ tendancy impact ETEC
* significant impact starvation
▪ tendancy impact starvation

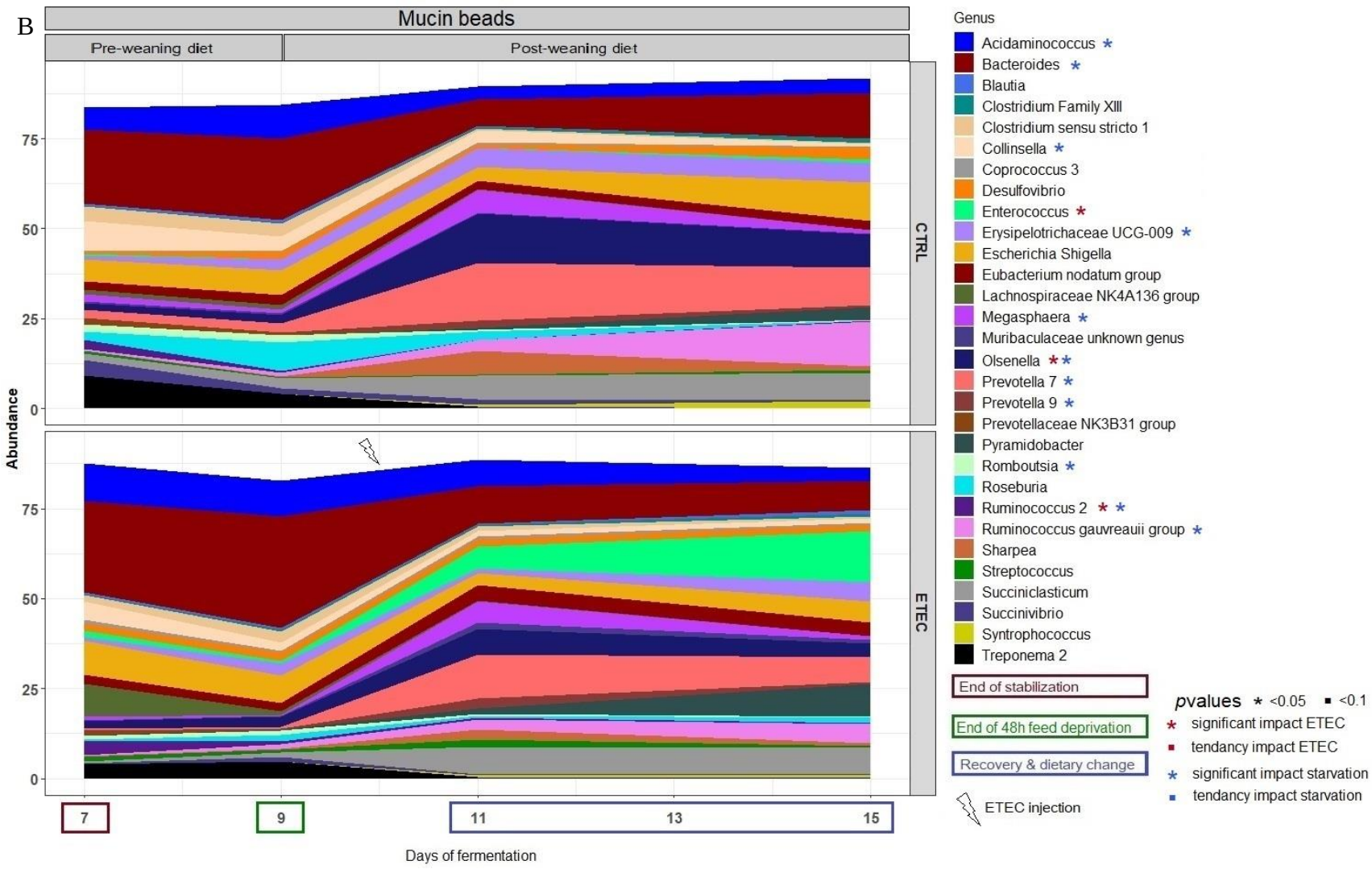


Figure 9. Mean relative abundance of the 30 more abundant bacterial genera in the bioreactor medium (A) and the mucin beads (B) of MPigut-IVM (n = 3).

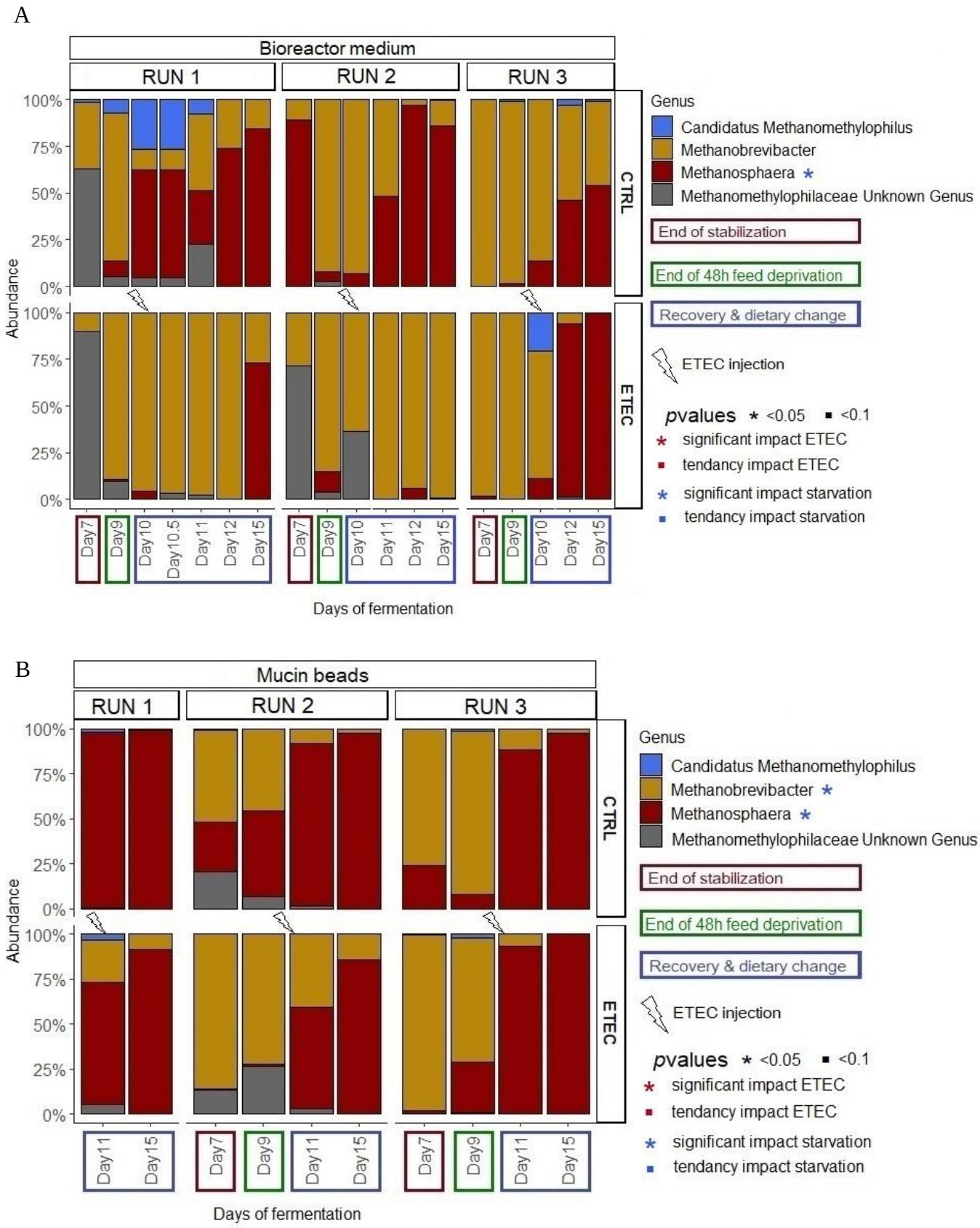


Figure 10. Relative abundance of the archaeal genera in the bioreactor medium (A) and mucin beads (B) of the MPigut-IVM. Day 7 and 9 of run #1 were removed due to their low number of sequences.

Survival of Ec105 in the MPigut-IVM

The presence of the ETEC strain was monitored in all the MPigut-IVM samples by quantifying the LT gene using qPCR. In the bioreactor medium of the ETEC condition, the LT gene was quantified at a mean of 8 ± 0.2 log of gene copy/g of sample at day 10 on the time of injection prior to slowly decrease over time to reach a mean of 4.9 ± 1.1 log of gene copy/g of sample at day 15. On the mucin beads of ETEC condition, the LT gene was quantified at mean values of 4.5 ± 1.6 and 3.3 ± 2.9 log of gene copy/g of sample respectively at day 11 and 15. However, the LT gene was quantified in higher quantity in the bead medium at mean values of 7.6 ± 1.6 and 6.4 ± 2.1 log of gene copy/g of sample respectively at day 11 and 15. It can be noticed that the LT gene was quantified in much lower quantity from day 11 in all the samples for run #1 (Figure 11). Finally, a low copy number of LT gene could also be detected in the CTRL condition at mean values of 2.9 ± 1.6, 3.4 ± 1.6 and 3.9 ± 0.2 log of gene copy/g of sample respectively in the bioreactor medium, mucin beads and bead medium, however, these values are close to the detection limit (Figure 11).

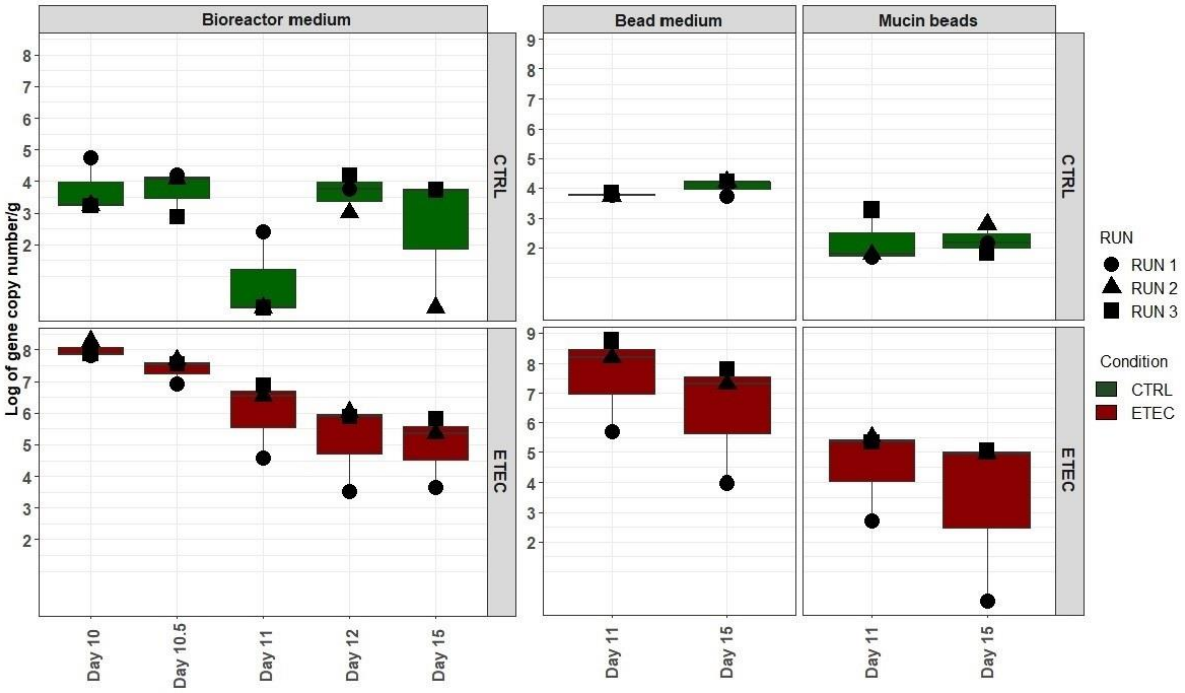


Figure 11. Quantification of the ETEC LT gene in the MPigut-IVM by qPCR (n = 3).

The activity of the ETEC strain in the MPigut-IVM was evaluated by quantification of virulence gene expression. The EAST1, LT and K88 genes were actually expressed in the

bioreactor medium and bead medium in the ETEC condition on the day 10, 11 and 15 and proved that the ETEC Ec105 was active in the MPigut-IVM (not shown). The expression of these virulence genes was not detected in the CTRL condition.

Effects of the ETEC challenge on microbiota composition

The presence of the ETEC Ec105 strain had no significant effects on bacterial populations quantified by qPCR (Figure 7). However, a significant impact of the ETEC challenge on the microbiota was highlighted by the Illumina MiSeq data between the samples from day 10 to 15 which were subjected or not to the injection of the ETEC Ec105 strain. At the phylum level in the bioreactor medium, the presence of the ETEC Ec105 during the recovery period led to significant higher mean relative abundance of the *Bacteroidetes* phylum from 35.4 ± 15.4% for the CTRL condition to 50.7 ± 10.5% for the ETEC condition from day 10 to 15 and lower mean relative abundance of *Actinobacteria* from 9.2 ± 7.4% in the CTRL condition to 2.7 ± 2.7% in the ETEC condition during the same period (Supplementary Figure 2A). On the mucin beads, the *Firmicutes* were significantly increased from 48.9 ± 7.7% in the CTRL condition to 58.1 ± 6.6% in the ETEC condition from day 10 to 15 (Supplementary Figure 2B). At the family level in the bioreactor medium (Figure 8A), the *Prevotellaceae* was significantly increased from mean relative abundance of 23.2 ± 23% in the CTRL condition to 30.9 ± 18.8% in the ETEC condition from day 10 to 15 (Figure 8A). In contrary, *Acidaminococcaceae, Atopobiaceae, Veillonellaceae* and *Erysipelotrichaceae* were significantly reduced from mean relative abundances of respectively 24.2 ± 8.4%, 6.3 ± 7%, 4.1 ± 2.8% and 6 ± 4.8% in the CTRL condition to 18.2 ± 7.4%, 0.3 ± 0.4%, 2.2 ± 2.1% and 2.7 ± 2.8% in the ETEC condition during the same recovery period (Figure 8A). On the mucin beads, the *Atopobiaceae* family was significantly decreased from mean relative abundance of 11.5 ± 3.2% in the CTRL condition to 5.8 ± 2.1% in the ETEC condition while the *Enterococaceae* was significantly increased from 0.5 ± 0.6% in the CTRL condition to 10.3 ± 5.7% in the ETEC condition from day 11 to 15 (Figure 8B). At the genus level, as shown in the Figures 4C and 4F, the presence of the ETEC strain significantly affected the abundance of several genera. For example, the genera *Eisenbergella, Peptoniphilus, Morganella, Tyzzerella* and *Enterococcus* were particularly enhanced by the injection of ETEC both in the bioreactor and the mucin beads (Figure 12). The MPigut-IVM archaeal microbiota was not significantly impacted by the ETEC challenge (Figure 11).

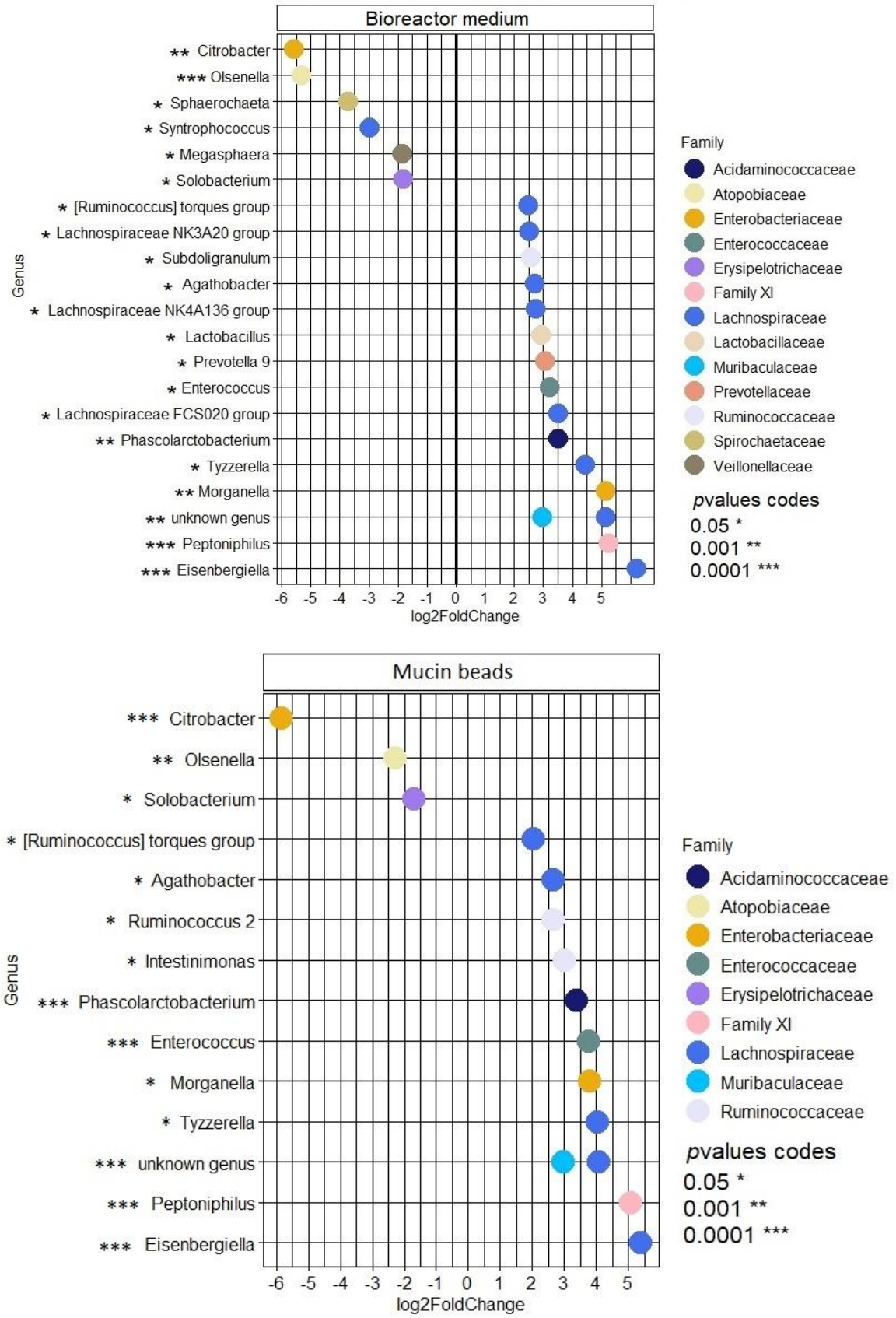


Figure 12. Differentially abundant genera between the samples of the recovery period containing the ETEC Ec105 strain or not in the bioreactor medium and mucin beads of the MPigut-IVM. Only significative Log2 fold changes are represented on the figure.

Positive Log2 fold changes indicates genera that were significantly more abundant in the presence of the pathogen. Pvalues were corrected for multiple testing.

Analysis of microbiota diversity

The MPigut-IVM microbiota alpha diversity indices were not significantly modified by any of the treatment (Supplementary Figure 4). Yet, beta diversity analysis using principal component analysis on Bray-Curtis distance showed that samples of MPigut-IVM clustered by diet (Figure 13).

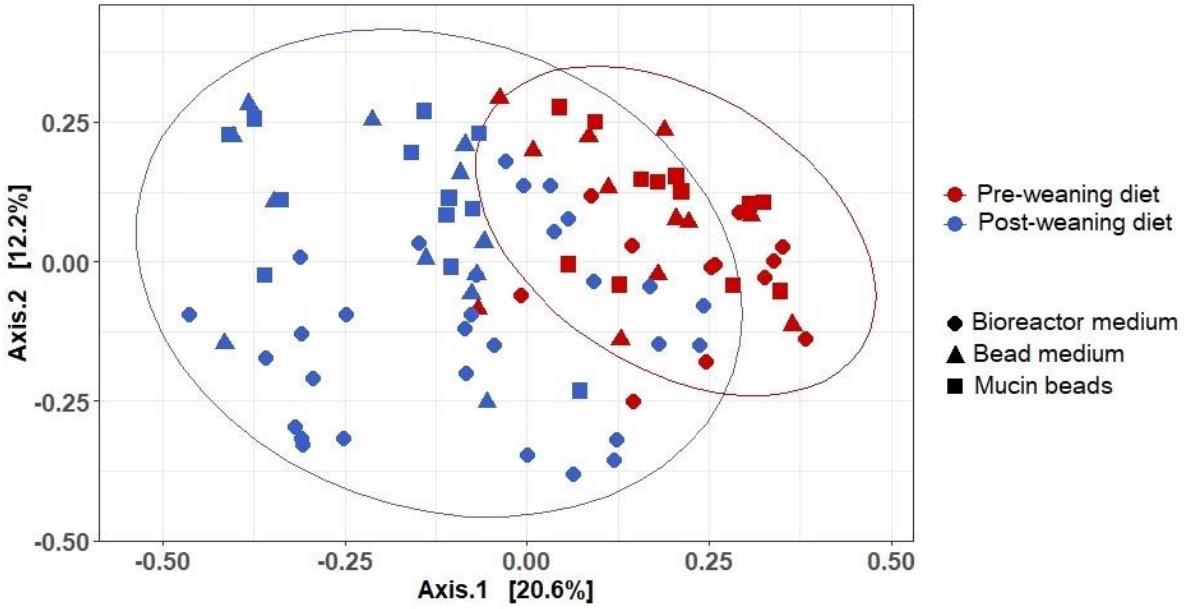


Figure 13. Principal Component analysis (PCoA) plot with Bray-Curtis dissimilarity on the bacterial communities separated by the type of diet in the bioreactor medium, bead medium and mucin beads of the MPigut-IVM.

The bead medium of MPigut-IVM challenged by ETEC induces changes in gene expression in IPI-2I cells

The expression of selected genes involved in innate inflammatory immune response targeting inflammatory cytokines and chemokines, tight junctions or mucus secretion (Supplementary

Table 4) of IPI-2I porcine cells incubated with the bead medium of MPigut-IVM were quantified using RT-qPCR (Figure 14). The supernatants from the bead medium of MPigut-IVM from day 15 of the ETEC conditions induced a significant increase in expression of TNFα, MYD88, MUC1 and CLDN4 genes compared to that of the CTRL conditions. Differences in IPI-2I gene expression caused by supernatants from other days of fermentation remained non-significant.

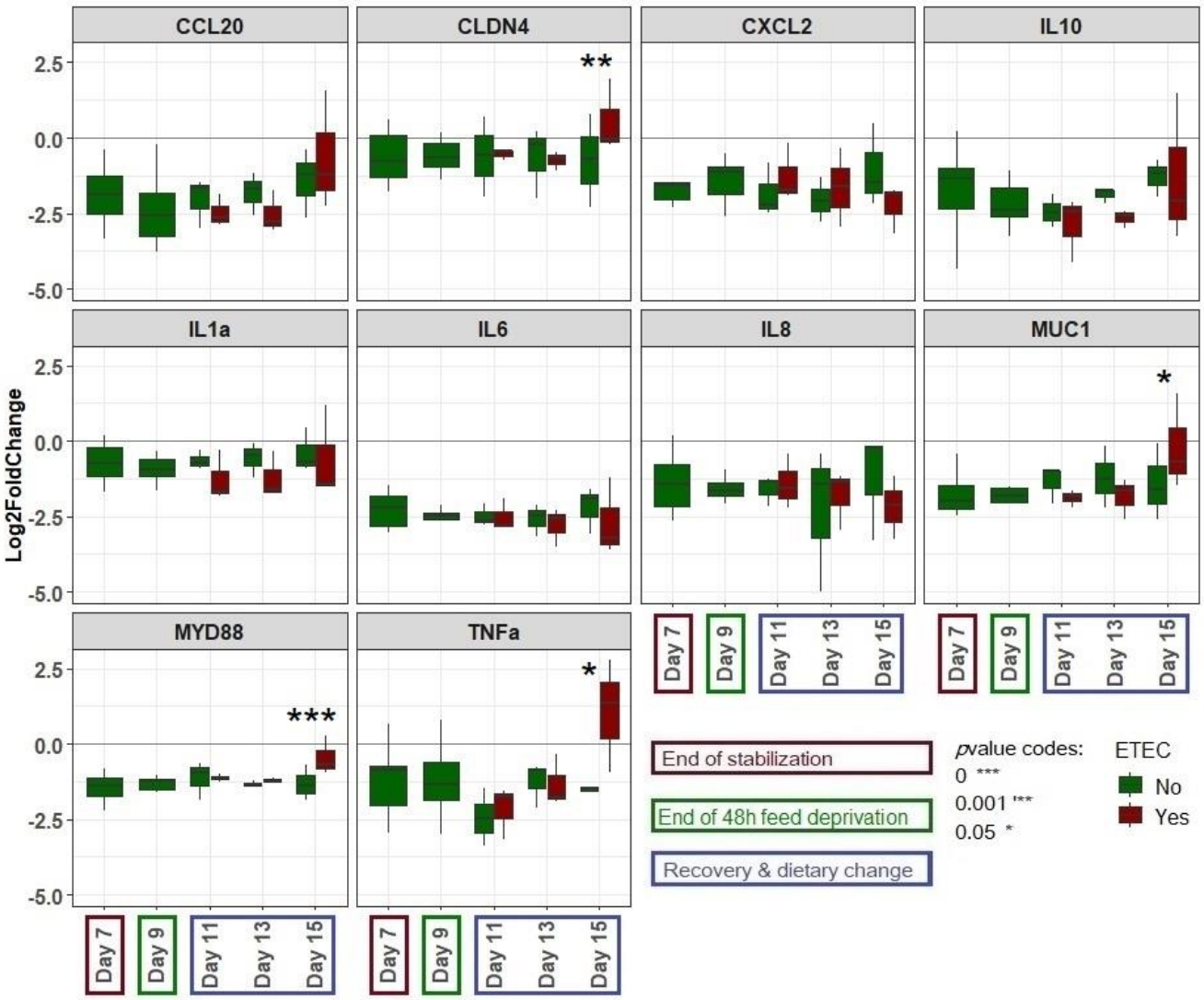


Figure 14. Log2 fold changes gene expression of IPI-2I cells incubated with bead medium supernatants of the MPigut-IVM collected at day 7, 9, 11, 13 and 15 and subjected to a 48h feed deprivation stress, diet shift and challenged or not with the ETEC Ec105 strain. Null values are IPI-2I cells incubated with their usual glutamine and FCS complemented DMEM medium. (n= 12, 6 technical replicates and 6 biological replicates, for day 11, 13 and 15; n= 6, 3 technical replicates and 3 biological replicates, for day 11, 13 and 15).

DISCUSSION

The MPigut-IVM has been previously developed to simulate the gut microbiome and mucobiome of weaning piglet (Chapter 9). Our previous work demonstrated that, due to unique features such as the presence of mucin beads and a self-maintained anaerobiosis, the MPigut-IVM harboured an *in vitro* microbiota very close to that of the proximal colon of piglets originating from the same farm, after a stabilization period of 7 days (Chapter 9). To further understand the impact of weaning stressors on the colonic microbiota of piglets and their roles in the etiology of post-weaning diarrhea, a diet change focused on the introduction of more diversified plant protein sources and of higher quantities of these nutrients into the MPigut-IVM right after the feed deprivation period to evaluate the impact of events close to weaning stressors found in commercial pig farms. First, in the present work, the dietary change applied after the feed deprivation stress induced modifications in the archaeal microbiota of the MPigut-IVM, shifting from a *Methanobrevibacter* to a *Methanosphaera* dominant microbiota. Modifications associated with weaning transitions are not yet well documented in piglets. This shift could however impact the metabolites present in the piglet gut, considering the high abundance of archaea populations in piglet lower gut (Gresse et al., 2019). Indeed, *Methanobrevibacter* and *Methanosphaera* are mainly known as hydrogenotrophic and methylotrophic archaea genera, respectively, although recent genomic studies suggest that they may have other metabolic differences (Poehlein et al., 2018). Second, regarding bacterial microbiota, the simulated weaning transition induced an increase in the relative abundance of several bacterial communities in the MPigut-IVM, including *Prevotellaceae* and *Enterobacteriaceae* family members while the *Bacteroidiaceae* was decreased, consistently with *in vivo* findings. Several studies reported a shift from a high relative abundance of *Bacteroides* in pre-weaning piglet feces towards a high relative abundance of *Prevotella* coupled with a reduced *Bacteroides* proportion in post-weaning piglet fecal samples (Alain B. Pajarillo et al., 2014; Frese et al., 2015; Guevarra et al., 2018; Trckova et al., 2018; Yang et al., 2019) and Adhikari et *al.* reported similar results in the colon (Adhikari et al., 2019). *Prevotella* is a common commensal genus which plays important roles in the digestion of nutrients, particularly in the degradation of starch, proteins and other plant polysaccharides (Ivarsson et al., 2014; Sandberg et al., 2019). In the study from Yang et *al.*, *Prevotella* was the most abundant genus in both healthy and diarrheic weaning piglets (Yang et al., 2019). However, piglets which developed diarrhea after weaning harboured a higher relative abundance of *Prevotella* and less *Escherichia coli* in the pre-weaning period compared to piglets which

remained healthy after weaning (Yang et al., 2019). A reduced number of *Bacteroides* in weaned piglet feces was also associated with diarrhea (Yang et al., 2019). Therefore, disturbed ratios between *Prevotella, Bacteroides* and *Escherichia* populations in early life could be associated with the onset of PWD. Also, the simulated weaning transition in the MPigut-IVM led to a decrease of the *Collinsella* genus from the *Coriobacteriaceae* family. A decreased proportion of *Collinsella* in human gut was previously linked to the development of a dysbiotic microbiota in inflammatory bowel disease (Kassinen et al., 2007) while an increase of *Collinsella* was positively correlated with protection against rotavirus diarrhea in gnotobiotic pigs (Twitchell et al., 2016) suggesting of potential role of *Collinsella* in pigs' health. In our present study, microbial activity was also significantly modified by the simulated weaning transition probably associated to microbiota changes. Redox potential was considerably impacted by the feed deprivation period which is in agreement with our previous findings (Chapter 9). Butyrate, acetate and isovalerate relative abundances significantly decreased after the feed deprivation stress period and dietary change in contrary to propionate, valerate and caproate both in the bioreactor and on the mucin beads of the MPigut-IVM. Butyrate proved to be very beneficial to piglet health by improving the performance of piglets around weaning due to the stimulation of intestinal epithelium (Lu et al., 2008), improved immune response (Melo et al., 2016) and modulation of intestinal microbiota (Castillo et al., 2006; López-Colom et al., 2019; Xu et al., 2016). Also, supplementation of early weaned piglets with sodium butyrate was shown to attenuate diarrhea symptoms and decrease intestinal permeability (Feng et al., 2018). Thus, fluctuations of both bacterial composition and activity were observed in the MPigut-IVM following the feed deprivation period/diet shift which could favour the emergence of opportunistic pathogens such as ETEC.

To further simulate the conditions leading to PWD at weaning (Gresse et al., 2017), an porcine ETEC strain was inoculated to the MPigut-IVM after the simulated weaning transition. Interestingly, two days after the injection of ETEC Ec105, the Labile Toxin (LT) gene was detected in high concentration in the bead medium of the MPigut-IVM, showing a preferential localisation of ETEC Ec105 close to an area rich in mucins which are indeed known to be a privileged site of adhesion *in vivo* (González-Ortiz et al., 2014). Only one replicate displayed a strong decrease in the pathogenic strain concentration after few days in the MPigut-IVM. This difference could be explained by a variability in the microbial composition of the fecal inocula, probably reflecting the *in vivo* situation. A stronger balance between microbial communities could lead to less susceptibility to the introduction of a pathogen. The ETEC challenge did not

influence the relative abundance of *Escherichia/Shigella* genus, probably because the concentration of the ETEC strain after inoculation was in the range of that of commensal *Escherichia/Shigella*. Indeed, the LT gene was quantified at ~ 8 Log_{10}/mL in the bioreactor and bead medium of the MPigut-IVM, and the commensal *Escherichia/Shigella* group was quantified at ~ 9 Log_{10}/mL. In addition, López-Colom et *al.* reported previously that an ETEC challenge was not associated with the increase of commensal *E. coli* in the ileum and ileal mucosa of weaned piglets (López-Colom et al., 2019). In this study, piglets which received butyrate and heptanoate preventive treatments to ETEC F4 displayed higher quantity of commensal *Enterobacteria* in the ileum and ileal mucosa few days after the infection (López-Colom et al., 2019). One hypothesis, supported by Leatham *et al.* (Leatham et al., 2009), could be due to the phenomenon of colonization resistance described as an increased of coliforms against colonization of pathogenic strains in the gut. In the present work, several families and genera were however significantly impacted by the injection of ETEC after the simulated weaning transition in the MPigut-IVM. The most spectacular change was the increase in *Enterococcus* in the ETEC conditions, especially on the mucin beads. Some *Enterococcus* members are known to bind to mucus *in vivo* (Hendrickx et al., 2015; Tytgat et al., 2016). Consistently with our findings, several *in vivo* studies reported a higher relative abundance or quantity of *Enterococcus* and *E. coli* or ETEC strains in the digestive content or mucosa of neonatal diarrheic piglets (Cheon and Chae, 1996; Hermann-Bank et al., 2015; Jonach et al., 2014; Larsson et al., 2014). A co-occurrence of *Enterococcus* and pathogenic *E. coli* could be thus involved in the pathogenesis of diarrhea episodes in piglets. Considering the several reports of this observation, we could hypothesised that *Enterococcus* and *E. coli/*ETEC members are able to naturally cooperate possibly by mechanisms of cross feeding or mutualism (Germerodt et al., 2016). The *Morganella* genus which was detected finally 4 times more abundant in the ETEC condition both in the mucin beads and the bioreactor by the differential analysis has been previously associated with diarrhea in humans (Ikeobi et al., 1996; Jertborn and Svennerholm, 1991; Müller, 1986) and was even positively correlated with diarrhea indices during an ETEC F4 challenge in mice (Xu et al., 2020). The MPigut-IVM is thus able to reproduce several characteristics of ETEC pathogenesis consistent with *in vivo* data. In consequence, the other populations which correlated with the ETEC conditions but are not currently described in the literature such as *Peptoniphilus* or *Eisenbergiella* should be considered as populations of interest for future investigations.

To increase our understanding of the etiology of PWD and how microbiota disruptions and pathogen presence affect host cells, we incubated diluted supernatants of bead medium from MPigut-IVM with the IPI-2I pig intestinal cell line. *In vitro* models of the human colon were previously successfully coupled with eukaryotic cell cultures (Defois et al., 2018; Marzorati et al., 2014) but, up to date, this was for the first time performed with an *in vitro* model of the piglet colon. In this study, supernatants from the bead medium of the ETEC condition induced significant increases in expression of genes coding for the myeloid differentiation primary response 88 (MyD88), tumor necrosis factor α (TNFα), claudin 4 (CLDN4) and mucin 1 (MUC1) in IPI-2I cells. The relation between mucins, commensals and pathogens have been widely studied(Etienne-Mesmin et al., 2019). MUC1 is a high molecular mass glycoprotein expressed at the apical surface of mucosal epithelial cells. MUC1 secretion can be stimulated by lipopolysaccharides produced by Gram negative bacteria and mucin is thought to play an important role in the fight against infections- by epithelial cells (Kato et al., 2017). Expression of the MUC1 gene notably limited the access of *Helicobacter pylori* in infected mice (McGuckin et al., 2007). In our study, claudin 4 gene was over-expressed in cells exposed to supernatants collected at day 15 of ETEC conditions. This gene belongs to the family of claudins family which are, with occludin proteins, tight junction proteins located at the apical side of piglet enterocytes (Pasternak et al., 2015) and thus play a role in intestinal permeability and infection. Consistently with our findings, claudin protein higher expression was previously associated with ETEC K88 infection in the porcine cell line IPEC-J2 in several studies (Luo et al., 2019; Wu et al., 2016). We could hypothesize that the higher expression of claudin genes from enterocytes could be induced in order to repair disrupted tight junctions due to the presence of toxins or virulence factors in the supernatants which, as reported by RT-qPCR expressed in the bead medium on the MPigut-IVM. The MyD88-mediated innate immune response has been already proven to be primarily important for protection against microbial pathogen infection via the induction of inflammatory cytokine production (Campos et al., 2004; Issac et al., 2018, 2013; Scanga et al., 2002; von Bernuth et al., 2012). Indeed, MyD88 deficient mice showed to be profoundly susceptible to infection (Issac et al., 2013). In weaning piglets, an increased level of MyD88 was already reported in enterocytes both *in vivo* and *in vitro* by several studies after infections with an ETEC K88 strain (Chytilová et al., 2014; Finamore et al., 2014; C. Xu et al., 2014). TNFα is one of the most widely studied proinflammatory cytokine involved in numerous bacterial, parasitic and viral infections and was suggested as a biomarker of digestive pathologies in weaned piglets due to its correlation with villi/crypt ratio damages (Barba-Vidal et al., 2017; Gustavo Hermes et al., 2013; López-Colom et al., 2019; Van Reeth

et al., 2002). Indeed, increased concentration of TNFα or increased expression of the associated gene were previously reported in the intestine of piglets orally challenged with ETEC K88 (López-Colom et al., 2019; Pu et al., 2018) as well as in IPEC-J2 cells co-incubated with ETEC K88 (Xia et al., 2017b, 2017a). Considering the matches between our results and literature, the incubation of MPigut-IVM bead medium supernatants on IPI-2I cells seems to reproduce, finally partially, the effects of ETEC infection on piglet enterocytes. However, it is important to notice that significant differences were detected only with supernatant collected at day 15. These findings could highlight that, the introduction of a porcine ETEC strain in the MPigut-IVM led to modifications of microbial metabolites which could be secreted in sufficient quantity to induce differential inflammatory responses in porcine epithelial cells at day 15 only.

To conclude, our studies reported that the simulation of a feed deprivation stress and dietary changes in the MPigut-IVM impacted the microbiota composition and functionality in a consistent manner compared to previous *in vivo* findings. The introduction of a porcine ETEC strain after the simulated weaning transition was positively correlated with the increase in several genera which should be considered in future investigations. If further research need be undertaken to fully understand the interactions, our results confirmed that members of *Bacteroides, Prevotella, Escherichia* and *Enterococcus* genera could play a role in the onset of post-weaning diarrhea in piglets. The incubation of the *in vitro* supernatants with pig intestinal cell lines, which was studied for the first time, indicated that MPigut-IVM was able to report the effect of microbiota metabolites change on host cells. The MPigut-IVM seems to reproduce the effects of weaning transition and ETEC colonization on the microbiota and will thus be used to evaluate preventive strategies against post-weaning intestinal dysbiosis.

Supplementary table 1. Composition of the fermentation medium mimicking ileum chyme of 28 days old piglets in the Chapter 10.

	Pre-weaning diet	**Post-weaning diet**
Concentration	g/L	g/L
Wheat starch	5	5
Corn starch	0	6
Pectin	2	2
Arabinogalactan	2	2
Cellulose	2	3
Soy protein isolate	10	12
Peptone from potatoes	0	5
Tryptone from casein	4.5	1.5
Mucin from porcine stomach type II	2	2
Whey powder	5	2
$FeSO_4$, $7H_2O$	0.005	0.005
L-cysteine HCl monohydrate	0.80	0.80
Porcine bile extract	0.40	0.40
KH_2PO_4	0.5	0.5
$NaHCO_3$	1.5	1.5
Hemin from porcine	0.005	0.005
NaCl	4.5	4.5
KCl	4.5	4.5
$MgSO_4$ anhy. (120.37 g mol^{-1})	0.64	0.64
$CaCl_2$ $2H_2O$ (147.02 g mol^{-1})	0.15	0.15
$MnCl_2$ $4H_2O$ (197.91 g mol^{-1})	0.20	0.20

Supplementary table 2. Primers and probes used for quantification of bacteria and methanogenic archaea in the Chapter 10.

Target	Type	Sequences	References	Concentration	Annealing temperature
***Escherichia coli*/ *Shigella* group**	Taqman	5'-CAT GCC GCG TGT ATG AAG AA-3' 5'-CGG GTA ACG TCA ATG AGC AAA-3' (6-FAM)-5'-TAT TAA CTT TAC TCC CTT CCT CCC CGC TGA A-3'(TAMRA)	(Huijsdens et al., 2002)	300 nM 300 nM 100 nM	61°C
Total Bacteria	SYBRGREEN	5'-ACT CCT ACG GGA GGC AG-3' 5'-GTA TTA CCG CGG CTG CTG-3'	(Yu et al., 2005)	500 nM 500 nM	61°C
Methanogenic archaea	SYBRGREEN	5'-GAG GAA GGA GTG GAC GAC GGTA-3' 5'-ACG GGC GGT GTG TGC AAG-3'	(Ohene-Adjei et al., 2008)	500 nM 500 nM	61°C
Labile enterotoxin (LT)	SYBRGREEN	5'- GGC GTT ACT ATC CTC TCT AT 3'—TGG TCT CGG TCA GAT ATG T	(Madoroba et al., 2009)	500 nM 500 nM	55°C

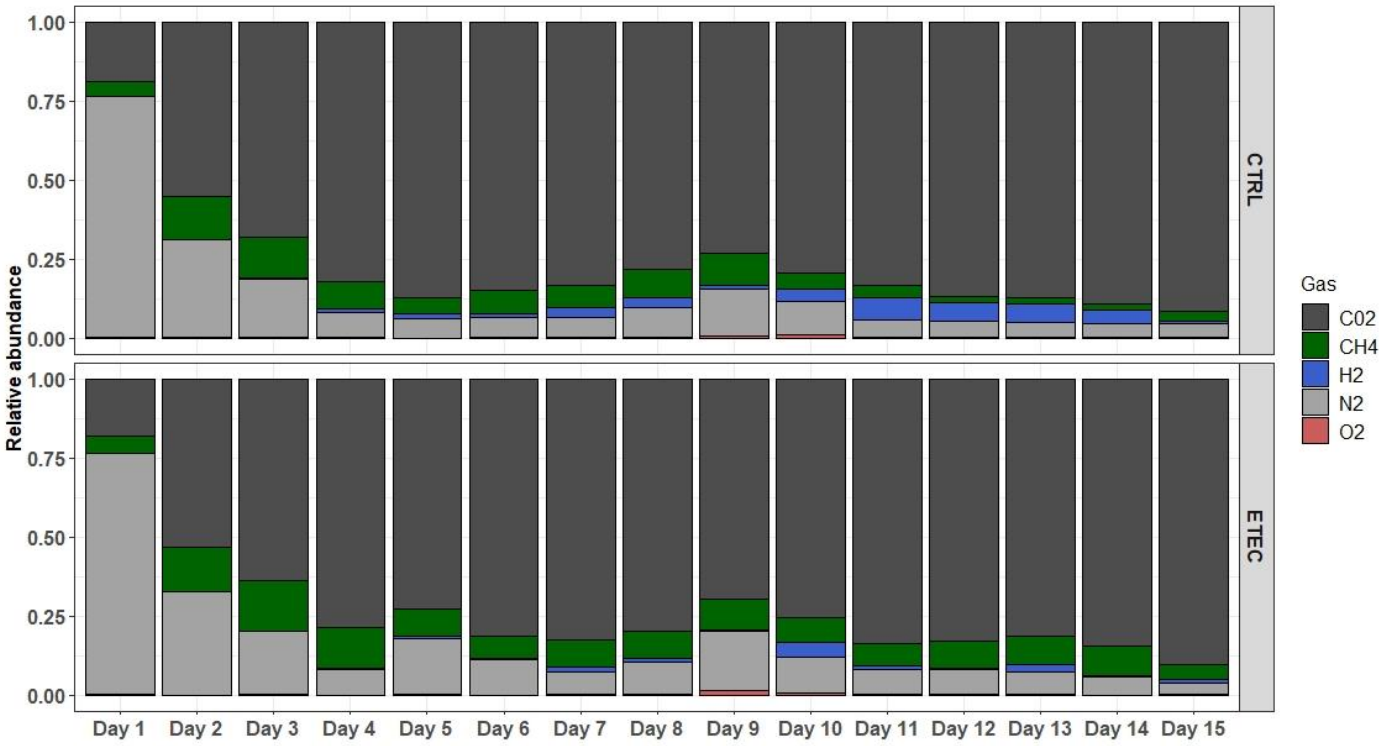
Supplementary table 3. Primers used for quantification of expression of selected ETEC virulence genes.

