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Lactobacillus sakei modulates mule duck microbiota in ileum and ceca during overfeeding

F. Vasai,* K. Brugirard Ricaud,*¹ L. Cauquil,†‡§ P. Daniel,# C. Peillod,|| K. Gontier,* A. Tizaoui,¶
O. Bouchez,** S. Combes,†‡§ and S. Davail*

*Institut pluridisciplinaire de recherche sur l'environnement et les matériaux–Equipe Environnement et Microbiologie UMR5254, IUT des Pays de l'Adour, Rue du Ruisseau, BP 201, 40004 Mont de Marsan, France; †Institut National de la Recherche Agronomique (INRA), UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, F-31326 Castanet-Tolosan, France; ‡Université de Toulouse, Institut National Polytechnique de Toulouse (INPT)–Ecole Nationale Supérieure Agronomique de Toulouse, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, F-31326 Castanet-Tolosan, France; §Université de Toulouse INPT Ecole Nationale Vétérinaire de Toulouse, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, F-31076 Toulouse, France; #Laboratoires des Pyrénées et des Landes, 1 rue Marcel David, BP219, 40004 Mont de Marsan, France; ||Institut Technique de l'Aviculture, 28 rue du Rocher, 75008 Paris, France; ¶Institut Universitaire de Technologie des Pays de l'Adour, Rue du Ruisseau, BP 201, 40004 Mont de Marsan, France; and **Plateforme Génomique Bâtiment Centre de Ressources de Génotypage & Séquençage-Centre National de Ressources Génomiques Végétales, INRA Auzeville, Chemin de Borderouge-BP 52627, 31326 Castanet-Tolosan Cedex, France

ABSTRACT The supplementation with *Lactobacillus sakei* as probiotic on the ileal and cecal microbiota of mule ducks during overfeeding was investigated using high-throughput 16S rRNA gene-based pyrosequencing and real-time PCR. The ducks were overfed with or without *L. sakei* for 12 d with 56% ground corn and 42% whole corn. Samples were collected before the overfeeding period (at 12 wk), at 13 wk (meal 12 of overfeeding), and at 14 wk (meal 24), 3 h postfeeding. Whatever the digestive segment and the level of intake, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant phyla in the bacterial community of mule ducks (at least 90%). Before overfeeding, ileal samples were dominated by *Clostridia*, *Bacteroidia*, and *Gammaproteobacteria* (80% and up), and cecal samples by *Bacteroidia* and *Clostridia* (around 85%). The richness

and diversity decreased in the ileum and increased in the ceca after overfeeding. Overfeeding increased the relative abundance of *Firmicutes* and especially the *Lactobacillus* group in ileal samples. Nonmetric multidimensional scaling profiles separated the bacterial communities with respect to overfeeding only in cecal samples. Richness indicators decreased after *L. sakei* has been added at mid-overfeeding only in the ileum. In the ceca, the decrease of these indexes only occurred at the end of overfeeding. The addition of *L. sakei* triggers major changes in the ileum, whereas the ceca are not affected. *Lactobacillus sakei* decreased the relative abundance of *Bacteroides* at mid-overfeeding and the relative abundance of *Enterobacteria* at the end of overfeeding in the ileum.

Key words: duck microbial diversity, overfeeding, probiotic, 454 pyrosequencing, real-time PCR

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INTRODUCTION

The intestinal microbiota play a major role in animal physiology and form a complex ecosystem. For example, intestinal bacteria can affect gut morphology and nutrition and are known to stimulate the immune response and to protect against pathogens (Moreau et

al., 1982; Leser and Mølbak, 2009). In chickens, the bacterial activity is more intense in the crop, the small intestine (jejunum, duodenum, ileum), and the ceca in comparison with proventriculus, gizzard, and pancreas (Fuller, 1984). Culture-dependent methods have shown 2 major bacterial populations: facultative anaerobes from the crop to the small intestine and obligate anaerobes in the ceca (Fuller, 1984; Mead, 1989). In accordance with culture-dependent methods, molecular methods identified *Lactobacillus* spp. as the major bacterial group in the ileum (over 70% were *Lactobacillus* related) and 65% of the sequences in the ceca were

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¹Corresponding author: karine.brugirardricaud@univ-pau.fr

Clostridiaceae related in chickens (Lu et al., 2003). Molecular approaches have shown that the composition of intestinal bacteria is modified by diets (Knarreborg et al., 2002; Hammons et al., 2010). The different phyla identified in bird microbiota are also present in mammals, but molecular inventory in the crop of hoatzin (a tropical bird) showed genera and species specific to birds over 94% of all sequences (Godoy-Vitorino et al., 2010). Furthermore, archaeobacteria have been identified in the chicken cecum (Saengkerdsut et al., 2007) and the crop of hoatzin (Wright et al., 2009). In goose feces, *Bacilli*, *Clostridia*, and *Bacteroidetes* were predominant (Lu et al., 2009). A study of the 2 parental genetic types (Pekin and Muscovy) showed that the ileal and cecal microbiota were mainly composed of *Firmicutes* and *Bacteroidetes*. Furthermore, overfeeding has an effect on the bacterial composition of the microbiota of these ducks (Vasai et al., 2013). During this study, the nonoverfed animals at the same age have shown no evolution in their microbiota composition between 12 and 14 wk. Culture-dependent methods have shown that obligate anaerobes dominate their ceca (Ridsdale and Corry, 1999). During overfeeding (OF), the birds are exclusively fed with corn, which is rich in carbohydrates (especially starch). This diet induces a hepatic steatosis resulting in storage of fatty acids in the liver (named foie gras; Davail et al., 2003). We can hypothesize that this diet could affect the bacterial community.