Target	Gene	Sequence 5' 3'	Concentration nM	Reference
ST toxin B	*Stb*	F- TGCCTATGCATCTACACAAT	150	(Madoroba et al., 2009)
		R- CTCCAGCAGTACCATCTCTA	150	
Labile enterotoxin	*LT*	F- GGCGTTACTATCCTCTCTAT	500	(Madoroba et al., 2009)
		R- TGGTCTCGGTCAGATATGT	500	
Fimbriae 4	*K88*	F- GAATCTGTCCGAGAATATCA	200	(Madoroba et al., 2009)
		R- GTTGGTACAGGTCTTAATGG	200	
Enteroaggregative heat stable enterotoxin 1	*EAST1*	F- TCGGATGCCATCAACACAGT	500	(Ngeleka et al., 2003)
		R- GTCGCGAGTGACGGCTTTGTAG	500	
Sigma 38 protein	*rpos*	F- GCGCGGTAGAGAAGTTTGAC	500	(Rahman et al., 2006)
		R- GGCTTATCCAGTTGCTCTGC	500	
aerobic control respiration	*arcA*	F- GTTCTTACCCGGCAGATTGA	500	(Roussel, 2019)
		R- CAGACCCCGCACATTCTTAT	500	
GAPDH *E. coli*	*gapA*	F- CGTTGAAGTGAAAGACGGTCATC	200	(Nicklasson et al., 2012)
		R- CAACACCAACTTCGTCCCATTT	200	

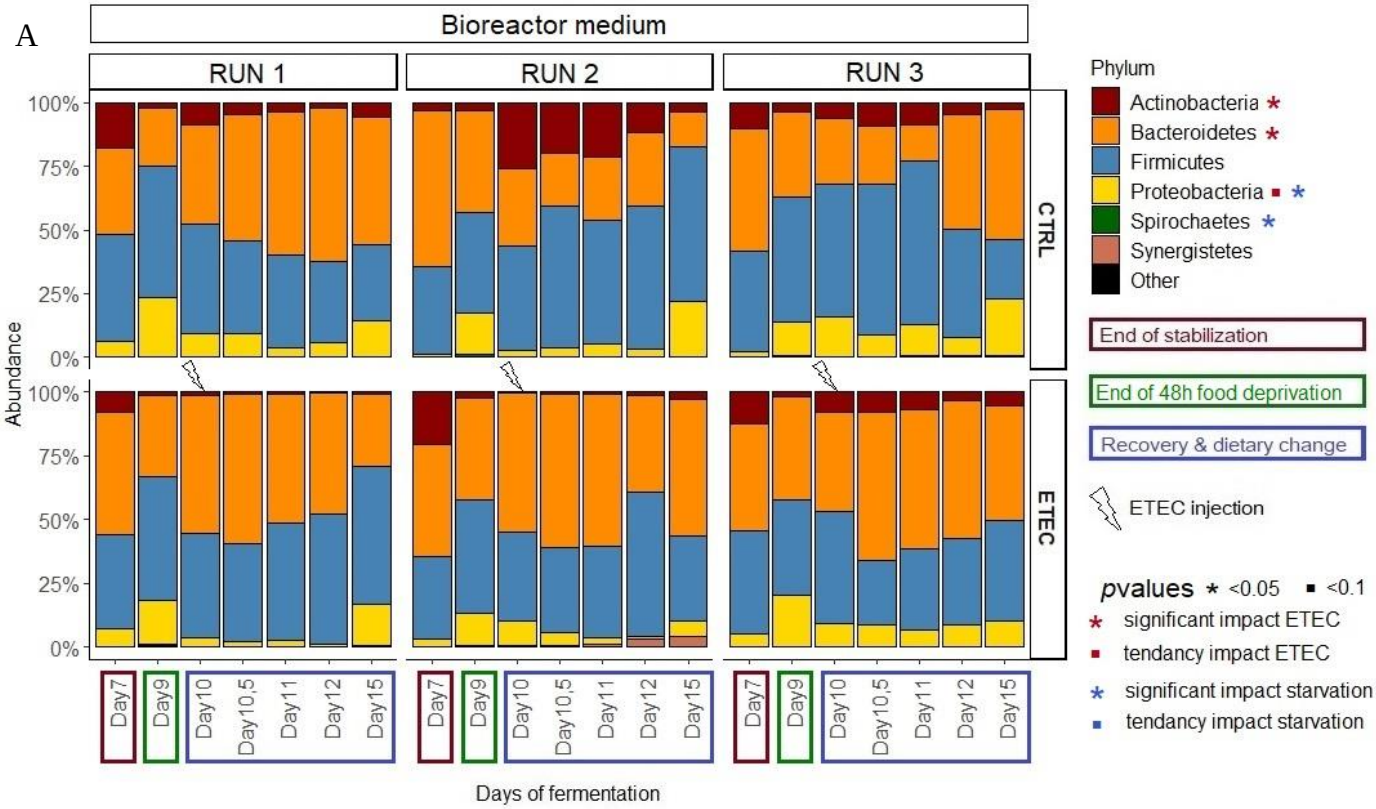
Supplementary table 4. Primer used for quantification of gene expression in IPI-2I cells exposed to MPigut-IVM supernatants.

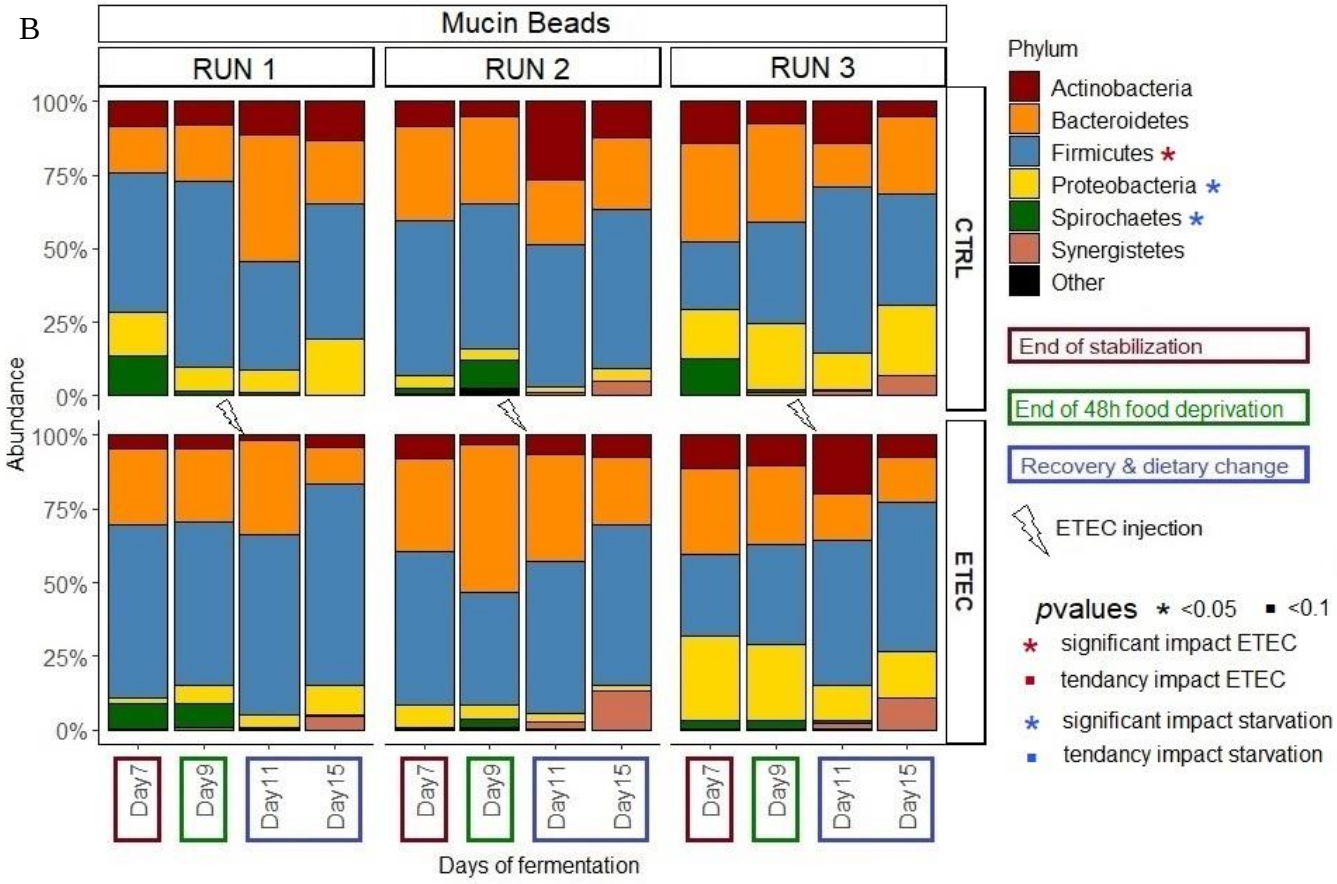
Target	Gene	Sequence 5' 3'	Annealing temperature	Reference
Cyclophilin A	*Cyclophilin-A*	CCT GAA CAT ACG GGT CCT G	57	Dr. J.J. Garrido
		AAC TGG GAA CCG TTT GTG TTG		
Beta actin	*β-actin*	CAGGTCATCACCATCGGCAACG	57	Dr. J.J. Garrido
		GACAGCACCGTGTTGGCGTAGAGGT		
Chemokine (C-C motif) ligand 20	*CCL20*	ACTT TGA CTG CTG CCT CCG ATA	54	Dr. J.J. Garrido
		TGC ATT GAT GTC ACA AGC TTC A		
Claudin 4	*CLDN4*	TAT CAT CCT GGC CGT GCT A	57	(Mariani et al., 2009)
		CAT CAT CCA CGC AGT TGG T		
Chemokine (C-X-C motif) ligand 2	*CXCL2*	GGA TAG CAC GCT GTA CCA TC	57	Dr. J.J. Garrido
		ACT GTC TCA ATA AAT AAC AAC CGA C		
Interleukin 10	*IL10*	CAG ATG GGC GAC TTG TTG	57	Dr. J.J. Garrido
		ACA GGG CAG AAA TTG ATG AC		
Interleukin 1a	*IL1a*	AAC GAA GAC GAA CCC GTG TTG CT	57	Dr. J.J. Garrido
		GGT CTC ATC TTT GAT GGT TTT GG		
Interleukin 6	*IL6*	TGG CTA CTG CCT TCC CTA CC	57	Dr. J.J. Garrido
		CAG AGA TTT TGC CGA GGA TG		
Interleukin 8	*IL8*	TTC GAT GCC AGT GCA TAA ATA	57	Dr. J.J. Garrido
		CTG TAC AAC CTT CTG CAC CCA		
Mucin 1	*MUC1*	CCC TGG CCA TCA TCT ATG TC	56	(Mariani et al., 2009)
		TGC CCA CAG TTC TTT CGT C		
Myeloid differentiation primary response 88	*MYD88*	TGG TGG TGG TTG TCT CTG ATG A	57	Dr. J.J. Garrido
		TGG AGA GAG GCT GAG TGC AA		
Tumor Necrosis Factor alpha	*TNF-α*	CGC CCA CGT TGT AGC CAA TGT	57	Dr. J.J. Garrido
		CAG ATA GTC GGG CAG GTT GAT CTC		

Supplementary figure 1. Relative abundance of gas produced by fermentation activity of the microbiota inhabiting the MPigut-IVM according to the different conditions applied to the model and time.

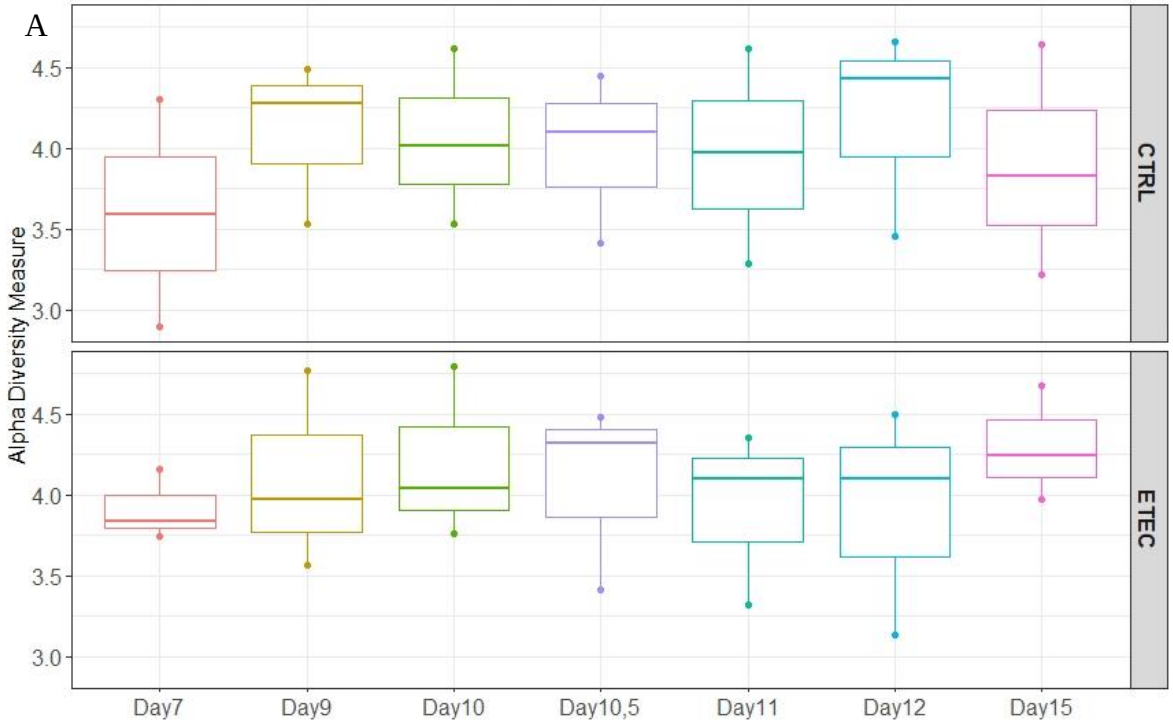


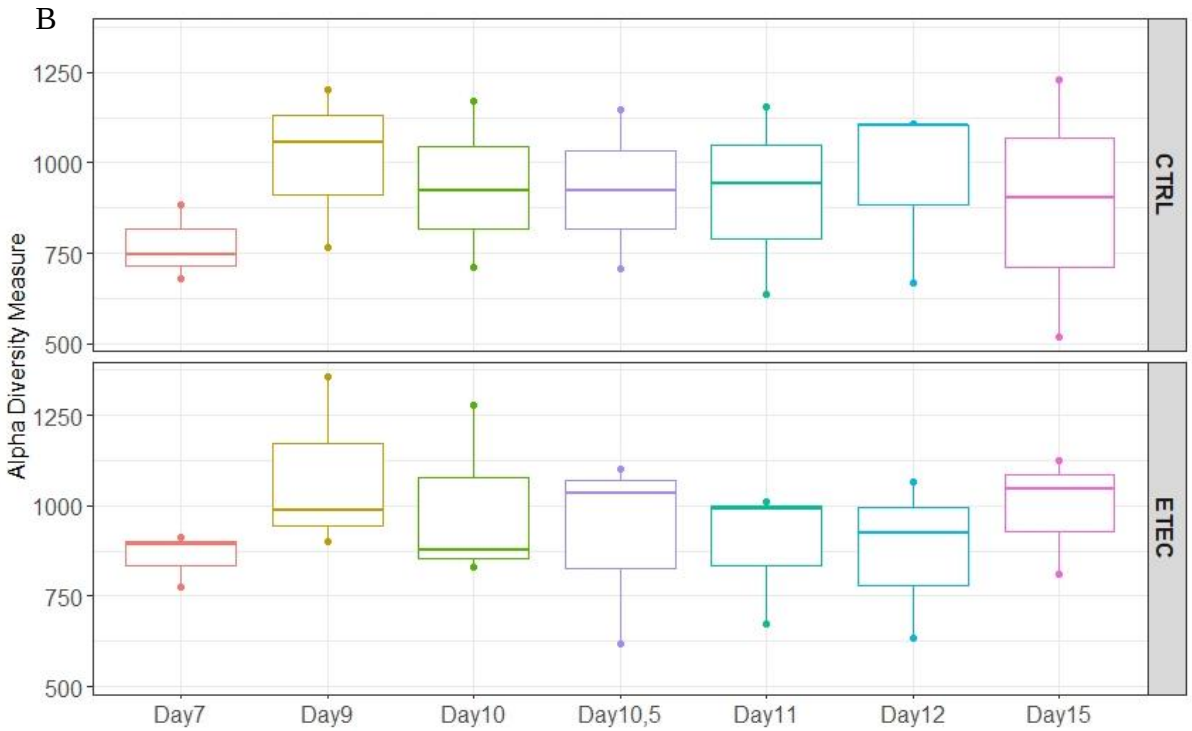
Supplementary figure 2. Relative abundances of the main bacterial phyla in the bioreactor medium (A) and the mucin bead (B) in MPigut-IVM during the runs 1, 2 and 3 which were subjected to a 48h feed deprivation stress, dietary change and challenged or not with the ETEC Ec105 strain.





Supplementary figure 3. Shannon (A) and observed (B) alpha diversity indices in the totally of MPigut-IVM samples during the runs 1, 2 and 3 which were subjected to a 48h feed deprivation stress, dietary change and challenged or not with the ETEC Ec105 strain. (n=3).





Chapter 11. Impact of SB CNCM I-1079 in the MPigut-IVM subjected to Weaning Simulation and Pathogen Exposure and on Porcine Epithelial Cells

As demonstrated in Chapter 10, the simulation of weaning transition by a feed deprivation period of 48h and dietary change coupled with pathogen exposure led to modifications of microbiota composition and activity in the MPigut-IVM. Additionally, fermentation supernatants from this condition could induce an inflammatory response in porcine epithelial cells. In this chapter, the effects of the yeast SB CNCM I-1079 on ETEC colonization and gut microbiota in the MPigut-IVM and on porcine epithelial cells was evaluated. The fermentation runs, here performed with 2 biological replicates, receiving SB supplementation before and after the simulation of weaning transition will be subjected to the ETEC strain used in Chapter 10 and compared with the fermentation starting from the same fecal inocula and subjected to pathogen exposure but without SB supplementation. Such as in the precedent Chapter, supernatants of the MPigut-IVM from the SB were coupled with porcine intestinal cell lines to study host responses towards microbiota changes, pathogen exposure and SB supplementation. At last, in order to investigate more accurately the mode of action of SB on ETEC infection of porcine epithelial cells, the effects of SB treatments was evaluated on ETEC adhesion and inflammatory response on another porcine epithelial cell line.

The part of the work on cell culture was performed during a PhD secondment at the Department of Animal Genetics of Cordoba University under the supervision of Pr. dr. Juan J. Garrido. This Chapter aims to answer to the following questions:

- Does SB has a protective effect against ETEC infections on porcine intestinal cells?

- Do the MPigut-IVM medium supernatants challenged with an ETEC strain but supplemented with SB mitigate inflammatory genes expression of porcine intestinal epithelial cells?

The graphical abstract presented below summarizes the principal results of this Chapter (Figure V). Briefly, the SB supplementation induced modification of the microbiota composition, could reduce ETEC adhesion on porcine epithelial cells and alleviate inflammatory response induced by pathogen exposure on epithelial cells.

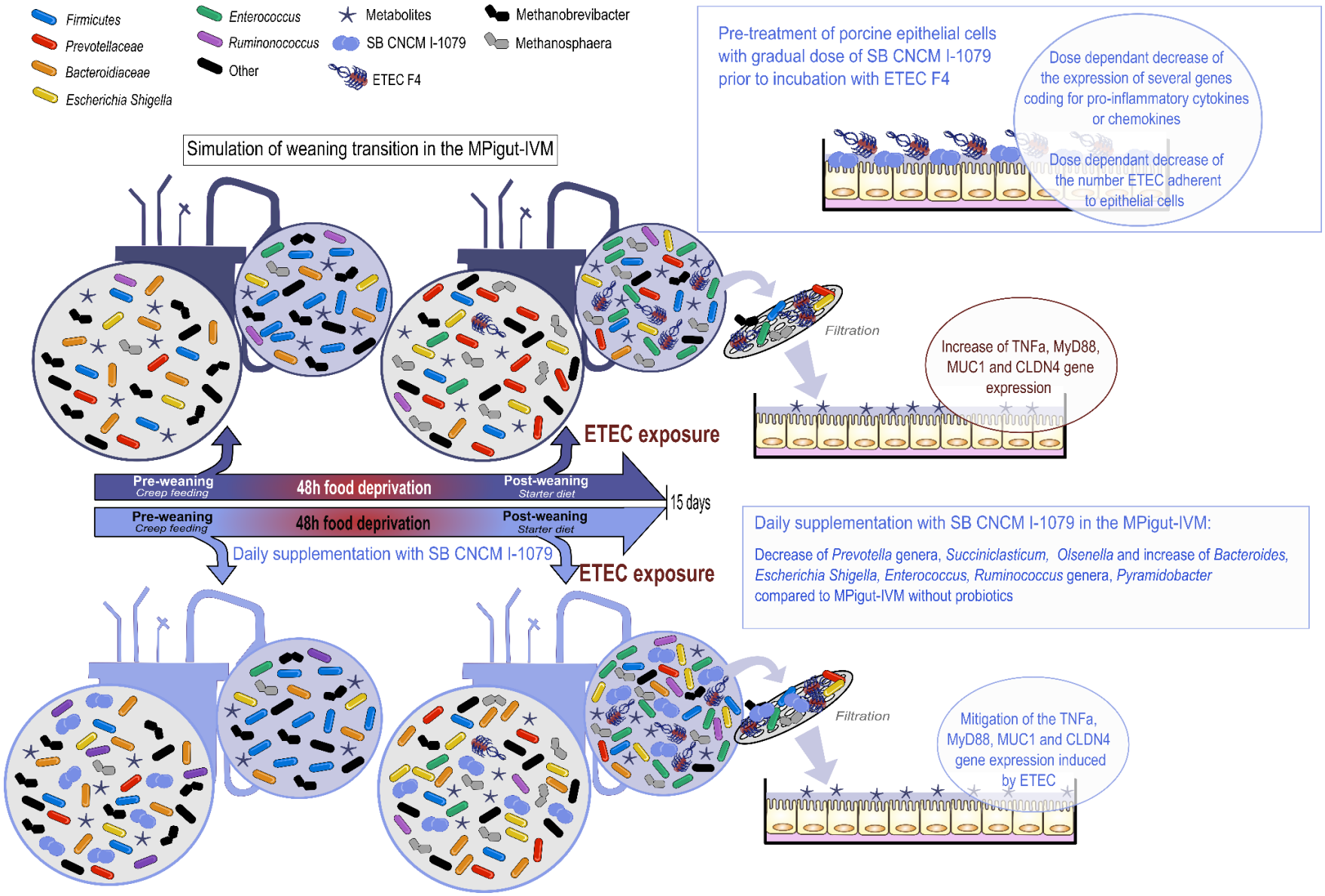


Figure V. Graphical abstract of the Chapter 11.

Saccharomyces cerevisiae var _Boulardii_ CNCM I-1079 reduces the expression of genes involved in inflammatory response in porcine cell lines challenged by enterotoxigenic _Escherichia coli_ (ETEC) and influences bacterial communities in an _in vitro_ model of the weaning piglet colon.

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BACKGROUND

Probiotics are living organisms that, when administered in adequate amounts, have the potential to confer health benefits to their hosts (Hill et al., 2014). If most of characterized probiotics belong to the bacterial domain such *Lactobacillus* or *Bifidobacterium* species, yeasts *Saccharomyces cerevisiae* ar. *boulardii* (SB) have shown to yield numerous positive effects for animal health including for example higher feed efficiency and digestibility, reduced diarrhea or reduced intestinal inflammation [2–6]. In livestock production, yeasts have been already commercialized as feed additives which may be able to replace the use of antibiotics as growth promoters (Kiros et al., 2019b). One of the next challenges of swine industry is to find non-pharmacological alternatives to reduce the incidence of post-weaning diarrhea while reducing the use of antibiotics in order to fight against the worrying increase of bacterial resistance towards antibiotics (Gresse et al., 2017). *In vivo* studies in piglets revealed that SB could be a potential candidate to help animals to resist to infections by Enterotoxigenic *Escherichia coli* (ETEC), the main pathogen responsible of post-weaning diarrhea (Dubreuil et al., 2016; Fairbrother et al., 2005; Rhouma et al., 2017), for instance by modulating intestinal microbiota,

reducing fecal ETEC shedding, decreasing diarrhea symptoms or improving intestinal morphology (Badia et al., 2012b; Brousseau et al., 2015; Che et al., 2017; Garcia et al., 2019; Kiros et al., 2019; Mathew et al., 1998; van Heugten et al., 2003; Zanello et al., 2011). *In vitro* trials started to give insights about *S. cerevisiae* and SB anti-infectious properties. In porcine intestinal cell lines challenged with ETEC strains, *S. cerevisiae* and SB were both able to inhibit the expression of pro-inflammatory cytokines induced by ETEC infection(Badia et al., 2012b; Zanello et al., 2011). However, further investigations are needed to unravel the yeast mode of action and beneficial effects as feed additive for weaning piglets.

In this study, we used an *in vitro* gut model of the weaning piglet colon (MPigut-IVM) to track the influence of SB CNCM I-1079 on the composition and activity of the resident microbiota challenged or not with an ETEC F4+ (K88) strain isolated from a diarrheic piglet . Fermentation supernatants from the ETEC challenged experiments were also used to evaluate if the introduction of SB CNCM I-1079 into the *in vitro* model could affect gene expression of an intestinal porcine cell line. Lastly, we evaluated the direct effects of this live yeast on bacterial adhesion and gene expression of intestinal porcine cells challenged by the ETEC strain.

MATERIAL AND METHODS

Effects of SB CNCM I-1079 in the MPigut-IVM

Fecal samples collection and treatments

All animals were housed in a conventional pig farm located in the Haute-Loire area of the Auvergne-Rhône-Alpes region in France. Piglets remained with their mother and siblings during the suckling period. None of the piglets had signs of enteric or metabolic disturbances. The animals didn’t receive any antibiotic in the 27 days prior to feces collection day. Fecal samples from 4-weeks old healthy male piglets (Landrace x Large White) were collected using sterile bottles and immediately stored under strict anaerobiosis conditions using GENbag anaer gas pack systems (Biomerieux, Marcy L’Etoile, France) and rapidly transported to the laboratory without freezing.

MPigut-IVM parameters

Five hundred mL MiniBio bioreactors (Applikon Biotechnology, Delft, The Netherlands) equipped with stirrers, ports and probes and fecal inocula were prepared as previously described (Chapter 9). To ensure anaerobic conditions at the beginning of fermentations, the same volume of fecal suspension was added per bioreactor to 150 mL of nutritive medium while flushing with O_2-free N_2 gas. Afterwards, during the fermentation course, the anaerobic conditions were maintained exclusively through the activity of the resident microbiota and by ensuring the airtightness of the system. The temperature of the fermentation was set up to 39°C and maintained inside the bioreactor medium using an incorporated panel heater and in the mucin bead compartment using a hot water bath. pH was maintained at 6.0 and recorded, together with the redox potential as previously described (Chapter 9). The fermentation medium was stirred at a constant speed of 300 rpm during the total duration of the experiment. The volume of bioreactors was maintained at a constant value of 200 mL by automatic withdrawal of the fermentation medium. Anaerobic conditions and gas composition were checked every day by analyzing O_2, CO_2, CH_4 and H_2 using a HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled with a TCD detector (Agilent Technologies, Santa Clara, USA) and two series columns, Molecular Sieve 5A and Porapack Q (Aligent Technologies, Santa Clara, USA).

Mucin bead production and compartment

Mucin from porcine stomach type II (Sigma-Aldrich, Saint-Louis, Missouri, USA) and sodium alginate (Sigma-Aldrich, Saint-Louis, Missouri, USA) were used to produce mucin beads of 4 mm diameter as described previously (Chapter 9). At the beginning of the fermentations, 350 ± 20 mucin beads were introduced in their specific glass compartments. Mucin beads were totally replaced every 48h to ensure a continuous availability of mucin adherent surfaces. During the time of bead replacement, the medium of the bead compartment was kept under CO_2 flushing to avoid oxygen entrance.

In vitro *fermentation procedures*

Experiments in the MPigut-IVM were designed as presented on figure 1. *In vitro* fermentation procedures aimed to simulate a weaning transition which starts by giving a pre-weaning diet

during the first seven days of the fermentations which were called stabilization period and, as previously described, correspond to the appropriate time to observe a stabilization of the microbiota composition and activity inside the MPigut-IVM (Chapter 9). At day 7 the flow of nutritive medium was interrupted during 48h to simulate feed deprivation. At day 9, the flow of nutritive medium restarted with a post-weaning diet and was called recovery period. Pre-weaning and post-weaning died were prepared as mentioned in Chapter 10. All samplings from day 7 were performed before the start of the feed deprivation period while samplings from day 9 were collected right at the end of the feed deprivation period and before the recovery period. Samples from the bioreactor medium and bead medium were centrifuged at 4°C, 10,000 g and 45 min. Pellets and supernatants were respectively stored at -20 and -80°C until analysis. After collection, mucin beads were gently washed 3 times in sterile 1X PBS and stored at -20°C.

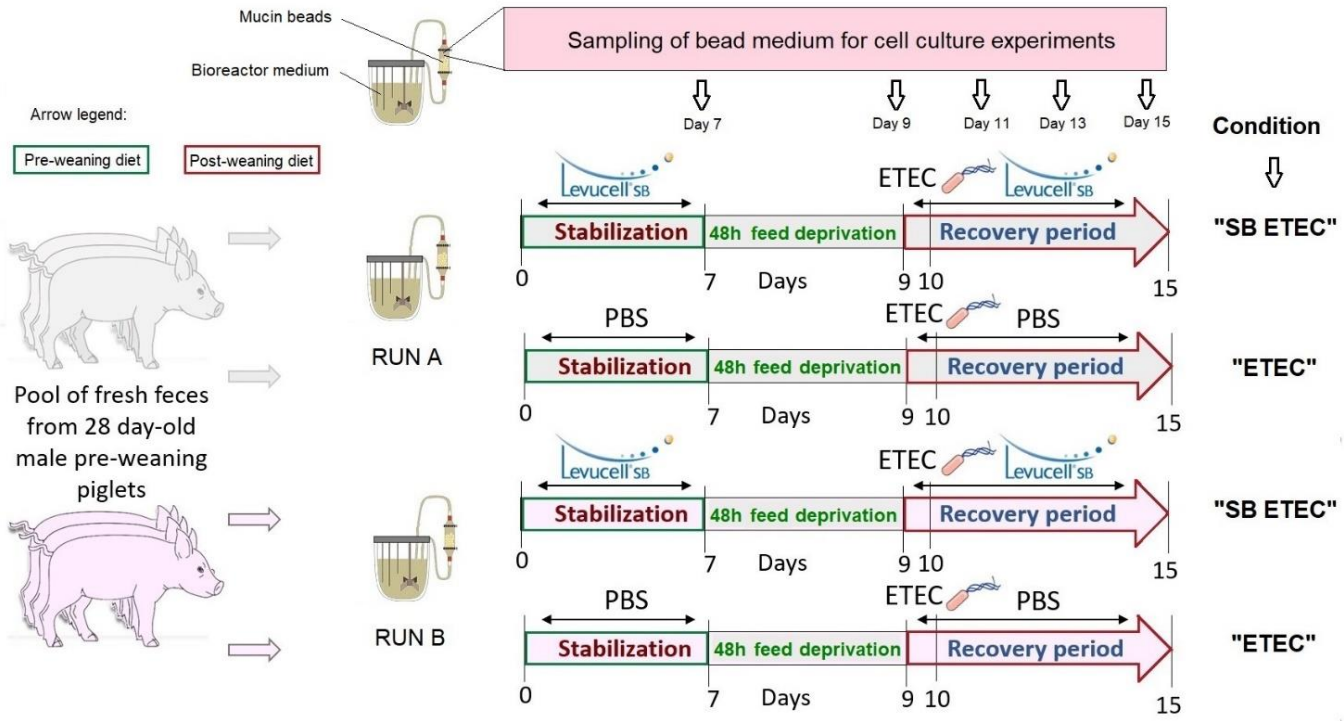


Figure 1. Experimental design of the *in vitro* fermentation procedures and denomination of the MPigut-IVM in the Chapter 11.

SB CNCM I-1079 supplementation

Every day, except during the feed deprivation period (48h), SB CNCM I-1079 active dry yeast (Levucell SB, Lallemand, Blagnac, France, 2 x 10^{10} CFU/g) was suspended into 1 mL of sterile

1X PBS and injected at a fixed time in the MPigut-IVM to reach a final concentration 10^7 CFU/mL. The ETEC condition received a daily dose of 1 mL of sterile 1x PBS as a control, except during the feed deprivation period.

ETEC culture conditions and challenge procedure for the MPigut-IVM

The ETEC strain was an Enterotoxigenic *E.coli* Ec 105 (F4, Stb+, East1+, LT+) isolated from a diarrheic piglet ETEC Ec 105 bacteria were grown at 39°C overnight, to be consistent with the temperature of the MPigut-IVM, in Luria Bertani (LB) broth (BD Difco, New Jersey, USA) until OD_{600nm}= 0.6. The bacteria were centrifuged at 4°C, 10,000 g and 15 min. The pellet was rinsed using sterile PBS 1X, suspended in 1 mL of sterile PBS and 2 x 10^9 CFU, corresponding to 10^7 CFU/mL, were injected inside the bioreactor medium of the "ETEC" and "SBETEC" conditions at day 10 of the fermentation right after the injection of SB CNCM I-1079 and just before the sampling time of day 10.

DNA extraction from MPigut-IVM samples

Total DNA was extracted from all collected samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The quality of the eluted DNA was assessed by agarose gel electrophoresis. Extracts were quantified using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, CarlSBad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, CarlSBad, CA, USA). Samples were stored at −20 °C prior to analyses.

Microbial quantification by qPCR

The list of primer pairs and their optimal conditions used for quantitative PCR are presented in Supplementary Table 1. Real-time PCR assays were performed on a Rotor-Gene Q (Qiagen, Venlo, NL) in 20 µL reactions with QuantiFast SYBR GREEN master mix (Qiagen, Venlo, NL) or Taqman Fast Advanced Master mix (Applied Biosystems, Foster City, California, USA) with the additions of each primer. The 16S rDNA genes were amplified using the conditions specified in Chapter 9 and 10.

MiSeq 16S rDNA sequencing and bioinformatic analysis

The DNA concentration of all samples was measured using the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, CarlSBad, CA, USA) with a Qubit 2.0 Fluorometer (Invitrogen, CarlSBad, CA, USA) and diluted to 2 ng/µL prior to PCR amplification. The Bacterial V3-V4 region of 16S rDNA and the Archaeal 16S rDNA were respectively amplified with primers 357F 5'-CCTACGGGNGGCWGCAG-3'(Yu et al., 2005) and 805R 5'-GACTACHVGGGTATCTAATCC-3'(Lane et al., 1985b) and primers 349F 5'-GYGCASCAGKCGMGAAW-3' and 806R 5'-GGACTACVSGGGTATCTAAT -3'(Ohene-Adjei et al., 2008). Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system (Illumina, San Diego, CA, USA) performed at the Carver Biotechnology Center of the University of Illinois (Urbana, IL, USA). The demultiplexed paired end Illumina sequence reads in the FastQ format were uploaded into the Galaxy instance (v.2.3.0) of the Genotoul bioinformatics platform (http://sigenae-workbench.toulouse.inra.fr) to be used in the FROGS (Find Rapidly OTU with Galaxy Solution) pipeline(Escudié et al., 2018). During the FROGS pre-process, sequences were depleted of barcode and the sequences with a non-appropriate length or containing ambiguous bases were removed. Next, reads were clustered into de novo operational taxonomic units (OTUs) using SWARM algorithm (Mahé et al., 2014) with, at first, a denoising step to build very fine cluster using the minimal distance equal to 1 and, secondly, with an aggregation distance equal to 3. Chimeras were then removed with VSEARCH (Rognes et al., 2016). Additionally, filters were applied to the OTUs in order to remove singletons(Auer et al., 2017; Bokulich et al., 2013). The OTUs selected were taxonomically assigned using the Silva release 132 reference database(Quast et al., 2013).

Quantification of short chain fatty acids (SCFAs) by gas chromatography

The SCFAs (acetate, propionate, isobutyrate, butyrate, caproate, isovalerate and valerate) were quantified in the bioreactor medium and bead medium by gas chromatography. Eight hundred µL of fermentation supernatants from bioreactor medium and bead medium were mixed with 500 µL of 0.4% (w:v) crotonic acid and 2% (w:v) metaphosphoric acid solutions. This mixture was centrifuged and the supernatant was injected in a PerkinElmer Clarus 580 gas chromatograph (Waltham, Massachusetts, USA) for quantification of SCFAs. A mixed-model one-way Anova (lmer and Anova functions) with time point (days of fermentation) or

conditions as a fixed effect and fermentation experiment as a random effect was used to compare the concentration of the main SCFAs between days of fermentation using the R packages lme4 and car.

Porcine intestinal cell line experiments

Adhesion assay of ETEC on IPEC-J2 cells and host gene expression

The IPEC-J2 cell line is a non-transformed intestinal cell line derived from the epithelium of the jejunum of a neonatal non-suckling piglet maintained as a continuous culture(Rhoads et al., 1994). This cell line is generally preferred to IPI-2I cell line for adhesion tests[15]. IPEC-J2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) medium (InvitrogenTM Life Technologies, CarlSBad, CA, USA) supplemented with 5% Fetal Calf Serum (FCS, PAA Laboratories GmbH, Austria). Cells were seeded onto 24-well tissue culture plates at 50,000 cell/well in a volume of 200 µL and grown for 24h in an atmosphere of 5% CO_2 at 37°C to allow for confluency the day of experiment.

Active dry yeast SB CNCM I-1079 was resuspended in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) medium (InvitrogenTM Life Technologies, CarlSBad, CA, USA) supplemented with 5% Fetal Calf Serum (FCS, PAA Laboratories GmbH, Austria) and rehydrated at 37°C under agitation for 30 min. ETEC Ec105 strain was grown at 37°C in LB broth (BD Difco, New Jersey, USA) until OD_{600nm}= 0.8-0.9 which corresponds to the concentration of $5*10^8$ CFU/mL. ETEC cells were pelleted, washed with sterile PBS and diluted with cell culture medium to reach the concentration of 100 ETEC CFU/ IPEC-J2 cell in a final volume of 100 µL/well. The IPEC-J2 cells on 24 well-plates were first pre-incubated 3h at 37°C and 5% CO_2 with SB at the concentrations of 10^8, 10^7 and 10^6 CFU/well, corresponding respectively to 625, 62.5 and 6.25 SB cell/ IPEC-J2 cell, or with 1mL of DMEM medium in triplicates. After 3h of incubation IPEC-J2 were infected with the ETEC Ec105 strain and incubated for additional 3 h. The entire experiment was run 4 times including 3 biological replicates and 1 technical replicate. After incubation, for the 3 biological replicates , IPEC-J2 cells were washed three times with sterile 1X PBS and lysed with 1% Triton X-100 (Sigma, St. Louis, USA) and serial dilutions were plated onto LB agar to determine the number of ETEC bacteria adhesive to the cells. For the last experiment, IPEC-J2 cells were washed three times with sterile 1X PBS and lysed with 1 mL of NucleoZOL (Machery-Nagel, Hoerdt, France) to extract RNA. Lysates were stored at -80°C prior to RNA isolation.

Incubation of ETEC-challenged MPigut-IVM samples with an intestinal porcine cell line and study of host gene expression

The IPI-2I cell line derived from the ileum of an adult male pig and immortalized by transfection with an SV40 plasmid (pSV3-neo)(Kaeffer et al., 1993), was used for this co-incubation assay due to its potential similarity with colonic enterocytes. IPI-2I cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) (InvitrogenTM Life Technologies, CarlSBad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS, PAA Laboratories GmbH, Austria) and 4 mM L-glutamine (Sigma, St. Louis, MO, USA).. Cells were seeded onto 48-well tissue culture plates at 25,000 cell/well in a volume of 200 µL and grown 24h in an atmosphere of 5% CO_2 at 37°C to allow for confluency at the day of experiment. Supernatants from fecal inocula and bead medium of fermentations from days 7, 9, 11, 13 and 15 were filtered using 0.2 µm sterile Minisart syringe filters (Sartorius, Göttingen, Germany) and 30 times diluted with DMEM (10% Fetal Calf Serum and 4mM L-glutamine). The diluted samples were added in duplicates to confluent monolayers of IPI-2I cells in 48-well plates, as described above. Plates were incubated during 2h at 37 °C, 5% CO_2. At the end of the incubation, the supernatants were removed and IPI-2I cells were lysed by addition of 500 µL of NucleoZOL (Macherey-Nagel, Hoerdt, France).

RNA isolation from cell IPEC-J2 and IPI2-I lysates

Total cellular RNA was extracted from IPEC-J2 and IPI-2I lysed cells following the NucleoZOL user manual (Macherey-Nagel, Hoerdt, France). The TURBO DNA-free^{TM} kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions to prevent DNA contamination. Purity and quality of the RNA extracts were controlled on 1% agarose gels. RNAs were then quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) using an optical density of 260 nm.

RT-qPCR on porcine intestinal cell RNA extracts

Reverse transcription was performed using the qScript cDNA Synthesis Kit (Quantabio, Beverly, MA, USA). Briefly, 350 ng of RNA per sample was added to 5 µL of sScript Reaction

Mix (5x) and 1 µL of qScript Reverse Transcriptase in a final volume of 15 µL. The reverse transcription mix was 5 min at 22°C, 30 min at 42°C and 5 min at 85°C. The synthetized cDNAs were stored at -20°C until used. Quantification of genes listed in Supplementary Table 4 of Chapter 10 was carried out using a QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The cyclophilin A and β-actin genes were used as reference genes. PCR reactions were carried out in 96 well plates using 3 µL of 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), 0.4 µL of forward and reverse primers (Supplementary Table 4, Chapter 10), 9.2 µL of milliQ water and 2 µL of cDNA. Tenfold dilution series of each primer pair were used as standard curves to determine primer efficiencies. Real time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. The appropriate reference gene and the Log_2 fold change of each gene were determined by GenEx software (http://genex.gene-quantifcation.info/). A mixed-model one-way Anova (lmer and Anova functions) with the different conditions as a fixed effect and replicates as a random effect was used using the R packages lme4 and car.

RESULTS

Live SB CNCM I-1079 impacts microbiota composition and activity of the MPigut-IVM challenged with a feed deprivation stress, a dietary change and an ETEC strain

We have previously described the effects of a simulated weaning transition including a feed deprivation period followed by diet change and a challenge with an ETEC strain on the microbiota composition and function in the MPigut-IVM (Chapter 10). Here, we report the effects on these parameters of a daily dose of SB CNCM I-1079 administrated before and after the weaning transition. These are preliminary results and experiments will need to be repeated to make statistically based conclusions.

Effects of SB on ETEC colonization in the MPigut-IVM

The quantification of the ETEC LT gene is presented in Figure 2. The quantification of this gene was used to monitor the presence of the inoculated ETEC strain in the MPigut-IVM and the impact of SB preventive treatment. At day 10, at inoculation time, the LT gene was quantified 0.5 $\log_{10}$ of gene copy numbers higher in the SBETEC condition compared with the ETEC condition in the bioreactor medium. Nevertheless, this difference disappeared from day 10.5, whatever the tested samples (bioreactor medium, bead medium or mucin beads), indicating no effect of SB on ETEC colonization (Figure 2). Overall, the LT gene level decreased for both conditions more quickly in the RUN A to reach 4 $\log_{10}$ of copy number in the bead medium on day 15 compared to the RUN B in which it stayed at 8 $\log_{10}$ of copy number in the bead medium.

Effects of SB on microbiota activity

No difference induced by the SB supplementation was noticed on the proportions of the seven SCFA measured in the bioreactor medium and in the bead medium but the SCFA total concentration was higher every day in the bioreactor medium (supplementary figure 1). At day 10, at the time of ETEC inoculation an increase in hydrogen proportion was noticed in the SBETEC condition (13.2%) compared to the ETEC condition (4.4%) as well as a decrease in methane proportion from 6.9% in the ETEC condition to 2.8% in the SBETEC condition but the gas mixture recovered similar profiles later on and these observation were visible on only one biological replicate out of the two (Supplementary figure 2). No effect was noticed on the maximum redox potential reached at the end of the feed deprivation period but the SBETEC

condition harboured a lower mean of -249 mV during the recovery period compared to -229 mV for the ETEC condition, especially emphasized in the Run B (Figure 3).

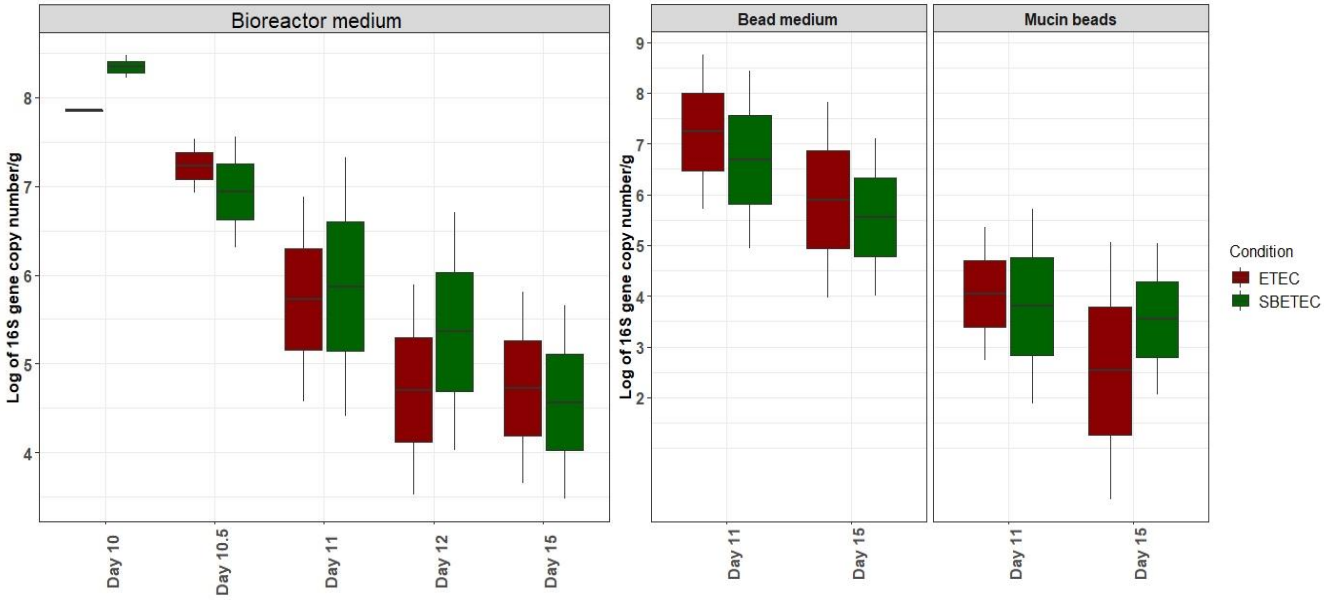


Figure 2. Quantification of the LT enterotoxin gene in the MPigut-IVM during the ETEC and SBETEC conditions (n = 2).

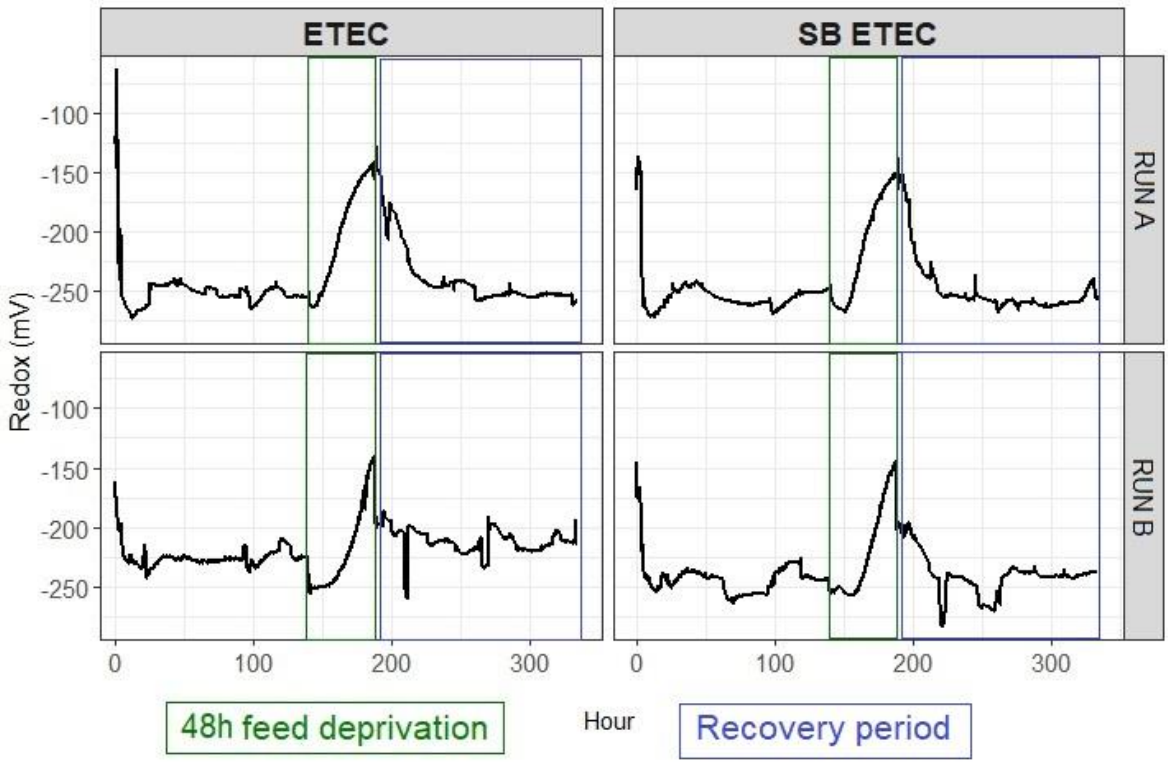


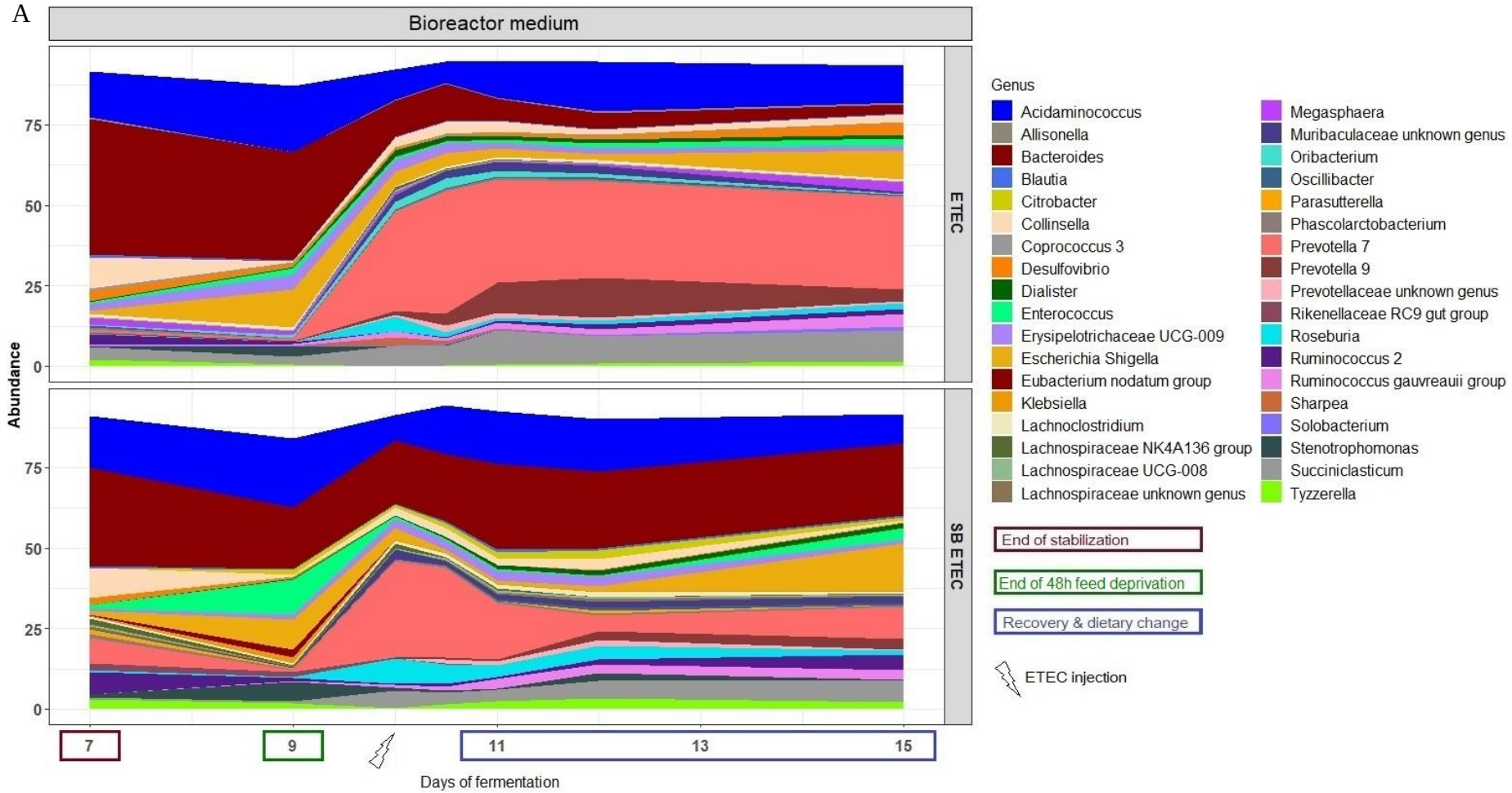
Figure 3. Redox potential measured in the bioreactor medium during the conditions ETEC and SBETEC.

Effects of SB on microbiota composition

At the phylum level, the bacterial composition of the *in vitro* microbiota of the MPigut-IVM challenged with a feed deprivation stress, a dietary change and an ETEC strain, and supplemented or not by SB displayed rather similar profiles in both the bioreactor medium and the mucin beads (Supplementary Figure 3). At the family level, the most distinguishable result was the higher relative abundance of *Bacteroidiaceae* and the lower *Prevotellaceae* during the recovery period in the SBETEC compared to the ETEC condition, especially in the bioreactor medium (Supplementary Figure 4). At the genus level, in the bioreactor medium the main difference observed between SBETEC and ETEC treatments was an evolution of the *Bacteroides / Prevotella* ratio (Figure 4A). Indeed, the mean relative abundances of respectively *Bacteroides* and *Prevotella* 7 & 9 genera were 16.1% and 13.7% for the ETEC condition and 23% and 7.5% in the SBETEC condition from day 10 to day 15. In the bioreactor medium, other differences were noticed such as a higher relative abundance of *Tyzzerella, Citrobacter, Enterococcus* at day 9, *Eubacterium nodatum* group at day 9, *Escherichia/Shigella* at day 15, *Ruminococcus* 2 at days 7 and 15 and *Roseburia* from day 9 to day 15 in SB inoculated fermenters (Figure 4A). On the mucin beads, when SB was daily distributed to the MPigut-IVM compared to control ETEC bioreactors, we measured higher relative abundances of *Streptococcus, Enterococcus, Ruminococcus gauvreauii* group and *Pyramidobacter* at day 15, *Treponema* 2 at days 7 and 9, an overall higher relative abundance of *Escherichia/Shigella* and a lower relative abundance of *Prevotella 7* after the feed deprivation period (Figure 4B).

Regarding archaeal microbiota, the SB supplementation reduced the relative abundance of *Methanosphaera* in aid of *Methanobrevibacter* during the recovery period especially for the Run B and in the bioreactor medium (Supplementary Figure 5). Besides the quantification of Saccharomyces cerevisiae at the expected level in the SBETEC condition, the quantification of bacterial and archaeal groups by qPCR was not impacted by the SB supplementation (Supplementary Figure 6).

No difference was observed in alpha diversity indices between the ETEC and SBETEC conditions (data not shown).


A
Bioreactor medium
Abundance
75
50
25
0
ETEC
SB ETEC
7
9
11
13
15
Days of fermentation
Genus
Acidaminococcus
Allisonella
Bacteroides
Blautia
Citrobacter
Collinsella
Coprococcus 3
Desulfovibrio
Dialister
Enterococcus
Erysipelotrichaceae UCG-009
Escherichia Shigella
Eubacterium nodatum group
Klebsiella
Lachnoclostridium
Lachnospiraceae NK4A136 group
Lachnospiraceae UCG-008
Lachnospiraceae unknown genus
Megasphaera
Muribaculaceae unknown genus
Oribacterium
Oscillibacter
Parasutterella
Phascolarctobacterium
Prevotella 7
Prevotella 9
Prevotellaceae unknown genus
Rikenellaceae RC9 gut group
Roseburia
Ruminococcus 2
Ruminococcus gauvreauii group
Sharpea
Solobacterium
Stenotrophomonas
Succiniclasticum
Tyzzerella
End of stabilization
End of 48h feed deprivation
Recovery & dietary change
ETEC injection

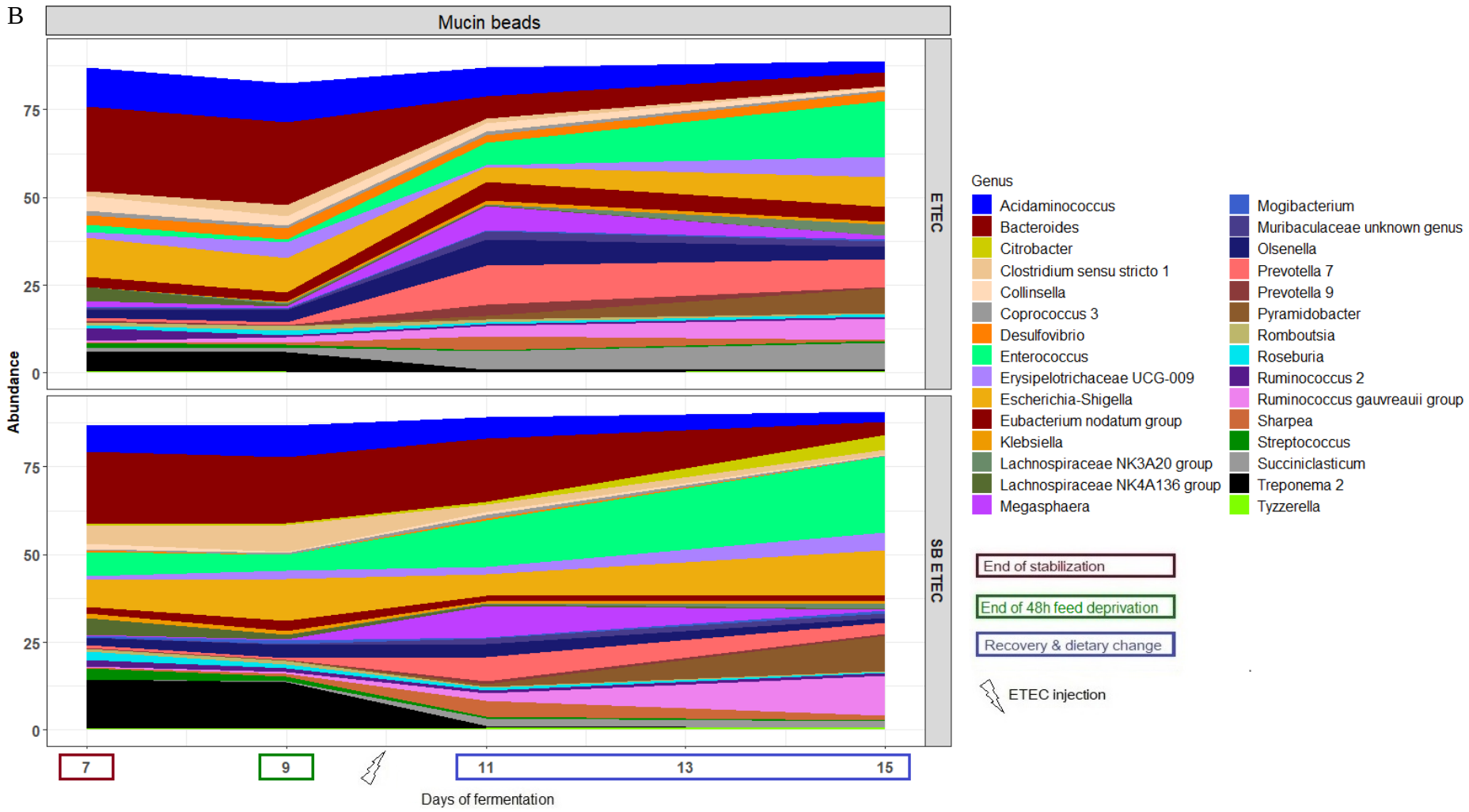


Figure 4. Relative abundance of the 30 main bacterial genera in the bioreactor medium (A) and on the mucin beads (B) of the MPigut-IVM during the ETEC and SBETEC conditions (n = 2).

SB live yeast supplementation of ETEC-challenged MPigut-IVM affects gene expression of IPI-2I cells

In the presence of supernatants collected from the bead medium of the bioreactors challenged with ETEC and which received a daily dose of SB, the expression of the genes encoding TNFα, MYD88, CLDN4 and MUC1 in IPI-2I was reduced at day 15 when compared to supernatants collected from non-supplemented bioreactors (Figure 5). On the opposite, the IL8 gene expression was increased in the presence of supernatants from the SBETEC condition compared to the ETEC condition at days 13 and 15.

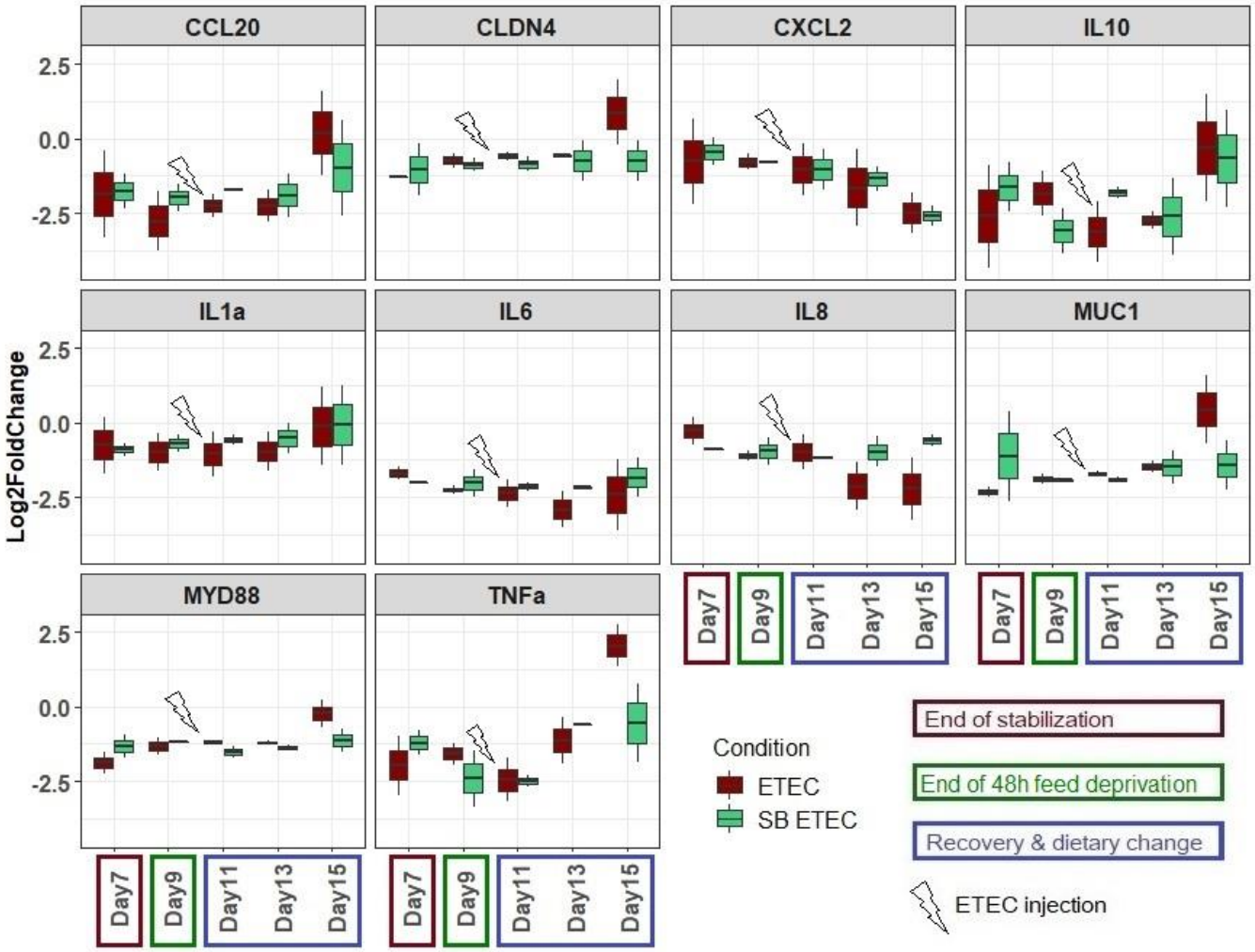


Figure 5. Log2 fold changes of IPI-2I cells gene expression when incubated with bead medium supernatants of the MPigut-IVM during the conditions ETEC and SBETEC. Fold changes are calculated compared to the control condition where IPI-2I cells are incubated with their usual glutamine and FCS complemented DMEM medium (n = 2).

SB CNCM I-1079 inhibits ETEC adhesion and ETEC-induced inflammatory response on IPEC-J2

ETEC adherent to IPEC-J2 cells were detected at an average of $1.1x10^7$, $4.3x10^6$ and $3.5x10^7$ cell/mL respectively for the three biological replicates (data not shown) and normalized to 100%. The pre-treatment of IPEC-J2 with live SB CNCM I-1079 significantly (*p*value < 0.05) reduced ETEC Ec105 adhesion, in a dose-dependent fashion, to reach respectively 69, 53 and 13% of adhesion for the 10^6, 10^7 and 10^8 SB doses (Figure 6). The pre-treatment of IPEC-J2 with SB yeasts had also numerous significant effects on the expression of inflammatory genes (Figure 7). For instance, the highest dose of SB significantly reduced the expression of CCL20, CXCL2, IL1a, IL6, IL8, TNFα or TLR4 genes of, respectively, -4.2 ± 1.5, -6.2 ± 1.6, -6.1 ± 1.7, -1.8 ± 0.3, -4.9 ± 0.1, -2 ±1 and -1 ± 0.3 log2fold changes comparatively to cells non pre-treated with SB (figure 7). All pre-treatments slightly, but yet significantly, increased the gene expression of MUC1.

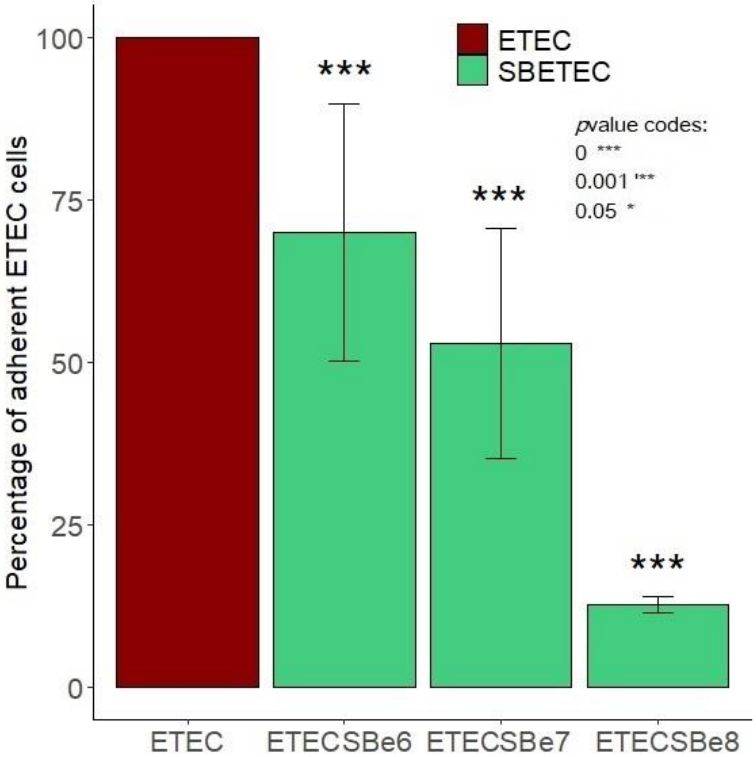


Figure 6. Percentage of ETEC adherent cells on IPEC-J2 cells treated or not with different doses of the SB CNCM I-1079. The number of ETEC adherent cells on control IPEC-J2 cells (non-treated with SB yeasts) was considered as 100% (n = 9). The denomination e6, e7 and e8 respectively correspond to the doses of 10^6, 10^7 and 10^8 CFU of SB incubated on the ETEC challenged IPEC-J2 cells.

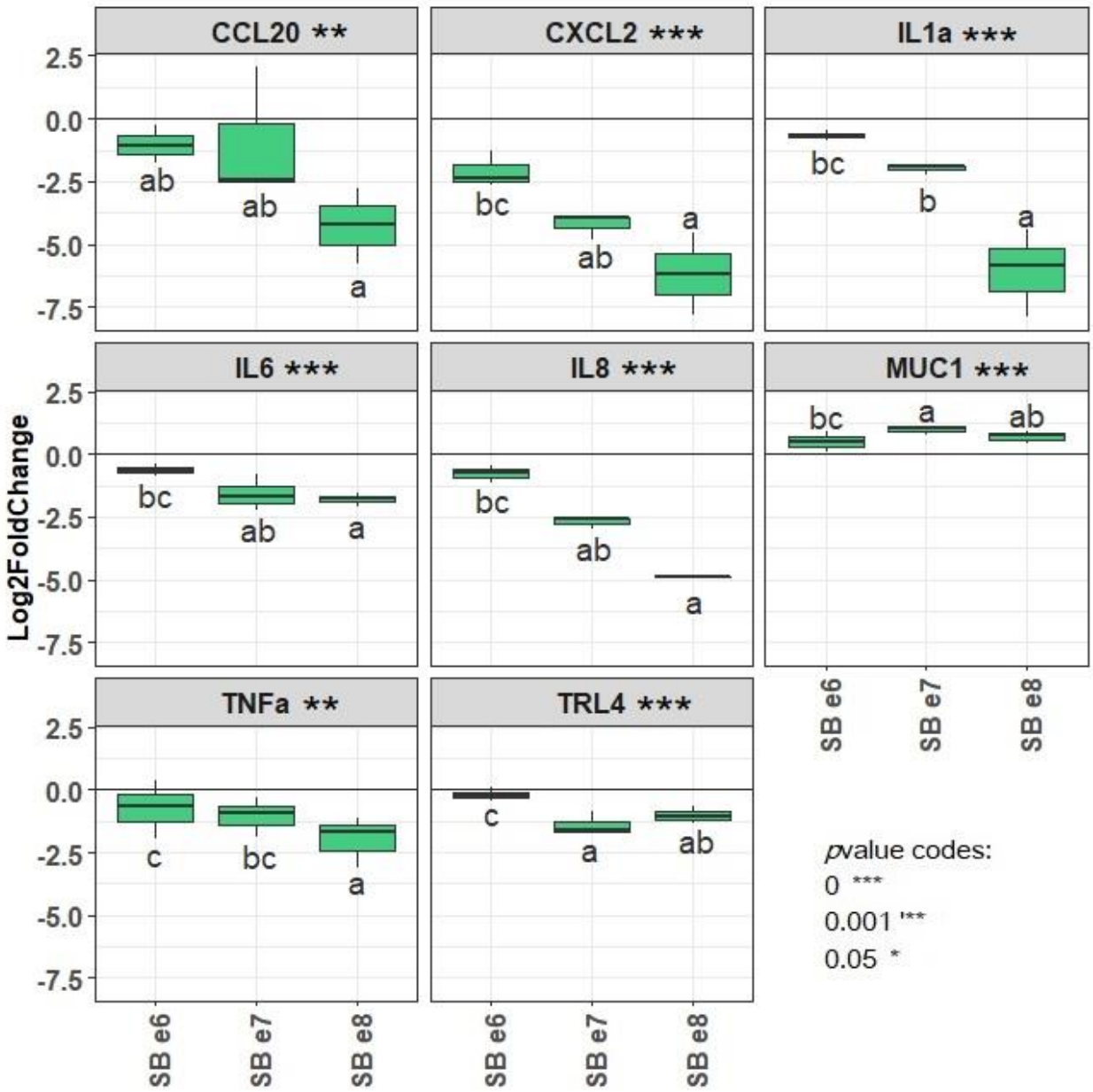


Figure 7. Gene expression of IPEC-J2 cells challenged with the ETEC Ec105 strain and treated or not with different doses of SB yeasts (n = 3). Log 2 fold changes represent the differential gene expression between non-pre-treated and SB treated ETEC-challenged IPEC-J2 cells. Conditions with the same letters are not statistically different from each other while different letters indicate that the conditions are statistically different from each other. The denomination e6, e7 and e8 respectively correspond to the doses of 10^6, 10^7 and 10^8 CFU of SB incubated on the ETEC challenged IPEC-J2 cells.

DISCUSSION

ETEC is the most common cause of diarrhea in farm animals (Dubreuil et al., 2016; Nagy and Fekete, 2005). In the last decades, common strategies to fight against infectious post-weaning diarrhea revolved in the massive use of antibiotics as preventive and curative treatments which is however not sustainable due to the increasing dissemination of resistant genes among bacteria (Barton, 2014; Gresse et al., 2017). More and more investigations now focus on finding non-pharmacological preventive and therapeutic solutions such as probiotic-based strategies. Several studies highlighted that strains from the *Saccharomyces* genus could be potential candidates for reducing ETEC colonization and signs of infections in weaning piglets (Che et al., 2017; Trckova et al., 2014; Trevisi et al., 2017). *Saccharomyces* yeasts showed beneficial impact on several bacterial infections such as the inhibition of *Citrobacter rodentium* adhesion to mice colonic epithelium (Wu et al., 2008), the modulation of ETEC-induced mitogen activated protein pathway on T84 intestinal cells, the inhibition of *Clostridium difficile* toxin production in rat ileum and the modulation of pro-inflammatory cellular response in *Shigella* infections via the secretion of proteases degrading pathogen toxins and receptors on T84 intestinal cells (Czerucka et al., 2000 ; Castagliuolo et al., 1999 ; Mumy et al., 2008). Another potential mode of action of probiotic yeasts could be the modulation of the intestinal microbiota of weaning piglets, as already demonstrated in *in vivo* studies (Brousseau et al., 2015). In the challenged MPigut-IVM with the ETEC F4 porcine strain, our preliminary results indicated that the daily supplementation of SB seemed to favour the relative abundance of *Bacteroides* while reducing *Prevotella* members after the *in vitro* simulation of weaning transition. As a decrease in relative abundance of *Bacteroidiaceae* in fecal microbiota was previously considered as the onset of post-weaning diarrhea in piglets (Yang et al., 2017), an increase in *Bacteroides* by the SB supplementation may help to limit the intestinal microbiota dysbiosis associated with weaning transition. *Bacteroides* are obligate anaerobes considered as one of the major genera of the core microbiota of mammals particularly known for their strong polysaccharides degradation systems and their beneficial interactions with their host (Tan et al., 2019). Studies performed on human microbiota reported that members of *Bacteroides* genus demonstrated their ability to treat intestinal colitis, immune dysfunctions and metabolic disorders and are considered as potential next-generation of probiotics (Tan et al., 2019). Consistently with our previous results (Annexe of Chapter 9), SB treatment in the MPigut-IVM also increased the relative abundance of *Escherichia/Shigella* in the MPigut-IVM. A previous study correlated a low relative abundance of commensal *E. coli* with a higher incidence of piglet post-weaning

diarrhea (Yang et al., 2019). The increase of commensal *Escherichia/Shigella* induced by the SB probiotic could lead to the occupation of the ETEC ecological niche by the commensal *E. coli* and constitute a protective effect against ETEC colonization in the gut of newly weaned piglets. In this study, the higher relative abundance of *Escherichia/Shigella* in the MPigut-IVM receiving SB was not correlated with a higher quantity of the LT toxin gene. However, this should be confirmed by targeting a larger panel of ETEC virulence genes to ensure the higher relative abundance of *Escherichia/Shigella* belongs to non-pathogenic commensal species. *Ruminococcus 2* and *Ruminococcus gauvreauii* group, beneficial SCFA producers (Macfarlane and Macfarlane, 2003), were also increased by SB treatments in the MPigut-IVM which could help to fight against dysbiosis and ETEC colonization by insuring an efficient microbiota functionality. A higher mean of total SCFA concentration was indeed detected every day in the SBETEC condition compared to ETEC condition in the bioreactor tank and should be confirmed by performing at last one other biological replicate. Also, considering the lower mean of redox potential during the recovery period, the SB treatment seemed to help restoring the microbiota activity quicker after the feed deprivation period. Indeed, redox potential reflects the reducing capacity of the gut environment which is influenced by several factors such as for instance diffusion of oxygen or release of reactive oxygen species or reactive nitrate species (Circu and Aw, 2011; Friedman et al., 2017; Pérez et al., 2017). However, in contrary to Chapter 9, which at this state of the experiment received the similar pre-weaning diet in this same condition, the SB treatment did not impact the maximal redox potential during the feed deprivation period. In comparison with the biological replicates from the Chapter 9, the maximal redox potential reached during the feed deprivation period in the present experiment was much lower and could indicate a better fulfilment of ecological niches and thus less disruptions of the microbiota metabolic activity from the transient lack of nutrients.

The MPigut-IVM has proved to be a relevant tool to assess the effects of probiotics. However, its principal limitation is the absence of host-microbiota crosstalk or immune regulations, which are of importance when evaluating the effects of probiotics. Therefore, modifications of the microbiota composition and activity induced by SB in the MPigut-IVM could have an impact on host cell metabolism or inflammatory response. In our study, we investigated the effects of supernatants from the ETEC-challenged MPigut-IVM bead medium supplemented with SB or not on gene expression in IPI-2I cells. Supernatants from the SB supplemented group could mitigate the expression of TNFα, MyD88, CLDN4 and MUC1 genes that was actually increased in the presence of ETEC. IL8 gene expression was down-regulated by the ETEC-challenged

samples (Chapter 10), and the SB supplementation seemed to restore basal gene expression of the cells. Although results from the literature about the effects of probiotics on host inflammatory genes are sometimes contradictory (Angulo et al., 2019; Che et al., 2017; Rajput et al., 2017; Saegusa et al., 2004), *in vivo* and *in vitro* studies reported some immunomodulatory effects of *Saccharomyces* species[4]. Wang et *al.* reported that SB supplementation could reduce the level of TNFα in mice colon (Wang et al., 2019). Dogs which received *S. cerevisiae* fermentation products also displayed a lower level of TNFα in their blood (Lin et al., 2019). Saegusa *et al.* reported that, in presence of butyrate, *S. cerevisiae* treatment of Caco-2 cells enhanced IL8 production suggesting that some yeast, hypothetically due to their cell wall component rich in glucans, are able to stimulate the immune system of the host *via* the increased production of specific cytokines (Saegusa et al., 2004). This last result matches with the increase of IL8 gene expression that we observed in our IPI-2I cells incubated with MPigut-IVM samples and also contained some butyrate. The light activation of IL-8 gene expression thus does not suggest necessarily that SB provokes inflammation which is moreover in contradiction with the reduction of TNFα and MyD88 gene expression. These data also suggest that the immunomodulatory effects of SB could be influenced by microbial activity (and especially SCFA production), however yeast modes of action are far from being fully understood and other studies will be needed to understand the complex molecular mechanisms of the dialogue between SB, gut microbiota and the host. Our results confirm that the MPigut-IVM is an effective tool to evaluate the effects of gut microbiota modulation due to pathogens and/or probiotics or other compounds on intestinal epithelial cells.

After orally entering into the piglet digestive tract, ETEC can attach to enterocytes using fimbrial adhesins (Luppi, 2017). The fimbriae 4 colonization factor is the most prevalent among ETEC responsible for post-weaning diarrhea in piglets (Dubreuil et al., 2016). As we wanted to confirm the efficiency of SB against our porcine isolated ETEC strain, IPEC-J2 cells, possessing the F4 receptor (Devriendt et al., 2010), were pre-treated directly with the active dry yeast SB CNCM I-1079 prior to ETEC challenge. Our results demonstrated a significant protective effect against ETEC F4 adhesion on IPEC-J2 porcine cells, in a dose response fashion. Additionally, SB also showed a positive effect on ETEC-challenged IPEC-J2 *via* lowering expression of several genes involved in pro-inflammatory cascade such as TNFα, TLR4, IL8, IL1a or IL6 and chemokines involved in immune responses like CCL20 and CXCL2. Then our results showed that the strain SB CNCM I-1079 could protect porcine enterocytes from ETEC infections by reducing bacterial adhesion and modulating the

inflammatory response. Only few studies investigated the effect of *S. cerevisiae* strains on porcine cell lines and found similar results than us. Zanello *et al*. showed that on top of its ability to agglutinate with ETEC, the strain *S. cerevisiae* CNCM I-3856 or its culture supernatant could inhibit expression of genes encoding for inflammatory cytokines such as IL6, IL8, CCL20 or CXCL2 of ETEC-challenged IPEC-1 and IPI-2I porcine cells (Zanello et al., 2011). Badia et *al.* evaluated the role of a SB strain on ETEC-challenged IPI-2I cells and also noticed a downregulation of TNFα, IL6, CCL2, CXCL8 and CCL20 gene expression(Badia et al., 2012b). However, as highlighted earlier the presence of SCFA, such as butyrate, in the microbiota could interact with the effects of a SB on epithelial cells.

To conclude, our study reported that SB CNCM I-1079 supplementation was able to modulate the MPigut-IVM microbiota and induce differential inflammatory gene expression responses on porcine intestinal epithelial cell experiments. This SB strain may be a potential candidate to reduce induced diarrhea in weaning piglets by favouring commensal *Escherichia coli* or by modulating piglet immune responses toward infection. Nevertheless, further investigations by repeating these *in vitro* experiments and later, confirming the results by *in vivo* trials will yet remain necessary.

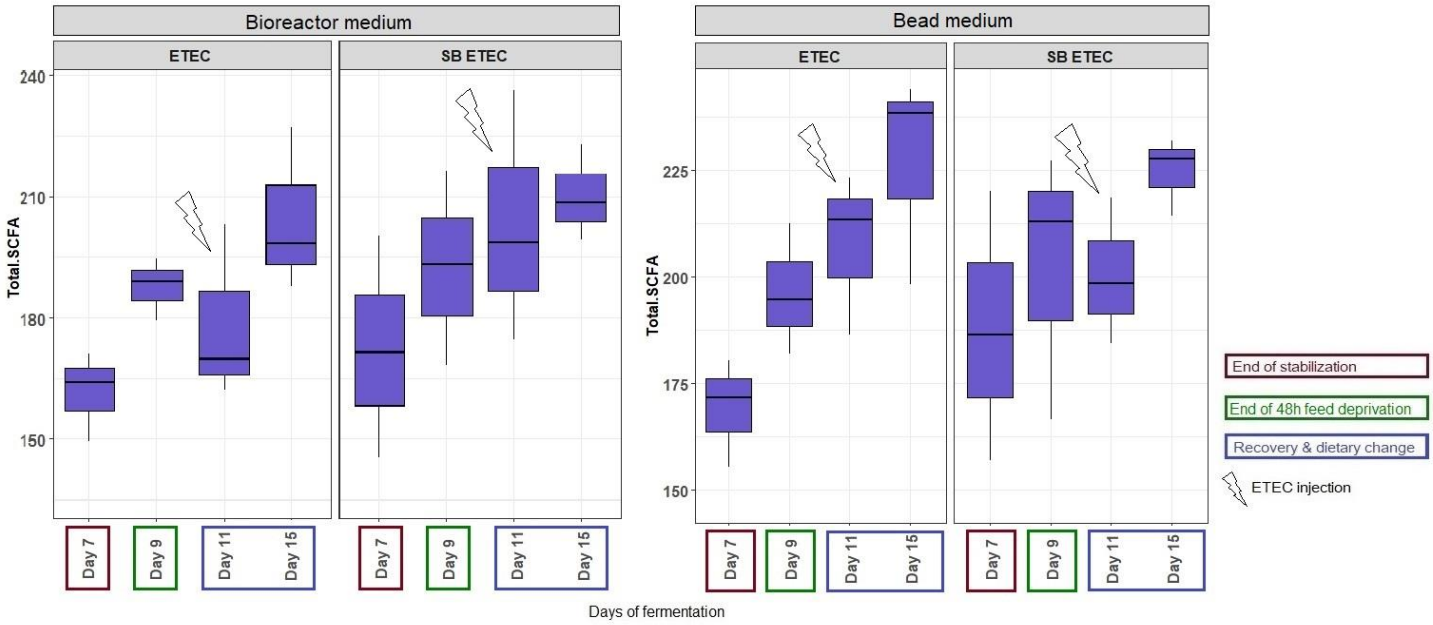
SUPPLEMENTARY MATERIAL

Supplementary table 1. Primers and probes used for quantification of bacteria and methanogenic archaea in the Chapter 11.