Probiotics (live microorganisms) are known as beneficial to their host (Fuller, 1995). A study with chickens showed that dietary supplementation of probiotics induced significant changes in the microbial community structure of the gastrointestinal tract (Netherwood et al., 1999). Different modes of action have been described; for example, probiotics can reduce the presence of pathogenic bacteria in the digestive tract presumably by the production of bacteriocins, lactic acid, or both (Czerucka and Rampal, 2002; Servin and Coconnier, 2003; Servin, 2004). Competition between probiotic and endogenous bacteria can also occur with respect to the occupation of ecological niches (La Ragione, 2003). By strengthening the lactic acid bacteria presence, probiotics can also help establishing stable environmental conditions with a low pH limiting the development of pathogens (van Winsen et al., 2001). In addition, some lactobacilli strains exhibit significant hydrolytic action and favor digestion and absorption of nutrients, and make certain compounds available to the host. The addition of lactic acid bacteria to feed increased its safety and favored endogenous digestive bacteria (van Winsen et al., 2001). In the poultry industry, the interest in probiotics is increasing because growers in many countries can no longer use antibiotics as growth stimulants (Nava et al., 2005). In the current study, the effect of adding a probiotic (*Lactobacillus sakei*) during overfeeding of male mule ducks was investigated using real-time PCR and high-throughput pyrosequencing. This is the first report of a molecular inventory of bacterial

community in ileum and ceca mule ducks during overfeeding.

MATERIALS AND METHODS

Experimental Design

All experimental procedures involving ducks were in accordance with the French national guidelines for the care of animals for research purposes. Male mule ducks (MMG × PKL) were raised in a breeding facility belonging to the French National Research Institute for Agronomy (INRA) at the Domaine d'Artiguères, Benquet, France, accreditation number B40-037-1. The birds were placed into one pen and reared under usual conditions of light [24 h of light for the first 3 d, then natural photoperiod and temperature (26°C for the first 3 d and then a decrease of 2°C every 3 d to reach 18°C)] at the Experimental Station for Waterfowl Breeding (INRA, Artiguères, France). From the first day of life to 4 wk of age, the ducks were fed ad libitum with 2 mm pellets of a diet (starter diet) providing 11.93 MJ/kg of feed and a CP of 17.5%. From 5 to 12 wk of age, birds were fed with a diet providing 11.7 MJ/kg of feed and a CP of 15% (grower diet). At 12 wk of age, all birds were weighed and 228 ducks were selected for OF studies based on their live weights being representative of the live weight variability of the population. Among the ducks selected, 20 were killed before OF. The remaining 208 birds were housed in collective cages of 4 ducks per box, outdoor access was denied during the overfeeding time. Birds were housed in a ventilated room. They were fed hydraulically with an automatic feed dispenser (Gaveuse Mg 300 Dussau, Distribution Sas, Pecorade, Landes, France) using a diet consisting of 56% cornflour and 42% corn grain (overfeeding diet) providing 13.9 MJ/kg of feed and a CP of 8.91% with or without probiotics (10^9 *L. sakei* live cells for one meal in the overfeeding food). The composition of the different feeds is listed in Table 1. During OF, birds were fed twice a day for 2 wk. Digestive contents from 20 ducks in the groups fed diets with and without probiotic were obtained after killing the birds at 13 wk (meal 12) and at 14 wk (meal 24). Ducks were killed by exsanguination after electric stunning 3 h following their last meal to homogenize the filling levels of the ducks' digestive tract. Ducks with similar weights were selected at 5 points. Depending on the time of analysis during OF, the experiment had the following 5 experimental groups, each having $n = 20$ ducks: baseline before OF at wk 12, 1 wk during OF without administration of *L. sakei*, 1 wk during OF with administration of *L. sakei*, 2 wk during OF without administration of *L. sakei*, and 2 wk during OF with administration of *L. sakei*. Ileum and ceca were collected immediately after slaughter and kept on ice. Digestive contents of the ileum and ceca were collected by gently applying pressure along the organ and stored at -20°C .

Table 1. Ingredients and chemical composition of the experimental diets

Item (% unless otherwise specified)	Starter diet	Grower diet	Overfeeding diet (dry corn) DM %
Ingredient			
Wheat	15	35	—
Corn	32.6	38.69	98
Rapeseed oil free	7	12	—
Sunflower cake	8	11.35	—
Sodium carbonate	1.17	1.375	—
Dicalcium phosphate	0.675	0.575	—
Lysine	0.336	0.298	—
Salt	0.25	0.36	—
Methionine	0.235	0.086	—
Choline chloride	0.06	0.04	—
Premix + vitamin	0.3	0.23	2
Nutrient			
ME, MJ/kg	11.93	11.7	13.9
Humidity	12.46	12.61	—
CP	17.5	15	8.91
Fat	2.66	2.26	3.68
Cellulose	5.46	5.28	2.77
Ashes	5.24	4.99	—
Starch	40.21	47.32	75.91
Nutrient level (% raw material)			
Lysine	0.92	0.701	—
Methionine	0.506	0.36	—
Methionine + cysteine	0.85	0.705	—
Threonine	0.672	0.548	—
Tryptophan	0.203	0.173	—
Linoleic acid	1.43	1.2	—
Calcium	0.948	0.95	—
Phosphorus	0.575	0.519	—
Sodium	0.139	0.15	—
Available phosphorus	0.358	0.299	—
pH	—	—	5.36

DNA Extraction

For DNA extraction from pure bacterial cultures (Table 2), the QIAamp DNA mini kit was used following the manufacturer's recommended procedure (Qiagen, Hilden, Germany). Genomic DNA from 220 mg of ileal and cecal samples were extracted employing the QIA DNA stool kit (Qiagen) according to the manufacturer's instructions. An additional lysis step using lysozyme (140 μ L of 10 mg/mL in Tris-EDTA buffer; Sigma, St. Louis, MO) to improve the DNA extraction of gram-positive bacteria (Johansen et al., 2007). The extracted DNA was eluted in 200 μ L of Tris-EDTA buffer and stored at -20°C .