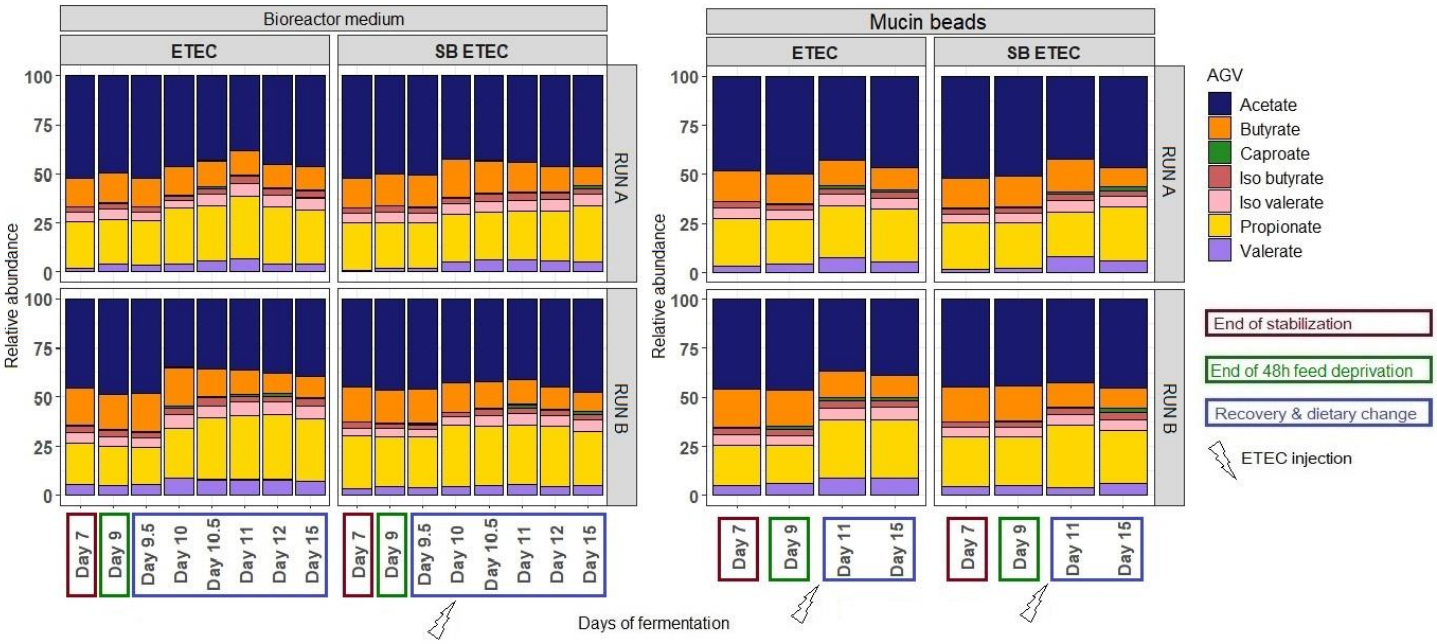
Target	Type	Sequences	References	Concentration	Annealing temperature
***Escherichia coli*/ *Shigella* group**	Taqman	5'-CAT GCC GCG TGT ATG AAG AA-3' 5'-CGG GTA ACG TCA ATG AGC AAA-3' (6-FAM)-5'-TAT TAA CTT TAC TCC CTT CCT CCC CGC TGA A-3'(TAMRA)	(Huijsdens et al., 2002)	300 nM 300 nM 100 nM	61°C
Total Bacteria	SYBRGREEN	5'-ACT CCT ACG GGA GGC AG-3' 5'-GTA TTA CCG CGG CTG CTG-3'	(Yu et al., 2005)	500 nM 500 nM	61°C
Methanogenic archaea	SYBRGREEN	5'-GAG GAA GGA GTG GAC GAC GGTA-3' 5'-ACG GGC GGT GTG TGC AAG-3'	(Ohene-Adjei et al., 2008)	500 nM 500 nM	61°C
S. cerevisiae	SYBRGREEN	5'- AGGAGTGCGGTTCTTTG -3' 5'- TACTTACCGAGGCAAGCTACA -3'	(Chang et al., 2007)	5µM	60°C
Labile enterotoxin (LT)	SYBRGREEN	5'- GGC GTT ACT ATC CTC TCT AT 3'—TGG TCT CGG TCA GAT ATG T	(Madoroba et al., 2009)	500 nM 500 nM	55°C

Supplementary Figure 1. Total SCFA concentration (A) and relative abundance of SCFA (B) during the SBETEC and ETEC conditions.

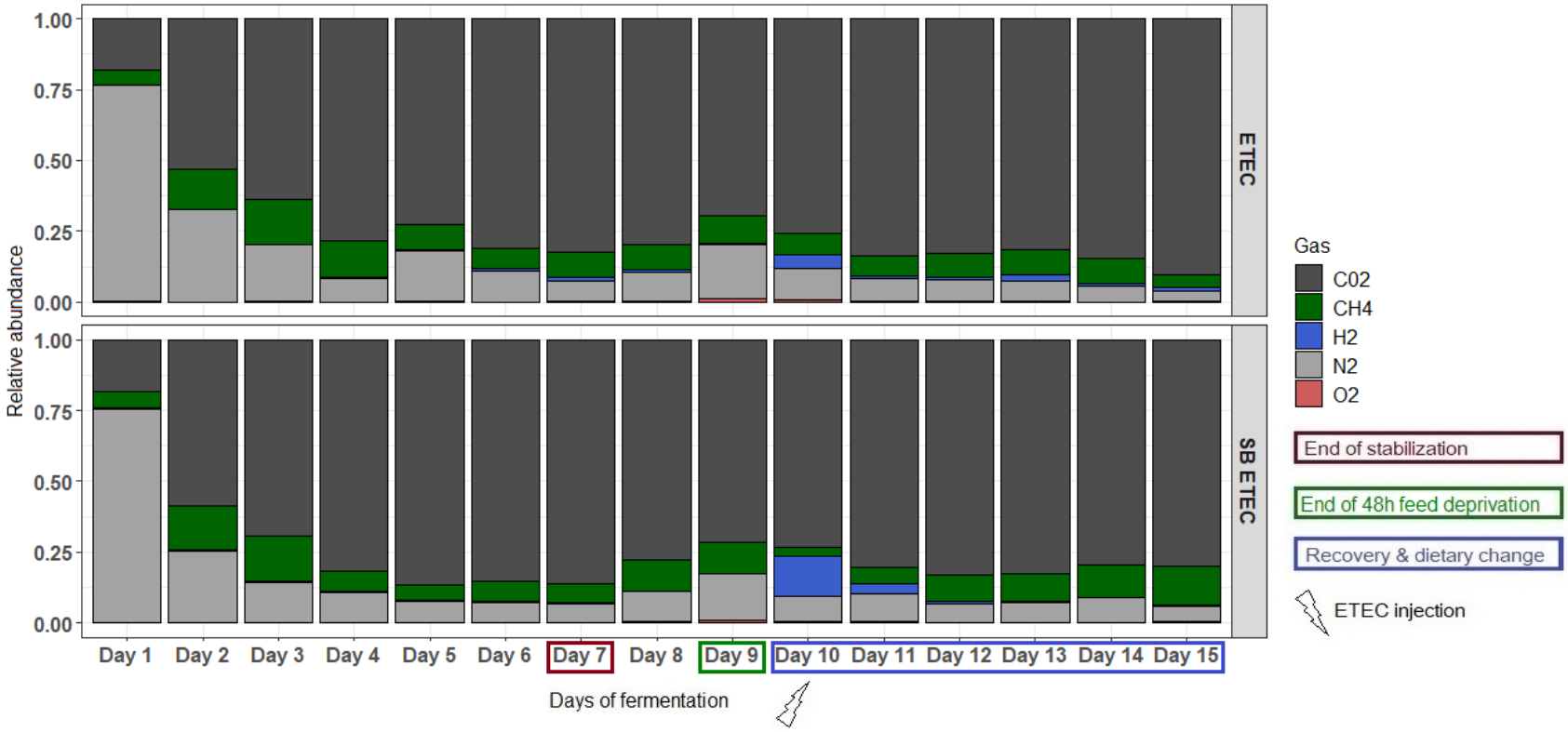
A



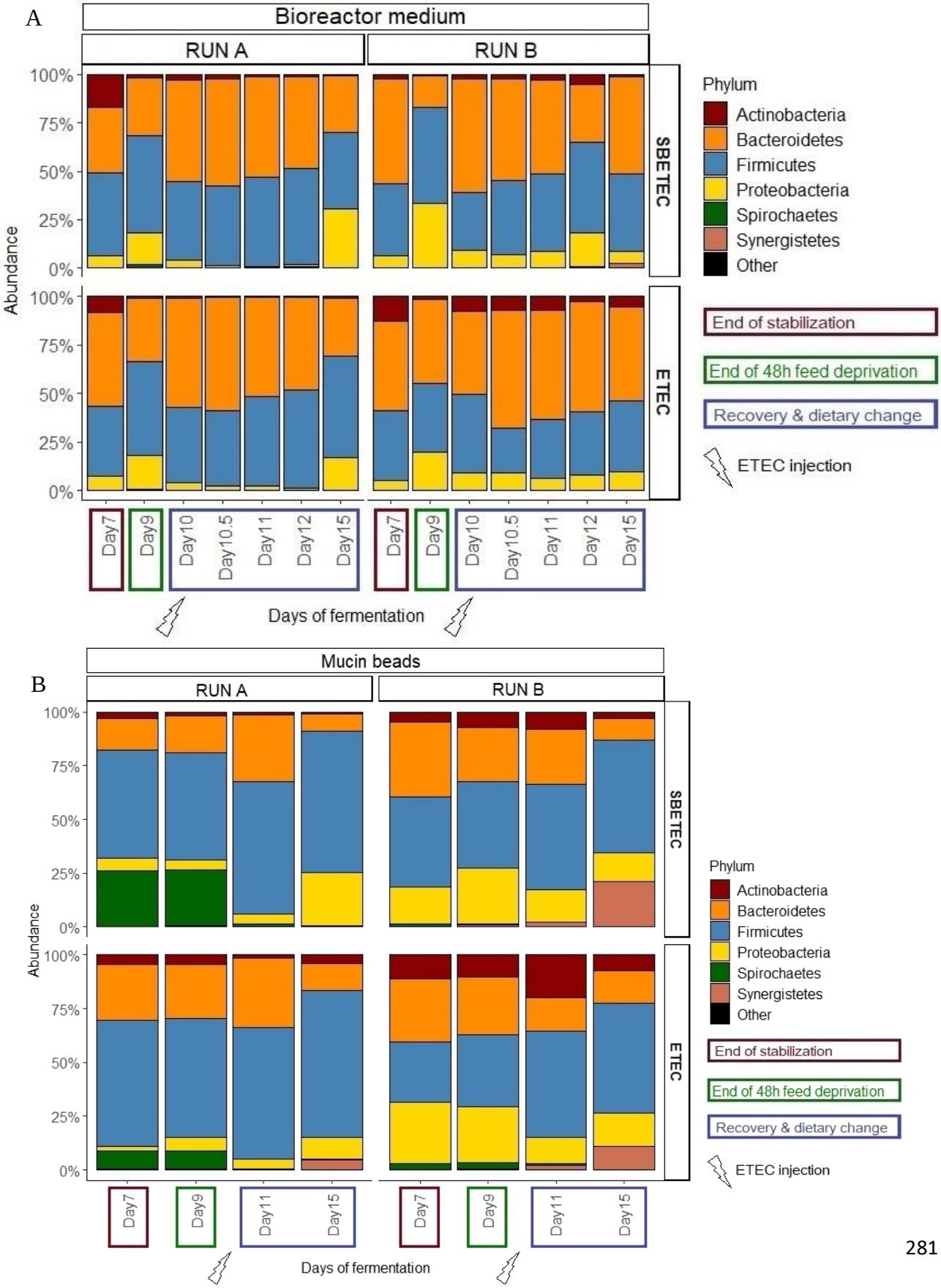
B



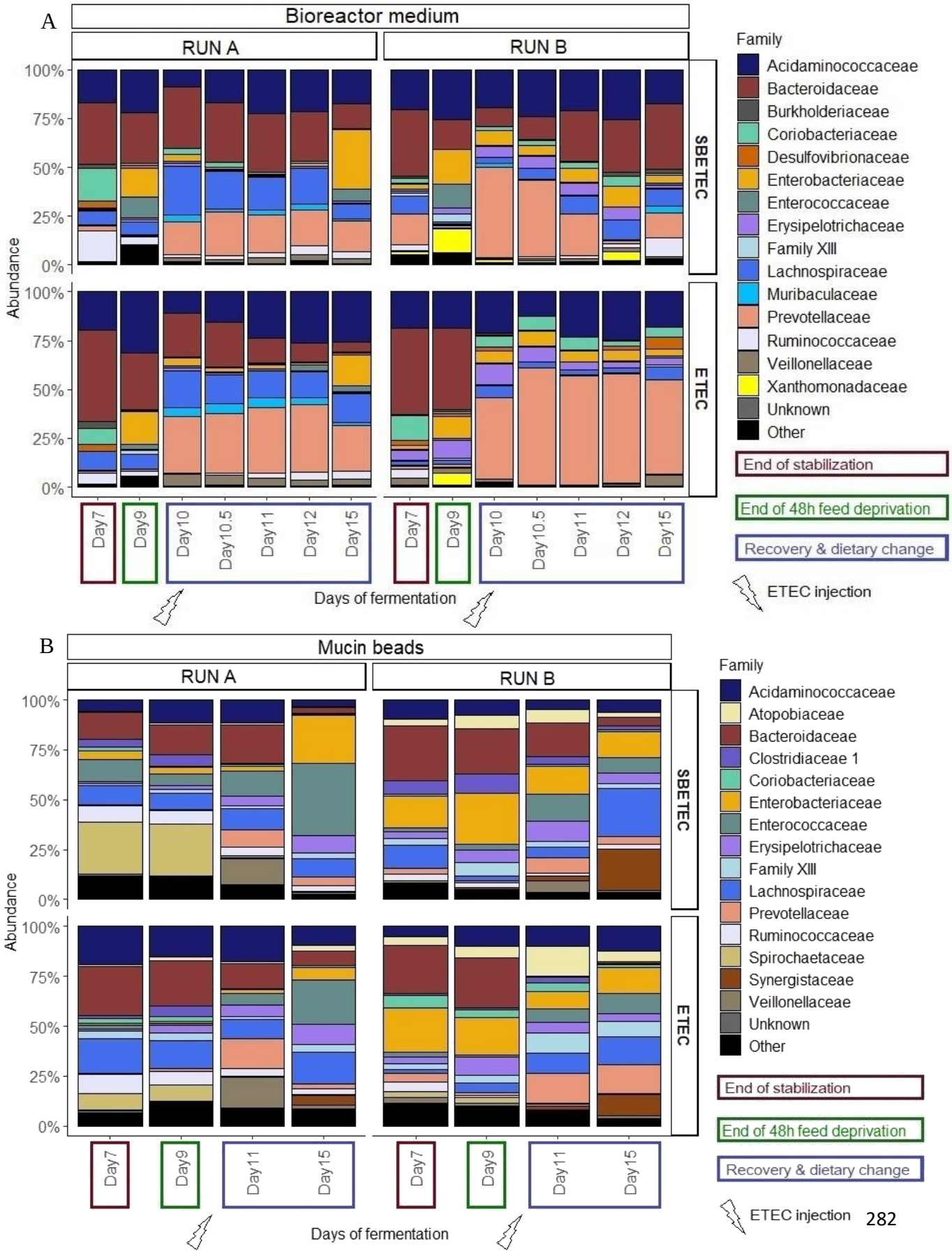
Supplementary figure 2. Mean relative abundances of gas produced by fermentation activity of the microbiota inhabiting the MPigut-IVM under SBETEC and ETEC conditions (n = 2).



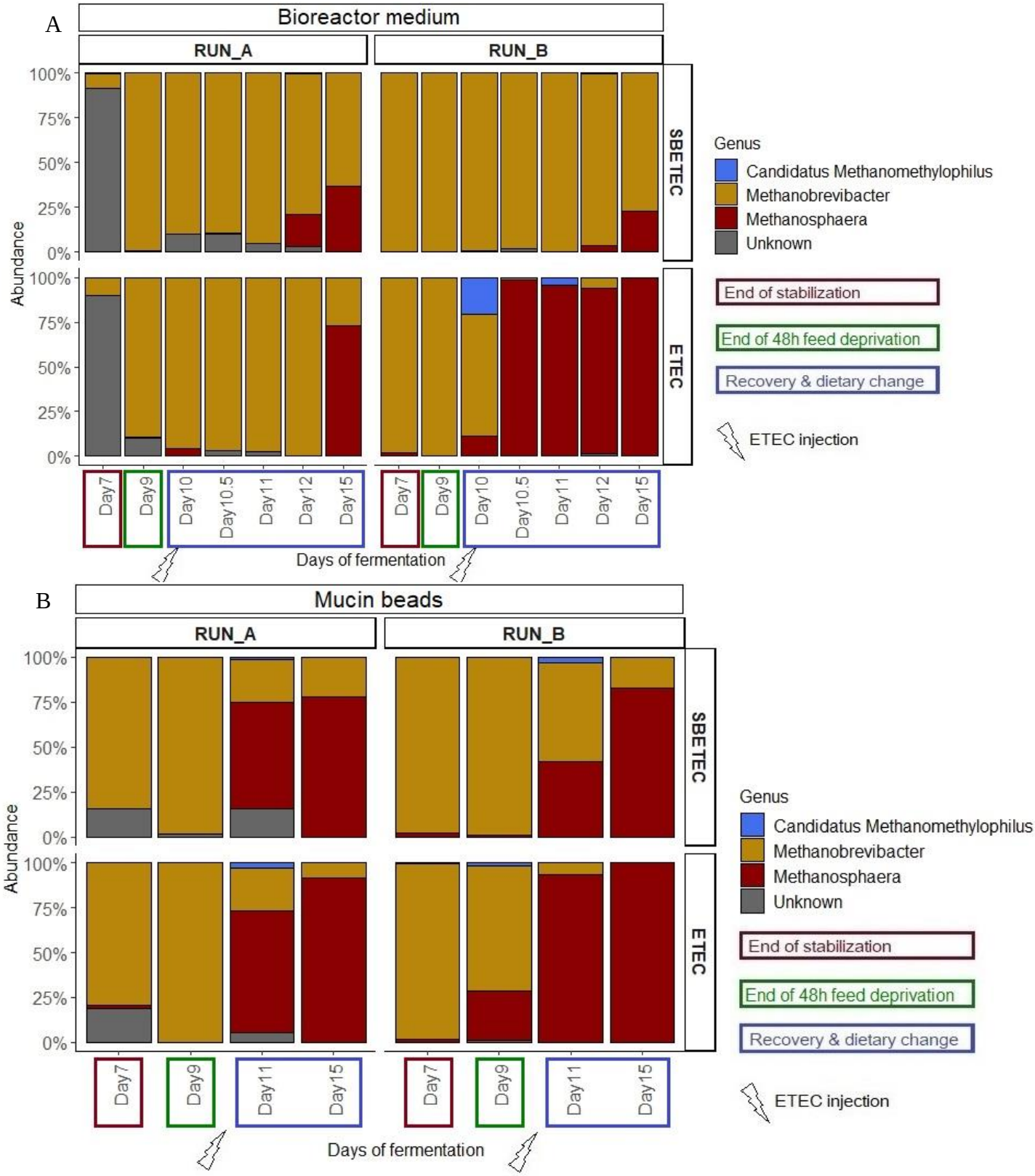
Supplementary figure 3. Relative abundances of the 5 main bacterial phyla in the bioreactor medium (A) and mucin beads (B) of the MPigut-IVM during the ETEC and SBETEC conditions.



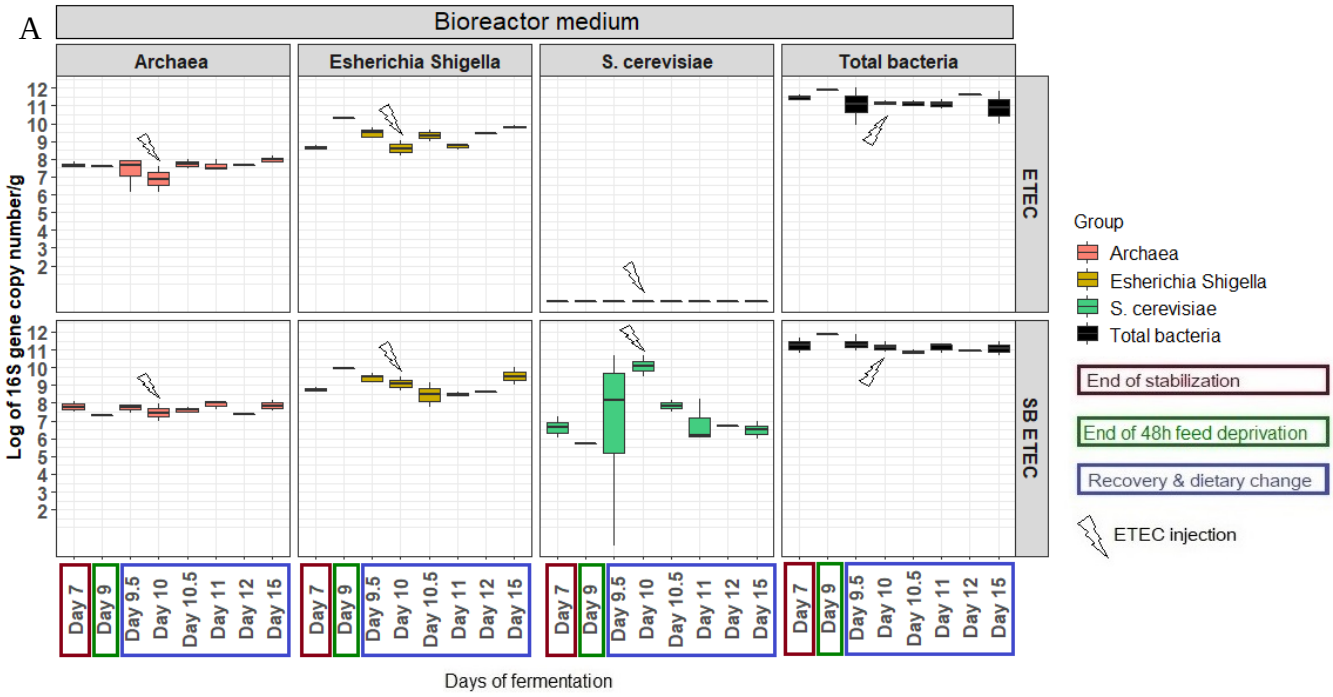
Supplementary figure 4. Relative abundance of the 15 main bacterial families in the bioreactor medium (A) and mucin beads (B) of the MPigut-IVM during the SBETEC and ETEC conditions.

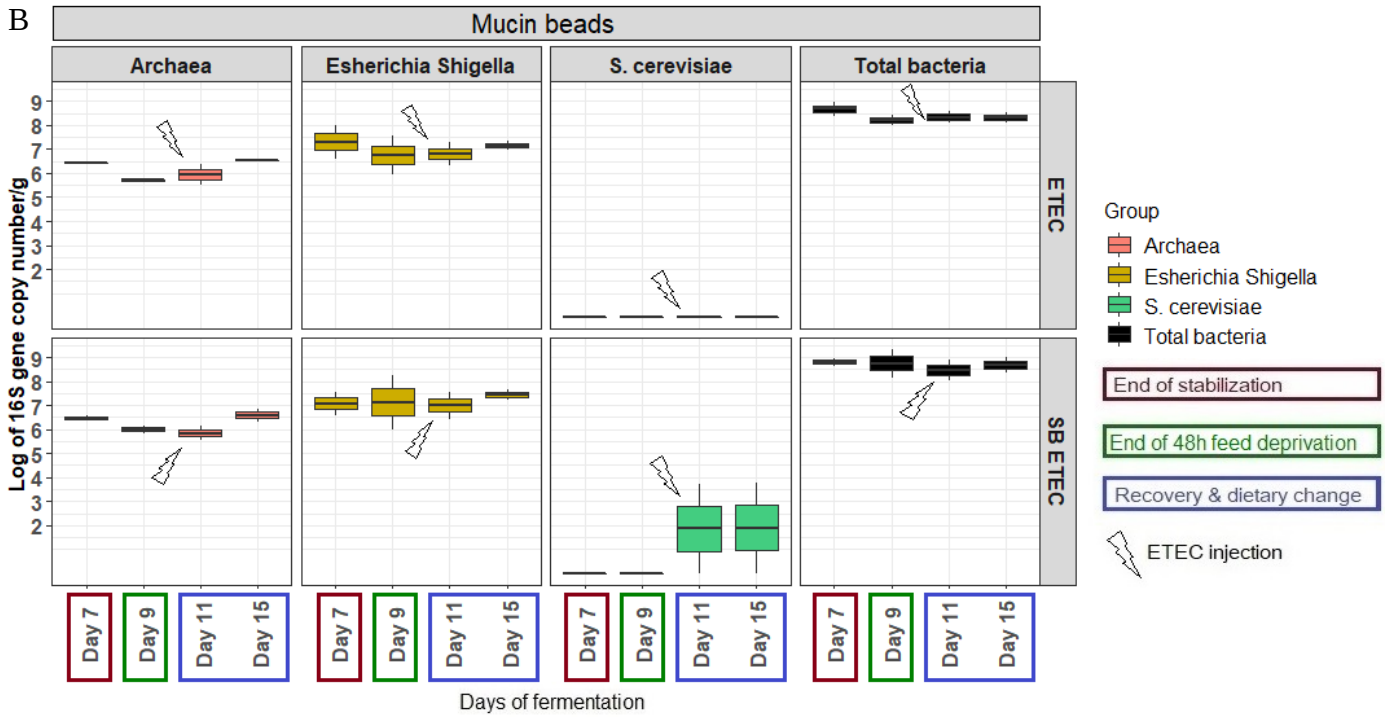


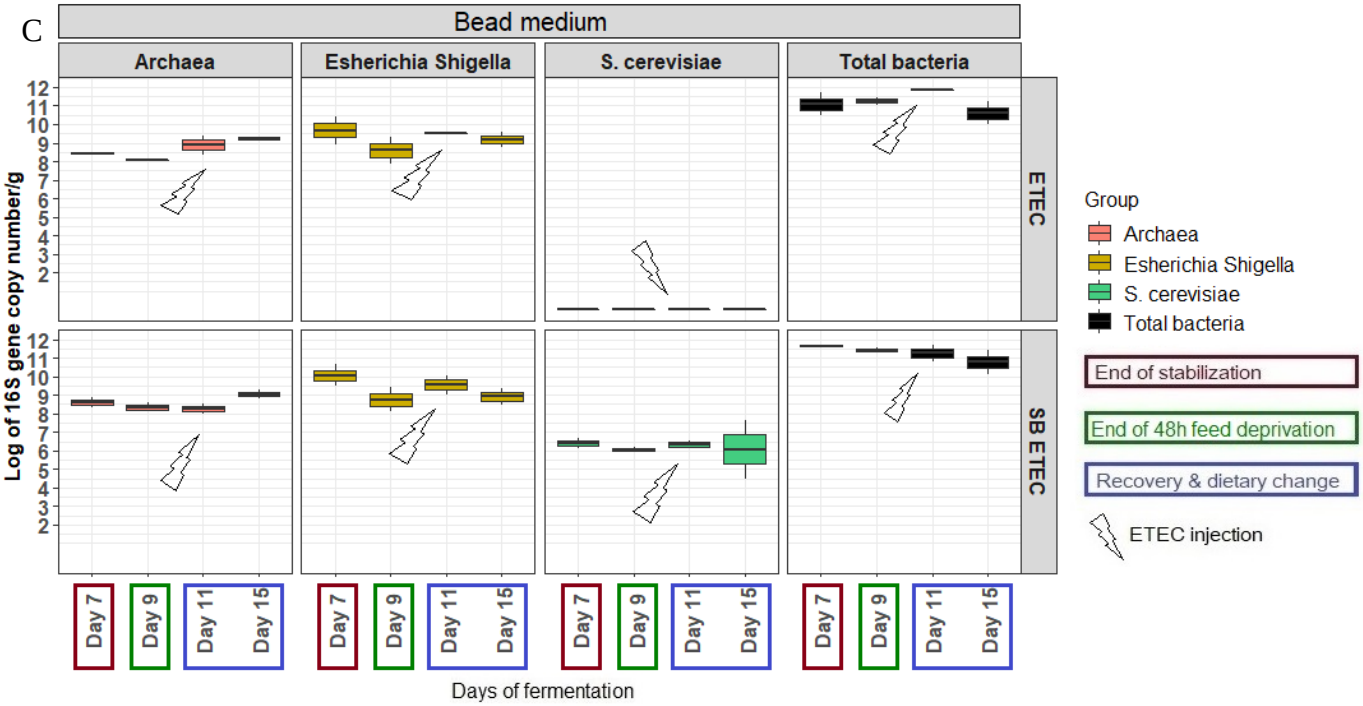
Supplementary figure 5. Relative abundance of the archaeal genera in the bioreactor medium (A) and mucin beads (B) of the MPigut-IVM during the SBETEC and ETEC conditions.



Supplementary figure 6. Quantification of bacterial and archaeal groups by qPCR in the bioreactor medium (A), mucin beads (B) and bead medium (C) of the MPigut-IVM during the SBETEC and ETEC conditions (n =2).






C
Bead medium
Archaea
Esherichia Shigella
S. cerevisiae
Total bacteria
ETEC
SB ETEC
Log of 16S gene copy number/g
Day 7
Day 9
Day 11
Day 15
Days of fermentation
Group
Archaea
Esherichia Shigella
S. cerevisiae
Total bacteria
End of stabilization
End of 48h feed deprivation
Recovery & dietary change
ETEC injection

Discussion

&

Perspectives

Chapter 12. General Discussion & Perspectives

1. Context of the research

ETEC-mediated diarrhea is one of the main cause of mortality of piglets worldwide (Lallès et al., 2007a). In addition to the enormous economic loss engendered by death from post-weaning diarrhea, the massive use of antimicrobials in livestock production now threatens even more health of humans and animals (Barton, 2014; Gresse et al., 2017; Lallès et al., 2007a; Pié et al., 2004). Numbers of bacteria have developed the ability of countering the action of a wide panel of antibiotics and spread those resistance genes via their persistence in the environment, water or food (Barton, 2014; Gross, 2013). The efficiency of last resort drugs such as colistin, the mostly used antibiotics in swine industry, will soon decline (Rhouma et al., 2017). Awareness about multi-resistance bacteria have been raised already several years ago in the EU. Following new European guidelines, the use of antimicrobials has started to decrease, but it is not yet associated with a decrease in the prevalence of antimicrobial resistance genes in pig gut microbiota (https://ema.europa.eu)(Pollock et al., 2020). The development of efficient preventive treatments for post-weaning diarrhea, such as essential oils, antioxidants, prebiotics or probiotics, to fight against ETEC colonization and virulence in piglet GIT becomes an urgent need. Importantly, this pessimistic context comes with the important issue that the etiology of post-weaning diarrhea remains far from clear. If numbers of predisposing, determining and contributing factors in piglets have been linked with higher risks of developing the diseases (Rhouma et al., 2017), the complex interplay between microbiota communities and the host leading to ETEC infections is still unknown which obviously impacts the efficiency of investigations aiming to find alternative preventive solutions. In parallel, in accordance to the EU directive aiming to replace, reduce and refine *in vivo* experiments, the use of animals in research raises more and more societal concerns. The pressure for developing *in vitro* alternative solutions to assess research questions is high and this option now emerges as unavoidable. *In vitro* gut models are efficient tools

allowing to reproduce different segments of the digestive tract from animals and humans. They represent great alternatives to investigate factors influencing the balance between resident microbiota communities, functional interactions, establishment of pathogen and effects of diverse compounds on the very complex gut microbiota. However, improvements of these in vitro gut models are continuously needed in order to stick better and better with *in vivo* conditions.

The principal goal of this PhD project set up by MEDIS research unit under industrial partnership with Lallemand Animal Nutrition company was to develop an *in vitro* model of the piglet colon at weaning, conscientiously set-up based on collected *in vivo* data and having the distinct feature of possessing mucin adherent surface. Once developed, the second aim of this project was to use the new in vitro model to start investigations about the etiology of intestinal dysbiosis in the post-weaning phase and evaluate the SB CNCM I-1079 commercialized by Lallemand under the active dry yeast Levucell SB product for its effect in thwarting the intestinal dysbiosis associated with weaning, limiting the ETEC colonization and having positive effects on the host.

2. Setting up the MPigut-IVM: discussion on features and parameters

The MPigut-IVM aimed to simulate the physicochemical and microbial parameters of the colonic environment of weaning piglets, corresponding to an age of around 28 days. In this PhD project, the focus was made on the colon due to its high fermentative activity and important microbiota which could, in case of dysbiosis, consequently impact piglet health. The transition from feces to *in vitro* colonic conditions obviously induced changes in the relative proportions of MPigut-IVM microbial populations such as observed in all *in vitro* models (Fleury et al., 2017; Rajilić-Stojanović et al., 2010; Tanner et al., 2014a; Van den Abbeele et al., 2012). The choice of fermentation parameters is responsible for shaping the *in vitro* microbiota during the stabilisation. Therefore, establishing most of parameters regarding *in vivo* data is a priority. The design of complex and dynamic *in vitro* models requires the adaptation of several parameters such as pH, transit time, digestive secretions or simulation of ileal chyme from the host which are sometimes not available in the literature or poorly described. Indeed, physico-chemical parameters of the gut are different depending on several criteria such as species or age and should always be taken into account in order to make reliable comparison with *in vivo* situation.

Prior to initiate the experimental work on the *in vitro* model, an *in vivo* study was needed in order to build the parameters of the MPigut-IVM on strong physiological basis. These *in vivo* data provided useful information on SCFA profile, microbiota composition and pH in the colon of piglets from the commercial farm collaborating with us in the frame of this PhD. Our collected *in vivo* data highlighted the presence of opportunistic pathogens throughout the GIT of piglets, the highly abundant, and most probably functionally important, archaeal microbiota, the differences between fecal samples and intestinal microbial communities and the differences between digesta and mucosa colonic microbiota. Information about transit time of the young pig is available in the literature (Wilson and Leibholz, 1981). However, this study was performed several decades ago and it would have been useful to update the data. Unfortunately, it was not possible to carry out this type of experiment on our commercial piglets.

Compared to the other existing model of weaning piglets (table 1), several characteristics of the MPigut-IVM were shown to stick more precisely to *in vivo* parameters.

Table 1. Comparison of the set-up and parameters between the two existing models of the weaning piglet colon.

	baby-SPIME (Dufourny et al., 2019)	MPigut-IVM This study
Multi-compartmental system	Yes	No
Adaptation of nutritive medium to the ileal chyme	No	Yes
Dietary change	Yes	Yes
Simulation of creep feeding in pre-weaning conditions	No	Yes
Physiological pH based on *in vivo* data	No	Yes
Adaptation of retention time based on in vivo data	No	Yes
Self-maintained anaerobiosis	No	Yes
Measurement of gas composition	No	Yes
Measurement of redox potential	No	Yes
Mucin adhesive surface	No	Yes
Host interactions	No	No

The principal characteristics of the MPigut-IVM, compared to other *in vitro* models, is the tailor-made mucin bead compartment aiming to reproduce the microbiota associated with the mucus layer by allowing adhesion to mucins and their degradation. In *in vitro* models, it is impossible to represent the extensive and extremely thin area from the *in vivo* mucus layer unless working with a very small volume of beads which would be very quickly degraded and thus unavailable for analysis. The most important function was to have a mucosal adhesion platform to study the specificity of mucosal microbiome and its dynamics as well as pathogen behaviour. The mucin beads were thus setup with a diameter of 4 mm and occupied the mucin bead compartment volume of 50 mL out of the 200 mL of the total MPigut-IVM volume. This bead diameter allowed an efficient microbial colonization and a reproducible degradation of the mucin beads incubated for 48h in the MPigut-IVM and thus, the beads were replaced every 48 hours. Additionally, observations by scanning electron microscopy confirmed that 48 hours was the appropriate time to observe a biofilm of strongly adherent microbiota on the mucin beads. A time of 24 hours was indeed too early to collect enough adherent microorganisms on the mucin beads while mucin beads staying 72 hours in the MPigut-IVM were completely degraded and were not suitable to collect samples for downstream analyses. To ensure the sampling of enough mucin beads every 48h and to avoid bias concerning the number of beads between experiments, the 50 mL compartment was fulfilled without compression with fresh mucin beads every 48h. If the use of gastric mucins is questionable to reproduce colonic environment due to their structural differences (Stanley et al., 1983), porcine gastric mucins are the only type of mucins commercially available. Additionally, Stanley *et al.* previously demonstrated that members of colonic *Bacteroides* and Clostridia were able to degrade similarly mucins of both origins (Stanley et al., 1986). *In vivo*, the luminal and mucosal microbiota are balanced between bacteria growing in the mucus layer and the degradation of mucus by mucin-degrading bacteria which diffuse into the gut lumen (Probert and Gibson, 2002). In the MPigut-IVM, the mucin bead compartment was able to successfully reproduce this phenomenon due to the constant flow of medium diffusing degraded mucins in the entire bioreactor. In the meantime, the establishment of a specific microbiota on the mucin beads was demonstrated as reported in Chapter 9. Indeed, in the bioreactor medium the principal bacterial populations were *Bacteroidiaceae, Prevotellaceae, Lachnospiraceae, Desulfovibrionaceae, Enterobacteriaceae, Acidaminococcaceae* and *Ruminococcaceae*. In the mucin beads, members of *Lachnospiraceae* were also belonging to the most prevalent families but also *Peptostreptococcaceae, Clostridium* Family XIII or *Clostridiaceae* 1 which were exclusively present in the mucin bead compartment. Fourteen out of the 15 principal bacterial families on piglet colon mucosa were able to colonize the mucin beads of the MPigut-IVM while 10 out 15 of the principal families found in colonic digesta were maintained in the bioreactor medium of the MPigut-IVM.

The MPigut-IVM was inoculated with a pool of fecal samples collected from several, mostly 6 to 8, piglets of 28 days old. Our principal constraint was the impossibility, regarding the size of the animals to obtain enough fecal material from one single donor to start several bioreactors run in parallel. In the past, starting *in vitro* fermentation from a pool of feces was questioned due to the hypothesis that the obtained microbiota would be reflecting only the most dominant species as a result of some competition for ecological niches (Roberts et al., 2015). Yet, several studies comparing the use of a pool of feces with inocula from single donors in *in vitro* models provided contradictory results by showing that pooled microbiota was able to reflect similar metabolic profile and bacteria composition compared to what was observed from a single donor (Aguirre et al., 2014; Auchtung et al., 2015). In the present project, we made the choice to prepare MPigut-IVM inocula from fresh fecal samples maintained in anaerobic jars until their transport to the lab, without freezing. Freezing of the fecal samples allows the preparation of a standardized inoculum to ensure better reproducibility and good comparison between studies (Rajilić-Stojanović et al., 2010). However, Metzler *et al.* demonstrated that freezing pig feces could influence the relative abundance of *Lactobacillus, Enterococcus, Streptococcus, Clostridium, Bacteroides, Prevotella* and *Enterobacteriaceae* analysed by 16S sequencing (Metzler-Zebeli et al., 2016). Therefore, if freezing influences results from DNA-based downstream analyses, we can easily imagine a strong impact on bacteria functions and cultivability in *in vitro* models. A study of Pastorelli *et al.* confirmed that fresh or frozen pig fecal inocula induced differences on *in vitro* fermentation capacities for degradation of various feed ingredients (Pastorelli et al., 2014). Consequently, to stick more closely to the composition and functionality of piglet microbiota we chose to use exclusively fresh inocula during this PhD project. The drawback of this choice was the need to accept variability brought by the use of different pools to inoculate the MPigut-IVM. However, this variability reflects better the *in vivo* situation and is needed to assess *in vitro* for example the effect of inter individual variability on the efficiency of preventive treatment such as probiotics on the microbiota.

Once inoculated in the *in vitro* model, fecal microbial communities need to adapt to the available nutrients and fulfil ecological niches. During the first few days, the absence of the host and the transition from fecal to colonic environment, represented for example by a decreased pH, change the microbiota structure compared to feces prior to reach a day-to-day stability. The MPigut-IVM parameters (Figure 1) shaping the structure of the *in vitro* microbiota are discussed below.

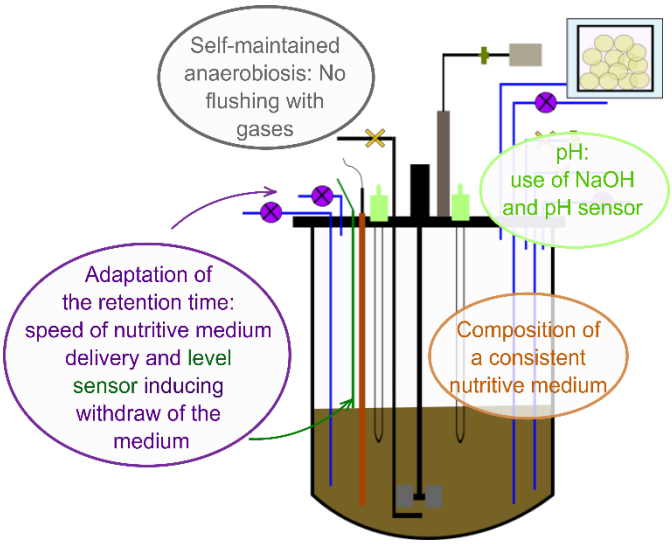


Figure 1. Important parameters shaping the microbiota in the MPigut-IVM.

- **NUTRITIVE MEDIUM:** The choice of nutritive medium components has of course a great impact in modelling *in vitro* microbiota. For example, a nutritive medium which contains too many carbohydrates enhances the augmentation of *Bacteroidetes* which is often observed in *in vitro* models (Kettle et al., 2015). Similar phenomenon was described *in vivo* where *Bacteroidetes* generally displayed a greater abundance in proximal colon where carbohydrates are more available while decreasing in the distal colon where less carbohydrates are present (Kettle et al., 2015). In the MPigut-IVM, a nutritive medium mimicking the ileal chyme of piglets was set up based on the protocols used by MacFarlane *et al.* and Tanner *et al.* which calculated, using digestibility indices, the percentage of starch or proteins which should be not digested and absorbed by the host and reach intact the colon. The second point which was of importance was the widely spread practice of creep feeding which was taken into account by formulating a pre-weaning diet medium already containing vegetable proteins or fibres besides a high proportion of dairy product. As a result, we showed notably a greater relative abundance of *Bacteroidetes* compared to the fecal inocula after the stabilization phase which was consistent with our *in vivo* data where colonic digesta of weaning piglets displayed more *Bacteroidetes* than the fecal samples (Gresse et al., 2019).

- **pH**: *In vivo* colonic pH influences the solubility and availability of nutrients and electrolytes. The pH also shapes the composition of the microbiota especially *Ruminococcus, Clostridium, Enterobacteriaceae* or *Bacteroides* members (Duncan et al., 2009). Duncan *et al.* (2009) started two fermentors with pH fixed respectively at 6.5 and 5.5 and reported that the major taxa in the pH 6.5 bioreactor was *Bacteroidetes* while the pH 5.5 bioreactor was dominated by *Clostridium* cluster XIVa members. Globally, Gram negative bacteria showed to be inhibited by low pH while some Gram

positive bacteria displayed greater tolerance to low pH (Duncan et al., 2009). Setting up the pH in *in vitro* model is thus of a great importance. In our *in vivo* study we measured the pH of all the GIT compartments of weaning piglets, and the colonic pH values were all between 6 and 6.5 (Gresse et al., 2019). For the setup of the MPigut-IVM we choose to fix the pH to 6, the lower range of our *in vivo* data, in order to limit the expansion of the *Bacteroidetes* and *Enterobacteriaceae* which is most of the time observed in *in vitro* models during the stabilization period (Fleury et al., 2017; Tanner et al., 2014a; Van den Abbeele et al., 2012).

- **RETENTION TIME**: The retention time, which is analogous to transit time, is also an important factor affecting microbial activity and composition in intestinal microbiota in animals as well as in *in vitro* models (Macfarlane et al., 1998; Rodes et al., 2011). In piglets, small intestine harbours much faster transit time compared to the lower part of the intestine (Wilson and Leibholz, 1981) as well as much more inter-individual variable microbial communities (Gresse et al., 2019). Similarly to what is observed *in vivo*, the overtime stability of bacteria in *in vitro* models is positively correlated with a slow transit time (Macfarlane et al., 1998; Rodes et al., 2011). Additionally, higher retention time increases protein breakdown and amino acids while decreasing SCFA production (Macfarlane et al., 1998). This parameter is crucial for the establishment of a reliable *in vitro* microbiota and should be accurately defined. However, very few data about retention time in the piglet large intestine are available and its definition in young animals is even more difficult due to over time changes (Wilson and Leibholz, 1981). The set-up of the MPigut-IVM retention time was based on the global colonic retention time measured on piglets from 35 days of age but could be better adjusted if new data are available. Another possibility is to distinguish proximal and distal colon using adapted retention times. However, these data are not available for weaning piglet yet and would have deserved more *in vivo* investigations.