Real-Time PCR and Primers

Twenty birds per experimental groups (with or without probiotic according to overfeeding period) were used for quantitative PCR analysis. All primers sets are listed in Table 3. Reactions were run in duplicate in 48-well plates in a final volume of 15 μ L. The PCR mix consisted of 7.5 μ L of SybrGreen Universal PCR Master Mix (Quanta Bioscience, Gaithersburg, MD), 5.5 μ L of 500 nM primers, and 2 μ L of template DNA of appropriate dilutions or water for the negative control. Real-time PCR were performed in a StepOne instrument (Applied Biosystems, Saint Aubin, France) with an initial 10-min step at 95°C , 35 cycles for 15 s at 95°C , annealing/extension for 1 min at the temperature specific for each primer set, and 1 final cycle at 95°C for 15 s. Melt curve analyses were done by slowly heating the PCR mixtures from 60 to 95°C , and the cycle threshold (Ct) was determined with the StepOne Applied Biosystems software. Standard curves were generated from 10-fold serial dilutions in water of known concentrations of genomic bacterial DNA (Table 2) and used to quantify the copy number of the respective PCR reaction, with cycle threshold determined by the second derivative maximum method (Tichopad et al., 2003). Results are presented as the number of 16S rDNA copies and expressed per gram of fresh samples and \log_{10} transformed for statistical analysis.

High-Throughput Sequencing and Analysis of 16S rRNA Gene Amplicons

For pyrosequencing, 3 birds (yielding 3 ileums and 3 ceca) per experimental groups were used except for ileal sample before overfeeding where only one sample was analyzed. Amplicons from the V3 to V4 regions of 16S rRNA genes (corresponding to a 460-bp region of *Escherichia coli* 16S rDNA, GenBank number J01695) were produced using bacterial forward 343F (TACGGRAG-GCAGCAG; Liu et al., 2007) and reverse 784R (TAC-CAGGGTATCTAATCCT; Andersson et al., 2008) primers. The PCR was performed in a total volume of 100 μ L containing $1\times$ PCR buffer, 200 μ M of dNTP, 1 U of Isis DNA polymerase (MP Biomedicals, Illkirch, Graffenstaden, France), 0.5 μ M of each primer, and 1 to 5 ng of DNA template. The amplification program consisted of an initial denaturation step at 94°C for 2 min;

Table 2. Strains used for the preparation of standard curves for real-time PCR analysis

Strain	Primer targeted	Reference
<i>Escherichia coli</i>	<i>Enterobacteriaceae</i>	CIP548T
<i>Lactobacillus plantarum</i>	All bacteria	NCIMB8826
	<i>Firmicutes</i>	
	<i>Lactobacillus</i> group	
<i>Enterococcus faecium</i>	<i>Enterococcus</i>	CIP5432
<i>Bacteroides dorei</i>	<i>Bacteroides</i>	NSMZ17855
	<i>Bacteroidetes</i>	
<i>Clostridium</i> spp.	<i>Clostridium</i>	Laboratory strain
	<i>Firmicutes</i>	

Table 3. Targets and list of primers used for the enumeration of 16S rDNA sequences representing different bacterial groups by real-time PCR

Target	Sequence (5'–3')	Temperature (°C)	Reference
All bacteria	TCCTACGGGAGGCAGCAGT GACTACCAGGGTATCTAATCCTGTT	58	Walter et al. (2001)
<i>Firmicutes</i>	TGAAACTYAAAAGGAATTGACG ACCATGCACCACCTGTC	61.5	Bacchetti De Gregoris et al. (2011)
<i>Bacteroidetes</i>	CRAACAGGATTAGATACCCT GGTAAGGTTCCCTCGCGTAT	61.5	Bacchetti De Gregoris et al. (2011)
<i>Bacteroides</i>	GAGAGGAAGGTCCCCAC CGCTACTTGGCTGGTTTCAG	60	Layton et al. (2006)
<i>Lactobacillus</i> group	CACCGCTACACATGGAG AGCAGTAGGGAATCTTCCA	58	Walter et al. (2001)
<i>Clostridium</i>	TTACTGGGTGTAAGGG TAGAGTGCTCTTGCGTA	60	Van Dyke and McCarthy (2002)
<i>Enterococcus</i>	CCCTTATTGTTAGTTGCCATATT ACTCGTTGTACTTCCCATTGT	61	Rinttilä et al. (2004)
<i>Enterobacteria</i>	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	63	Bartosch et al. (2004)

32 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; and a final extension step at 72°C for 7 min. The PCR products were purified with the QIAquick PCR Purification kit (Qiagen) followed by DNA yield quantification and quality estimation using a Nano-Drop instrument. Sequencing of the 16S rRNA genes was performed with a 454 GS FLX instrument (454 Life Sciences, Roche, Branford, CT) and Titanium chemistry according to the manufacturer's instructions. Amplicons of 450 bp were pooled using equal amount of each PCR product. For the analysis, samples were recognized by couples of multiplex identifier (mid: 11-bp nucleotide sequence), which were fixed on the universal primers provided by Roche during the amplification. All sequences that did not have a length between 150 and 600 bp, contained 10 homopolymers, 1 *N* (ambiguous base), or had a mismatch in the primer sequence were removed using Python script developed by the bioinformatics platform of Toulouse (<https://mulcyber.toulouse.inra.fr/>).

The remaining sequences were treated with the R software (<http://www.r-project.org/>) to obtain the composition of the microbiota with respect to treatment conditions. Sequences were analyzed using Mothur software v. 1.24 (Schloss et al., 2009) and were aligned with the Silva database (14,956 sequences).

Pyrotag Processing and Analysis

A total of 292,043 16S rRNA gene sequences (also referred to as 16S pyrotags) were obtained with the 454 Titanium pyrosequencing run for the 28 samples. The 16S pyrotags were sorted based on their respective barcodes to form a total of 28 pyrotag library representing the 28 collected ileal and cecal samples. Sequences were sequentially filtered using a Python script developed by the bioinformatic platform of Toulouse (<https://mulcyber.toulouse.inra.fr/>), first by removing those sequences with a sequencing length less than 150 nt (35,880 sequences), those with at least one ambiguous base (8,887

sequences) or with a homopolymer stretch longer than 8 bases (140 sequences), those that did not match the proximal PCR primer sequences (with 2 mismatches allowed; 801 sequences), and finally those including both primer sequences but with a length shorter than 350 pb (295 sequences). A total of 246,090 sequences were retained corresponding to 8.789 ± 1.510 sequences per samples.

Taxonomic Classification and Statistical Analysis

Filtered sequences were analyzed using MOTHUR software v. 1.24; (Schloss et al., 2009) Readings were aligned with the SILVA alignment database provided by the MOTHUR software (14,956 sequences corresponding to unique sequences in the SSU REF database v102; Pruesse et al., 2007), and an alignment quality was calculated using the SILVA secondary structure map file (1,072 sequences were removed). After calculating a pairwise distance between aligned sequences, the sequences were clustered into operational taxonomic units (OTU, cutoff 0.05 using a farthest neighbor clustering). Rarefaction curves, abundance-based coverage estimator (ACE; defines the coverage of sampling), and Chao1 richness (calculates the estimated true species diversity of a sample) were calculated with the Rarefaction and Chao1 Estimator. Shannon α -diversity index (measure biodiversity) was calculated according to Hayek and Buzas (1996), and the results of diversity indexes were obtained using the Vegan library on the R software (Oksanen et al., 2010). For real-time PCR results, the Mann-Whitney test (nonparametric test) in the R software was used. Two independent samples of respective sizes n_1 and n_2 were available. If n_1 and $n_2 \geq 8$, the U statistic follows a normal distribution. The null hypothesis (H_0) is the sum of the ranks for the sample X is analogous to Y. $U_{X, Y} = \min(U_X, U_Y)$ was defined for testing the hypothesis (H_0). If the size n_1 and n_2 are lower than 20 and higher than 8, for a

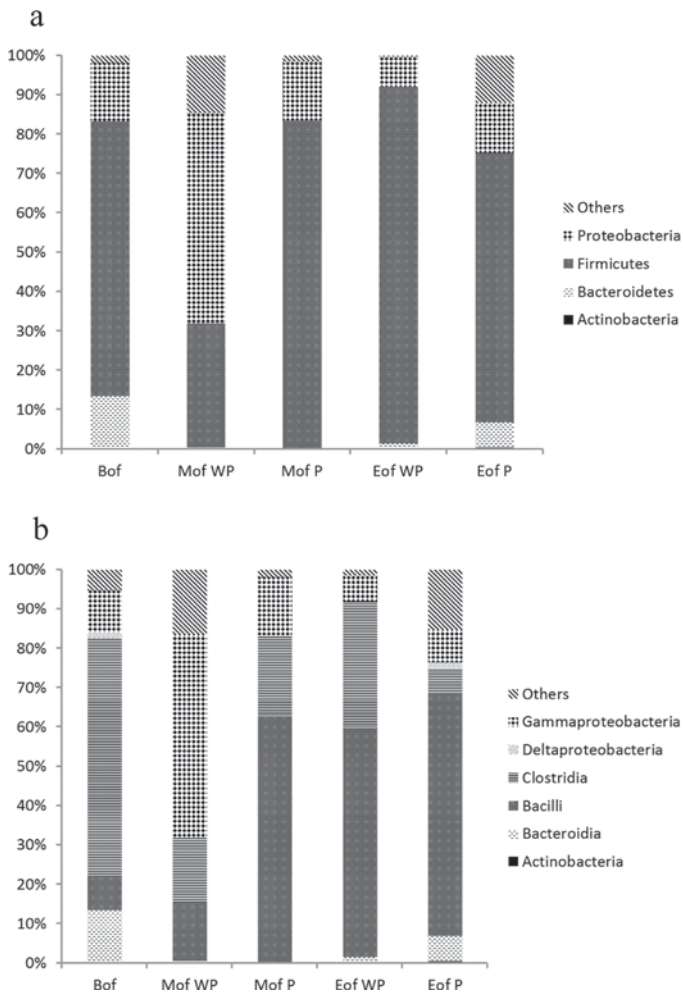


Figure 1. Percentage contribution of sequences (%) evaluated at the phylum (a) and class (b) levels to the total number of sequences in ileum during overfeeding (OF) and with or without probiotics (3 samples by experimental groups). Before OF (Bof), mid-OF without probiotics (Mof WP), mid-OF with probiotics (Mof P), end OF without probiotics (Eof WP), end OF (Eof P).

significance level α ($\alpha = 5\% = 0,05$), Mann-Whitney tables provide a critical value c to decide if $U_{X,Y,obs} > c$, H_0 was accepted; if $U_{X,Y,obs} \leq c$, H_0 was rejected.