- **ANAEROBIOSIS**: In addition, the distinct feature of *in vitro* colonic models from MEDIS unit is the self-maintained anaerobiosis compared to the alternative methods consisting in flushing the bioreactors with nitrogen or carbon dioxide. This particular characteristic allows to accurately monitor the redox potential in real time and thus to get information about *in vitro* microbiota reducing capacity and activity as well as the gas produced in bioreactors. The continuous flow of nitrogen to maintain anaerobiosis, used by other *in vitro* colon systems, could be responsible of the disappearance of populations growing under the presence of carbon dioxide or hydrogen, such as archaea methanogens (Kettle et al., 2015) which were to our knowledge never observed in continuously

flushed *in vitro* models, and yet functionally important in pigs notably through methanogenesis pathway. The methanogenesis is the production of methane by archaea, mainly through carbon dioxide reduction by hydrogen in the gut, and could be important when reproducing the gut environment. For example, the reproduction of methanogenesis in ruminal *in vitro* models, such as Rusitec (Czerkawski and Breckenridge, 1977), is essential to be able to investigate feed additives and products to inhibit methane production in cattle industry and limit greenhouse effects. In the rumen, the elimination of methanogenesis is linked with the accumulation of hydrogen which is produced by fermentation byacetate or butyrate producers (Dridi et al., 2011; Leng, 2014). The accumulation of hydrogen due to the absence of methanogens has indeed been linked with less efficient fermentation activity both in *in vitro* co-culture and *in vivo* ruminal microbiota of lambs (Fonty et al., 2007; Iannotti et al., 1973; Latham and Wolin, 1977). The presence of methanogenesis in the MPigut-IVM reinforce is representativeness toward *in vivo* gut environment.

Regarding the criteria listed above, several parameters lead to the *in vitro* transformation of the fecal microbiota to a colonic microbiota in the few days following inoculation of the *in vitro* models. Therefore, it is particularly important to let the microbial ecosystem the time to adapt and stabilise in order to get reliable results when applying any treatments or testing any compounds. In the MPigut-IVM, a period of 7 days was setup to allow the stabilisation of microbial communities (composition and activity). The overall day-to-day stability of the MPigut-IVM microbiota was satisfying after the stabilisation period. The optimization made by Tanner *et al.* consisting in the immobilization of the microbiota in polymer beads considerably improved the overtime stability of *in vitro* microbiota in PolyFermS of the pig colon up to several months (Tanner et al., 2014a). However, in this project, the aim was to focus on the relatively short period of weaning and to keep the reactivity of the microbiota towards the changes applied to the MPigut-IVM.

Finally, the MPigut-IVM displayed a good reproducibility between replicates from the same fecal inoculum. Biological replicates, therefore coming from different fecal inocula, were able to maintain specific profiles in term of bacterial composition all along the fermentation runs (chapter 9). Even if most of the studies aim to erase variability, it is actually important to take into account that our *in vitro* models, as *in vivo* assays, should be representative of the individual variability exhibited by animals. Indeed, *in vivo,* some animals are more responsive to a treatment, feed additives or pathogens, and tend to be more impacted by any changes. Too many standardization procedures can hide individual variations and thus affect the representativeness of the results.

Considering the results and parameters discussed above, the MPigut-IVM was validated and ready to be used to simulate weaning transition.

3. Simulation of weaning transition

3.1. Effects of feed deprivation

Piglets encounter a wide variety of stressors at weaning including mixing litters, fighting, separation from the mother, diet change and sometimes transportation (Bian et al., 2016; Gresse et al., 2017; Lallès et al., 2007a). However, many of these stresses are not reproducible in an *in vitro* model deprived of host. Transient anorexia is a behavioural consequence of all these events happening at weaning, consequently impacts host physiology and microbiota (McCracken et al., 1999), and is easily reproducible in an *in vitro* model. To accurately reproduce *in vivo* situation, the simulation of transient anorexia *via* feed deprivation in the MPigut-IVM was experimented prior to dietary change. The duration of the feed deprivation period was chosen regarding literature indicating that weaning associated anorexia lasted mostly between 24h to 48h (Lallès et al., 2007a; Sutherland et al., 2014). The impact of 12h, 24h and 48h feed deprivation period was thus examined using replicates from the same fecal inoculum and revealed gradual changes in bacterial composition. Considering both the importance of changes, the consistency with literature and the capacity of resilience of the *in vitro* microbiota, the feed deprivation of 48h was chosen and replicated using 4 biological fecal inocula. The modifications of the MPigut-IVM microbiota composition due to feed deprivation led to transient changes, principally of *Bacteroides* and *Prevotella* which were mainly erased few days after (Chapter 9). Mucin beads-associated microbiota showed distinct changes, for example in *Clostridiaceae* family, compared to that of the bioreactor medium, which demonstrated again the specificity of this micro-environment. The decrease in butyrate producing *Clostridium* members in the mucus layer could, *in vivo*, directly impact the quantity of absorbed butyrate by enterocytes and contribute to the disruptions of intestinal morphology observed during weaning transition (Brown et al., 2006; Cera et al., 1988). Rather small effects were detected in SCFA proportions maybe due to their accumulation during feed deprivation which could have masked the actual changes. Analysis of the metabolome, which was performed for the time, indicated a clusterisation of bacterial metabolites within the stabilisation, feed deprivation and recovery periods and a decrease of biogenic amines and amino acids during the feed deprivation indicating a reduction in proteolytic and peptidolytic microbial activity. The impact of feed deprivation on the microbiota activity was also characterised by a remarkable increase in the redox potential. In control conditions, fermentative activity drives down

the redox potential by degrading sugars and reducing electrons present in the medium. However, the lack of nutrients during feed deprivation considerably reduces fermentative activity of the microbiota which leads to an increase of redox potential. Despite the use of fecal pools to inoculate the MPigut-IVM, our results highlighted some variability between biological replicates, especially toward the expansion of *Escherichia/Shigella* on the mucin beads. *Escherichia* species are known to be particularly resistant to oxidative stress (Xu et al., 2014). Therefore, in microbiota profiles harbouring more susceptible populations to oxidative stress, *Escherichia/Shigella* could take advantage of the situation to bloom.

3.2. Impact of feed deprivation and dietary change

In order to simulate more closely the weaning transition, the next bunch of fermentation experiments consisted in introducing a dietary change after the feed deprivation period. The dietary change consisted in an important reduction of milk-derived product as well as a higher quantity and a more diverse source of plant proteins. Interestingly, the introduction of dietary change after feed deprivation (Chapter 10) mostly modulated *Bacteroides* and *Prevotella* ratio among several other genera but led to more profound and long-lasting changes compared to the previous experiment subjected to feed deprivation only. Our principal results, being the decrease of *Bacteroidiaceae* and the increase of *Prevotellaceae* was totally consistent with several animal studies reporting changes in the microbiota composition of weaning piglets. Even though most of the results from the simulated pre-weaning microbiota composition cannot be compared to our data due to large differences in nutritive medium setting, the simulated dietary weaning transition in the bioreactor medium of the baby-SPIME also led to a decrease of *Bacteroides* and an increase of *Prevotella* (Dufourny et al., 2019). These results could overall indicate that the *Prevotella* genera present in piglet feces harbour members possessing various metabolic capacities such as degradation of sugars and complex carbohydrates in addition to amino acids and protein degradation and are probably more versatile and willing to adapt to any changes compared to other populations occupying one specific ecological niche, both *in vivo* and *in vitro*, which explains why this genus is generally found among the dominant members in most digestive ecosystem (Alauzet et al., 2010; Ley, 2016). *In vivo* data about changes of mucus-associated microbiota at weaning have not been described in the literature yet. However, mucin beads from control fermentation runs showed a high similarity with the microbiota of mucosa from piglet colon by sharing 14 out of 15 of the main bacterial families. Therefore, it seems reasonable to think that changes observed on the mucin beads after feed deprivation and dietary changes such as modifications of *Erysipelotrichaceae*, *Megasphaera*, *Olsenella*, *Ruminococcus* or *Romboutsia*, could

provide a good indication of microbiota modifications which would happen *in vivo.* Getting *in vivo* data about the evolution of mucosal microbiota communities of piglets around weaning would have been helpful for the development of the model. However, this approach would have needed the slaughter of a much higher number of animals.

3.3. Emphasis on individual susceptibility

Individual susceptibility of piglets toward weaning transition was previously reported *in vivo* and was related to differential abundances of *Prevotella, Bacteroides* and *Escherichia coli* in early life between healthy weaned piglets and diarrheic weaned piglets (Yang et al., 2019). In this study, piglets which developed diarrhea after weaning harboured a higher relative abundance of *Prevotella* and less *Escherichia coli* in the pre-weaning period compared to piglets which remained healthy after weaning while a reduced number of *Bacteroides* in weaned piglet feces was also associated with diarrhea (Yang et al., 2019). Although future studies need to be performed in order to define “normal” and “abnormal” ratios of these three taxa, our results confirm the awareness about the importance of a good balance between these populations in weaning piglets. We suggest that abundances of *Prevotella, Bacteroides* and *Escherichia coli* could be considered as biomarkers of a healthy piglet microbiota versus a microbiota having higher risk to develop dysbiosis and thus to encounter diarrhea after weaning. In the MPigut-IVM, it would be interesting to simulate weaning transition in fermentors inoculated with individual fecal samples instead of pooled feces in order to compare individual susceptibility to dietary change and especially to feed deprivation. The impact of maternal imprinting on piglet microbiota and its response towards weaning stress could be also investigated by using fecal samples from different sows subjected for example to different diets or feed additives. This approach will need several repetitions to make sure to test different microbiota profiles and couldn’t be performed within the timeframe of this PhD mainly focusing on the MPigut-IVM development and etiology of post-weaning dysbiosis and infections. Additionally, due to the small amount of fecal material per piglet, the investigations of technical replicates would require smaller bioreactors. Individual susceptibility driven by early life microbiota could further play a role both in ETEC colonization of piglet GIT as well as emergence of opportunistic pathogens.

4. ETEC colonization in the MPigut-IVM

The mucin rich environment created by the mucin bead compartment is of particular interest to study the colonization of pathogens such as ETEC in the gut microbiota as well as their interactions with mucus-associated commensal microorganisms. Indeed, ETEC strains are known to colonise the mucus layer through attachment to the intestinal epithelium and this strengthen their virulence and colonization efficiency (Dubreuil et al., 2016; Luppi et al., 2016). The 2 major pathogens in swine industry are *Salmonella* serovar Typhimurium and *E. coli* pathogenic strains (Tran et al., 2018). Among pathogenic *E. coli,* ETEC F4 is the main pathotype of post-weaning diarrhea in piglets (Dubreuil et al., 2016; Luppi et al., 2016). The ETEC F4 strain used during this PhD was isolated from Spanish diarrheic piglets and kindly provided by Dr J.J. Garrido (Department of Animals Genetics, Universidad de Cordoba, Spain).

4.1. Effects of an ETEC strain on the weaning piglet gut microbiota

Sun *et al.* determined that 10^9 to 10^{10} CFU of ETEC are required to induce diarrhea in nursery pigs typically lasting 1 to 5 days after ETEC challenge (Sun and Kim, 2017). Therefore we decided to set up the dose of ETEC to inoculate in the MPigut-IVM at 7 Log_{10}/mL corresponding to 9 Log_{10} for the total volume of fermentation medium. The first challenge when studying the effect of ETEC colonization on piglet gut microbiota using the MPigut-IVM was the assessment of ETEC survival among commensal *E. coli* population. Our first idea was to make an ETEC mutant able to resist to rifampicin or chloramphenicol in order to be able to monitor and quantify it by culture on selective medium. However, this was unachievable because we found that, after sampling feces of 10 weaning piglets and plating dilutions on LB medium containing rifampicin or chloramphenicol, all of them harboured resistant bacteria (data not shown). ETEC survival and virulence were thus followed by DNA and RNA based techniques. A small expression level of LT, F4 and EAST1 virulence genes was detected in the initial fecal inocula by RT-qPCR, indicating that some endogenous *E. coli* from our fecal samples expressed virulence genes similar to our ETEC F4 strain. Both F4, LT and ST genes are plasmid encoded (Dubreuil et al., 2016; Ochi et al., 2009) and EAST1 can be carried by commensal *E.coli.* The LT gene is located on a large low copy number plasmid (one or two copies) with a low-self transmissibility (Ochi et al., 2009). Due to its known low copy number characteristic limiting the bias of targeting a plasmid encoded gene, the LT gene was chosen to monitor the ETEC strain in the MPigut-IVM. Probably due to the low proportion of endogenous bacteria bearing the LT

gene, the total number of LT gene in the ETEC conditions was quantified at 8 Log_{10} / g of fermentation medium despite the 7 Log_{10} / mL initially calculated (Chapter 10). Interestingly, few days after the injection of the ETEC strain in the bioreactor, the LT virulence gene was clearly quantified in higher quantity in the mucin bead compartment compared to its abundance in the bioreactor which considerably decreased with time. This highlighted a specific attraction of the ETEC strain for mucin-rich environments, maybe due to the fact that ETEC possess the ability to degrade MUC2 mucin proteins using EatA and YghJ (Dubreuil et al., 2016). In 2 out of 3 repetitions, the ETEC strain was maintained at a level close to 8 Log/g until the end of the fermentation runs in the bead medium. However, in the run #1 (Chapter 10), ETEC population (as monitored by the LT gene) declined to 3.5 Log/g in the bioreactor medium within 48 hours and 0.2 Log/ g of bead medium at day 15 in the MPigut-IVM and thus reached a level close to that found in the control condition. This finding may indicate that the microbiota of the run #1 was more prone compared to the one in runs #2 and 3, to limit the implantation of the ETEC strain in the ecosystem. Repetitions have to be performed to investigate more accurately the mechanisms of inter-inocula or individual susceptibility towards implantation of ETEC in the microbiota which could be also linked for example to environmental factors or genetic background. We could assume that an individual more susceptible to let the pathogen colonize its microbiota would have also more chance to develop the disease. The testing of different doses of inoculation as well as assessing different modes of inoculation to mimic for example chronic exposure to lower dose of ETEC could also be performed using the MPigut-IVM. This type of experiment could provide interesting insights about ETEC colonization and the response of the microbiota. Also, chronic exposure of low dose of ETEC will involve longer time of experiment but hypothetically stick more closely to the natural contamination from the environment in farms were piglets might not ingest 9 or 10 Log of an ETEC strain at the same time. In the MPigut-IVM, a unique dose of ETEC F4 led to higher differential abundances of several bacterial genera such as *Eisenbergiella, Peptoniphilus* and *Morganella* (Chapter 10). Also, specifically on the mucin beads, the *Enterococcus* genus relative abundance increased to a mean relative abundances of 14.2% on day 15 compared to 0.9% in the control condition. These changes could be related to the fact that the introduction of a new species could lead to associations with some commensal bacteria and to different fulfilment of nutrient ecological niches. This phenomenon which also occurs *in vivo* is thought to contribute to successful colonization and persistence of pathogen in the mammalian gut (Pereira and Berry, 2017). Processes shaping the intestinal microbiota are indeed thought to be essentially niche-driven and depending on environmental factors such as the composition of nutrients available which considerably determine if an organism can persist in the ecosystem (Pereira and Berry, 2017). On the other side, the introduction of a new species, such as ETEC, being more

competitive for a certain type of nutrient could disadvantage commensal species from the same ecological niche and provoke their diminution or even disappearance. This phenomenon could thus trigger microbiota dysbiosis. The nutrient-niche theory, proposed by Rolf Freter, postulate that in a situation where nutrients are perfectly mixed and there is balanced microbial growth, an organism can survive only if it is able to use one nutrient more efficiently than its competitors (Freter et al., 1983b, 1983a). Whereas in animal gut nutrients vary depending on the location, the MPigut-IVM medium, constantly mixed and supplied with an equal amount of nutrients, would be particularly well adapted for the observation of this phenomenon. The MPigut-IVM being able to maintain the differences in microbiota competition between the fecal inocula, this phenomenon could explain the difference in ETEC persistence during the fermentation depending on the biological replicates.

In piglets, ETEC strains are known to preferentially trigger pathogenesis in the small intestine (Dubreuil et al., 2016; Luppi, 2017). However, modifications of microbiota due to post-weaning ETEC infections and diarrhea are most of the time detected in piglet feces indicating that colonic microbiota could be also impacted, provoking possible consequences on the host. The expression of LT, F4 and EAST1 was detected from day 10 in samples exclusively from the ETEC condition from the bioreactor tank and bead medium, indicating that the inoculated ETEC strain could be metabolically active in the MPigut-IVM. The expression of virulence genes in the MPigut-IVM suggests that the pathogenic potential of the inoculated ETEC F4 strain could also be expressed under colonic conditions and without being in contact with host cells. Indeed, if the adhesion of ETEC via the fimbriae F4 to enterocytes stimulates the production of toxins, LT and ST toxins are mostly modulated by environmental factors (Dubreuil et al., 2016). For example, LT toxins can be produced in a wide variety of conditions though optimal production of the LT gene was detected in conditions close to the small intestine environment such as 37°C, pH of 8, in the presence of glucose and with 0.2M of salt concentration (Dubreuil et al., 2016; Gonzales et al., 2013; Hegde et al., 2009). However, during pathogenesis, LT delivery to host cells most probably occur via vesicles which implies close contact with the enterocytes (Dubreuil et al., 2016).

In the MPigut-IVM, the absence of interactions with the host to study the colonization and the virulence of an ETEC strain constitutes at the same time both an advantage and a limit. Indeed, the ability to distinguish modifications of the microbiota without the potential influence of the host can provide new insights about interactions between microbial families which could lead to a dysbiotic state. In the meantime, the use of *in vitro* models, despite the presence of digestive secretion and mucins, is not suitable to collect information about the whole pathogenesis process of the ETEC strain.

4.2. Impact on the host

Host associated factors toward ETEC colonization of MPigut-IVM microbiota were assessed using the coupling of mucin bead effluents on porcine epithelial cells (Chapter 10). The choice of performing culture cell experiments exclusively with bead medium supernatants within the frame of this project was because of the localisation of the ETEC strains close to the mucin rich area. Additionally, the microbiota composition of the bead medium showed to be very similar to the mucin beads and distinct from the bioreactor medium. Unfortunately, pig intestinal cell lines from a colonic origin are not yet available. IPI-2I cell lines were therefore chosen to investigate the effects of MPigut-IVM samples on intestinal epithelial cells due to their ileal origin, closer to the colon, compared to other cell lines with duodenal or jejunal origins and the site of ETEC pathogenesis which takes place in the ileum *in vivo* (Mariani et al., 2009). Coupling fermentation medium with intestinal epithelial monolayer can be biased by the induced toxicity for cells (Payne et al., 2012). Prior to start our cell culture experiments we therefore investigated the effects of dilution series of MPigut-IVM supernatants incubated with IPI-2I cells at different time points. This preliminary experiment concluded that the incubation of a dilution to 1/30 of the MPigut-IVM supernatants during 2 hours was optimal to avoid a too important mortality of the cells as well as maintaining a suitable load and quality of RNA for analysis (data not shown). Supernatants from days 7, 9, 11 and 15 were tested. Interestingly, the sole presence of MPigut-IVM bead medium supernatants of the ETEC condition induced a significant increase in expression of TNFα, MYD88, MUC1 and CLDN4 genes compared to that of the CTRL condition on IPI-2I cells. However, it is important to notice that significant differences were detected only with supernatant collected at day 15. This delay of 5 days after ETEC inoculation to produce an effect on the cell cultures could be related to a required time for the ETEC strain to synthetize and secrete metabolites in a sufficient amount to induce effects on IPI-2I cells. TNFα, MYD88, MUC1 and CLDN4 genes respectively play a role in induction of inflammatory responses, mediation of innate immune responses, secretion of the non-gel-forming mucin MUC1 protein, and secretion of claudin 4 one of the proteins forming tight junctions (Issac et al., 2013; Kato et al., 2017; López-Colom et al., 2019; Pasternak et al., 2015). While the presence of ETEC cause no important mucosal destruction or inflammation, ETEC secreted toxins and virulence factors disrupt enterocyte functions and can induce pro- and anti-inflammatory responses that favour bacterial survival and growth (Dubreuil, 2017). The MyD88-mediated innate immune response has already been proven to be primarily important for protection against microbial pathogen infection via the induction of inflammatory cytokine production (Campos et al., 2004; Issac et al., 2018, 2013; Scanga et al., 2002; von Bernuth et al., 2012). Indeed, MyD88 deficient mice showed to be profoundly

susceptible to infection (Issac et al., 2013). In weaning piglets, an increased level of MyD88 was already reported in enterocytes both *in vivo* and *in vitro* by several studies after infection with an ETEC K88 strain (Chytilová et al., 2014; Finamore et al., 2014; C. Xu et al., 2014). TNFα is one of the most widely studied proinflammatory cytokine involved in numerous bacterial, parasitic and viral infections and was suggested as a biomarker of digestive pathologies in weaned piglets due to its correlation with villi/crypt ratio damages (Barba-Vidal et al., 2017; Gustavo Hermes et al., 2013; López-Colom et al., 2019; Van Reeth et al., 2002). Increased concentration of TNFα or increased expression of the associated gene were previously reported in the intestine of piglets orally challenged with ETEC K88/F4 (López-Colom et al., 2019; Pu et al., 2018) as well as in IPEC-J2 cells co-incubated with ETEC K88/F4 (Xia et al., 2017b, 2017a). The incubation of MPigut-IVM bead medium supernatants on IPI-2I cells seemed to reproduce partially, the inflammatory effects of ETEC infection on piglet enterocytes. The relation between mucins, commensals and pathogens have been widely studied (Etienne-Mesmin et al., 2019). MUC1 is a high molecular mass glycoprotein expressed at the apical surface of mucosal epithelial cells. Its secretion can be stimulated by lipopolysaccharides of Gram negative bacteria and mucin is thought to play an important role in the fight against infections- by epithelial cells (Kato et al., 2017). The production of the MUC1 protein notably limited the access of *Helicobacter pylori* in infected mice (McGuckin et al., 2007). One hypothesis could be that metabolites or compounds associated with the presence of the ETEC strain in the MPigut-IVM supernatants could induce a mechanism of defence by porcine epithelial cells implying a higher expression of MUC1 in order to form a tighter physical barrier on the apical surface of the cells through the increased production of MUC1 protein. CLDN4 gene belongs to the family of claudins which are, with occludins, tight junction proteins located at the apical side of piglet enterocytes (Pasternak et al., 2015) and thus play a role in intestinal permeability and infection. STb purified toxins, which might be present in the supernatants of the MPigut-IVM, were previously shown to increase the epithelium permeability (as measured by transepithelial resistance) and to redistribute claudin-1 proteins of T84 human colonic cells (Nassour and Dubreuil, 2014; Ngendahayo Mukiza and Dubreuil, 2013). Although the ETEC STb virulence factor seems to impact tight junction proteins, the two last mentioned studies did not investigate the gene expression of claudin proteins. Consistently with our findings, higher claudin gene expression was previously associated with ETEC K88/F4 infection in the porcine cell line IPEC-J2 in several studies (Luo et al., 2019; Wu et al., 2016). We could hypothesize that the higher expression of claudin genes from enterocytes could be induced in order to repair disrupted tight junctions due to the presence of toxins or virulence factors in the supernatants which, as illustrated by our RT-qPCR data, were expressed in the bead medium of the MPigut-IVM. The coupling of *in vitro* model effluent with intestinal cells is therefore useful in order

to study the host cell response to the presence of a specific component, here a pathogen, within the microbiota. Indeed, in contrary to a simple co-incubation of a pathogen with intestinal epithelial cells, the microbiota can influence the pathogen-host interactions for example by secreting metabolites such as SCFA or by degrading molecules secreted by the pathogenic strain. However, when those interactions are still unknown, the study of only a restricted number of genes by RT-qPCR represent a clear limitation. The use of omics techniques such as proteomics and transcriptomics of both epithelial cells and *in vitro* model effluents would be recommended to highlight interesting interactions, but such techniques are still extremely expensive. The coupling of *in vitro* model effluents with cell culture cannot totally replace *in vivo* studies. However, *in vitro* techniques, can, for example, help to select the best additives to fight against effect of ETEC on host cells, prior to run animal experiments and thus participate to the reduction of the number of animals used in research.

At last, it could have been very interesting to perform NMR analysis of the metabolome from MPigut-IVM supernatants of the 15th day. Only analyses of the days 7 and 11 were performed, but analysis of the day 15 could represent a very short-term perspective.

5. The application of a probiotic strategy to the MPigut-IVM in the context of multiple challenge

A vast amount of research is currently undertaken to find probiotics able to improve gastrointestinal health by notably fighting against digestive pathogens in piglets. Probiotics must possess several characteristics in order to be considered as potentially effective candidates such as resisting to gastric acids, bile salts and pancreatic enzymes and adhering to the intestinal mucosa to enhance their persistence within the host GIT (de Melo Pereira et al., 2018; Dubreuil, 2017; Gibson et al., 2017). Three principal mechanisms are acknowledged to probiotics to fight against enteric pathogens: i) immunomodulation via interactions with host immune system to enhance innate or adaptive immune responses or limit pro-inflammatory responses induced by pathogens, ii) direct antagonism via for example the inhibition of virulence factor secretion or the blocking of colonization sites, or pathogen binding mechanisms and iii) indirect modes of action such as modulation of the gut microbiota, improvement of epithelium barrier function or stimulation of host defence factors (Roussel et al., 2017).

5.1. Modulation of the microbiota

The use of gut *in vitro* models is well adapted to study the modulation of the gut microbiota by probiotics due to absence of microbiota modulation by host cells. The SB CNCM I-1079 strain is already used in swine industry to improve performances, digestive health, milk quality, litter size, feed efficiency and limit incidence of diarrhea (Chaucheyras-Durand and Durand, 2010). Additionally, daily supplementation of SB CNCM I-1079 in the diet of 21 day-old weaning piglets showed to modulate their colon microbiota by increasing the abundance of *Porphyromonadaceae* and *Ruminococcus* (Brousseau et al., 2015). The ability of SB CNCM I-1079 to modulate a microbiota subjected to a feed deprivation stress was thus evaluated in the MPigut-IVM. Most of the time, probiotics are inoculated at a high dose, reaching for example 10^{10} CFU/mL in *in vitro* models to emphasize their effects and decipher their mode of action. However, our principal idea was to evaluate the potential of SB CNCM I-1079 under settings close to *in vivo* conditions, and therefore to use a dose reflecting the proportion of live SB actually reaching the colon of piglets. Internal Lallemand Animal Nutrition data as well as published information indicated that 10^5 to 10^6 CFU of SB CNCM I-1079 per gram of digestive content could be enumerated from piglet colon after a daily supplementation with 3 x 10^9 CFU/ day (Le Bon et al., 2016). The dose of 10^6 CFU/mL was therefore chosen to inoculate daily the MPigut-IVM by injection during the stabilisation phase and after the feed deprivation period, to mimic *in vivo* preventive supplementation prior to weaning transition. Also, a preventive supplementation with probiotics prior to weaning should be more efficient as it could possibly enhance microbiota metabolism during the risky early life period. Indeed, probiotics can be also used in-feed via the administration of creep feed from one week after birth. Our results reported a beneficial effect of SB treatment on microbial metabolism via the maintenance of lower redox potential values during feed deprivation compared to the control condition. As stated above, an increase in redox potential relates a reduced fermentative activity of the microbiota. Thus, although SB was not detected by qPCR at the end of the feed deprivation stress, the SB treatment was able to confer a metabolic advantage to some microbial populations allowing them to maintain metabolic activities. Despite the low dose of inoculation, the SB treatment induced some modulation of the MPigut-IVM microbiota. *Streptococcus* was increased by more than 2 folds on the mucin beads and *Escherichia/Shigella* was quantified with one additional Log compared to the non-SB supplemented condition after the feed deprivation period. If the increase of *Escherichia/Shigella* may appear not beneficial considering that some members of this genus group are well known enteric pathogens, the great majority are normal commensals of the pig GIT. It will thus be interesting to know which types of *E.coli* (pathogens or commensals) are actually increased, with further biological repetitions and

quantifications of a panel of virulence genes specific to this group. If non-pathogenic *E.coli* commensals are promoted, it would actually represent a strong advantage to avoid the colonization of pathogenic *E. coli.* Indeed, it is appropriate to consider that non-pathogenic *E. coli* occupy rather similar ecological niches than pathogenic *E. coli* such as ETEC. In antibiotic-treated hamsters, the colonization with non-toxigenic *Clostridium difficile* could protect against a subsequent challenge with epidemic *Clostridium difficile,* most likely due to the occupation of the vacant nutrient niche by the non-pathogenic strain (Nagaro et al., 2013). Therefore, a higher abundance of non-pathogenic *E. coli* in the microbiota could contribute to avoid a strong establishment of ETEC strains. In agreement with this hypothesis, Bin et *al.* highlighted that the relative abundance of *E. coli* was reduced from 86% to 49% in the jejunum of ETEC-challenged diarrheic piglets compared to healthy individuals (Bin et al., 2018). This finding was supported by Yang et *al.* who reported that *E. coli* was decreased in the fecal microbiota of pre-weaned diarrheic piglets (Yang et al., 2019). In addition, the well-known probiotic *E. coli* Nissle 1917 has shown efficient suppression of clinical signs against ETEC-induced diarrhea in an infection model of pre-weaned piglets (Schroeder et al., 2006) and could suppress clinical signs, histopathological changes and induction of inflammatory cytokines associated with *Salmonella* Typhimurium infections in germ-free piglets (Splichal et al., 2019).

5.2. Effects of SB during a simulated weaning transition

The effects of a preventive daily dose of SB CNCM I-1079 was then evaluated during a more accurately simulated weaning transition consisting in a 48h period of feed deprivation followed by dietary change and pathogen exposure. As described above the inoculation of the ETEC strains being fixed at 10^7 CFU/mL, the daily dose of probiotic inoculated in the MPigut-IVM was thus expanded to 10^7 CFU/mL in order to emphasize the impact of SB. Two biological repetitions out of the 3 initially planned could be conserved for data analysis because of technical issues with the third replicate. Therefore, this experiment should be repeated to get stronger results and perform statistical analysis. The preliminary data showed that SB was not able to reduce ETEC establishment in the bioreactor medium and bead medium of MPigut-IVM. However, modulations of the microbiota were highlighted such as an increase of *Escherichia/Shigella,* consistently with our findings from the Chapter 9, and *Ruminococcus* genera relative abundance. As explained earlier, a higher proportion of *Escherichia/Shigella* would not be necessarily negative, and a higher proportion of *Ruminococcus* members is considered beneficial as some species of this genus are fibre-degraders and butyrate producers (Macfarlane and Macfarlane, 2003). The most important effect of SB treatment was the increase of *Bacteroides / Prevotella* ratio after the feed deprivation and the dietary change, especially

in the bioreactor medium. The increase of the relative abundance of *Bacteroides* could represent a beneficial modulation of the microbiota to fight against the onset of post-weaning dysbiosis. Indeed, as evocated above a low relative abundance of *Bacteroides* in the fecal microbiota of piglets was associated with post-weaning diarrhea (Yang et al., 2019).

5.3. Impact on the host

The principal limit of the use of MPigut-IVM to study the effects of probiotics is the absence of host interactions with epithelial cells. Investigations about immunomodulation properties of SB CNCM I-1079 was thus conducted by incubation of bead medium supernatants which were subjected to SB treatments as well as ETEC exposure on IPI-2I cells in comparison with supernatants coming from the ETEC condition without probiotics. The preliminary results of the coupling of MPigut-IVM supplemented supernatants on IPI-2I inflammation gene expression revealed that supernatants from the SBETEC condition could mitigate the gene expression of TNFα, MyD88, MUC1 and CLDN4 induced by bead medium supernatants from the day 15 of ETEC conditions. A hypothesis could be that the SB strain could act against effects of ETEC on epithelial cells via immunomodulation or by direct antagonism via the production of proteases degrading ETEC virulence factors present in the supernatants of the MPigut-IVM. Both mechanisms of action have been documented in diverse *Saccharomyces* yeasts against ETEC or other enteric pathogens (Badia et al., 2012b; Buts et al., 2006; Castagliuolo et al., 1996; Czerucka et al., 1994; Czerucka and Rampal, 2019; Hermes et al., 2011; Zanello et al., 2011). However, such as highlighted in these studies, yeasts demonstrate strain dependent properties and further investigations will be needed to fully understand the mode of action of SB CNCM I-1079.

5.4. Immunomodulation and direct antagonism properties

Direct antagonism properties of SB CNCM I-1079 against our ETEC F4 strain were investigated using additional cell culture experiments without including MPigut-IVM supernatants. These experiments were performed on the IPEC-J2 cell line, originated from the jejunum of piglets, because, in contrary to IPI-2I, they possess the receptor for the F4 fimbriae allowing the ETEC strain to attach to the cells (Dr J.J. Garrido, personal communication). Pre-treatment with several doses of SB: 10^6, 10^7 and 10^8 CFU, corresponding respectively to 6.25, 62.5 and 625 SB CFU/ IPEC-J2 cell, prior to inoculation of ETEC (MOI 1:100) induced a dose-dependent inhibition of the adhesion of ETEC to

the IPEC-J2 hypothetically due to the hiding or blocking of adhesion sites. Dose dependent down regulation of the gene expression of cytokines and chemokines such as CCL20, CXCL2, IL1α, IL6, IL-8 and TNFα was also observed after pre-treatments of SB prior to co-incubation with the ETEC strain. Zanello *et al*. showed that on top of its ability to agglutinate with ETEC, the strain SC CNCM I-3856 or its culture supernatants could inhibit expression of genes coding for inflammatory cytokine such as IL6, IL8, CCL20 or CXCL2 of ETEC-challenged IPEC-1 and IPI-2I porcine cells (Zanello et al., 2011). Badia et *al.* evaluated the role of SB on ETEC-challenged IPI-2I cells and also noticed a downregulation of TNFα, IL6, CCL2, CXCL8 and CCL20 gene expression (Badia et al., 2012b). Thus, SB confirmed to possess immunomodulation as well as competitive exclusion properties against our ETEC F4 strain (Chapter 11). Investigations about the ability of SB to down regulate virulence factors could have been easily assessed during this *in vitro* experiment by RT-qPCR and qPCR but were unfortunately not fitting in the time frame of the PhD secondment at Cordoba University. These experiments will have to be performed in the near future. Also, investigations were limited to the use of RT-qPCR to monitor the expression of a specific set of ETEC virulence genes and of intestinal cell inflammatory genes. Other genes could be investigated for example ETEC genes coding for the mucin proteases YghJ and EatA (Luppi, 2017) or SB genes coding for proteases which were shown to degrade toxins or receptors from other enteric pathogens (Castagliuolo et al., 1996; Czerucka et al., 1994; Czerucka and Rampal, 2019).The study of both epithelial cells, SB and ETEC interactions by the RNA-Seq approach (Herrera-Uribe et al., 2018; Westermann and Vogel, 2018) would have been particularly suitable due to its wide spectrum, and represents a perspective of our preliminary work.

6. Future perspectives

6.1.Short term perspectives

Technical perspectives about the MPigut-IVM should focus on the optimisation of the mucin beads compartment (Figure 2). The actual system is opened using articulated clamps which can be tricky to manipulate and need a certain time to get used to the system leading to user-dependent results especially concerning the maintenance of anaerobiosis inside the compartment. A more user-friendly opening system would be to replace the articulated clamp by a screw cap system. Additionally, the

replacement of the water bath by a tailor made heating cover would benefit to the model by leading to economy of water, the use of shorter pipes to connect the mucin beads compartment with the model, and the avoidance of light. At last, an appendix allowing the sampling of mucin bead compartment would be of a particular interest to study the implantation of pathogens in the mucin-rich area of the model with tighter time points (Figure 3). A test of screw caps opening systems is currently carried out in the UMR MEDIS unit.

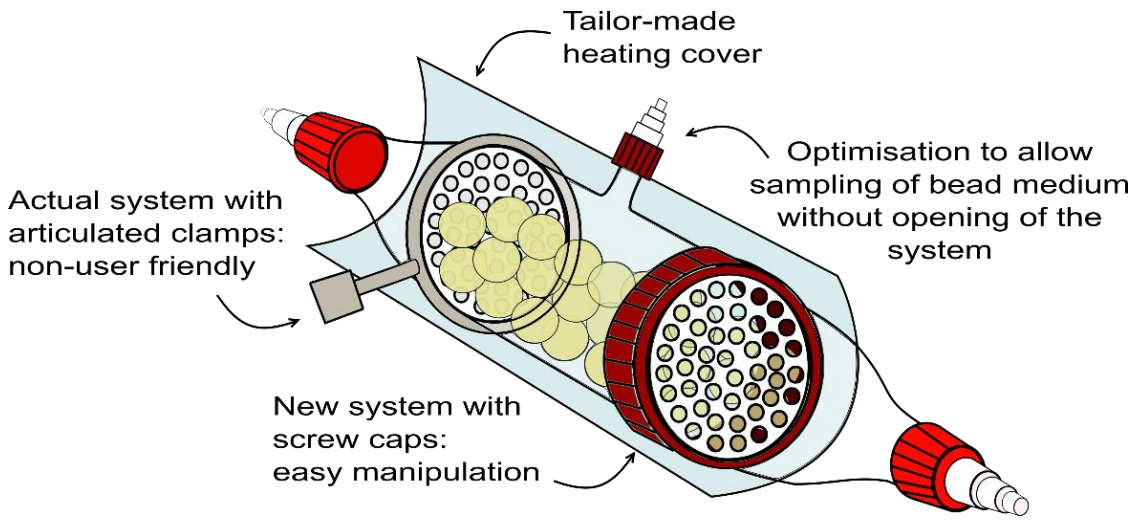


Figure 2: Possible optimizations of the mucin bead compartment of the MPigut-IVM.

- **REPETITIONS OF SBETEC CONDITION:** Scientific perspectives should first focus on the repetitions of the experiments performed in Chapter 11 to confirm the modulating effect of SB on the microbiota subjected to weaning transition and pathogen exposure as well as coupling experiments with porcine epithelial cells lines to confirm the mitigation of the non-SB-treated supernatant induced immune responses. At last one additional biological replicate would allow the application of statistical analysis in order to confirm changes of MPigut-IVM microbiota and host responses.

- **MODE OF INOCULATION AND DOSE OF PROBIOTICS:** Experiments about the effects of a curative treatment of SB, that is daily supplementation of probiotics after the simulation of feed deprivation and dietary shift, was also performed but not analysed due to lack of time. Priority was made on the preventive mode of inoculation throughout this PhD which was closer to the *in vivo* situation and conform to the recommendation for practical application of probiotics at the farm level. It would be tempting to use higher dose of probiotics, like 10^{10} or 10^{11} CFU such as most *in vitro* studies, to emphasize their effects but that approach would be questionable regarding the number of in-feed probiotics reaching the large intestine of piglets in farm conditions. The oral inoculation of

one high single dose of $3x10^9$ CFU showed to reduce diarrhea score and incidence in neonatal piglets (Hancox et al., 2015). Therefore, this approach could also be tested *in vitro* by the inoculation of one high dose right before the simulated weaning transition in the MPigut-IVM.

- **COMPARISON WITH ANTIBIOTICS AND ZINC OXIDE (ZnO):** Antibiotics are the current treatment used in swine industry to fight against post-weaning ETEC infections in piglets (Barton, 2014; Rhouma et al., 2017). In-feed zinc oxide has been previously proposed as growth factor to improve weight gain and feed efficiency as well as an alternative to antibiotics to reduce post-weaning diarrhea incidence due to its ability to reduce enterobacteria, coliforms and *E. coli* in the microbiota (Broom et al., 2006; Shen et al., 2014; Starke et al., 2014; Vahjen et al., 2011). Piglets challenged with ETEC F4 and receiving ZnO in their post-weaning diet displayed better fecal consistency, intestinal morphology or reduce expression of pro-inflammatory genes (Kim et al., 2015; Sargeant et al., 2010). However, decrease of microbial diversity and impairment of other bacterial groups was also noticed in some of these studies which could be linked to the onset of a dysbiosis. Again, a particular attention should be given to the reduction of *E. coli* species which are, one the one side opportunistic pathogens, but on the other side one of the first commensal colonizers of the piglet gut. Additionally, the European Medicines Agency concluded that the ZnO could have harmful impact on the environment and recommended the withdrawal of ZnO containing products (https://ema.europa.eu/). Therefore, it would be interesting to compare the effects of an antibiotic such as colistin or of zinc oxide treatment in the MPigut-IVM after the simulation of weaning transition and the ETEC exposure with the effects of preventive administration of probiotics. Such experiments would need fermentation runs of around 1 month to allow the study of longer term effects such as the resilience of the microbiota after the cessation of zinc oxide, antibiotics or probiotic supplementation. In case of promising effects of the preventive probiotics treatment compared to antibiotics or ZnO in the MPigut-IVM, an *in vivo* experiments could be considered.

- **COUPLING WITH CELL CULTURE**: The coupling of in vitro digestive system effluents with porcine cell culture was for the first time applied here. Combined omics approaches such as transcriptomics or proteomics of MPigut-IVM medium, ETEC, probiotics and host cells could help to correlate bacterial metabolites with virulence factors, probiotics metabolism and host responses. An adaptation of porcine intestinal cells in the HMI module (Marzorati et al., 2014) could also be an option to investigate crucial time for the MPigut-IVM such as the 48h of feed deprivation, first hours of dietary change or ETEC injection. The use of HMI type of system favours *in situ* coupling and avoid the freezing of fermentation supernatants which can degrade the metabolites and lead to under

estimated results. Gut organoids are cultured from intestinal stem cells and differentiated to various intestinal cell types such as enterocytes, Paneth cells, goblet cells and endocrine cells and reproduce the production of mucus and the microanaerobiosis of the epithelium (Verhoeckx et al., 2015). Such culture models could be co-incubated with MPigut-IVM effluents in order to better investigate host-microbiota cross talk and feedback mechanisms to weaning transition, ETEC infections or probiotics use and could be especially used prior to consider investigations in animals.

6.2.Long term perspectives

- **EFFET OF SB ON SALMONELLA:** *Salmonella enterica* serovar Typhimurium is the second pathogen responsible for diarrhea in piglets (Bonardi, 2017). Therefore, the MPigut-IVM could be used in the same conformation to study the impact of this pathogen on the weaning microbiota as well as on porcine cell lines. Additionally, an SB strain was shown to be able to modulate the immune response against *Salmonella enterica* serovar Typhimurium on porcine cell lines and thus could be a potential candidate to fight against *Salmonella* diarrhea in piglets (Badia et al., 2012a). Also, screening of the most effective live yeast strains could be performed thanks to our model.

- **INVESTIGATION OF INDIVIDUAL SUCEPTIBILITY**: As shown during this discussion the inter-individual differences of piglet microbiota composition during early-life seems to influence the susceptibility to develop infections and diarrhea after weaning, especially the proportion of *Bacteroides, Prevotella* and *Escherichia/Shigella* which were impacted by the simulated weaning transition in the MPigut-IVM. The investigations of the responses of fecal samples from individual piglets in the MPigut-IVM could help to find biomarkers of risky profile highly susceptible to be efficiently colonized by ETEC and thus develop diarrhea. Additionally, the identification of profiles responding better to one particular probiotic treatment could help to develop effective combined probiotic strategies taking into account the inter-individual variability to fight against post-weaning diarrhea.

- **SET UP OF AN ILEAL MPIGUT-IVM**: The use of a colonic *in vitro* model in the present project was justifiable due to the choice to focus on microbiota dysbiosis. However, enteric pathogens such as pathogenic *E. coli* and *Salmonella* were previously shown to colonize and induce pathogenesis in the host in the small intestine where they also provoked changes in the microbiota (Argüello et al., 2018; Dubreuil et al., 2016). Therefore, in further projects, the adaptation of the MPigut-IVM to the

ileal piglet microbiota would be of great interest to study ETEC colonization mechanisms, effect of probiotics, and effects on the host, and to compare the results with those obtained in this project on the colonic environment. However, setting up a reliable piglet ileal *in vitro* model would be a greater challenge, due to the adaptation from a fecal microbiota to an ileal microbiota (Dufourny et al., 2019; Roussel, 2019; Stolaki et al., 2019). Ileum and feces are two environments considerably different in terms of physico-chemical parameters. One option could be to start bioreactors wit simplified consortium of isolated strains. In the Chapter 9, we could indeed observe that the microbiota from piglet ileum was relatively poorly diversified and mainly composed of twelve different genera. However, this option would need the use of a more precise metagenomic technique in order to accurately identity the species from each genus and the investigations of the microbiota from ileal mucosa which was not done in this PhD thesis.