RESULTS

Bacterial Community in Mule Ducks

Microbial diversity in samples was estimated by calculating the number of OTU. For all samples of the ileum, the number of OTU with a cutoff of 0.05 was 791 ± 63 with coverage by sample of $96.09 \pm 0.97\%$. The average number of sequences was 10,100; 8 different phyla were recorded for 118 different taxa. Furthermore, the Chao1, the ACE, and the Shannon index were, respectively, $1,472 \pm 105.3$, $1,943 \pm 136.3$, and 3.98 ± 0.17 (Table 4). The majority of the diversity of the ileum before OF was represented by *Firmicutes* sequences (70%). The rest was composed of *Proteobacteria* (14.98%) and *Bacteroidetes* (13.27%) sequences. Other phyla such as *Actinobacteria* or *Fusobacteria* represented less than 2% (Figure 1a). At the class level, the sequencing data indicated that the microbiota were mainly composed of *Clostridia* (60.50%) and *Bacilli* (8.63%) from the *Firmicutes*, *Bacteroidia* (13.14%) from the *Bacteroidetes*, and *Gammaproteobacteria* (10.73%) in the phylum *Proteobacteria*.

Regarding ceca samples, the number of OTU at a cutoff of 0.05 was $2,111 \pm 622$. The average number of sequences was 14,031 with 9 phyla represented by 138 different taxa. The Chao1, the ACE, and the Shannon index were, respectively, $4,129.49 \pm 1,166.61$, $6,246.55 \pm 1,760.35$, and 5.88 ± 0.27 (Table 4). The sequences retrieved from the ceca were mainly composed of *Bacteroidetes* sequences (57.03%). *Firmicutes* sequences amounted to 33.95% of the total, and *Proteobacteria* sequences accounted for 7.26% (Figure 2a). The sequences from 2 classes were dominant in the ceca, *Bacteroidia* (56.34%) from *Bacteroidetes*, and *Clostridia* (32.85%) from *Firmicutes*.

Effect of Overfeeding on the Microbial Community

Overfeeding triggered changes in bacterial microbiota in the ileum because before OF, the microbiota

Table 4. Estimators of diversity during overfeeding (OF) in ileum and ceca of mule ducks

Item	Period ¹					SEM
	Bof	Mof WP	Mof P	Eof WP	Eof P	
Ileum						
Number of operational taxonomic unit	791	441	302	320	569	63
Chao1 estimated richness	1,472.1	723.7	530.4	577.3	884.3	105.3
ACE ²	1,943.2	956.6	766.4	770.3	1,052.1	136.3
Shannon diversity index	3.98	3.52	2.59	2.94	3.22	0.17
Ceca						
Number of operational taxonomic unit	2,111	654	1257	780	361	209
Chao1 estimated richness	4,129.5	1,237.2	1,826.8	1,598.5	721.5	403.4
ACE	6,246.6	1,759.5	2,758.8	2,297.8	1,079	609.2
Shannon diversity index	5.88	4.74	5.15	4.63	4.46	0.19

¹Bof: before OF; Mof WP: mid-OF without probiotics; Mof P: mid-OF with probiotics; Eof WP: end OF without probiotics; Eof P: end OF. Three samples by experimental groups.

²ACE = abundance-based coverage estimator.

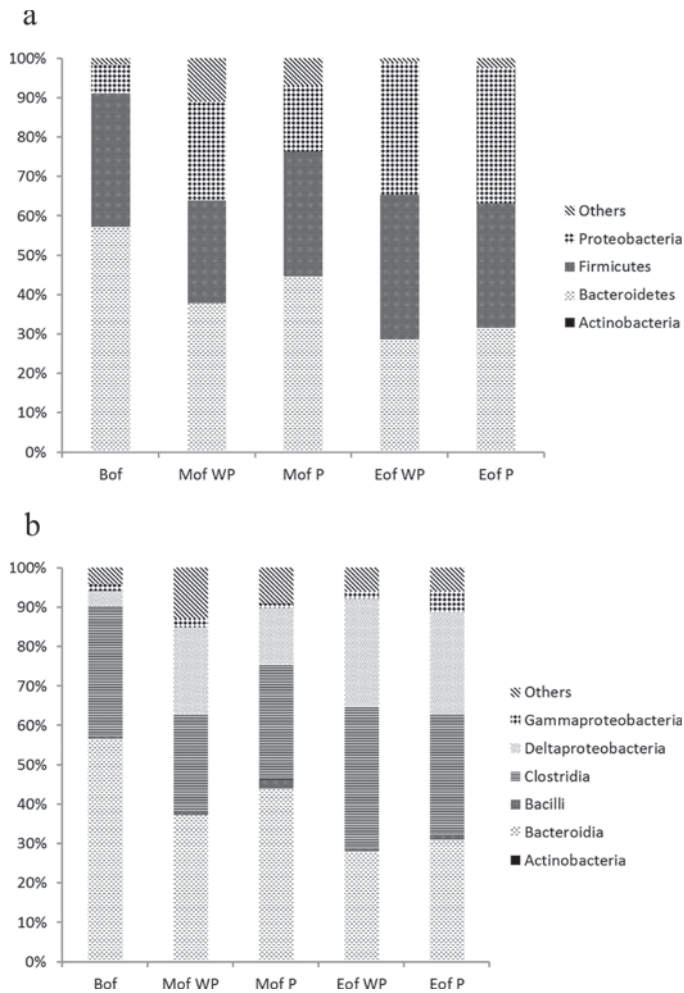


Figure 2. Percentage contribution of sequences (%) evaluated at the phylum (a) and class (b) levels to the total number of sequences in ceca during OF and with or without probiotics (3 samples by experimental groups). Before overfeeding (OF; Bof), mid-OF without probiotics (Mof WP), mid-OF with probiotics (Mof P), end OF without probiotics (Eof WP), end OF (Eof P).

was mainly composed of *Clostridia* (60.5%), followed by *Bacteroidia* (13.1%), *Bacilli* (8.6%), and *Gammaproteobacteria* (10.7%) as indicated by the sequencing data. At mid-OF, the *Gammaproteobacteria* were most represented (51.6%), whereas *Clostridia* represented only 16.3% of the population and *Bacteroidia* almost disappeared (0.4%). At the end of the OF phase, *Bacilli* (58.1%) were dominant but *Clostridia* still were an important component of the bacterial population with 32.3% (Figure 1b). Significant changes were observed using real-time PCR analysis of different targets. Toward mid-OF, a statistically significant decrease in *Bacteroides* and *Clostridium* was indicated by the pyrosequencing results. Between mid-OF and the end of OF, *Bacteroidetes* including *Bacteroides*, *Firmicutes* including *Enterococcus* numbers apparently decreased, whereas *Clostridium* number increased (Table 5).

The cecal microbiota consisted essentially of *Bacteroidia* (56.34%) and *Clostridia* (32.85%). Some changes were detected during OF but these 2 classes stayed dominant during OF. At mid-OF, there was a signifi-

cant increase in *Deltaproteobacteria* (21.79%), concomitant to a decrease in *Bacteroidia* (37.01%) and *Clostridia* (24.85%), but these 3 classes were still dominant at the end of OF, representing 90% of the total bacterial population (Figure 2b). Nonmetric multidimensional scaling (nMDS) profiles (representing the repartition of different families) between different periods of OF in ceca indicated that the bacterial communities in cecal samples were separated with respect to the 3 different OF periods (Figure 3). Analyses of similarity yielded an r of 0.346 and a P -value of 0.09 between before and mid-OF, $r = 0.143$ and a $P = 0.09$ between mid and end OF and $r = 0.636$ and a $P = 0$ between the first and the last period of OF. These changes were confirmed by real-time PCR as a statistically significant decrease in *Bacteroidetes* and *Enterococcus* after 1 wk of OF. Between mid-OF and the end of OF the total bacterial load, *Bacteroides*, *Firmicutes* including *Clostridium*, and *Lactobacillus* group decreased (Table 5).

Effect of a Probiotic on the Microbial Community

In samples from probiotics-fed ducks, richness indicators decreased at mid-OF only in the ileum whereas in the ceca, the decrease of these indexes only occurred at the end of OF. In the ileum at mid-OF, the addition of *L. sakei* (which is part of the *Bacilli* class) triggered an increase in the *Bacilli* class from 15.05% without probiotics to 62.6% with them. This increase coincided with a decrease in *Gammaproteobacteria* from 51.57 to 14.89%, whereas the *Clostridia* increased slightly from 16.34 to 20.35%. At the end of OF, when *L. sakei* was added to the meal, we observed a decrease in the percentage of *Clostridia* (−25.95%), whereas conversely there was an increase in *Bacteroidia* of around 6.5% and of the *Fusobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Epsilonproteobacteria*. At the end of OF, the *Bacilli* represented more than 60% of the total population (Figure 1b). Analyses by nMDS showed that the bacterial community can be separated regarding the addition of *L. sakei*, since an r -value of 0.349 and a P of 0.025 were obtained. These data indicate separated but strongly overlapping groups (Figure 4). Real-time PCR data confirmed this effect on bacterial composition: *Bacteroides* statistically decreased at mid-OF with or without probiotics but with a strong effect when *L. sakei* was administrated (Table 5). A slight decrease in total bacterial load and the representatives of the *Firmicutes* including the *Lactobacillus* group was observed. Sequences representing the *Enterobacteria* decreased strongly (Table 5).

Treatment with *L. sakei* did not trigger changes in the number of sequences representing the *Bacilli* in the ceca. At mid-OF, only a small percentage of corresponding *Bacilli* sequences (2.36%) were found. In contrast, *Deltaproteobacteria* sequences decreased from 21.79 to 14.40% whereas *Clostridia* and *Bacteroidia* sequences increased by 4.20 and 6.83%, respectively. At the