7. Use of *in vitro* fermentation models in research

For a scientific point of view, *in vivo* experiments are and will remain the gold standard for a majority of research subjects. Indeed, despite the constant optimizations of *in vitro* alternatives, there are no possibilities to reproduce the complete functionality of immune or neuroendocrine systems for examples. Regarding the problematic of this PhD thesis, the social stresses encountered by piglets at weaning which are separation with the mother, mixing litter, fighting or transportation are impossible to reproduce in the MPigut-IVM. These models are not appropriate to study the pathogenesis of invasive pathogens or the effects of compounds aiming to impact host physiology first. Thus, prior to consider the use of *in vitro* fermentation models, this is mandatory to review accurately the objectives of the undertaken research. *In vitro* fermentation models are particularly well adapted to assess questions related to the dynamics of the resident microbiota in response to probiotics, prebiotics, feed additives, pathogens or physico-chemical parameters, as well as the survival or activity of armful or beneficial microbes in a digestive environment. Additionally such models, being a close and mixed environment with the possibility of continuous nutrient supply are ideal studies to decipher the organisation of ecological niches. Scientifically, to obtain mechanistic insights, this is interesting to avoid interactions with the host while investigating on the microbiota when for instance, animal behaviour or stress engendered by manipulations can influence the microbiota. When microbiota-host crosstalk is needed, the coupling of *in vitro* gut models with cell cultures can provide

good information and can be used to refine the strategy and experimental design for the *in vivo* assays. This point is of particular importance to respect the European directive aiming to replace but also reduce and refine the number of animals used in research. For the manipulator, the use of *in vitro* fermentation models can be tedious and need long time of experiments to assess enough technical and biological replicates. However, *in vitro* fermentation models harbour considerable practical pros such as a good reproducibility, technical flexibility, the possibility to carry out experiments in a virtual indefinite manner, the possibility to quickly perform a replicate in case of technical issues with the initial experiment, rather low cost once the material is financed and the possibility to quickly adjust the parameters.

To conclude, this PhD project led to the development of an innovative *in vitro* model of the weaning piglet colon. Current results provided interesting insights about modifications of the piglet microbiota induced by weaning, pathogen exposure or yeast supplementation. The MPigut-IVM is now ready to be applied to numbers of other projects (Figure 3).

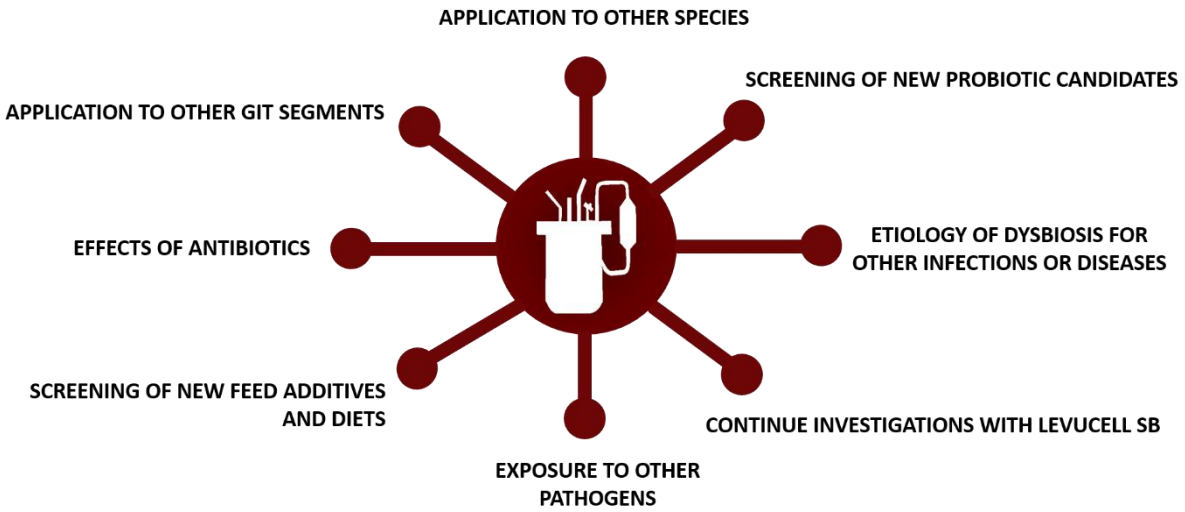


Figure 3. Example of possible future investigations using the newly designed MPigut-IVM.

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Appendix

During this PhD project, a participation to a published review focusing on the experimental models to study intestinal microbes-mucus interactions, a topic close to the subject of this PhD, was also provided. This review is presented here after.

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REVIEW ARTICLE

Experimental models to study intestinal microbes–mucus interactions in health and disease

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One sentence summary: The review summarises the state of the art for studying gut microbes-mucus interactions using *in vitro*, *ex vivo* and *in vivo* experimental models.

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ABSTRACT

A close symbiotic relationship exists between the intestinal microbiota and its host. A critical component of gut homeostasis is the presence of a mucus layer covering the gastrointestinal tract. Mucus is a viscoelastic gel at the interface between the luminal content and the host tissue that provides a habitat to the gut microbiota and protects the intestinal epithelium. The review starts by setting up the biological context underpinning the need for experimental models to study gut bacteria-mucus interactions in the digestive environment. We provide an overview of the structure and function of intestinal mucus and mucins, their interactions with intestinal bacteria (including commensal, probiotics and pathogenic microorganisms) and their role in modulating health and disease states. We then describe the characteristics and potentials of experimental models currently available to study the mechanisms underpinning the interaction of mucus with gut microbes, including *in vitro*, *ex vivo* and *in vivo* models. We then discuss the limitations and challenges facing this field of research.

Keywords: intestinal mucus; gut microbiota; experimental models; mucin *O*-glycosylation

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INTRODUCTION

The human gastrointestinal (GI) tract harbours a complex and diverse community of microbes, including 10 trillion of microorganisms, collectively referred to as the gut microbiota (Sender, Fuchs and Milo 2016). Several regulatory mechanisms cooperate to maintain intestinal homeostasis and a disturbance of the relationship between the gut microbiota and the host can result in several disorders including chronic inflammatory diseases and metabolic syndromes (Rooks and Garrett 2016). While the intestinal microbiota provides important benefits to the host, such as calorie extraction and immune system maturation, it also holds the power to activate various innate and adaptive immune signalling which can lead to uncontrolled and deleterious intestinal inflammation (Pickard *et al.* 2017). A key component in maintaining a beneficial relationship between the commensal microbes inhabiting the intestine and the host is the presence of an appropriate barrier that prevents bacteria to reach and persist on the epithelial surface (Johansson and Hansson 2016; Sicard *et al.* 2017; Bretin, Gewirtz and Chassaing 2018). It is well acknowledged that intestinal epithelial cells (IECs) provide a physical and biochemical barrier that prevents the translocation of commensal bacteria to the underlying host tissue. In addition, there is an emerging paradigm that the mucus layer is an important modulator of human health in mediating the homeostatic relationship between the gut microbiota and the host. On the luminal side, the mucus layer provides the first physical, chemical and biological line of defence against large particles, including commensal bacteria and invading pathogens, segregating them from IECs (Turner 2009; Peterson and Artis 2014). Furthermore, mucus provides a biological niche for a microbial community, referred to as mucus-associated microbiota, which is likely to have a major influence on human health (Martens, Neumann and Desai 2018). However, advances in this field of research have been hampered by the lack of suitable model systems recapitulating all the interactions occurring at the mucosal interface. This review provides an overview of currently available experimental models to study the interplay between gut bacteria and intestinal mucus at a mechanistic level, and summarizes their main applications and the challenges remaining in this field of research.

OVERVIEW OF MUCUS STRUCTURE AND FUNCTION IN THE GASTROINTESTINAL (GI) TRACT

Mucus structural organisation

Mucus structure

Mucus is a highly hydrated gel made up of more than 98% water that makes it totally transparent, microscopically invisible and difficult to study. This aqueous viscoelastic secretion also contains electrolytes, lipids and various proteins (Bansil and Turner 2018). Mucus is found throughout the entire GI tract from the stomach to the large intestine, with its thickness and structure varying depending on the location, reflecting its various protective functions.

The mucus in the small intestine consists of one layer, while the stomach and colon have a bi-layered mucus. In human stomach, the mucus is about 200–400 μm in thickness and consists of an inner layer loosely attached to the epithelial surface, keeping the surface neutral (pH 7) while the gastric lumen pH is acidic (pH 2), and an outer layer which is mobile on the luminal side. Only few bacteria have evolved strategies to colonise the stomach, among which *Helicobacter pylori* is a specialist (Atuma *et al.* 2001; Juge 2012). In the small intestine, mucus fills up the space between the villi but is not attached to the epithelium and is somewhat permeable to bacteria (Atuma *et al.* 2001). In the colon, the two layers mediate opposite interactions with the microbiota; whereas the outer layer (up to 800 μm) is densely colonised by an important microbial biomass, the inner layer (> 200 μm in humans) is virtually devoid of bacteria leaving a space virtually free of microbes (commensals and or pathogens) leaving a space virtually free of microbes above the epithelium (Johansson, Sjovall and Hansson 2013). However, single-cell imaging at tissue scale in mice revealed the presence of bacteria in close proximity of the epithelium (Earle *et al.* 2015). Among commensal microorganisms, Segmented Filamentous Bacteria (SFB) are immunomodulatory commensals with the ability to adhere to IECs and to invade this mucus layer without invading the host (Hedblom *et al.* 2018; Ladinsky *et al.* 2019). Of note, a recent study revealed differences in mucus organization between the proximal and distal colon of rodents (Kamphuis *et al.* 2017): in the later, the mucus layer is attached to the faecal pellet and absent from the surface of the epithelium (Kamphuis *et al.* 2017).

Other studies demonstrated that the mucus thickens as the microbiota become more diverse, as particularly evident in the colon (Jakobsson *et al.* 2015). This is also supported by studies using germ free mice showing an impairment in mucus structure (Johansson *et al.* 2008; Johansson, Sjovall and Hansson 2013; Jakobsson *et al.* 2015). Gnotobiotic mice colonized with human faecal microbiota present a mucus layer structure resembling that of conventional mice by day 7 post-colonization (Hayes *et al.* 2018). Animals housed in distinct rooms of the same animal facility exhibit distinct microbiota profiles that are associated with large differences in the inner colon mucus layer, thereby affecting mucus barrier properties (Jakobsson *et al.* 2015). Also, it has been demonstrated in mice that mucus becomes thinner with age (Elderman *et al.* 2017). Variations in the mucus thickness and spatial organisation of the gut microbiota in mice were also found to be dependent of the diet (Earle *et al.* 2015). Interestingly, the thickness of the mucus layer has been shown to undergo circadian fluctuations, with highest microbial proximity to the mucosal surface during the dark phase (Thaiss *et al.* 2016).

Mucus secretion

The mucus is produced and secreted by specialized cells namely goblet cells located in the crypt in the small intestine and in higher numbers in the upper crypt in the colon (Johansson and Hansson 2013; Johansson and Hansson 2016; Sicard *et al.* 2017). Before secretion in the gut lumen, mucin polymers are stored in mucin granulae within the goblet cells (Johansson, Larsson and Hansson 2011; Johansson, Sjovall and Hansson 2013). The function of goblet cells varies depending on their localisation in the small intestinal or colonic crypts (Pelaseyed *et al.* 2014). Apart from their role in secreting mucus, small intestinal goblet cells can play a role in delivering luminal material to the immune system (Pelaseyed *et al.* 2014). Interestingly, a study from Gunnar Hanson's laboratory identified a subpopulation of goblet cells called 'sentinels' goblet cells (Birchenough *et al.* 2016). These cells are able to sense Toll-like receptor (TLR) microbial ligands at the entrance of colonic crypts and trigger the activation of

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NLRP6 inflammasome, leading to mucus secretion from neighbouring goblet cells to defend the colon against bacterial invasion (Birchenough *et al.* 2016).

Renewal of the mucus is an important factor to preserve epithelial damage and bacterial exposure. The colonic mucus has a rapid turnover, since the inner mucus layer is renewed within 1 hour (Johansson 2012), while the gut epithelium renewal takes around 4–5 days (De Weirdt and Van de Wiele 2015).

Gastro-intestinal mucins

The main structural components of mucus are large glycoproteins called mucins. The protein sequences of mucin domains share a common core structure rich in the amino acids prolin (P), threonine (T) and serine (S) called the PTS domain. These domains are then decorated by *O*-linked glycans made up of *N*- acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal) and usually terminated by sialic acid and fucose (Juge 2012; Johansson and Hansson 2016; Sicard *et al.* 2017). These *O*-glycans render the mucin domains highly resistant to protease degradation and confer mucins their high-water binding capacity.

Mucins are produced as transmembrane mucins or secreted gel-forming mucins (Juge 2012; Johansson, Sjovall and Hansson 2013). In the stomach, MUC1 and MUC5AC are produced by the superficial epithelium, while MUC6 are secreted by the stomach glands (Johansson, Sjovall and Hansson 2013; Johansson and Hansson 2016). In the small intestine and colon, mucus is structurally built around the mucin-2 glycoprotein (MUC2). The folding and dimerization of MUC2 is a demanding process owing to the large number of disulfide bonds, and a defect during this process may affect the structure and function of intestinal mucus (Johansson, Larsson and Hansson 2011). Proteolytic cleavages of MUC2 catalysed by the host as well as bacteria enzymes favour the transition from firm to loose layer form and allow bacteria to penetrate into the mucin net-like structure of the outer mucus layer (Johansson *et al.* 2008). In addition to this proteolytic activity, the degradation of mucin glycan chains by bacterial glycosidases contribute to the establishment of a microbial community in the outer mucus layer (Johansson *et al.* 2008; Pelaseyed *et al.* 2014).

Mucin glycosylation

Glycosylation is the most frequent post-translational modification of proteins and can occur in *N*-linked and *O*-linked form, and *O*-glycosylation is the main modification of mucins (Arike and Hansson 2016). Mucin-type *O*-glycans are built from eight core structures, with core 1, core 2, core 3 and core 4 glycans most commonly found in intestinal mucins (Brockhausen, Schachter and Stanley 2009). *O*-glycosylation is initiated in the Golgi apparatus by the addition of a GalNAc residue to the hydroxyl group of serine and threonine of the mucin backbone. Further elongation and branching of the *O*-glycan chains is governed by a large family of glycosyltransferase enzymes (Bennett *et al.* 2012). The oligosaccharides can be further modified by addition of histo-blood group antigens (ABO, Lewis), secretor (H) epitopes and sialic acids and sulfate (Rossez *et al.* 2012; Bansil and Turner 2018). Mucin glycosylation varies along the GI tract (Robbe *et al.* 2003; Robbe *et al.* 2004; Holmen Larsson *et al.* 2013) and is linked to microbial colonization (Juge 2012; Bergstrom and Xia 2013; Tailford *et al.* 2015; Arike, Holmen-Larsson and Hansson 2017). Mounting evidence suggests that mucin glycosylation is critical to the biological and physical role played by mucus in the gut by influencing the physico-chemical properties and penetrability of mucus and by modulating the composition of the associated mucus-associated microbiota (see section 1.3). Not surprisingly, an alteration of mucin *O*-glycosylation profile has been reported in intestinal diseases associated with an impaired gut barrier function such as inflammatory bowel disease (IBD) and colorectal cancer (Larsson *et al.* 2011; Theodoratou *et al.* 2014) as also supported by work in animal models (Bergstrom *et al.* 2017) (see also section 4.5).

Mucus function in the gut

For decades, mucus has been considered to act as a simple physical barrier protecting the host, but mounting evidence suggests that mucus plays additional biological and immunological roles in maintaining gut homeostasis. The coating gel of mucus is acting, in concert with the immune system, the intestinal epithelium and the gut microbiota, to provide a physical, biological and chemical line of defence against potentially harmful invaders while harbouring a distinct microbial community having a major influence on host health.

Throughout the gut, the viscous mucus secretion acts as a lubricant that helps the progress of digestive matter along the GI tract and protects the underlying epithelium from excessive mechanical or chemical stresses. In the stomach, the mucus coating creates a pH gradient that protects the epithelium against the crude acidic gastric environment. Mucus acts as a size exclusion filter for larger compounds while selectively allowing transport of small molecules such as gases, ions, nutrients and many proteins to reach the enterocytes (De Weirdt and Van de Wiele 2015), but the mucus lining would prevent digestive enzymes from attacking these cells.

In the colon, the outer mucus layer serves as a biological habitat for various microorganisms. Indeed, the glycan structures in the mucus provide potential binding sites and constitute a carbon and energy source to support the growth of commensal but also pathogenic bacteria (Tailford *et al.* 2015) (see sections 2.1 and 2.2). It is believed that the mucin glycosylation patterns along the GI tract contribute to the microbial tropism of certain taxa in the mucus (Tailford *et al.* 2015).

The mucus layer also helps in the protection of the epithelium and, in association with the immune system, plays a crucial role in intestinal homeostasis. This gel is an important retention matrix for non-mucin proteins with immune regulatory molecules such as antimicrobial molecules (e.g. bactericidal RegIIIγ, α-defensins, secretory immunoglobulins IgAs, etc), therefore limiting the number of bacteria that can reach the epithelium and the underlying immune system (Peterson and Artis 2014; Johansson and Hansson 2016). This physical and biological barrier helps to keep the tremendous amount of bacteria that reside in the lumen as well as enteric pathogens at a safe distance from the epithelium (Chassaing, Ley and Gewirtz 2014; Johansson *et al.* 2014; Chassaing *et al.* 2015a). However, this system can be subverted and invading pathogens or pathobionts have evolved strategies to circumvent this barrier by e.g. degrading mucins and/or influencing mucin secretion (Rolhion and Chassaing 2016). In summary, mucus has a dual role in relation to the gut microbiota, it is an ecological niche for bacteria by providing adhesion sites and nutrients, while protecting the underlying epithelium from microbial aggressors that can breach this barrier.

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The mucus-associated microbiota

The gut microbiota composition is known to differ along the longitudinal axis of the GI tract but it also varies transversally from the lumen to the mucosa due differences in key physiological parameters such as nutrient availability or oxygen gradient. The colonic epithelium is made of crypts with specific oxygen conditions and various concentrations of glycans that is a niche for mucin-degrading bacteria such as *Bacteroides fragilis* (Pereira and Berry 2017). The use of Carnoy fixative to preserve the mucus layer has been a crucial step for the detection of bacteria in the mucosal environment (Johansson *et al.* 2008). It is now well appreciated that the faecal microbiota community differs from the luminal, mucosa- or mucus-associated bacterial communities (Swidsinski *et al.* 2005; Li *et al.* 2015).

Studies in humans demonstrated that the abundance of Bacteroidetes appears to be higher in faecal/luminal samples than in the mucosa (Eckburg *et al.* 2005). Members of Firmicutes phylum and in particular *Clostridium* cluster XIVa are significantly enriched in the mucus layer compared to the lumen (Van den Abbeele *et al.* 2013). Analysis of human colonic biopsies have also shown a distinct mucosal community enriched in Actinobacteria and Proteobacteria compared to the luminal community (Albenberg *et al.* 2014). Certain species such as *Bacteroides acidifaciens*, *B. fragilis* and *Akkermansia muciniphila* are enriched in the outer layer of colon mucus (Derrien *et al.* 2004; Donaldson, Lee and Mazmanian 2016).

Similar findings have been observed in animals. Indeed, mice studies have shown that Firmicutes were enriched in the mucosa-associated microbiota, especially members of the Lachnospiraceae and Ruminococcaceae families (Tailford *et al.* 2015). Bacterial species such as *Bacteroides thetaiotaomicron* or *Escherichia coli* display specific genomic repertoires to persist in the outer mucus layer compared with the same species in the intestinal lumen (Li *et al.* 2015). This spatial localisation may be reflective of the radial oxygen gradient that shapes the mucus-associated and faecal microbiota, since oxygen can favour or impede certain microorganisms (Albenberg *et al.* 2014). Moreover, laser capture microdissection (LCM) in combination with metagenomics studies provided new insights into the composition of the mucus-associated microbiota (Wang *et al.* 2010). The use of LCM in mouse models revealed that this microbial community is especially dominated by Acinetobacter in the colonic crypts (Pedron *et al.* 2012). Using LCM coupled to DNA sequencing based analysis, Chassaing and Gewirtz recently reported profound differences at the phyla level between the inner mucus communities comprising 20% – 60% Proteobacteria and a concomitantly marked reduction in Bacteroidetes as compared to faecal microbiota (Chassaing and Gewirtz 2019).

Due to a high polysaccharide content (up to 80% of the mucin biomass), mucus provides an ecological niche for the intestinal microbiota. Mucus-associated bacteria are able to use oligosaccharides from mucins as binding sites through specific bacterial adhesins that promote their colonisation (Section 2.1) or as an energy source to support their growth (Section 2.2). Robbe and colleagues first suggested that the important repertoire of potential ligands and/or carbon sources in mucins could explain the pattern of bacterial colonisation in the different gut regions (Robbe *et al.* 2004). Mucin degradation has been extensively studied in pathogenic bacteria and more recently investigated in commensal bacteria including *A. muciniphila*, *Bacteroides* spp., Bifidobacteria and *Ruminococcus* spp. (Derrien *et al.* 2004; De Weirdt and Van de Wiele 2015). A disproportion of bacterial taxa able to invade mucus could further play a role in the development of the dysbiotic microbiota associated with the onset of various intestinal diseases (see section 3).

MUCIN-BACTERIA INTERACTIONS

Mechanisms of mucin binding by commensal and pathogenic microorganisms in the gut

Cell-surface proteins of pathogens and probiotics/commensal strains have been implicated in mediating the binding of microbes to intestinal mucus (Fig. 1). These include (i) specialized cell-surface adhesins or lectins, (ii) appendages such as pili and flagella or (iii) moonlighting proteins (see (Juge 2012) for a review). In particular, a considerable amount of research has been devoted to the characterization of these adhesins in *Lactobacillus* species (as extensively reviewed in (Van Tassell and Miller 2011; Nishiyama, Sugiyama and Mukai 2016)).

Mucus binding proteins

Mucus-binding proteins (MUBs) containing a variable number of Mub repeats are unique to gut inhabiting Lactobacilli and these proteins have been thoroughly characterised in *Lactobacillus reuteri*, a gram-positive bacterial species inhabiting the GI tract widely used as a probiotic (Frese *et al.* 2011). MUB from *L. reuteri* ATCC 53608 is one of the best-studied examples of mucus adhesins in commensal bacteria. It is a large protein consisting of six type 1 repeats (Mub1) and eight type 2 repeats (Mub2) with each repeat divided into a mucin binding (MucBP) domain and an immunoglobulin binding protein domain (Kuznetsova ; MacKenzie *et al.* 2009; Etzold *et al.* 2014b). The Mub repeats mediate binding to mucin glycans, through interactions with terminal sialic acid (Etzold *et al.* 2014a; Gunning *et al.* 2016), and Igs (MacKenzie *et al.* 2009). MUB has the shape of a long, fibre-like structure, of around 180 nm in length (Etzold *et al.* 2014b), and forms appendices reminiscent to pili found in pathogenic and, more rarely, other commensal bacterial species. However, in contrast to pathogenic pili which adhesin is restricted to the N-terminal tip, MUB interactions with mucin glycans occur through its long and linear multi-repeat structure, as shown by atomic force spectroscopy (Gunning *et al.* 2016). This multivalent binding would restrict penetration through mucus and limit access of the bacteria to the epithelium surface. In addition, MUB from *L. reuteri* ATCC 53608 was recently shown to modulate inflammatory responses in human monocyte-derived dendritic cells *via* interaction with DC-SIGN (Bene *et al.* 2017). The presence of mucus adhesins was also shown to mediate the binding of *L. reuteri* strains to both HT-29 and mucus-producing LS174T cells. The binding of *L. reuteri* to mucus led to a decreased enteropathogenic *E. coli* (EPEC) adherence to small intestinal biopsy epithelium (Walsham *et al.* 2016). Recombinant Mub proteins containing Mubs5s6 domains from Lp-1643 protein of *L. plantarum* Lp9 have been shown to adhere to human intestinal tissue sections (Singh *et al.* 2017) and inhibited the adhesion of enterotoxigenic *E. coli* (ETEC) to cultured intestinal HT-29 and Caco-2 cell lines, probably through the recognition of cell-surface mucins (Singh *et al.* 2018). Together, these findings show that the nature and function of these adhesins are strain-specific with the potential to target either the epithelium or the mucus layer and compete with pathogens.

Flagella

Several microorganisms have evolved strategies, in particular extracellular appendages such as flagella, pili and fimbriae, to

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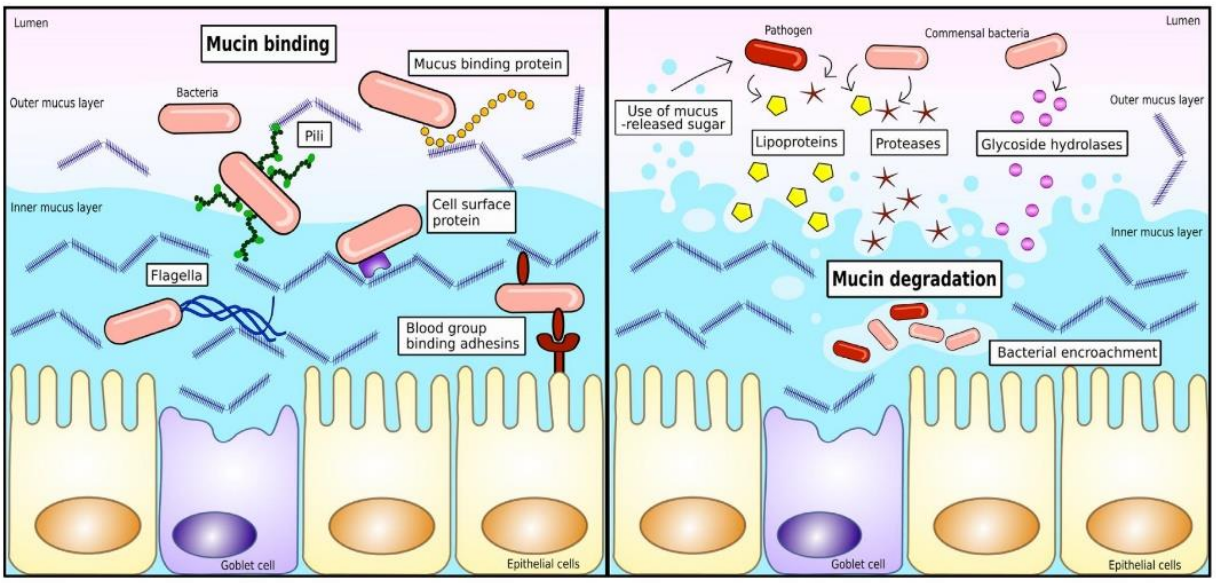

Figure 1. Mucin-bacterial interactions in the digestive tract.

Left panel: Mucins display various and diverse oligosaccharide structures representing potential binding sites for microbial adhesion. Commensal and pathogenic microbes can use cell-surface appendages, such as pili, flagella or fimbriae or adhesins to bind to mucus.

Right panel: Mucin glycans are an important energy source for microbes inhabiting the mucus niche that further confer them with an ecological advantage over other members of the gut microbiota. Commensal and pathogenic microorganisms can degrade mucin glycan chains leading to the release of mono- or oligosaccharides from that can be subsequently metabolized by other gut microbes in the mucosal environment.

attach to and to penetrate the mucus layer (Juge 2012). Pili and flagella are large polymeric proteins that form long surface structures involved in bacterial adhesion. Flagella are composed of several thousand copies of flagellin subunits and have been extensively studied in EPEC and enterohemorrhagic *E. coli* (EHEC) for their role in virulence and motility, but their role in mucus binding remains unclear. The adhesive properties of bacterial flagella to mucus were previously reported for *Clostridium difficile* where crude flagella, recombinant flagellar FliC and FliD proteins were shown to bind to murine mucus (Tasteyre *et al.* 2001). In pathogenic *E. coli* strains, the H6 and H7 flagella EPEC E2348/69 and EHEC EDL933 and their flagellin monomers were shown to bind to mucins and to bovine mucus (Erdem *et al.* 2007). Further studies then showed that EPEC and EHEC O157:H7 adherence to HT-29 cells is related to mucin-type core 2 *O*-glycan, facilitating invasion into host cells (Ye *et al.* 2015; Ye *et al.* 2015). However, flagella are involved in the ability of these pathogenic strains to cross the mucus layer, conferring a selective advantage in penetrating the mucus layers and reaching the epithelial surface, as demonstrated with Adherent-Invasive *E. coli* (AIEC) LF82 (G368). It is therefore tempting to speculate that in EPEC and EHEC, the flagella have a preference for cell-surface mucins rather than secreted mucus, in line with their ability to penetrate the mucus layer and attach onto the cell surface before invasion. In the probiotic *E. coli* strain Nissle 1917, a direct interaction was observed between isolated flagella from EcN and porcine MUC2 and human mucus but not murine mucus. The mucus component gluconate was identified as one receptor for the binding of EcN flagella (Troge *et al.* 2012). EcN was therefore proposed to confer the probiotic strain the ability to compete for binding sites on host tissue with bacterial pathogens.

Pili

Pili have been identified in *Lactobacillus rhamnosus* GG where they confer binding to mucus (Kankainen *et al.* 2009; von Ossowski *et al.* 2011) and are predicted to exist in other *Lactobacillus* species including *L. casei* and *L. paracasei*, based on genomics analyses (Douillard *et al.* 2013; Aleksandrzak-Piekarczyk *et al.* 2015; Nissila *et al.* 2017). In *L. rhamnosus* GG, these are composed of a three-protein complex SpaCBA, which has been involved in adhesion to mucus, IECs, and immunomodulatory interactions with IEC (Lebeer *et al.* 2012; von Ossowski *et al.* 2013; Ganguli *et al.* 2015; Vargas Garcia *et al.* 2015; Bene *et al.* 2017). The mucus-binding pili of *L. rhamnosus* GG shares immunological and functional similarities with those of the clinical *Enterococcus faecium* strain E1165. The binding of *E. faecium* E1165 to mucus could be prevented by the addition of the mucus-binding SpaC protein or antibodies against *L. rhamnosus* GG (Tytgat *et al.* 2016). Collectively, these studies show the potential of using mucus adhesins from probiotic strains to prevent the binding of enteric pathogens to the host.

Although not a resident member of the gut microbiota, several *Lactococcus lactis* strains have also been shown to exhibit mucus-binding properties through bacterial surface proteins such as mucin-binding proteins and pili (as recently reviewed in (Mercier-Bonin and Chapot-Chartier 2017)). The mechanisms of adhesion have been extensively studied by atomic force spectroscopy demonstrating a comparable role played by these two surface proteinaceous components in adhesion of *L. lactis* TIL448 to pig gastric mucin (PGM) neutral oligosaccharides under static conditions, whereas a more important contribution of the MUBs than the pili one was observed under shear flow (Le *et al.* 2013).

Other cell surface proteins

Other cell surface proteins implicated in the binding of commensal bacteria to mucin include aggregation-promoting factors (APFs) from *L. plantarum* NCIMB 8826 (Bolonkin 1990) or *L. lactis* (Lukic *et al.* 2012; Lukic *et al.* 2014), mucus-binding protein A (CmbA) from *L. reuteri* ATCC PTA 6475 (Etzold *et al.*

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2014a; Jensen *et al.* 2014), Lam29 from *L. mucosae* ME-340 (Watanabe *et al.* 2010), mucus adhesion-promoting protein (MapA) from *L. fermentum/reuteri* 104R (Rojas, Ascencio and Conway 2002), a mucus-binding factor (MBF) from *L. rhamnosus* GG (von Ossowski *et al.* 2011; Nishiyama *et al.* 2015), a MucBP-containing mannose-specific adhesin protein (Msa) from *L. plantarum* WCFS-1 (Pretzer *et al.* 2005), a 32-Mmubp from *L. fermentum* BCS87 (Macias-Rodriguez *et al.* 2009), an extracellular transaldolase (Tal) from *Bifidobacterium bifidum* DSM20456 (Gonzalez-Rodriguez *et al.* 2012) and a recently-characterised serine rich repeat protein (SRRP) from *L. reuteri* ATCC 53 608 (Sequeira *et al.* 2018). It is expected that adhesion of these commensal or probiotic bacteria to mucus may favour their persistence within the gut in order to exert their beneficial effects to the host. Furthermore, it was recently suggested that carbohydrate binding modules (CBMs) appended to glycoside hydrolases could contribute to the tropism of gut bacteria to glycan-rich area of mucins in the colon, as shown for *Ruminococcus gnavus* sialic-acid-specific CBM40 (Owen *et al.* 2017).

Blood group binding adhesins

In addition, several human enteric pathogens bind to human histo-blood group antigens (HBGAs) expressed on the gut mucosa, including *Campylobacter jejuni*, *Norwalk virus* and *H. pylori*. The role of HBGA recognition to mucin binding has been extensively studied in the gastric pathogen *H. pylori* where *Helicobacter* adhesins have been reported to play a critical role in the attachment of the pathogen to both the glycosylated gastric epithelial cell surface and to glycosylated mucins. The binding of *H. pylori* to gastric mucins through blood group binding adhesin (BabA) and sialic acid-binding adhesin (SabA) revealed a complex charge/low pH-dependent mechanism involving four modes of *H. pylori* adhesion to MUC5B, MUC7 and MUC5AC mucins (Linden *et al.* 2008; Skoog *et al.* 2017). More recently, a novel outer membrane protein adhesin named LabA has been identified in *H. pylori* and shown to bind to LacdiNAc, a structure, which is also expressed on MUC5AC (Rossez *et al.* 2014). Binding of *H. pylori* to gastric mucins therefore is determined both by the mucin glycosylation and also by the adhesins expressed by individual strains. A chitin-binding protein GbpA from *Vibrio cholerae* shown to bind to N-acetyl-D-glucosamine residues of intestinal mucin has been proposed as an important factor mediating intestinal colonisation and pathogenesis by *V. cholerae* (Bhowmick *et al.* 2008; Wong *et al.* 2012).

Moonlighting proteins

Unexpectedly, several primarily cytoplasmic proteins have been reported to play a role in mucin binding. Due to their dual function, these proteins are referred to as moonlighting proteins (Henderson and Martin 2011; Henderson and Martin 2013; Henderson 2014). In *L. acidophilus*, *L. plantarum* and *Mycoplasma genitalium* for instance, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was clearly demonstrated to play a role in bacterial adhesion and bind mucins (Alvarez, Blaylock and Baseman 2003; Kinoshita *et al.* 2008; Patel *et al.* 2016). While the exact domain responsible for mucin binding remains to be elucidated, GAPDH is suggested to play a similar role in other commensal or pathogenic microorganisms (Kinoshita *et al.* 2013). In *L. reuteri*, elongation factor-Tu (EF-Tu) was found to bind the PGM when exposed at the bacterial cell surface (Nishiyama *et al.* 2013). Here, the sulfated carbohydrate moieties of mucins were demonstrated to play a significant role in EF-Tu-mediated bacterial adhesion to PGM and mucosal surfaces (Nishiyama *et al.* 2013). Proteosurfaceome analyses in a range of microorganisms have revealed a large repertoire of cytoplasmic proteins present at the bacterial cell surface but their implications in binding to various extracellular matrix (ECM) proteins, including mucins, remain to be more systematically investigated (Chagnot *et al.* 2012; Desvaux, Candela and Serror 2018).

Mechanisms of mucin degradation by commensal and pathogenic microorganisms in the gut

Several enzymatic activities are required for the degradation of mucins by pathogens or commensal bacteria including glycoside hydrolases (GHs), sulfatases, or proteases (Fig. 1) as described below.

Glycoside hydrolases

Mucin glycan degradation in bacteria relies on the expression of GHs such as sialidases (GH33), α-fucosidases (GH29, GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, GH42), α-N-acetylglucosaminidases (GH89), endo-β1,4-galactosidases (GH98) and α-N-acetylgalactosaminidases (GH101, GH129) (www.cazy.org). These enzymes have been functionally characterised in resident members of the gut microbiota able to forage on mucins, including *A. muciniphila*, *B. thetaiotaomicron*, *B. bifidum*, *B. fragilis* and *R. gnavus*, as recently reviewed (Tailford *et al.* 2015; Ndeh and Gilbert 2018). The released mono- or oligosaccharides derived from mucus degradation by these commensal bacteria can be utilised by the bacteria itself or scavenged by other bacteria inhabiting the mucus niche including pathogenic species such as *Salmonella* species, *C. difficile*, diarrhoeagenic *E. coli* or *Vibrio cholerae* through cross-feeding interactions (Fabich *et al.* 2008; Abyzov *et al.* 2012; Ng *et al.* 2013). In addition, some of these pathogens have the glycolytic potential to release mucus-derived sugars for their own consumption (Mondal *et al.* 2014; Arabyan *et al.* 2016).

Sulfatases

Sulfatases are being increasingly investigated for their role in modulating the gut microbial ecosystem in health and disease. Some members of the gut microbiota such as *B. thetaiotaomicron*, *Bacteroides ovatus* and Prevotella sp. strain RS2 *Bifidobacterium breve* UCC2003, or *B. fragilis* possess mucin-desulfating sulfatases or glycosulfatases (Salyers *et al.* 1977; Berteau *et al.* 2006; Benjdia *et al.* 2011; Egan *et al.* 2016; Praharaj *et al.* 2018). Mucin sulfatase activity of these species may provide them a competitive advantage in the infant gut and/or the adult gut. The mucin-desulfating sulfatases that have been characterised so far include sulfatases specific for the -D-galactopyranosyl 3-sulfate, -Dgalactopyranosyl6-sulfate and 2-acetamido-2-deoxy-D-glucopyranosyl6-sulfate (6-SO3-GlcNAc) building blocks of the oligosaccharide chains. GlcNAc-6-S can be found in terminal or branched positions of mucin oligosaccharide. The desulfation of mucin by bacterial sulfatases may be a rate-limiting step in mucin-degradation mechanism, allowing glycosidases to access and act on the mucins by other members of the gut microbiota. The release of sulfate from mucins may also contribute to the expansion of Sulfate-reducing bacteria (SRB) in the gut (Rey *et al.* 2013). SRB are able to produce hydrogen sulfide (H_2S) which can reduce disulfide bonds present in the mucus network, leading to mucus erosion and access of bacteria to the epithelium, therefore contributing to epithelial damage and inflammation This mechanism has been proposed to be involved in the aetiology and/or severity of IBD (Ijssennagger, van der Meer and van Mil 2016). In addition, Hickey and colleagues showed that sulfatases

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of *B. thetaiotaomicron* are required for its outer membrane vesicles to transit to underlying host immune cells and cause colitis (Chatzidaki-Livanis and Comstock 2015). Together these data highlight the complex role of bacterial sulfatases in the gut.

Proteases

Bacterial proteases from commensal or pathogenic *E. coli* have also been implicated in the recognition and degradation of mucins. In EHEC, StcE (secreted protease of C1 esterase inhibitor from EHEC) was originally described as specifically cleaving C1 esterase inhibitor (C1-INH) (Lathem *et al.* 2002; Grys, Walters and Welch 2006) but later showed to be even more active against MUC7 (Lathem *et al.* 2002). This soluble enzyme is important in reducing mucin levels. StcE has been suggested to have a dual role during human infection, (i) by promoting the penetration of bacterial cells through the mucus barrier lining the GI tract and thus facilitating the intimate EHEC adherence to IECs, which is an essential step in colonisation (Hews *et al.* 2017), and (ii) by acting as an anti-inflammatory agent protecting bacterial and host cell surfaces from complement-mediated lysis (Grys *et al.* 2005; Abreu and Barbosa 2017). StcE is secreted by a Type II, subtype a, secretion system (T2aSS) (Monteiro *et al.* 2016; Hay *et al.* 2018). This mucinase is a metalloprotease belonging to the peptidase M66 family (IPR019503) carrying one zinc atom per protein but no structural calcium, which is a reported feature of metalloproteases (Yu, Worrall and Strynadka 2012). Recently, EHEC StcE metalloprotease was shown to reduce the inner mucus layer in human colonic mucosal biopsies and the MUC2 glycoprotein levels in mucin-producing LS174T colon carcinoma cells (Hews *et al.* 2017).

Pic (protein involved in intestinal colonisation), also previously known as Shmu (*Shigella* mucinase), is a secreted protease identified in *Shigella flexneri* and enteroaggregative *E. coli* (EAEC) (Henderson *et al.* 1999a). Pic is secreted by a Type V, subtype a, secretion system (T5aSS) and belongs to the subfamily of serine protease autotransporters (SPATEs), with a catalytic domain corresponding to the peptidase S6 family (IPR030396). This enzyme was reported to display proteolytic activity against gelatin as well as bovine and murine mucin but not hog gastric mucin (Henderson *et al.* 1999a). PicU was also shown to exhibit mucinolytic activity in uropathogenic *E. coli* (Parham *et al.* 2004).

Hbp (hemoglobin-binding protease), also previously known as Tsh (temperature-sensitive haemagglutinin), is capable of cleaving bovine submaxillary mucin but not hog gastric mucin, which so far would appear as a feature of mucinolytic serine protease autotransporter of Enterobacteriaceae (SPATE) of the peptidase S6 family (Dutta *et al.* 2002). In some EHEC strains, a SPATE of the peptidase S6 family exhibiting mucinolytic activity was identified on plasmid pO113, namely EpeA (EHEC plasmid-encoded autotransporter) (Leyton *et al.* 2003). In AIEC, a Vat (vacuolating autotransporter) homologue belonging to the SPATE of the peptidase S6 family was demonstrated to exhibit a mucinolytic activity (Gibold *et al.* 2016). Vat-AIEC appears to significantly contribute to the colonisation ability of AIEC by decreasing mucus viscosity as well as enhancing bacterial penetration in mucus and access to IECs (Gibold *et al.* 2016).

In some non-O157 EHEC strains, a subtilase cytotoxin (SubAB) was identified (Paton *et al.* 2006; Wang, Paton and Paton 2007) and appeared to contribute to mucin depletion as shown with a Shiga-toxin encoding *E. coli* (STEC) O113:H21 strain (Gerhardt *et al.* 2013). While the A subunit harbours the enzymatic activity with a subtilase-like serine protease domain belonging to the peptidase S8/S53 family (IPR000209), the mucinolytic activity of SubAB remains to be clearly established.

Other proteins have been described in *V. cholerae*. Among them, TagA is a secreted protease of *V. cholerae* that specifically cleaves mucin glycoproteins (Szabady *et al.* 2011). The *V. cholerae* extracellular chitinase ChiA2 secreted in the intestine hydrolyzes intestinal mucin to release GlcNAc, and the released sugar is successfully utilized by *V. cholerae* for growth and survival in the host intestine (Mondal *et al.* 2014).

SslE (secreted and surface associated lipoprotein), previously known as YghJ, is a secreted and cell-surface lipoprotein degrading the major mucins in the small intestine, namely MUC2 and MUC3, thus facilitating bacterial penetration of the mucus layer and ultimately adhesion to host cells (Luo *et al.* 2014; Valeri *et al.* 2015; Tapader, Bose and Pal 2017). SslE is secreted via a T2aSS and appears inactive against the mucin-like CD43, bovine submaxillary mucin, gelatin, or IgG (Luo *et al.* 2014). This Zn-metalloprotease, belonging to the peptidase M60 family (IPR031161), is found in pathogenic and commensal *E. coli*, including ETEC, EHEC O104:H4, *E. coli* SE-11 or Nissle 1917 strains. AcfD (accessory colonisation factor D) from *V. cholerae* is homologous to SslE but its putative mucinolytic activity remains to be investigated (Peterson and Mekalanos 1988). Of note, SslE is also considered as a relevant target for the development of vaccines against intestinal pathogenic *E. coli* (Nesta *et al.* 2014; Naili *et al.* 2016; Naili *et al.* 2017).