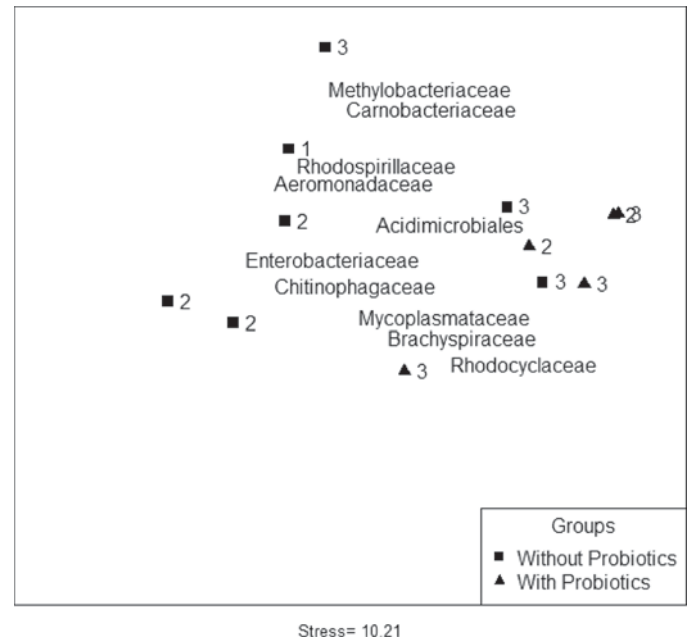
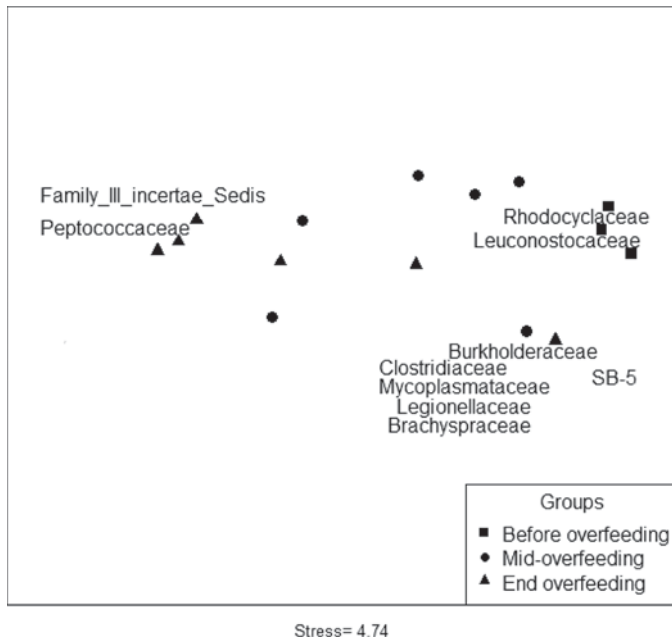


Figure 3. Nonmetric multidimensional scaling (nMDS) profile showing the distribution of different families of the different samples studied before, mid, and end of overfeeding in ceca.

Figure 4. Nonmetric multidimensional scaling (nMDS) profile showing the distribution of different families of the different samples studied without or with probiotics in 3 periods of overfeeding (OF) in the ileum. Before (1), mid (2), and end (3) of OF in the ileum.

end of OF there was a decrease in *Clostridia* numbers (-5.23%), whereas there was a slight increase in *Bacteroidia* ($+2.95\%$) and *Gammaproteobacteria* numbers ($+3.34\%$) in samples from probiotics-fed birds (Figure 2b). The nMDS analysis did not show a separation of

groups with respect to probiotics-fed and probiotic-free birds (data not shown). Statistical changes due to the probiotics used were also not observed with the real-time PCR analysis.

Table 5. Statistical effect of overfeeding (OF) and addition of probiotics on different phyla, genus, or groups in ileum or ceca of mule ducks¹

Item	Period ²					SE	Significance ³	P-value
	Bof	Mof WP	Mof P	Eof WP	Eof P			
Ileum (20 samples by experimental groups)								
All bacteria	8.39	8.95	9.98	9.32	8.74	0.61	d	0.019
<i>Bacteroidetes</i>	6.25	6.38	6.36	5.37	5.43	0.51	b	<0.001
<i>Bacteroides</i>	6.31	5.79	5.60	4.74	4.72	0.69	a	0.011
							c	0.026
							b	<0.001
<i>Firmicutes</i>	9.85	10.00	10.75	10.05	9.25	0.54	b	0.018
							d	0.0031
<i>Clostridium</i>	8.74	6.60	6.57	8.6	8.79	1.17	a	0.04
							b	<0.001
<i>Enterococcus</i>	4.90	5.01	5.33	3.69	4.32	0.65	b	0.0002
<i>Lactobacillus</i> group	8.33	8.66	9.43	9.04	8.74	0.41	d	0.033
<i>Proteobacteria</i>								
<i>Enterobacteria</i>	6.73	8.00	8.76	8.23	6.82	0.89	d	<0.001
Ceca (20 samples by experimental groups)								
All bacteria	11.27	11.78	11.88	10.10	10.4	0.81	b	<0.001
<i>Bacteroidetes</i>	9.98	9.19	8.96	8.46	8.87	0.56	a	<0.001
<i>Bacteroides</i>	10.07	10.47	10.30	8.29	8.85	0.97	b	<0.001
<i>Firmicutes</i>	10.92	12.23	12.04	11.05	11.07	0.62	b	0.0001
<i>Clostridium</i>	11.92	12.18	12.34	11.01	12.23	0.59	b	<0.001
<i>Enterococcus</i>	5.36	3.53	4.27	4.89	4.86	0.71	a	<0.001
<i>Lactobacillus</i> group	8.87	8.91	10.05	7.82	7.97	0.90	b	<0.001
<i>Proteobacteria</i>								
<i>Enterobacteria</i>	8.24	9.53	9.03	9.27	9.05	0.48	—	—

¹The median is expressed as \log_{10} copies number of 16S rDNA/g of fresh digestive content.

²Bof: before OF; Mof WP: mid-OF without probiotics; Mof P: mid-OF with probiotics; Eof WP: end OF without probiotics; Eof P: end OF.

³Overfeeding effect: a: Bof versus Mof WP, b: Mof WP versus Eof WP. Probiotic effect: c: Mof WP versus Mof P, d: Eof WP versus Eof P.

DISCUSSION

As in many other studies on intestinal microbiota of birds and mammals, the current study provides evidence for the *Firmicutes* and *Bacteroidetes* as the major phyla, a finding that suggests their important role in metabolism and host physiology (Ley et al., 2008; Kohl, 2012). The *Proteobacteria* were the second most common phylum after *Firmicutes* in ileal samples based on 454 pyrosequencing, as was also observed in feces of Canada geese (Lu et al., 2009) and in Muscovy ducks (*Cairina moschata*; Vasai et al., 2013). Interestingly, mule ducks are a hybrid of a male Muscovy and a female Pekin duck (*Anas platyrhynchos*). Pekin, Muscovy, and mule ducks differ in their ability to produce a fatty liver because Mule and Muscovy ducks show a lower capacity for fat storage than Pekin ducks. In contrast to chickens, where obligate anaerobes dominated ceca samples (the *Clostridia* class from *Firmicutes* and *Bacteroidetes*) and facultative anaerobes (*Bacilli* class and especially *Lactobacillus*) are dominant in the ileum (Lu et al., 2008), in mule ducks, obligate anaerobes were dominant in both ileal (class *Clostridia* and *Bacteroidia*) and cecal samples. Higher numbers of sequences representing the *Clostridia* in ducks than in chickens suggest that the bacterial digestive metabolism in chicken and ducks could be somewhat different.

Overfeeding increased the relative abundance of *Firmicutes* in the ileum and especially a component of the *Bacilli* class, the *Lactobacillus* group as previously described for Pekin and Muscovy ducks (Vasai et al., 2013). These bacteria are well known as amylolytic bacteria and frequently increase in pigs and rats fed with diets rich in starch (Wang et al., 2002; Regmi et al., 2011). Interestingly, in cattle, it has been shown that *Lactobacillus* increase with diets containing more than 71% of starch on concentrate with a composition very close to that the feed used in the present study (Brown et al., 2006). According to the PCR results, *Bacteroides* and *Clostridium* numbers decreased, but interestingly, for *Clostridium* this decrease was reversed at the end of OF. The clustering of the results for the cecal samples shows a good separation into different groups regarding the OF period, before, mid, or end of OF, probably due to the negative effect on the total bacterial load especially the *Bacteroidetes* including *Bacteroides*, *Firmicutes* including *Clostridium*, *Lactobacillus* group, and *Enterococcus*. *Bacteroides* is well known to ferment a variety of plant polysaccharides like amylose starch (Salyers et al., 1977). Interestingly, when *Enterococcus faecium* was used as probiotic in piglets, it increased the nutrient availability and improved the absorption of glucose (Lodemann et al., 2006).

Lactobacillus sakei reduced the diversity indexes at mid-OF only in the ileum, whereas in the ceca, the decrease of these indexes only occurred at the end of OF. In newborn chicks and ducks inoculated intragastrically with *Lactobacillus* species, growth performance as well

Firmicutes/Bacteroides ratios increased in comparison with those of controls (Angelakis and Raoult, 2010), but in the current study no significant effect on growth performance and weight of the fatty liver was observed (data not shown). Feeding of *L. sakei* triggered an increase in ileal *Bacilli* numbers and a decrease in sequences representing the phylum. In poultry species, the use of lactobacilli alone or in mixtures of bacteria reduced the numbers of coliforms including *Escherichia coli*, *Salmonella enterica*, and *Clostridium perfringens* as determined by culture-dependent methods (Fuller, 1977; Francis et al., 1978; Watkins et al., 1982; Watkins and Kratzer, 1983; Mountzouris et al., 2007). Molecular methods confirmed the changes induced by probiotics on bacterial communities (Netherwood et al., 1999). Real-time PCR has been used to confirm the beneficial effect of *L. sakei* by decreasing *Enterobacteria*, suggesting that *L. sakei* can protect against potential pathogens (Chaucheyras-Durand et al., 2006). The number of sequences representing *Bacteroides* significantly decreased with probiotic supplementation, suggesting some level of antagonisms between *L. sakei* and these bacteria. The separation of the sequence data into 2 separated groups based on the diets with and without the probiotic (Figure 4) suggests that the bacterial communities were more affected by *L. sakei* than by overfeeding. Because a decrease in the total bacterial load at the end of OF in the ileum was observed, it is possible that *L. sakei* inhibited growth of several other bacteria. The cecal samples showed a very different pattern than the ileal samples in response to probiotics. First of all, the probiotics did not change the microbial communities enough to see an effect based on nMDS analysis and bacterial composition. In chickens, it is well known that *Lactobacillus* spp. (facultative anaerobes) are an important component of the ileal microbiota, whereas *Clostridia* and *Bacteroides* (obligate anaerobes) are predominant in cecal samples (Lu et al., 2003). This observation could explain why feeding *L. sakei* did not have a noticeable effect in the ceca. Perhaps, the complex anaerobic environment is very refractory to changes in the microbial community. Overall, the real-time PCR data were in agreement with the sequencing results; however, for samples from the mid-OF and end OF periods, pyrosequencing showed an increase whereas real-time PCR revealed a slight decrease in *Firmicutes* numbers. The reason(s) for the discrepancy are not known. To conclude, this work, using both real-time PCR and high-throughput pyrosequencing based on 16S rRNA genes in ileal and cecal samples, gives a first report on the microbial community in ducks and the effect of administering probiotics during overfeeding on the abundance of major groups. The microbial diversity of mule ducks is dominated by *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, and OF modifies bacterial communities of ceca samples, whereas probiotics show an important effect on ileal samples. The increase in lactobacilli through the OF process

when adding *L. sakei* as a probiotic could potentially improve the birds' health and could be of great interest to the poultry industry.

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