Importance of mucus-bacteria interactions in health and disease

In the colon, the outer mucus layer offers a niche to commensal bacteria by providing preferential binding sites (Section 2.1) and nutrients (Section 2.2). Due to its proximity to host cells and the immune system, the mucus-associated microbiota, sometimes also referred to as the mucobiome (Belzer *et al.* 2017), has been proposed as an important modulator of health. The integrity of the mucosa relies on a combination of factors including the gut microbiota composition, the diet and host genetic factors (Fig. 2) (Martens, Neumann and Desai 2018). The mucus and mucus-associated bacterial community play a key role in limiting access of invading pathogens to the underlying epithelial cells and in limiting the progression of intestinal and extra-intestinal diseases (Donaldson, Lee and Mazmanian 2016).

Effect of bacteria and bacterial products on mucus production

A number of animal studies (using antibiotic-treated, germ-free or gnotobiotic mice) suggest that the presence of bacteria triggers the development of the protective mucus layer. Mice treated with the antibiotic metronidazole, but not streptomycin, display an altered goblet cell function and thinning of the inner mucus layer (Wlodarska *et al.* 2011). However, another study reported that depletion of the intestinal microbiota following a 3 week-antibiotic period (cocktail of four antibiotics) did not modify mucus penetrability (Johansson *et al.* 2015).

Compared to conventionally housed animals, germ-free mice have fewer goblet cells, which are smaller in size (Kandori *et al.* 1996) and harbour an impaired mucus layer, indicating that the formation of the protective mucus layer depends upon the presence of bacteria (Rodriguez-Pineiro and Johansson 2015). Johansson and colleagues demonstrated that the mucus of germ-free mice displayed a significant decrease in Muc2 level and was more penetrable to bacterium-size fluorescent beads as compared to conventionally raised mice (Johansson *et al.* 2015). The gut microbiota composition of germ free animals is normalized two weeks after colonisation in terms of microbiota composition, but up to 8 weeks are needed to reach

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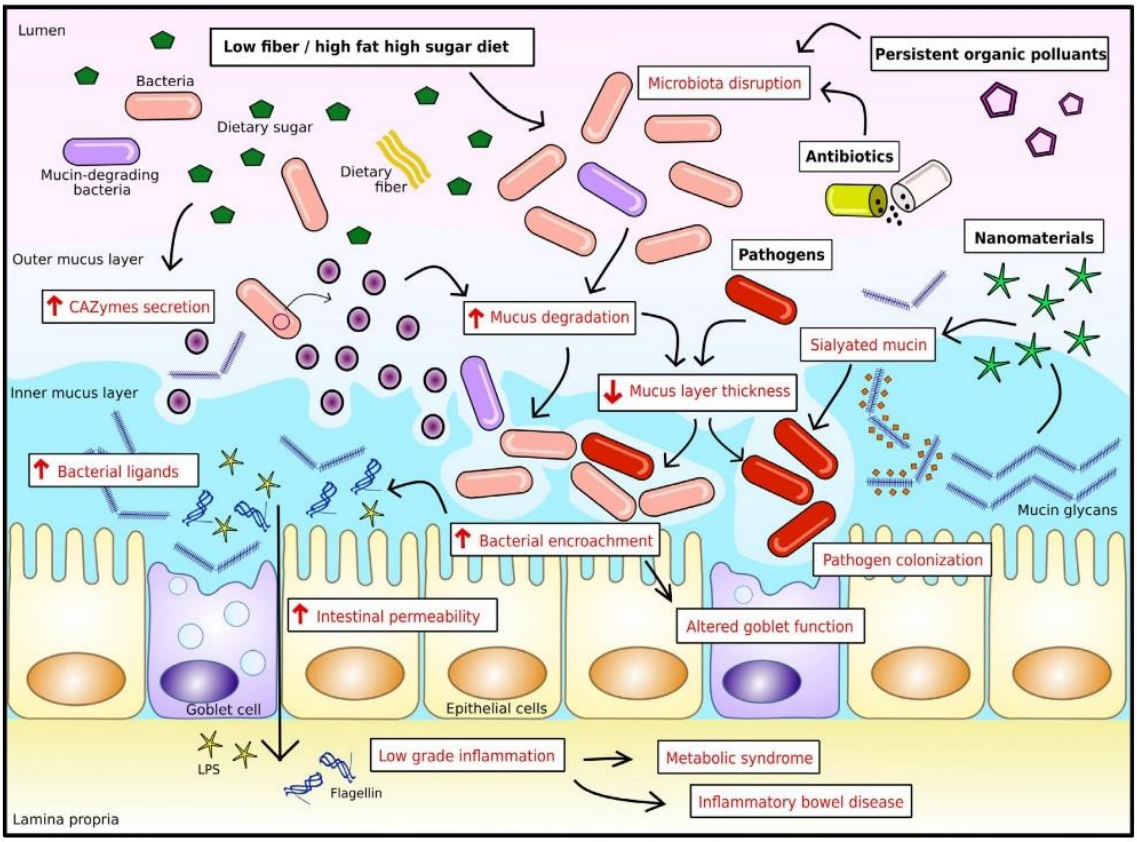

Figure 2. Perturbations of the mucus barrier in response to environmental and microbial stimuli.

This figure represents an overview of the various factors (diets, nanomaterials, pollutants, antibiotics or invading pathogens) affecting the gut microbiota composition and/or the thickness, structure and composition of the mucus barrier. Disruption of the mucus layer promotes bacterial encroachment leading to the subsequent development of low-grade inflammation, associated with inflammatory bowel diseases and metabolic disorders.

a normalized mucus phenotype (Johansson *et al.* 2015; Hayes *et al.* 2018). In support of this, fortification of the mucus layer and increased diversity of mucin glycosylation was observed within 48 hours of human intestinal organoid colonization with human-derived, non-pathogenic *E. coli* (Hill *et al.* 2017). Some bacteria, in particular Anaerostipes, have been shown to display mucus-stimulating properties (Jakobsson *et al.* 2015). *Lactobacillus* species can also stimulate MUC2 production and secretion by the goblet cells in the human gut (Sicard *et al.* 2017). Representative members of the two main phyla of the gut microbiota, *B. thetaiotaomicron* and *Faecalibacterium prauznitzii* can modulate goblet cell differentiation and thus mucus production (Wrzosek *et al.* 2013). A recent study showed that *Streptococcus thermophilus*, a transient food-borne bacterium, was able to induce mucus pathway in gnotobiotic rodents despite its poor capacity for mucus adhesion and mucin glycan degradation *in vitro* (Fernandez *et al.* 2018). Some of the mechanisms mediating mucin production and secretion by gut bacteria have been elucidated as described below.

Pathogen associated molecular patterns such as lipopolysaccharide (LPS) or peptidoglycan are known to induce mucus production (Petersson *et al.* 2011). LPS and flagellin purified from Gram-negative bacteria as well as lipoteichoic acid from Gram-positive bacteria have been shown to induce mucin upregulation via the Ras-signalling pathway (McNamara and Basbaum 2001). LPS also increases the production of interleukin (IL)-8 by goblet cells, which further promotes mucin secretion (Smirnova *et al.* 2003). TLR family members play an important role in mucus formation. Mice lacking the TLR adaptor protein MyD88 show a decreased production of mucus (Bhinder *et al.* 2014). Mice engineered to lack the flagellin receptor, TLR5 deficient mice, have a disorganised mucus layer and lack a well-defined inner layer when compared to wild type animals with an increase abundance of Proteobacteria in close contact with the epithelial surfaces (Carvalho *et al.* 2012; Chassaing, Ley and Gewirtz 2014; Chassaing *et al.* 2015a). Lastly, it has been shown *in vitro* using various human-derived cell lines that bacterial metabolites such as short-chain fatty acids (SCFA) and especially butyrate can stimulate MUC2 production in the absence of other energy sources (Willemsen *et al.* 2003; Gaudier *et al.* 2004). The effect of butyrate on MUC2 gene expression is mediated by epigenetic modifications (acetylation/methylation of histones) on the MUC2 promoter as demonstrated *in vitro* using human goblet cell-like LS174T cells (Burger-van Paassen *et al.* 2009). Fernandez and colleagues suggested that lactate produced by *S. thermophilus* in the GI tract could stimulate mucus production via a signalling pathway dependent of KLF4, a transcription factor involved in the differentiation of goblet cells (Fernandez *et al.* 2018). Some other bacterial effectors have been identified to mediate mucin expression and glycosylation such as small peptides from *R. gnavus* and *B. thetaiotaomicron* (see section 2).

Interactions of pathogens with mucus

The mucus barrier provides a bulwark against intestinal pathogens (Johansson, Sjovall and Hansson 2013; Sicard *et al.* 2017; Martens, Neumann and Desai 2018). The importance of

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intestinal mucus in controlling enteric infection has been widely documented in *Muc2* knockout mice (*Muc2*$^{-/-}$ mice) (see section 4.5), which do not produce mucus in the small and large intestine, thus leading to a close contact between bacteria and the epithelium. Bergstrom and colleagues reported that *Muc2*$^{-/-}$ mice exhibit an increase susceptibility to murine bacterial pathogen *Citrobacter rodentium* (Bergstrom *et al.* 2010). Likewise, Muc2 plays a crucial role in controlling *Salmonella* infection (Zarepour *et al.* 2013). In a similar way, *H. pylori* has evolved mechanisms allowing its residence in the gastric mucus layer (Moore, Boren and Solnick 2011). As previously described for bacteria, *Muc2*$^{-/-}$ mice are also more susceptible to enteric parasitic infection with *Trichuris muris* since they exhibit a delayed expulsion of the parasite compared to wild type animals (Hasnain *et al.* 2010). Clearance of parasitic infection is associated with exclusion of helminths via a TH2 cell-mediated goblet cell increase and mucus release (Artis and Grencis 2008). *Entamoeaba histolytica* also possesses lectins binding to mucins and secretes proteases responsible for the cleavage of Muc2, allowing the protozoan to invade the underlying epithelium (Lidell *et al.* 2006). Recently, a detailed investigation of the cooperative roles for colonic microbiota and Muc2 in mediating innate host defence against *E. histolytica* was carried out using *Muc2*$^{-/-}$ mice, germ free mice and mucus-secreting LS174T cells, demonstrating that mucus secretion and pro-inflammatory responses were microbiota-specific (Leon-Coria *et al.* 2018). Lastly, as shown with *S. flexneri* and *H. pylori*, some pathogenic bacteria are able to reshape mucin structures by remodelling their glycosylation pattern in a type III secretion system-dependent manner (Sperandio *et al.* 2013; Magalhaes *et al.* 2015).

Emerging data suggest that pathogenic bacteria can benefit from the capacity of commensal microorganisms to release mucin degradation products that can be used to support their proliferation within the mucus niche. For example, *B. thetaiotaomicron* can release free sialic acid from colonic mucus glycans that can be utilized by *C. difficile* and *Salmonella* Typhimurium to promote their own colonisation and persistence in the gut (Ng *et al.* 2013). Another study indicates that EHEC bacteria colonise the mucus layer within the cooperation of local bacterial communities including *B. thetaiotaomicron* and other anaerobes which are able to cleave host glycan-derived sugar and produce fucose (Pacheco *et al.* 2012). EHEC then senses fucose produced by *B. thetaiotaomicron* to control expression of its type III secretion system (Pacheco *et al.* 2012; Cameron *et al.* 2018).

Mucus-pathogen interactions have also been evidenced in the extra-digestive area. *Pseudomonas aeruginosa*, a Gram-negative-flagellated pathogen, is the main causal agent for the development of pneumonia in immunocompromised patients and patients with cystic fibrosis (CF). This infection is associated with a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate ion channel protein with a key role in protecting the small intestine from bacterial invasion. CF conducts to blockage airway, mucus hypersecretion leading to chronic bacterial lung infections and inflammation. It has been shown that *P. aeruginosa*, via LPS, upregulate MUC2 and MUC5AC gene expression contributing to the excessive mucus production and airway blockage seen in CF (Bellu *et al.* 2013).

Effect of diet on mucus

Recent evidences have demonstrated that the diet can influence the properties of colonic mucus and thereafter interfere with the gut microbiota.

Fibres

Living in symbiosis with the host, the gut microbiota depends mostly on non-digestible fibres and polysaccharides as energy source. In the absence of fibres in the diet, the gut microbiota shifts towards the utilisation of host glycans such as those provided by mucins, resulting in a thinner protective colonic mucus (Sonnenburg and Sonnenburg 2014; Earle *et al.* 2015). Accordingly, Desai and colleagues demonstrated that a low-fibre diet promotes the enrichment of mucin-degrading bacteria and the overexpression of carbohydrate-active enzymes (CAZymes) that degrade the colonic mucus barrier (Desai *et al.* 2016; Martens, Neumann and Desai 2018). In these mice fed with a deprived-fibre diet, infection with *C. rodentium* promotes greater epithelial access and lethal colitis (Desai *et al.* 2016).

A lack of fermentable fibres in the diet also leads to a reduction in epithelial cell proliferation resulting in a thin mucosa with encroached bacteria (Chassaing *et al.* 2015b). Further studies in mice reported that inulin supplementation increases the number of colonic goblet cells, which correlates with a thicker mucus layer and an increase proportion of the *Akkermansia* genus (Kleessen, Hartmann and Blaut 2003; Everard *et al.* 2013). Similarly, studies in mice showed that a diet enriched in inulin fibre prevents mucus deterioration (Schroeder *et al.* 2018). It was recently showed that inulin but not cellulose protects against diet-induced obesity by reducing microbiota encroachment in a cytokine IL-22-dependent manner (Zou *et al.* 2018), demonstrating the importance of dietary factors, especially soluble fibre, in the homeostasis of host–microbiota relationship. Considering the increased mucus foraging activity occurring when mice are fed with a low-fibre diet, a recent study showed that supplementation with probiotic bifidobacteria (*B. longum*) or prebiotic fibre (inulin) could reduce such mucus defect. Notably, administration with *B. longum* was sufficient to restore mucus growth, while administration with inulin could prevent the increase of mucus penetrability in mice fed a western style diet (WSD) (Schroeder *et al.* 2018).

Western diet and food additives

Besides fibres, other nutrients within a WSD can modulate intestinal barrier function. A WSD is a rich in saturated fats and simple carbohydrates but depleted in dietary fibres. As a result, a diet-induced obesity in mice leads to colon mucosal barrier dysfunction with a thinner mucus layer (as described above) and treatment with A. muciniphila appears to counteract this effect by improving mucus thickness (Everard *et al.* 2013). Similarly, mice fed a high-fat and high-sugar diet exhibit an increased abundance of mucin-degrading species leading to a decrease in mucus thickness (Martinez-Medina *et al.* 2014). The diet of modern societies has dramatically changed as evidenced by a steady increase in the consumption of processed foods concomitantly with an increase in the use of food additives (Chassaing *et al.* 2015a). Mice treated with dietary emulsifiers (polysorbate 80 or carboxymethylcellulose) show a reduced mucus thickness and increased gut permeability. In these animals, some bacteria appear in close contact with the epithelium. Emulsifier-treated mice have an altered microbial composition associated with increased levels of mucolytic bacteria including *R. gnavus* and a marked reduction in microbial diversity, with a bloom in *Verrucomicrobia* phyla, especially *A. muciniphila* (Chassaing *et al.* 2015a). This may further contribute to the intestinal passage of bacterial constituents such as LPS and flagellin, which participates in the development of low-grade inflammation and metabolic disorders in wild type mice and of colitis in susceptible host animals (Chassaing, Ley and Gewirtz 2014; Chassaing *et al.* 2015a;

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Chassaing *et al.* 2017b). Mice fed with diets enriched in maltodextrin, a filler and thickener used in food processing, show a reduction of *Muc2* expression, making the host more sensitive to low-grade inflammation but with no significant change in mucosa-associated microbiota (Laudisi *et al.* 2018).

Effect of food contaminants on mucus

The intestinal mucosa is increasingly appreciated as a key player in the emerging field of gut toxicology of environmental pollutants, as recently reviewed (Gillois *et al.* 2018). Human contamination mainly occurs via the oral route through consumption of food but also through polluted water and soil exposure.

Nanomaterials

The use of nanotechnology in many common consumer products, especially in food products, is growing. Scarce studies have evaluated the interactions of food nanoparticles with the microbiota and mucus (Mercier-Bonin *et al.* 2018). It was shown *in vitro* that common nanoparticles of Titanium dioxide (TiO_2) are trapped into mucus, leading to areas with a high local concentration (Talbot *et al.* 2018). Silver nanoparticles are widely used in food industry to colour the surface of confectionary and pastries. Rats fed with these particles exhibit higher numbers of goblet cells and a modification of the glycosylation pattern of mucins with a decreased proportion of sulfated mucins and an increased proportion of sialyated mucins (Jeong *et al.* 2010). Repeated silver nanoparticle-exposure may therefore produce pathological regions in the lamina propria (Jeong *et al.* 2010).

Persistent Organic Pollutants

A recent study showed that mice chronically exposed to benzo[a]pyrene (BaP) which is the most toxic member of the polycyclic aromatic hydrocarbons family display significant shifts in the composition and relative abundance of stool and mucosa-associated bacterial communities (decrease of *Verrucomicrobiaceae*, represented by *A. muciniphila*) (Ribiere *et al.* 2016). Furthermore, exposure to perfluorooctane sulfonate (PFOS, environmental contaminant used as a surfactant and repellent) in a mouse model of *C. rodentium* infection led to a significant reduction in mucin gene expression and a failure to clear the bacterial infection (Suo *et al.* 2017). Smoke exposure also significantly affects the mucosa-associated bacterial community and alters the expression of mucins in the murine gut (Allais *et al.* 2016).

Mucus and inflammatory-related diseases

Inflammatory bowel diseases (IBD)

Barrier disturbances including alterations in the thickness or composition of the intestinal mucus layer are recognized to play a crucial role in the onset of GI disorders such as Crohn disease (CD) or ulcerative colitis (UC). The mucus layer in UC patients is thinner and has an altered glycosylation profile making it more penetrable to bacteria (Johansson *et al.* 2014). To better understand the onset of IBD, several murine models of intestinal inflammation (genetically or chemically induced) have been established. The most common experimental model of colitis relies on the administration of Dextran Sodium Sulfate (DSS) in the drinking water. Mice orally administered with DSS display an inner mucus layer which is more penetrable by bacteria within 12 hours (Johansson *et al.* 2010). Similarly, *IL-10*$^{-/-}$ and *TLR5*$^{-/-}$ mice that develop spontaneous colitis have a thicker mucus layer and more penetrable inner mucus layer when compared to wild type animals (Johansson *et al.* 2014). *Muc2*$^{-/-}$ mice develop intestinal inflammation with diarrhoea, rectal bleeding and prolapse (Johansson *et al.* 2008) and are more susceptible to DSS-induced colitis; these animals exhibit a massive number of bacteria in close contact with host tissues, further promoting inflammation (Van der Sluis *et al.* 2006). Moreover, abnormal mucin *O*-glycosylation has been associated with an increased inflammation, highlighting the importance of mucin glycans in the maintenance of gut homeostasis (Johansson *et al.* 2014) (Bergstrom and Xia 2013; Bergstrom *et al.* 2016).

These changes in mucus composition were also mirrored by changes in the gut microbiota composition at the mucosal surface. IBD patients exhibit a disproportion of mucin-degrading (or mucinolytic) bacteria with an increased abundance of *Ruminococcus torques* and *R. gnavus*, but a decreased abundance of *A. muciniphila*. In addition, the expansion of certain pathobionts and in particular AIEC exhibiting mucinolytic activity has been reported to favour gut colonisation and further induce inflammation in CD (Palmela *et al.* 2018). Taken together, these data suggest that mucus-bacteria interactions contribute to the intestinal barrier dysfunction in IBD patients and future work is needed to better understand the influence or consequence of these interactions on the disease.

Obesity and metabolic-related disorders

A correlation between adiposity, dysglycemia and microbiota encroachment has been reported in a number of animal studies. *Muc2*$^{-/-}$ mice fed a High Fat Diet (HFD) are protected from diet-induced weight gain, fatty liver, and insulin resistance as they displayed less inflammation and increased systemic levels of IL-22 (Hartmann *et al.* 2016). This study supports a role of Muc2 during obesity and highlights the importance of the crosstalk between microbiota, mucus and immune mediators. In mice fed a HFD, mucus secretion is altered in the ileum but not in the duodenum and jejunum, largely in response to an alteration of PPAR-γ signalling. In these mice, Muc2 accumulates at the apical side of goblet cells, leading to a reduction in the expansion capacity of the mucins, thus strongly altering the phenotype of the mucus layer (Tomas *et al.* 2016). Studies by Chassaing and colleagues in different mouse models of metabolic syndrome and in humans demonstrated that bacteria have the ability to infiltrate the mucus layer and reach the epithelium (Chassaing, Ley and Gewirtz 2014; Chassaing *et al.* 2015a; Chassaing *et al.* 2017a). Further, measurement of bacterial-epithelial distance reveals that microbiota encroachment is a feature of insulin resistance-associated dysglycemia in humans that may promote inflammation (Chassaing *et al.* 2017a). Several studies demonstrated that *A. muciniphila* is less abundant in the intestinal microbiota of both genetic and diet-induced obese and diabetic mice, as well as in individuals with obesity, when compared to the faecal microbial population of healthy individuals (Everard *et al.* 2013; Shin *et al.* 2014). *A. muciniphila* treatment has been shown to reverse fat gain, serum LPS levels, gut barrier function and insulin resistance. In addition, oral administration of an outer-membrane protein from *A. muciniphila* led to reduced fat mass and metabolic syndrome in mice fed an obesity-induced diet (Plovier *et al.* 2017). Conversely, anti-diabetic treatments such as metformin administration led to an increase in the *Akkermansia* spp. population (Shin *et al.* 2014).

Human studies have shown that alcohol abuse induced alcoholic liver diseases (ALD) are associated with an increase in intestinal mucus thickness in patients, using wheat germ agglutinin staining on duodenal biopsies (Hartmann *et al.* 2013). Animal studies demonstrated that when compared to wild type

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animals, *Muc-2*$^{-/-}$ mice are protected from alcoholic steatohepatitis in an experimental alcohol-induced liver disease model (Hartmann *et al.* 2013). In addition, *Muc-2*$^{-/-}$ mice are protected from Non Alcoholic Fatty Liver Disease (NAFLD) when fed a high-fat diet inducing liver steatosis (Hartmann *et al.* 2016). Altogether, these data highlight the role of mucus and mucins in the gut-liver axis.

Cancer

The role of mucins in cancer progression has been extensively reviewed (Hollingsworth and Swanson 2004; Kufe 2009). *Muc2*$^{-/-}$ mice displayed spontaneous development of adenomas in the small intestine that progressed to invasive adenocarcinoma, as well as rectal tumours (Velcich *et al.* 2002). In humans, high levels of expression of MUC2 by pancreatic and biliary tumours has been associated with a low degree of invasiveness, malignancy and a better prognosis as compared to tumours not expressing MUC2 (Hollingsworth and Swanson 2004). An abnormal mucin O-glycosylation has been associated with an increased inflammation that could contribute to the development of colitis-associated colon cancer in mice (Bergstrom and Xia 2013; Bergstrom *et al.* 2016). Together these studies support the role of MUC2 as a tumour suppressor.

POTENTIAL OF EXPERIMENTAL MODELS TO STUDY MUCUS/MUCIN INTERACTIONS WITH GUT MICROBES

As mounting evidences highlight the importance of mucus in the cross-talk between the gut microbiota and the host, a wide range of experimental models has been developed to study mucus-bacteria interactions (Table 1). These include the use of purified mucins, mucin-secreting cells or tissues, or mucin-containing fermentation models, as described below.

In vitro mucus/mucin binding assays

Microplate assays

Several microtiter plate assays have been developed for testing bacterial adhesion to mucus and/or mucin (McNamara, Sack and Fleiszig 2000; Gusils, Morata and Gonzalez 2004). These generally rely on the immobilisation of mucins or mucus to the wells of microtiter plates following incubation overnight at 4°C or at room temperature in buffers such as such as PBS (pH 7–7.5), HBSS (pH 7–7.5) or carbonate buffer (pH 9.6) (Gusils, Morata and Gonzalez 2004; Dague *et al.* 2010; Mackenzie *et al.* 2010; Chagnot *et al.* 2013). BSA (bovine serum albumin) is generally used as a negative control for assessing the specificity of the binding to mucus and/or mucin. Binding assays are usually performed at 37°C and the contact time with bacterial cells generally ranges between 30 min to 3 hours before washing to remove non-adhered bacteria. Antibiotic at growth inhibiting concentration, such as chloramphenicol, or sometimes thermic treatment can be applied to prevent the growth of microorganisms in the course of the adhesion assay. Binding can be determined using crystal violet staining of the adhered microbial biomass (Azeredo *et al.* 2017) or by Enzyme-linked Immunosorbent Assay (ELISA) when specific antibodies against bacteria are available (Skoog *et al.* 2012), by measurement of viable counts after plating of the cells (McNamara, Sack and Fleiszig 2000) or by quantitative PCR (Skoog *et al.* 2012). Alternatively, bacteria can be labelled with a radioactive probe or a fluorescent dye before inoculation and the binding quantified using a scintillation counter or a fluorometer, respectively (Gusils, Morata and Gonzalez 2004; Mackenzie *et al.* 2010). Microbial cells can also be labelled by biotinylation and further assayed using streptavidin-HRP by ELISA (Sheng *et al.* 2012). Quantification of microbial binding to mucin can also be performed by flow cytometry, where microbial cells are put in contact with mucin labelled with a fluorescent tag (de Repentigny *et al.* 2000).

Dot blot assay

More recently, a dot-blot method was developed for the sensitive and rapid detection of microorganisms able to bind to mucins (Ringot-Destrez *et al.* 2018). In brief, purified mucins were spotted on a nitrocellulose membrane, whereas the bacterial cells were labelled using a fluorescent dye, such as 4',6-diamidino-2-phenylindole (DAPI), Syto9 or Fluorescein isothiocyanate (FITC), before being overlaid (Ringot-Destrez *et al.* 2018). The adhesion capacities of the microorganisms tested differed depending on the nature of the mucins including purified GI tract mucins, PGM and mucins from the mucus-secreting cell line such as HT29-MTX (see detailed description of this cell line in Section 4.2) (Ringot-Destrez *et al.* 2018).

Mucin microarrays

The carbohydrate microarray technology offers a powerful platform where natural or synthetic glycans are immobilized onto a solid support. (Poole *et al.* 2018). Microarrays incorporating mucins from various sources onto different chips surfaces provide a high-throughput approach to screen bacteria-mucin interactions as well as identify glycan-binding proteins and glycan epitopes involved in this interaction (Clyne *et al.* 2017). For example, the use of mucin microarrays revealed that *C. jejuni* and *H. pylori* recognised distinct mucin receptors despite being closely related phylogenetically (Naughton *et al.* 2013). Recently, *H. pylori* was shown to interact with trefoil factor family (TFF) protein TFF1 (Reeves *et al.* 2008), and that TFF1 specifically interacts with human gastric mucin but not with human colonic mucins nor mucins from other animal sources as shown using mucin microarrays (Dunne *et al.* 2018). This indicates that TFF1 may play an important role in the development of gastric cancer in *H. pylori* infections (Reeves *et al.* 2008; Dunne *et al.* 2018). Mucin microarrays were also used to identify the interactions of commensal strains with mucus (Lactobacillus salivarius AH102 and Bifidobacteria longum AH1205), highlighting the importance of mucin glycans in the preference of the two bacteria to mucins (Naughton *et al.* 2013; Flannery *et al.* 2015).

Binding assays in flow chamber

As a consequence of fluid shear gradient in the gut, the bacteria located in the outer mucus layer are exposed to a more turbulent flow compared to those that reside between the microvilli of the epithelial cells and therefore less exposed to physical perturbation (De Weirdt and Van de Wiele 2015). While the assays described above correspond to adhesion under static conditions, experiments can also be performed in dynamic conditions using flow chamber, where the shear force can be controlled (Le *et al.* 2013). Low-fluid shear environments and high shear rates are known to provide laminar pattern. Over time and with different laminar flow rates, the surface coverage of microbial cells to coupons coated with mucin provides an estimate detachment profile as a function of the shear stress.

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Table 1. Experimental models available to study mucus-bacteria interactions.

GI : Gastro-Intestinal, HMI : Host–Microbe Interactions, IBD : Inflammatory Bowel Ddiseases, IVOC : *In vitro* organ culture, M-SHIME : Mucus Simulator of the Human Intestinal Microbial Ecosystem.

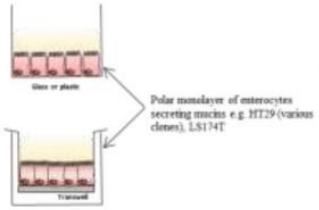
Types of models	Description	Applications	Advantages	Limitations	References
In vitro mucus/mucin binding assays Microplates—Flow chambers 	∗ Immobilization of mucus/mucin on the microtiter plate ∗ Microtiter plate: adhesion in static conditions ∗ Flow chambers: adhesion under dynamic conditions (fluid shear)	∗ Evaluation of bacterial adhesion (commensals and pathogens) to mucins and molecular mechanisms associated	∗ Fast, quantitative and high throughput method to study mucus-microbe interactions independently from other *in vivo* conditions ∗ Identification of molecular determinants involved in adhesion of microbes ∗ Coupling with biophysical techniques (Surface Plasmon Resonance, Atomic Force Microscopy)	∗ Influence of experimental conditions (antibiotics, mechanical treatments, growth conditions, hydrophobic interactions) ∗ Limited availability of purified mucins (mainly use of pig gastric mucin) ∗ Absence of gut microbiota	McNamara *et al.* 2000; Gusils *et al.* 2004; Ringot-Destrez *et al.* 2018; Clyne *et al.* 2017; Dunne *et al.* 2018
In vitro cell models Monoculture models 	∗Gut–derived epithelial cells resembling intestinal tissue consisting mainly of mature goblet cells that secrete an adherent	∗Adherence of commensal and pathogenic bacteria to host cells ∗ Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer	∗Reproducible and easily handled in laboratories ∗ Identification of molecular determinants involved in adhesion of microbes and host cell mucin synthesis ∗ Good platform for screening and characterizing probiotic activity	∗Derived from cancer cells, different from healthy tissue ∗ Not representative of various cell types recovered in mucosal epithelial tissues ∗ Not representative of appropriate *MUC* gene expression ∗ Modulation of mucus production by culture conditions ∗ Absence of gut microbiota ∗ Difficulty to maintain for long-term experiments (> 1 month) ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Linden *et al.* 2007; Navabi *et al.* 2013; Hews *et al.* 2017

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Table 1. Continued

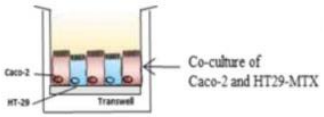
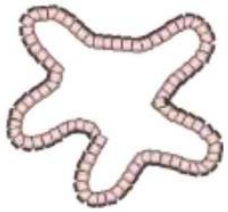
Types of models	Description	Applications	Advantages	Limitations	References
Co-culture models 	∗Mixed culture of enterocytes and mucin secreting cells	∗Adherence of commensal and pathogenic bacteria to host cells ∗ Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer	∗Better representation of cell-type ratio recovered in mucosal epithelial tissues ∗ Simple model, well described in literature	∗Absence of M-cells (development of triple co-culture Caco-2/HT29-MTX/Raji B) ∗ Variations in seeding ratios of HT29 MTX/Caco-2 can impede results interpretation ∗ Modulation of mucus production by culture conditions ∗ Absence of gut microbiota ∗ Difficulty to maintain for long-term experiments (> 1 month) ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Hilgendorf *et al.* 2000; Lozoya-Agullo *et al.* 2017
***Ex-vivo* organ cultures** *Intestinal organoids* 	∗Generation of self-propagating spheres of primary intestinal epithelial cells ∗ Enteroids = derived from adult stem cells isolated from the crypts of human small intestinal ∗ Colonoids = derived from adult stem cells isolated from the crypts of human colonic tissue	∗Study of advanced aspects of mucus development in a more complex scenario ∗ Study of host–commensals and pathogens interactions	∗Often collected from mice tissues, possible use of patient-derived tissues ∗ Assay that more accurately mimics *in vivo* conditions ∗ Amenable to long-term culture	∗Highly expensive and requires specialized expertise ∗ Requires access to biopsies/tissues ∗ Donor-to-donor variability ∗ Requirement of injection to infect organoids with bacteria ∗ Absence of gut microbiota ∗ No reproduction of peristalsis motions and GI stressful events ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Jung *et al.* 2011; Sato *et al.* 2011

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Table 1. Continued

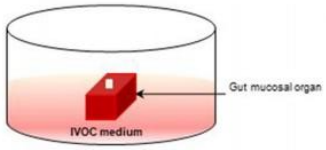
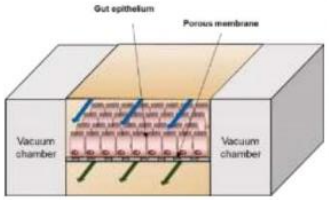
Types of models	Description	Applications	Advantages	Limitations	References
In vitro organ culture (*IVOC*) 	∗Whole organs maintained *in vitro*	∗Study of host–commensals and pathogens interactions	∗Better maintenance of tissue architecture ∗Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) ∗ Often collected from animal tissues, possible use of patient-derived tissues ∗ Possible use of biopsies from disease patients (e.g. IBD)	∗Requires access to biopsies/tissues ∗ Expensive and requires expertise ∗Donor-to-donor variability ∗ Difficulty to maintain for long-term experiments ∗ No reproduction of peristalsis motions and GI stressful events ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Browning and Trier 1969; Schüller *et al.* 2007
Gut- on-a chip 	∗Reproduction of the multicellular structures, cell–cell and tissue–tissue interactions, and the native microenvironment ∗ Closely reproduction of the *in vivo* situation	∗Study of the complex physiological and pathophysiological responses of tissues at an organ level ∗ Study of host–commensals and pathogens interactions	∗Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) ∗ Reproduction of peristalsis like motions ∗ Possible use of biopsies from disease patients (e.g. IBD)	∗Expensive and requires dedicated expertise and instrumentation ∗ Stem cell differentiation is difficult to achieve ∗ Flow rate of the medium can influence cell metabolism ∗ Absence of gut microbiota ∗ No input from immune and nervous system ∗ Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes) ∗ No reproduction of the full complexity of the human gut microbiota	Kasendra *et al.* 2018

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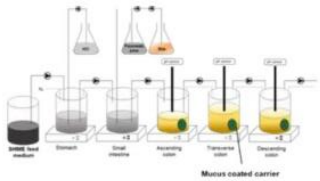
Table 1. Continued

Types of models	Description	Applications	Advantages	Limitations	References
In vitro human colonic models involving a mucosal phase					
M-SHIME 	∗Series of bioreactor modeling the different parts of the human gut ∗ Introduction of mucus-coated carriers (Mucus SHIME)	∗Study the fine-scale spatial organization of the gut microbial ecosystem ∗ Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	∗Integration of human GI -related parameters and possibility to modulate them depending on diet, age and diseases (e.g. ulcerative colitis) ∗ Capture dynamics by time-resolved analyses ∗ Capture inter-individual variability of human gut microbiota ∗ Possible long term experiments ∗ Possible coupling with cell culture models and Host-Microbe Interactions (HMI) module	∗Expensive and requires expertise and specialized instrumentation ∗ Use of pig gastric mucin ∗ No reproduction of immune and nervous system ∗ No reproduction of the full complexity of the human gut microbiota ∗ Donor-to-donor variability	Van den Abbeele *et al.* 2012; Van den Abbeele *et al.* 2013; De Paepe *et al.* 2018
In vivo animal models					
	∗Whole organism models ∗ Development of genetically modified mice with impaired mucin production (comparison with wild type animals)	∗Study of the functional roles of mucin and mucus under physiological or pathological conditions at the level of entire organism ∗ Investigation of downstream consequences of mucin modulation in mucosal barrier defense ∗ Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	∗Physiological model ∗ Allow targeting of a specific gene/pathway in the complex gut-microbiota—host interactions ∗ Amenable to diet or microbiome-based interventions ∗ Possible long-term experiments	∗Requires housing facility and adequate agreements ∗ Expensive to maintain colonies ∗Housing husbandries and diets can modulate mouse microbiota ∗ Murine gut microbiota different from the human gut microbiota ∗ Mucin glycosylation profile of mice different from human intestinal mucins ∗ No reproduction of the full complexity of the human gut microbiota ∗ Limited translational capacity to human situation ∗ Mice generally inbred so no reproduction of the genetic variations found in the human population	Velcich *et al.* 2002; Van der Sluis *et al.* 2006

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Biophysical assays

In order to gain further molecular insights into the interactions of microbial cells with mucin, various biophysical techniques have been developed and applied over the years. Optical biosensors based on resonant mirrors have been used to determine the binding kinetics of *H. pylori* cells to mucin (Hirmo *et al.* 1999). Following competition binding assays, the recognition of sialylated and sulphated moieties of mucin by *H. pylori* was demonstrated. Surface plasmon resonance (SPR) has been used to evaluate the adhesion abilities of a range of *Lactobacillus* species (Uchida *et al.* 2004; Kinoshita *et al.* 2007). In these studies, human colonic mucin (HCM) was immobilised on the sensor chip whereas bacterial cells were eluted as analytes. Using sialidase or sulfatase, it was further possible to discriminate some strains of lactobacilli and bifidobacteria that could specifically bind to the sialic acid or sulphate residues of HCM respectively (Huang *et al.* 2013). Single-cell force spectroscopy (SFCS) has been used to quantify the adhesion forces of *L. rhamnosus* with mucin at a single-cell level, pinpointing heterogeneities in the bacterial population (Sullan *et al.* 2014). More recently, further molecular details of mucin-bacteria interactions were investigated using atomic force microscopy (AFM). Such an approach was used for the first time to accurately quantify the force of adhesion of *L. lactis* cells immobilised on the AFM tip to PGM at nanoscale level (Dague *et al.* 2010). Surprisingly, it was found that PGM coating strongly reduced the bacterial adhesion force compared to bare polystyrene, highlighting the interplay between electrostatic, hydrophilic and steric repulsions, and that both specific and non-specific interactions need to be considered (Dague *et al.* 2010). These results were consistent with a previous investigation of the muco-adhesive properties of *L. lactis* using quartz crystal microbalance with dissipation monitoring (Le *et al.* 2012). Using bacteria mutant strains, AFM was also used to provide molecular insights into the respective role and contribution of mucus-binding proteins and surface organelles (pili or flagella) in muco-adhesion (Le *et al.* 2013). Interactions at the protein-protein level were further investigated by AFM to study the adhesive properties of *L. reuteri* Mub with mucins (Gunning *et al.* 2016).

In vitro mucin-secreting cell models

Monoculture models

While many colon carcinoma cell lines express mRNAs encoding surface-associated and/or secreted intestinal mucins (Deplancke and Gaskins 2001), few of them secrete MUC2 or form a mucus layer (Linden, Driessen and McGuckin 2007; Navabi, McGuckin and Lindén 2013; Hews *et al.* 2017). Most mucus-secreting cell lines are derived from the heterogeneous adenocarcinoma cell line HT-29 which can be differentiated into a mucus-secreting phenotype by growth under metabolic stress conditions. After an initial phase of cell mortality, adapted subpopulations of highly differentiated cells emerge (Lievin-Le Moal and Servin 2013). HT29–18N2 cells are often used as a model system for goblet cell differentiation and mucin secretion; these cells have been established by growth under glucose deprivation in galactose-containing culture medium (Phillips *et al.* 1988). In contrast, HT29-MTX cells and their clonal derivatives have been obtained by sequential adaptation to increasing concentrations of methotrexate (Lesuffleur *et al.* 1990). When grown on Transwell filter supports, some HT-29 MTX clones (e.g. MTX-D1 and MTX-E12) form polarised monolayers mostly constituted of mature goblet cells secreting an adherent mucus layer of 50–150 μm thickness as revealed by Alcian Blue staining (Behrens *et al.* 2001). In addition, the mucin-secreting clonal cell line HT-29.cl16E emerged from parental HT-29 cells after subculture in sodium butyrate whilst HT29-FU cells were established by treatment with 5-fluorouracil (Lesuffleur *et al.* 1991).

These mucus-producing HT-29 derivatives have been widely used to investigate the adherence of commensal and pathogenic bacteria to host cells (Coconnier *et al.* 1992; Bernet *et al.* 1993; Eveillard *et al.* 1993; Bernet *et al.* 1994; Kerneis *et al.* 1994; Favre-Bonte, Joly and Forestier 1999; Gopal *et al.* 2001; Schild *et al.* 2005; Barketi-Klai *et al.* 2011; Dolan *et al.* 2012; Gagnon *et al.* 2013; Naughton *et al.* 2013; Martins *et al.* 2015; Martins *et al.* 2016) and/or evaluate the effect of commensal bacteria on infection with enteropathogens (Bernet *et al.* 1993; Bernet *et al.* 1994; Coconnier *et al.* 1998; Gopal *et al.* 2001; Alemka *et al.* 2010; Zihler *et al.* 2011; Zivkovic *et al.* 2015; Vazquez-Gutierrez *et al.* 2016). Some studies investigated the direct effect of commensal or pathogenic bacteria on host cell mucin synthesis and/or composition of the mucus layer. Infection with atypical EPEC increased expression of secreted MUC2 and MUC5AC as well as membrane-bound MUC3 and MUC4 in HT29-MTX cells, thereby enhancing bacterial growth by providing nutrients for adherent bacteria (Vieira *et al.* 2010). Another study showed that apical infection with *Listeria monocytogenes* stimulated mucus secretion by polarised HT29-MTX cells. This effect was mediated by binding of the toxin listeriolysin O to a receptor on the epithelial brush border (Coconnier *et al.* 1998) and reduced bacterial invasion and colonisation of the host epithelium (Lievin-Le Moal, Servin and Coconnier-Polter 2005). Interestingly, probiotic *Lactobacillus* strains which adhering to mucus-producing HT-29 cells upregulated the transcription and secretion of MUC3 which reduced adherence of EPEC in co-incubation experiments (Mack *et al.* 2003). Modulation of mucus production and mucin glycosylation by commensal bacteria can also occur independently of adhesion. For example, a small soluble peptide of the gut commensal *R. gnavus* E1 strain has been shown to increase HT-29 MTX cell glycosylation via enhanced transcription of glycosyltransferases and MUC2-encoding genes (Graziani *et al.* 2016). Similarly, a soluble low molecular weight compound from *B. thetaiotaomicron* has been reported to enhance galactosylation in HT29-MTX cells. While no change in transcription was detected, galactosyltransferase activity was increased in HT29-MTX cells treated with soluble bacterial extract suggesting post-translational mechanisms of regulation (Miguel *et al.* 2001).

In addition to HT-29 cell derivatives, mucus-producing LS174T colon carcinoma cells have been used to study host-bacteria interactions. LS174T cells secrete mature MUC2, MUC5AC and human gallbladder mucin (van Klinken *et al.* 1996) but do not produce an organised adherent mucus layer (Navabi, McGuckin and Lindén 2013). Recent studies using this cell line showed that the secreted metalloprotease StcE reduced MUC2 levels during infection with EHEC and thereby facilitated bacterial adherence to the intestinal epithelium (Hews *et al.* 2017). In addition, the soluble protein p40 from *L. rhamnosus* GG stimulated MUC2 mRNA and protein expression in LS174T cells, and this effect was dependent on the epidermal growth factor receptor (Wang *et al.* 2014). Furthermore, treatment with butyrate, a product of bacteria fermentation, increased mucin production in LS174T cells (Burger-van Paassen *et al.* 2009; Jung *et al.* 2015). Recently, LS174T cells were used to decipher *E. histolytica*-elicited suppressed goblet cell transcription (Leon-Coria *et al.* 2018).

Co-culture models

To model human intestinal epithelia, mixed cultures of enterocyte-like Caco-2 cells and mucus-producing HT29-MTX

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cells have been widely used in drug absorption and permeability studies (Hilgendorf *et al.* 2000; Lozoya-Agullo *et al.* 2017). Co-cultures prepared with different ratios of Caco-2 and HT29-MTX cells seeded out on Transwell inserts formed a continuous mucus layer similar to cultures of HT-29-MTX cells grown alone (Poquet, Clifford and Williamson 2008; Beduneau *et al.* 2014). Notably, the probiotic strains *L. rhamnosus* GG or bifidobacteria as well as pathogenic strains of *E. coli* or *L. monocytogenes* adhered better to mucus-deficient Caco-2 cells than to mucus-producing HT-29MTX cells or Caco-2/HT-29 co-cultures (Laparra and Sanz 2009). Considering the 'closed' oxygen-restricted environment in the human gut, Chen and colleagues developed a 3D porous silk scaffolding in the shape of a hollow tube. While the inner tube wall was coated with Caco-2/HT29-MTX epithelia, primary human intestinal myofibroblasts were grown in the tube scaffold space underneath to support epithelial growth and differentiation. Notably, epithelia grown on 3D scaffolds demonstrated increased MUC2 production compared to Transwell cultures resulting in the formation of a mucus layer of 11–17 μm thickness (Chen *et al.* 2015).

Organ-on-a-chip

Another approach to simulate a mucin-producing human intestinal epithelium is the 'Gut-on-a-Chip' system, where Caco-2 cells are grown on a porous membrane support in a microfluidic device. While the cell membrane support is maintained under cyclic strain mimicking peristaltic motion, the chambers above and below the cell membrane are constantly perfused with medium, thereby generating low shear stress. This environment stimulates the formation of 3D intestinal villi similar to those found in the small intestine (Kim *et al.* 2012), and the differentiation of Caco-2 cell into absorptive enterocytes, and also includes enteroendocrine cells, Paneth cells and mucus-producing goblet cells (Kim and Ingber 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model display enhanced barrier function and mucus production as compared to static Caco-2 cell cultures (Kim and Ingber 2013). This system has recently been developed further to allow co-culture with strict anaerobes (Shin *et al.* 2019). Although the Gut-on-a-Chip devices have been mostly used for long-term co-culture of IECs with commensal microbes under healthy conditions, they are now being employed to model intestinal inflammation (Kim *et al.* 2016). Using a Gut-on-a-Chip model, the pathophysiological manifestation and dysregulated barrier function observed during inflammation could be recapitulated which may help to gain insights into disease mechanisms and assess potential therapeutic strategies (Shin and Kim 2018). Notably, probiotic VSL#3 targeted restoration of the mucosal barrier did not effectively control the local inflammation nor improve mucus production (Shin and Kim 2018). The HuMix (Human Microbial Cross-talk) model is another microfluidic device enabling the co-culture of Caco-2 cell monolayers with commensal bacteria under anaerobic conditions. In contrast to the Gut-on-a-Chip system, the epithelial cells which do not produce mucus are separated from the bacteria by a membrane coated with porcine gastric mucin (Shah *et al.* 2016).

Ex vivo organ cultures

As described above, traditional culture of human cells represents a valuable predictor of human physiology, pathology, and therapeutic responses but is limited by the absence of the tissue microenvironment. Culture approaches using human intestinal biopsy samples therefore represent an upscale platform to investigate the involvement of the mucus layer in healthy conditions or in the onset of various diseases.

In vitro organ culture (IVOC)

In 1969, Browning and Trier were the first to establish a technique to culture human mucosal biopsies *ex vivo*. By using a specific culture medium and incubation of the samples in 95% O_2, 5% CO_2 at 37°C, mucosal biopsies from the duodeno-jejunal junction were kept alive for up to 24 hours demonstrating epithelial cell proliferation, fat absorption and active mucus secretion by goblet cells (Browning and Trier 1969). The advantages of IVOC of intestinal biopsies versus cell line culture models include the presence of healthy non-transformed cells including all major IEC types (enterocytes, goblet cells, Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue, and the production of mucus. While it is problematic to maintain the loose outer mucus layer of colonic biopsies during sampling, the inner colonic and small intestinal mucus layers are generally well preserved as evidenced by microscopy (Haque *et al.* 2004; Walsham *et al.* 2016; Hews *et al.* 2017). IVOC of biopsy samples has been used to investigate adherence of pathogenic bacteria such as EPEC (Knutton, Lloyd and McNeish 1987; Schüller *et al.* 2007), EHEC (Phillips *et al.* 2000; Fitzhenry *et al.* 2002; Lewis *et al.* 2015), ETEC (Knutton *et al.* 1989; Baker, Moxley and Francis 1997) and *C. jejuni* (Grant, Woodward and Maskell 2006) to human intestinal mucosa. In addition, IVOC demonstrated cytotoxic effects of bacterial toxins, such as Pet toxin from EAEC (Henderson *et al.* 1999b), Shiga toxin from EHEC (Schüller, Frankel and Phillips 2004) and *C. difficile* toxin A (Mahida *et al.* 1996) on intestinal epithelium or mucosa. Interactions of enteropathogenic bacteria with mucus production were observed in small intestinal and colonic biopsy tissue infected with EAEC where bacteria were predominantly associated with a thick mucus layer above the epithelium, which was not present in non-infected control samples (Hicks, Candy and Phillips 1996; Andrade, Freymuller and Fagundes-Neto 2011). This suggests that EAEC stimulates mucus secretion which agrees with the production of mucoid stools during EAEC diarrhoea (Croxen *et al.* 2013). Similarly, stimulation of mucus secretion and bacterial binding to the mucus layer were observed in biopsy samples from the terminal ileum infected with S. Typhimurium. This was followed by *Salmonella* adherence and invasion of the epithelium accompanied by ruffling of the host cell membrane (Haque *et al.* 2004). Recently, the IVOC system was used to show that the metalloprotease StcE diminishes the inner mucus layer and enhances EHEC adherence to human colonic biopsy epithelium (Hews *et al.* 2017).

Polarised IVOC (pIVOC)

While the traditional IVOC system allows bacterial access to the mucosal and submucosal side of the biopsy, polarised organ culture models have been developed which limit bacterial contact to the mucosal side of the tissue. This is particularly relevant when studying host responses to bacterial infections where artificial interactions with immune cells in the lamina propria might confound experimental readouts. Using a pIVOC approach by mounting colonic tissue explants between two Perspex disks in a Snapwell plate, Raffatellu and colleagues demonstrated that *Salmonella* Typhi reduced mucosal expression of the pro-inflammatory cytokine interleukin (IL)-8 by production of a capsule which masked pathogen-associated molecular patterns such as LPS and flagellin (Raffatellu *et al.* 2005). In addition, pIVOC showed that apical exposure to EPEC or purified H6 flagellin induced IL-8 expression in duodenal biopsies (Schüller *et al.*

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2009). Furthermore, infection with *C. jejuni* stimulated the production of reactive oxygen species (ROS) in duodenal and colonic mucosa (Corcionivoschi *et al.* 2012). The pIVOC system has also been used to study the interaction of probiotic bacteria with mucosal tissue, and incubation of duodenal explants with *L. reuteri* demonstrated localisation of bacteria in the mucus layer but not in the epithelium. Nevertheless, pre-incubation with *L. reuteri* reduced EPEC adherence to the epithelium (Walsham *et al.* 2016). A different approach to restrict bacterial access to the epithelial surface was developed by Tsilingiri and colleagues by gluing a perspex cylinder to the mucosal side of colonic resection tissue (Tsilingiri *et al.* 2012). Surprisingly, apical incubation with probiotic *L. plantarum* resulted in degeneration of mucosal tissue from healthy donors, whilst all three strains studied (*L. paracasei, L. rhamnosus, L. plantarum*) caused tissue damage in resections from patients with IBD. In contrast, supernatants from *L. paracasei* reduced inflammation in *Salmonella*-infected and IBD tissue. As the maintenance of larger tissue samples requires incubation in high levels of oxygen (95–99%), the use of IVOC to study interactions of oxygen-sensitive bacteria with human intestinal mucosa remains problematic. However, a novel murine 3D-intestinal organ culture system was recently developed whereby an intact intestinal fragment was luminally perfused with degassed medium containing anaerobic bacteria while the serosal side of the tissue was maintained under humidified oxygenated conditions. Whilst preserving gut tissue architecture, the system also supported the growth of commensal microbes (*Clostridium ramosum* and SFB) and allowed assessment of their impact on the immune and nervous system (Yissachar *et al.* 2017).

Human enteroids/colonoids and intestinal organoids

New technologies have been developed which enable the generation of self-propagating spheres of primary intestinal epithelial cells ('mini-guts'). Enteroids or colonoids are derived from adult stem cells isolated from the crypts of human small intestinal or colonic tissue, respectively (Jung *et al.* 2011; Sato *et al.* 2011). In contrast, human intestinal organoids (HIOs) are established by differentiation of embryonic or, more often, induced pluripotent stem cells (genetically reprogrammed adult stem cells) (Spence *et al.* 2011). In comparison to enteroids, HIOs lack maturation and more closely resemble foetal than adult intestine. In addition, they are devoid of functional intestinal stem cells and surrounded by a mesenchyme which is absent in enteroids (Sinagoga and Wells 2015; Leslie and Young 2016). As the apical side of the epithelium is facing inwards, infection of spheroid enteroids/HIOs with bacteria requires microinjection. Studies on the anaerobic pathogen *C. difficile* showed that injected bacteria remained alive in HIOs for up to 12 hours and caused disruption of epithelial barrier function *via* secretion of the toxin TcdA. Interestingly, oxygen measurements indicated reduced oxygen levels in the lumen of HIOs (5 to 15%). Furthermore, infection with *C. difficile* resulted in reduced MUC2 and mucus production in HIOs (Engevik *et al.* 2015). HIOs also supported growth of EHEC and commensal *E. coli*. Infection with EHEC induced ROS production and an inflammatory response associated with recruitment of external neutrophils into HIO spheres (Karve *et al.* 2017). Interestingly, colonisation of HIOs with commensal *E. coli* (ECOR2) stimulated enterocyte maturation, antimicrobial peptide secretion, production of a MUC2-containing mucus layer and increased epithelial barrier function, thereby indicating the establishment of stable host–microbe symbiosis (Hill *et al.* 2017).

To facilitate incubations with bacteria, 2D enteroid systems have now been successfully developed where primary intestinal cells are grown as monolayers on permeable membrane supports. Previous studies showed that differentiated human enteroid and colonoid monolayers contained MUC2-producing goblet cells and formed a mucus layer of more than 25 μm thickness (VanDussen *et al.* 2015; In *et al.* 2016). Two-dimensional enteroids and colonoids supported binding of EAEC, EHEC and EPEC (VanDussen *et al.* 2015). More specifically, apical EHEC infection of colonoids resulted in the formation of characteristic attaching and effacing lesions, mucus degradation and reduced expression of the microvillar protein protocadherin 24, which was mediated by the secreted serine protease EspP (In *et al.* 2016). The 2D enteroid model was further refined by adding primary human macrophages to the basolateral side of the membrane support. Intriguingly, enteroid monolayers grown in the presence of macrophages exhibited increased cell height and barrier function. In addition, underlying macrophages were able to capture and kill EPEC and ETEC by extending projections across the epithelial monolayer (Noel *et al.* 2017).

In another approach to mimic the gut environment more closely, cells from human small intestinal enteroids were seeded on tubular silk sponge scaffolds and supported by primary human intestinal myofibroblasts as described for Caco-2/HT29-MTX (Section 4.2). The resulting intestinal model epithelium contained all four major epithelial cell types and exhibited tight junction formation, microvillus polarisation, digestive enzyme secretion and low oxygen tension in the lumen. Moreover, infection with a laboratory strain of *E. coli* resulted in a significant innate immune response (Chen *et al.* 2017). Recently, a Gut-on-a-Chip model based on primary intestinal epithelial cells has been developed which also includes co-culture of an underlying endothelium. Human enteroids are cultured on a side of a porous membrane within a microfluidic device whereas the intestinal microvascular endothelium is established on the other side of the filter. This device reproduces the epithelial cells proliferation and host defenses more accurately (Kasendra *et al.* 2018). Kim and colleagues showed that a human Gut-on-a-Chip micro device colonized by non-pathogenic bacteria (commensal and probiotic bacteria) was able to induce production of a key set of pro-inflammatory cytokines. This device enabled high level of mucus production on micro engineered intestinal villi, therefore providing a protective barrier to maintain long-term stable host–microbe coexistence (Kim *et al.* 2016).

In vitro human fermentation models involving a mucosal phase

As aforementioned, the spatial positioning of gut microorganisms in the mucus layer is important with respect to their functional role in the human gut ecosystem. The microbial community residing in the mucus layer across the length of the GI tract is, however, hard to study given the difficulty to sample this region *in vivo*, especially in human (Macfarlane, McBain and Macfarlane 1997; Flint *et al.* 2012; Donaldson, Lee and Mazmanian 2016). *In vitro* colonic models involving a mucosal phase are a valuable alternative to study the fine-scale spatial organisation of the gut microbial ecosystem.

Multiple colon *in vitro* models have been developed over the years, ranging from simple, single stage batch incubations to more complex and representative three stage continuous and semi-continuous reactor models (Miller and Wolin 1981; Gibson, Cummings and Macfarlane 1988; Allison, McFarlan and MacFarlane 1989; Blanquet-Diot *et al.* 2012; McDonald *et al.* 2013; Van den Abbeele *et al.* 2013). These continuous fermentation models, inoculated with faecal samples of donors, recapitulate the main

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biotic and abiotic parameters of the human colon, such as temperature, pH, residence time, supply of nutritive medium reproducing the composition of ileal effluents, therefore enabling the study of a complex and metabolically active gut microbiota under anaerobiosis conditions. In these fermentation models, the bioreactors can be inoculated with fresh or frozen stools provided by individual or pool of different donors being healthy human volunteers with no history of antibiotic treatment 2 to 6 months before the beginning of the study.

Introduction of mucus carriers in human in vitro colonic models

Mucins in solution have been frequently included in these colonic models to provide a nutrient source to the gut bacteria, but the study of mucus colonisation by gut bacteria in these systems has been revolutionized by the ability to simulate the viscoelastic gel-like nature of the mucus layer through the incorporation of mucus carriers (Gibson, Cummings and Macfarlane 1988; Macfarlane, Hay and Gibson 1989; Macfarlane, Woodmansey and Macfarlane 2005; Van Herreweghen *et al.* 2017). MacFarlane first demonstrated a rapid colonisation of an agar-mucus layer during a 48-hour incubation in a two-stage continuous fermentor system by a mixture of *Bacteroides*, enterobacteria and facultative anaerobes (Macfarlane, Woodmansey and Macfarlane 2005). However, the use of glass tubes, containing this agar-mucus layer, in this set-up did not permit a practical long-term implementation (Van den Abbeele *et al.* 2009). Mucus-coated beads (mixture of 5% porcine mucin type II and 1% agar) have since been identified as crucial platforms in sustaining microbial diversity by selectively enriching species, which are not thriving in the luminal environment. This mucus interphase was introduced in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) resulting in the M-SHIME (Mucus SHIME) configuration (Van den Abbeele *et al.* 2012). This system was first used to assess the fitness of potential probiotic lactobacilli, revealing a pronounced enrichment of *Lactobacillus mucosae* and *L. rhamnosus* GG in the mucus beads compared to the luminal environment, contributing to their long-term persistence in M-SHIME (Van den Abbeele *et al.* 2012). The use of next-generation 16S rRNA gene amplicon sequencing methods and the incorporation of mucus beads in both proximal and distal colon conditions in the M-SHIME, further resulted in the detection of several additional mucus-associated species (De Paepe *et al.* 2018). Besides *Roseburia faecis/Enterococcus rectale*, a strong enrichment of *Ruminococcus inulinivorans*, *Clostridium*, *Bilophila*, *Anaeroglobus* and *Veillonellaceae* species was observed in the proximal mucus layer compared to the lumen (De Paepe *et al.* 2018). *A. muciniphila*, *Cloacibacillus evryensis*, *Pyramidobacter piscolens*, *Eubacterium contortum* and species belonging to *Odoribacter*, *Enterobacteriaceae* and *Desulfovibrio* were predominantly residing in the distal mucus layer (De Paepe *et al.* 2018). It can be expected that the list of species inhabiting the mucus layer will continue to expand in the future M-SHIME experiments, by continuing to explore the inter-individual variability of faecal samples.

Another major advantage of *in vitro* fermentation models is the possibility to capture dynamics by time-resolved analyses. To minimize disturbance of the system during such analyses, the M-SHIME system was adapted to facilitate a rapid, anaerobic, frequent sampling by mounting sampling ports with an airlock system on top of the SHIME lids. These sampling ports moreover enable the anaerobic addition, transfer and sampling of any insoluble dietary substrate. The adapted model was correspondingly termed Dietary Particle-Mucosal-Simulator of the Human Intestinal Microbial Ecosystem (DP-M-SHIME). The DP-M-SHIME offers a closer proxy for the diversity of *in vivo* GI microenvironments, as aside from the mucus layer, insoluble dietary particles present an interesting additional niche for microorganisms to physically interact with and colonise (De Paepe *et al.* 2018).

In addition, these colonic *in vitro*-fermentation models enable the study of severe gut microbiome perturbations such as antibiotic therapy or pathogen invasion, which cannot be performed in humans for obvious ethical reasons. Using M-SHIME, the mucus bead carriers were shown to confer resilience to lactobacilli species against a combined treatment of antibiotics including tetracycline, amoxicillin and ciprofloxacin (Van den Abbeele *et al.* 2012). In addition, the M-SHIME model has been used to demonstrate the antagonistic effects of probiotics and prebiotics such as *L. reuteri*, long-chain arabinoxylans or inulin towards AIEC colonisation in the mucosal environment (Van den Abbeele *et al.* 2016). Another study reported the importance of mucus for providing a protective environment to beneficial gut microbes, such as *L. reuteri*, to help them escape from stress induced by high loads of linoleic acid, the most common polyunsaturated fatty acid in a WSD (De Weirdt *et al.* 2013). The M-SHIME model has also been shown to preserve disease signatures, as illustrated by a reduced luminal and mucosal *Clostridium* cluster XIVa colonisation in colon vessels inoculated with the faecal material of ulcerative colitis patients compared to healthy donors (Vermeiren *et al.* 2012). The mucin-beads technology has recently been transferred into the Artificial Colon (ARCOL), with adaptations to avoid flushing the bioreactor with nitrogen when mucin-coated beads are being renewed (Cordonnier *et al.* 2015; Thevenot *et al.* 2015).

Combining colon fermentation models with in vitro cell culture

Recent advancements in the field of *in vitro* fermentation involve the combination of gut microbiota models with a host compartment to assess host–microbiota interactions (Bahrami *et al.* 2011; Marzorati *et al.* 2014; Defois *et al.* 2018). The three-way interactions between host, microbiome and dietary interventions can be examined by applying the supernatants of colonic samples onto human cell lines or combinations thereof, such as co-cultures of enterocytes and immune cells, in Transwell systems (Marzorati *et al.* 2014). Cytokine and TNF-α production are followed up as markers of intestinal inflammation, whereas the trans-epithelial electrical resistance (TEER) and Lucifer yellow translocation give an indication of epithelial barrier function (Geirnaert *et al.* 2017). While typically, in human cell line experiments, the short-term effects of a single treatment application onto differentiated cells with a disrupted barrier are being evaluated, recently, a method was developed to test the effects of a probiotic treatment on the development of epithelial barrier integrity during cell differentiation, which is more representative of the *in vivo* situation (Geirnaert *et al.* 2017). In order to further improve the *in vivo* relevance, a host module, such as the Host Microbiota Interaction (HMI) Module (Marzorati *et al.* 2014), can be coupled to the colonic *in vitro* fermentation systems described above to directly and continuously recirculate the colonic microbial suspension over a mucus layer that is in indirect contact with enterocytes and/or immune cells.

In vivo animal models

As described above, *in vitro* mucin-secreting cell cultures, *ex-vivo* organ cultures as well as *in vitro* fermentation models have yielded fundamental insights into the role of mucins and mucus in bacterial interactions with the host. However, the use of *in vivo* models is necessary to study the biological roles of mucins

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under physiological or pathological conditions at the level of entire organism. Genetically modified mouse models with an impaired mucin production or glycosylation have been developed to assess the role of mucus in the interaction between gut bacteria and the host *in vivo*.

Muc2$^{-/-}$ mouse model

Many *in vivo* animal studies investigating the role of mucus in gut homeostasis have relied on the use of *Muc2$^{-/-}$* mice, lacking the major intestinal mucin Muc2.

The first studies based on *Muc2$^{-/-}$* mice showed that these animals displayed an impaired epithelial barrier function characterised by aberrant intestinal crypt morphology and altered cell maturation and migration, and that the mice frequently developed adenomas in the small intestine, as well as rectal tumours (Velcich *et al.* 2002). The microscopic analysis of the colon indicated mucosal thickening, increased proliferation and superficial erosions (Van der Sluis *et al.* 2006). The development of spontaneous colitis in Muc2 deficient mice indicated that Muc2 is critical for colonic protection (Van der Sluis *et al.* 2006). A gut microbiota dysbiosis was also observed in the *Muc2$^{-/-}$* mice which harboured a pro-inflammatory-like microbiota profile, characterized by an increase in *Clostridiales* and a decrease in *Lactobacillaceae* (Huang *et al.* 2015). Furthermore, it was shown that the spatial compartmentalization of bacteria in the intestine of *Muc2$^{-/-}$* mice was compromised and transcriptomic analysis revealed a downregulation of TLR, immune and chemokine signaling pathways compared to wild type mice (Sovran *et al.* 2015) Also, the expression of the network of IL-22-regulated defense genes was increased in *Muc2$^{-/-}$* mice (Sovran *et al.* 2015). Recent work also confirmed a clear shift in the microbiota composition of *Muc2$^{-/-}$* mice, with the Firmicutes phylum enriched and the Bacteroidetes phylum decreased, as well as an increase in genera considered as potential pathogens also (Wu *et al.* 2018).

Muc2$^{-/-}$ mice have been used to test the effect of the probiotic mixture VSL#3 on colonic inflammation and intestinal barrier function (Kumar *et al.* 2017). This probiotic mixture contains eight strains belonging to *Lactobacillus*, *Bifidobacterium* and *Streptococcus* genera which are usually found in the human intestinal microbiota. In *Muc2$^{-/-}$* mice, VSL#3 reduced basal colonic proinflammatory cytokine levels and improved epithelial barrier function. In addition, VSL#3 reduced the level of proinflammatory chemokines and upregulated tissue regeneration growth factors leading to a faster resolution of colitis symptoms in *Muc2$^{-/-}$* mice with DSS-induced colitis. This was associated with the restoration of antimicrobial peptide gene expression in the small intestine, and an increased abundance of commensal bacteria in the gut. The authors proposed that these beneficial effects were mediated by acetate, produced by the gut bacteria (Kumar *et al.* 2017). Treatment of *Muc2$^{-/-}$* mice with *Lactobacillus* spp. could ameliorate spontaneous colitis and led to an increased production of SCFA (Morampudi *et al.* 2016).

Muc2$^{-/-}$ mice have also been used to investigate the role of this mucin to prevent bacterial and parasite infection. Upon infection with *C. rodentium*, a murine pathogen related to diarrhoeagenic attaching-effacing *E. coli*, *Muc2$^{-/-}$* mice exhibited a rapid weight loss and up to 90% mortality (Bergstrom *et al.* 2010). Mucin secretion was increased in wild type mice during infection as compared to the uninfected controls, suggesting that mucin production is critical to clear the mucosal surface from pathogenic bacteria. In *Muc2$^{-/-}$* mice, commensal bacteria were also found to interact with *C. rodentium* and host tissues, indicating that Muc2 regulates all forms of intestinal microbiota at the gut surface (Bergstrom *et al.* 2010). When *Muc2$^{-/-}$* mice were infected with *Salmonella*, they showed a dramatic susceptibility to infection, carrying significantly higher caecal and liver pathogen burdens, and developing significantly higher barrier disruption and higher mortality rates than wild type mice (Zarepour *et al.* 2013). Colonisation of *Muc2$^{-/-}$* mice by enterotoxigenic *B. fragilis*, a causative agent of acute diarrhoea in humans, led to lethal disease (Hecht *et al.* 2017). The protective function of Muc2 was also demonstrated in models of *T. muris* parasitic infection (Hasnain *et al.* 2010). *T. muris* is a murine infecting nematode which is used as model of T. trichiura infection in humans, a threat in developing countries. After infection, *Muc2$^{-/-}$* mice showed a delayed expulsion of the worms from the intestine compared to wild type mice. In addition, an increase in Muc2 production, observed exclusively in resistant mice, correlated with worm expulsion. The nematodes demonstrated a decrease in their energy status in wild type mice compared to the susceptible *Muc2$^{-/-}$* mice (Hasnain *et al.* 2010). *E. histolytica* is a human parasite infecting the colon and responsible of amoebic dysentery and/or liver abscesses. *E. histolytica* specifically colonises the mucus layer by adhering to galactose and GalNAc residues present in Muc2 (Kissoon-Singh *et al.* 2013). The parasite also induces potent hypersecretion from goblet cells. Kissoon-Singh and colleagues showed that *E. histolytica* induced a pronounced time-dependent secretory exudate with increased gross pathology scores and serum albumin leakage in *Muc2$^{-/-}$* mice. Colonic pathology, secretory responses and increased proinflammatory cytokine secretions were also correlated with altered expression of tight junction proteins (Kissoon-Singh *et al.* 2013). These results demonstrate that colonic mucins confer both luminal and epithelial barrier functions and that, in the absence of Muc2, mice are more susceptible to *E. histolytica*-induced secretory and pro-inflammatory responses. A recent study using antibiotic treated *Muc2$^{-/-}$* and *Muc2$^{+/+}$* littermates showed that *E. histolytica* elicited robust mucus and water secretions, enhanced pro-inflammatory cytokines and chemokine expression and higher pathology scores as compared to the modest response observed in non-antibiotic treated littermates. Host responses were microbiota specific as mucus secretion and pro-inflammatory responses were attenuated following homologous faecal microbial transplants in antibiotic-treated *Muc2$^{+/+}$* quantified by secretion of ^{3}H-glucosamine newly synthesized mucin, Muc2 mucin immunostaining and immunohistochemistry (Leon-Coria *et al.* 2018). The mechanism controlling mucus release in the presence of *E. histolytica* was further studied by Cornick and colleagues who identified vesicle-associated membrane protein 8 (VAMP8) present on mucin granules as orchestrating regulated exocytosis in human goblet cells in response to the presence of *E. histolytica* (Cornick *et al.* 2017). In *Vamp8$^{-/-}$* mice, *E. histolytica* induced enhanced killing of epithelial cells and aggressive proinflammatory response with elevated levels of IL-1α, IL-1β and TNF-α secretion, highlighting the downstream consequences of improper mucin secretion in mucosal barrier defence. Taken together, these results demonstrate the critical involvement of Muc2 in host protection from nematode infection, by constituting an effective physical and biological barrier against pathogenic infection.

Muc1$^{-/-}$ mouse model

Mice impaired in the production of cell surface mucins have also been engineered. The *Muc1$^{-/-}$* mouse model revealed the role played by Muc1 in *H. pylori* infection, a pathogen involved in gastric ulcers and adenocarcinoma (McGuckin *et al.* 2007; Linden *et al.* 2009). *Muc1$^{-/-}$* mice displayed a 5-fold increase in *H. pylori* colonisation as compared to wild type mice (McGuckin

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et al. 2007). This study further demonstrated the ability of *H. pylori* to bind to purified Muc1 *in vitro*, suggesting that Muc1 limits the access of *H. pylori* to the epithelial surface thereby providing protection from infection and proinflammatory bacterial products. Muc1 deficiency also resulted in increased epithelial cell apoptosis in *H. pylori* infected mice (Linden *et al.* 2009). More recently, the long-term consequence of Muc1 deficiency on *H. pylori* pathogenesis was investigated in *Muc1*$^{-/-}$ mice (Ng *et al.* 2016). *Muc1*$^{-/-}$ mice began to die 6 months after *H. pylori* challenge, indicating that a deficiency in Muc1 leads to lethal infection. This study also revealed that *Muc1* was an important, previously unidentified negative regulator of the NLRP3 inflammasome, and loss of this regulation resulted in the development of severe pathology (Ng *et al.* 2016). Consistent with these studies, *Muc1*$^{-/-}$ mice have a higher rate of systemic infection in a murine *C. jejuni* model of gastroenteritis (McAuley *et al.* 2007).

Muc13$^{-/-}$ *mouse model*

The MUC13 transmembrane mucin is highly and constitutively expressed in the small and large intestines and MUC13 polymorphisms have been associated with human IBD and susceptibility to *E. coli* infection in pigs. While Muc13-deficient mice did not show intestinal pathology, they developed more severe acute colitis than wild type mice after DSS challenge, as reflected by increased weight loss, rectal bleeding, diarrhoea and histological colitis scores (Sheng *et al.* 2011).

Mouse models with an altered mucin glycosylation

Loss of O-glycans impairs the expression and function of several intestinal mucins, thereafter causing more profound defects in the function of the intestinal barrier than a flaw caused by the deficiency of an individual mucin. A number of transgenic mouse models have been developed to decipher the mechanisms underpinning the role of mucin glycosylation in gut homeostasis. Mice lacking core 3–derived O-glycans (also known as *C3GnT*$^{-/-}$ mice) display a substantial reduction of Muc2 protein and an increased susceptibility to DSS-induced colitis and accelerated colorectal tumorigenesis (An *et al.* 2007). In addition, core 3 O-glycosylation was shown to play a major role in controlling Salmonella intestinal burdens in *C3GnT*$^{-/-}$ mice (Zarepour *et al.* 2013). Similarly, *C2GnT2*$^{-/-}$ mice (mice lacking core 2-derived O-glycans) displayed an increased susceptibility to DSS-induced colitis but with no change in Muc2 expression (Stone *et al.* 2009). Mice with intestinal epithelial cell–specific deficiency of core 1–derived O-glycans (IEC *C1galt1*$^{-/-}$) develop spontaneous colitis (Fu *et al.* 2011). Mice lacking both core 1- and core 3-derived O-glycans (DKO mice) have an impaired mucus barrier function and develop colitis-associated colon cancer in which the dysbiotic microbiota promote inflammation and cancer (Bergstrom *et al.* 2016). In a water avoidance model in rats, psychological stress lead to less-protective mucus layer. In particular, O-glycosylation of mucins was strongly affected and these changes were associated with flattening and loss of the mucus layer cohesive properties (Da Silva *et al.* 2014). Altogether, these data suggest that the lack of a proper O-glycosylation impairs Muc2 expression or secretion and alters gut barrier function of the mucus layer.

In addition to modifications of mucin core glycans, mouse models have been developed targeting epitope modifications of the mucin glycans chains. Dawson and colleagues reported that deletion of the sulfate transporter NaS1 in mice (*Nas1*$^{-/-}$ mice) resulting in a decrease in mucin sulfation, enhanced susceptibility to experimental DSS colitis and systemic infection by *C. jejuni* (Dawson *et al.* 2009). In addition, mice with a deletion of the sulfo-transferase GlcNAc6ST2 enzyme adding sulfate to GlcNAC residues on O-mucin glycan chains exhibited an increased susceptibility to DSS-induced colitis (Tobisawa *et al.* 2010). Mice deleted for Sat-1 (sulphate anion transporter-1) were more susceptible to chronic infection by parasite *T. muris* (Hasnain *et al.* 2017). Collectively, these findings indicate that mucin abnormalities can initiate the onset of inflammatory related diseases in the gut.

In addition to mice harbouring a deletion in genes encoding proteins directly involved in mucin expression or glycosylation, several transgenic mouse models have been shown to display alterations in mucus properties. These include the Winnie and Eeyore mice which carry single missense mutations in two different D-domains of Muc2 (Heazlewood *et al.* 2008). These mice display fewer goblet cells and a reduction in secreted mucus with O-glycosylated and non-O-glycosylated Muc2. The misfolding results in endoplasmic reticulum stress, goblet cell apoptosis, depletion of the secreted mucus layer and development of chronic intestinal inflammation (Heazlewood *et al.* 2008). In combination with mucus, a large population of intraepithelial lymphocytes (IELs) bearing the $\gamma\delta$ T cell receptor is mediating immune protection against invading bacteria. In $\gamma\delta$ T-cell-deficient (*TCR*$\delta^{-/-}$) mice, mucin expression and glycosylation is altered, mucus-secreting goblet cells are significantly reduced in number and those animals are more prone to DSS-induced colitis (Kober *et al.* 2014). Mouse models deficient in TLR5, IL-10 and Sodium hydrogen antiporter 3 (Slc9a3 or Nhe3) revealed bacteria in contact with the epithelium. Additional analysis of the less inflamed *IL-10*$^{-/-}$ mice revealed a thicker mucus layer but a more penetrable inner mucus allowing bacteria to penetrate and reach the epithelium (Johansson *et al.* 2014).

Non-rodent models

Differences in mucus thickness and composition have been observed between rats, pigs and rabbits, and suggests that the pig mucus pattern resembles more closely that of humans (Varum *et al.* 2012). The zebrafish larva is an emerging model system for investigating components of the innate immune system, including mucus physiology. It has been shown that five gel-forming secreted mucin genes are found in zebrafish with a high degree of homology to other vertebrate mucins regarding their genomic and protein domain organisation, as well as their tissue specific expression (Jevtov *et al.* 2014).

Limitations of current experimental models involving mucus and future challenges

Given the importance of the gut microbiota as a modulator of health and disease, increasing attention has been devoted to the role played by mucus in the interaction with gut bacteria (Juge 2019). As described above (section 4), various experimental models increasing in complexity from simple *in vitro* assays to cell lines, organ-on-chips, *in vitro* colon fermentation systems or animal models have been developed and successfully applied to the study of gut microbe-mucus interactions. However, one of the limitations common to most *in vitro* models is the origin of mucins used to assess the interactions with bacteria doubled by the inability to reproduce a colonic mucus gel recapitulating the *in vivo* situation. This is important as the nature and origin of purified mucins used in these assays greatly influence the outcome of binding as demonstrated using microtitre plate (Owen *et al.* 2017), dot-blot (Ringot-Destrez *et al.* 2018) or mucin microarrays (Clyne *et al.* 2017). Mucin glycosylation plays a critical role in the interaction between gut bacteria and mucus and significant

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glycosylation differences occur between purified mucins used in *in vitro* assays from different sites of the murine GI tract or from goblet cells (e.g. LS174T), as analysed by mass spectrometry (Leclaire *et al.* 2018; Ringot-Destrez *et al.* 2018). In addition, the purification steps alter the properties of native glycoproteins and purified mucins used in these assays lack the ability to form viscoelastic hydrogels (Kocevar-Nared, Kristl and Smid-Korbar 1997). A similar situation occurs with mucus secreting cell lines where the type of mucins and structure of mucus differ from the colonic environment. For example, the HT29 cell line secretes mostly MUC5AC whereas MUC2 is the main mucin secreted in the small and large intestines. These differences are due to the use of cancer cells which show an alteration in the expression and glycosylation of mucins. In addition, the production of mucus by epithelial cell lines can be influenced by culture conditions. For example, growing cells on Transwell filters with a small amount of apical medium (semi-wet interface culture) in combination with mechanical stimulation (on a rocking platform) and addition of the Notch γ-secretase inhibitor DAPT resulted in polarisation and secretion of MUC2 and MUC5AC by HT29 MTX-P8, HT29 MTX-E12 and LS513 cells (Navabi, McGuckin and Lindén 2013). Additionally, the mucus produced by goblet cells in *in vitro* co-culture cell models is not continuous nor homogenate which is not fully representative of the *in vivo* situation. Lastly, the formation of the bi-layered mucus found in the colon remains a challenge in these models. Novel strategies such as multiple cell layers, 2D-organoid techniques or Organ-on-a-Chip devices are currently being developed to better mimic the human intestinal epithelial microenvironment. Such multiple cell models exhibit intestinal villus morphogenesis associated with mucus production. These models are also needed to recapitulate antimicrobial defense and inflammatory reactions normally occurring in mucosal tissues. Another advantage of these systems is that, unlike cell lines, organoids can be used to evaluate long-term interactions between mucus and gut microbes. However, these more advanced biopsy- based models remain low throughput and expensive as compared to *in vitro* assays and are limited by the availability and variability of clinical specimens.

In addition, to the host side, several microbial factors must be taken into consideration when assaying the interactions between the gut bacteria and mucus. These include the handling and labelling microbial cells which may affect the surface molecular determinants potentially involved in mucus/mucin interactions (e.g. cell-surface adhesins, pili or flagella) (Chagnot *et al.* 2014). The growth conditions (e.g. growth media with different nutrient compositions; temperature, pH, osmolarity or redox potential) can also influence the expression of the bacterial receptors mediating the interactions with mucins. To date, most studies have focused on the interactions between mucus and probiotic or pathogenic strains and assessing strictly anaerobe gut symbionts or complex microbial communities remain a challenge in this field of research.

An alternative to the systems described above is the use of dynamic *in vitro* fermentation models of the human gut, such as the M-SHIME (Marzorati *et al.* 2014) or DP-M-SHIME (De Paepe *et al.* 2018) models. In these *in vitro* colonic models, the introduction of mucin-covered beads allows to study the long-term *in vitro* microbial colonisation of mucin, in the presence of a complex anaerobic intestinal microbiota (Marzorati *et al.* 2014; Shah *et al.* 2016). These models provide a mean to study gut microbiota functionality and niche differentiation, during treatments with xenobiotics (for example antibiotics, synthetic chemicals such as food additives, environmental pollutants like persistent organic pollutants (POPs)), pathogens or functional foods. Future developments in this field will be the introduction of mucus secretion and/or a mucus surface layer in dynamic *in vitro* models of the upper GI tract, such as in the gastric and small intestinal TNO Gastro-Intestinal model (TIM) (Guerra *et al.* 2012). This is important so to take into account the successive stressful events (e.g. acidic gastric pH, bile salts) that commensal or pathogenic microbes undergo in the human GI tract before reaching the intestinal epithelium and that may greatly influence their physiological stage, virulence and/or activity.

However, as mentioned above, a limitation of these *in vitro* GI models is that they rely on commercially available mucins used for the mucin bead technology. These secretory mucins, usually MUC5AC and MUC6 porcine gut gastric mucin, differ in terms of structure and glycosylation from intestinal MUC2 and cannot form a bi-layered mucus gel. It has been proposed that in the future, *in vitro* engineered mucus may be used to mimic human-derived mucus in a more reproducible manner. The colonic *in vitro* models could also be improved by including immunoglobulins, specific antimicrobial peptides, or secreted phosphatidylcholine, which have been shown to modulate mucus surface properties, thereby influencing bacterial adhesion (Martens, Neumann and Desai 2018). Future *in vitro* colon models should also better mimic the *in vivo* transit, and particularly retrograde movements (Hiroz *et al.* 2009), as back-flow was recently suggested to be crucial for the persistence of gut microbes in the GI tract (Cremer *et al.* 2016). Current technological advances include the coupling of these fermentation models to intestinal epithelial cells or more complex units such as the HMI module. A next step will be to couple the digestive/fermentation models with enteroids/colonoids or HIO. However, despite their increased complexity, most of these approaches remain limited by the absence of important host functions, such as variable peristalsis-like motions. This is a critical limitation because mechanical deformations resulting from peristalsis both influence normal epithelial cell differentiation and control microbial overgrowth in the living intestine (Gayer and Basson 2009; Benam *et al.* 2015). The development of microfluidic systems and organ-on-chips is currently addressing this important technological gap (Kim and Ingber 2013; Kim *et al.* 2016).

The development of these advanced *in vitro* systems is essential to help reduce dependence on animal studies. Due to the invasive nature of the experiments, the mechanisms underpinning microbe-mucus interactions *in vivo* have mainly been investigated in animal models, mostly rodents. Genetically engineered mice impaired in mucin secretion or glycosylation have been instrumental to decipher the role of mucins and mucus in the protection of the intestinal epithelium and the interactions between pathogenic bacteria, commensal microbiota and the mucus barrier. However, although the domain organisation and expression pattern of mucins appear largely conserved between human and mouse (Joshi *et al.* 2015), mucin glycosylation and gut microbiota (Nguyen *et al.* 2015) differ between these two species. It has been speculated that differences in mucin glycosylation between mammalian species may underlie some of the differences in infectivity and/or pathogenicity for individual microbial pathogens (Linden *et al.* 2008) or the different commensal microbiota (Thomsson *et al.* 2012). Therefore, caution should be applied when translating data obtained in mouse models to humans. Lastly, unlike *in vitro* assays, *in vivo* studies are restricted to end-point measurements.

Recent years have witnessed unprecedented technological advances in the development of *in vitro* GI models that more

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closely resemble the gut mucosal interface. Our next challenge will be to simulate these models at different stages of development or disease conditions (e.g. IBD, obesity or CF). Special attention should be paid to inter-individual differences and intra-individual variability in gut microbiota composition and intestinal biopsies from different donors or patients. This is important to better understand the role of gut microbe–mucus interactions in the aetiology of a particular disease or condition and determine the microbial and biochemical signature that could differentiate between diseased and healthy status. In particular, more research is warranted to determine how the physicochemical properties and/or thickness of the mucus layer and mucin glycosylation are altered during a specific disease. In the future, these pre-clinical models will help screen novel therapeutic strategies aimed at restoring gut barrier function and tailored to the individual patient as a step towards personalised medicine.

Conflict of Interest. None declared.

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ABSTRACT

In piglets, weaning is a critical event associated with acute social, environmental and dietary stress engendering fairly often post-weaning diarrhea and enterotoxigenic *Escherichia coli* infections. Current strategies imply the systematic use of antimicrobials now raising public health concerns due to the massive increase of antibiotic resistance genes among bacteria. Non-pharmacological preventive and curative strategies are necessary to fight against post-weaning diarrhea in piglets. In compliance with the European Union directive aiming to reduce, refine and replace the use of animals in research, the principal aim of this PhD project was to develop a new *in vitro* fermentation model of the weaning piglet colon implemented with mucin beads to simulate both luminal and mucosal microbiota. Following its development and validation towards *in vivo* data, the presently designed *in vitro* model was used to evaluate the impact of two of the major stress encountered at weaning: a dietary change and transient anorexia as well as an enterotoxigenic *Escherichia coli* strain responsible for post-weaning diarrhea on the composition and activity of the microbiota. Finally, the effects of a *Saccharomyces* live yeast strain to fight against microbiota dysbiosis and ETEC infections in weaning piglets was evaluated using the newly developed *in vitro* model and cell culture experiments. The data collected throughout this PhD thesis participated to a better understanding of microbiota dysbiosis associated with weaning and gave interesting insights about the interest of a probiotic strategy in the critical period of weaning in piglets.

RÉSUMÉ

Chez les porcelets, le sevrage est une étape cruciale associée avec d'importants stress d'ordres sociaux, environnementaux et alimentaires qui engendrent fréquemment des diarrhées post-sevrage majoritairement d'origine colibacillaire. Actuellement, ces infections sont traitées de manière systématique à l'aide d'antibiotiques. Toutefois, le nombre de bactéries multi-résistantes aux antibiotiques augmente de façon alarmante et le besoin de stratégies alternatives aux antibiotiques pour lutter contre ces diarrhées post-sevrage devient urgent. En accord avec la directive européenne visant à réduire, remplacer et raffiner le nombre d'animaux utilisés à des fins de recherche, le principal objectif de ce projet de thèse était de développer un modèle *in vitro* reproduisant les paramètres physicochimiques et microbiens de l'environnement colique du porcelet au sevrage, le MPigut-IVM. Ce système permet, de manière unique, de distinguer les microenvironnements coliques luminaux et mucosaux et leurs microbiotes associés. Une fois validé comparativement à des données *in vivo* chez le porcelet, ce modèle innovant a été utilisé pour étudier l'effet des principaux stress rencontrés au sevrage, un jeûne alimentaire, suivi d'un changement d'alimentation et d'une infection par une souche d'*Escherichia coli* entérotoxigénique responsable de diarrhées sur la composition et l'activité du microbiote. Enfin, l'intérêt d'un traitement préventif à base de levures *Saccharomyces* a été évalué *in vitro* en utilisant une approche originale combinant le modèle colique développé et des cellules épithéliales intestinales en culture. Les données de cette thèse ont permis à la fois de mieux comprendre l'étiologie de la dysbiose du microbiote observé au sevrage chez les porcelets et d'évaluer *in vitro* l'intérêt d'une stratégie probiotique lors de cette phase critique de la vie de l'animal.