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SUMMARY
Severe acute malnutrition (SAM) is a multifactorial disease affecting millions of children worldwide. It is associated with changes in intestinal physiology, microbiota, and mucosal immunity, emphasizing the need for multidisciplinary studies to unravel its full pathogenesis. We established an experimental model in which weanling mice fed a high-deficiency diet mimic key anthropometric and physiological features of SAM in children. This diet alters the intestinal microbiota (less segmented filamentous bacteria, spatial proximity to epithelium), metabolism (decreased butyrate), and immune cell populations (depletion of LysoDC in Peyer’s patches and intestinal Th17 cells). A nutritional intervention leads to a fast zoometric and intestinal physiology recovery but to an incomplete restoration of the intestinal microbiota, metabolism, and immune system. Altogether, we provide a preclinical model of SAM and have identified key markers to target with future interventions during the education of the immune system to improve SAM whole defects.

INTRODUCTION
The first 1,000 days of life are critical for optimal growth, physiological, neurological and immune development, and establishment of symbiotic relationships with the microbiota. At weaning, the introduction of solid food and subsequent changes of gut microbiota are critical for inducing an immune reaction to microbiota that educates the immune system and protects from immunopathologies later in life. In low- and middle-income countries, weaning is a high-risk period for malnourished children because of the intake of foods low in protein and essential micronutrients. Undernutrition is often associated with altered immunity resulting in an elevated risk of infection and a reduced response to oral vaccines. Thus, severe acute malnutrition (SAM) is a life-threatening condition affecting 13.6 million children under five worldwide in 2020. SAM condition is defined by a weight-for-height Z score lower than −3 SD and a mid-upper arm circumference less than 115 mm. Despite advances in the management of SAM with therapeutic foods, considering only anthropometric and clinical criteria to assess nutritional recovery correlates poorly with optimal long-term recovery. This is reflected by the high post-discharge mortality associated with malnutrition relapse, recurrent infections, intestinal inflammation, and enteropathy.

Children with SAM harbor an atypical immature microbiota composition that matches the one of younger healthy children. Furthermore, SAM is transmitted in germ-free mice on a malnutrition diet when receiving the microbiota or IgA-targeted bacterial taxa of children with SAM. These findings support the central role of microbiota composition in the pathogenesis of SAM. Moreover, increased susceptibility of children with SAM to diseases may be due to this abnormal microbiota unable to properly educate the mucosal immune system. The simultaneous assessment of physiological, microbial, and immune parameters at mucosal sites is limited in SAM children for obvious ethical reasons. Current preclinical models including inadequate diets, like dietary/calorie restricted or low protein ones, are pertinent to better understand the involvement of these parameters in SAM pathogenesis, but they do not recapitulate the full spectrum of SAM pathogenesis in children.
In this study, we developed a “SAM mouse” model starting at weaning and reproducing the main physiological features of SAM in children. Using this model, we investigated intestinal microbiota composition and location, metabolite production, and intestinal immunity. Nutritional intervention led to zoometric and physiological parameters recovery. However, microbiota, metabolic, and immune defects were prolonged, meaning that this murine model will not only help to dissect SAM pathogenesis but also to implement therapeutic foods with specific compounds restoring proper microbiota and immunity.

RESULTS

Severe growth and intestinal barrier defects in malnourished weanling mice

We aimed to develop a preclinical mouse model of SAM that would mimic as closely as possible the physiopathology of SAM in children. According to the literature, the diets of children suffering from growth stunting are high in carbohydrates and low in protein and fat. However, severe forms of undernutrition, including kwashiorkor, are not only associated with protein deficiency but also with type II nutrients (e.g., essential amino acids, zinc, and potassium) and micronutrients deficiencies. Exclusive cereal-based diets, including corn, are deficient in type I and II nutrients, which are essential for metabolic processes and growth. In rats, protein deficiency associated with high concentration of sodium and/or potassium was associated with the development of edema, a clinical sign of kwashiorkor. Similar to the study by Lykke et al., who used a corn-based diet to reproduce severe growth stunting in a piglet model, and in order to mimic real-life situation in which young children are fed a low-variety and cereal-based diet deficient in type II nutrients, we designed a pure corn-based diet deficient in type I and II nutrients, including kwashiorkor, are not only associated with protein deficiency but also with type II nutrients (e.g., essential amino acids, zinc, and potassium) and micronutrients deficiencies. Exclusive cereal-based diets, including corn, are deficient in type I and II nutrients, which are essential for metabolic processes and growth. In rats, protein deficiency associated with high concentration of sodium and/or potassium was associated with the development of edema, a clinical sign of kwashiorkor. Similar to the study by Lykke et al., who used a corn-based diet to reproduce severe growth stunting in a piglet model, and in order to mimic real-life situation in which young children are fed a low-variety and cereal-based diet deficient in type II nutrients, we designed a pure corn-based diet deficient in protein, fat, and most of essential minerals and vitamins to induce SAM in conventional mice (Table S1).

At weaning, male and female mice received either a regular control diet (Ctrl diet) or the isocaloric SAM diet during four weeks (Figure 1A). From the first week after weaning, males fed with the SAM diet gained significantly less weight than Ctrl males, reaching a 2.4 times lower weight gain (Figure 1B) and a mean weight 1.8 times lower (Figure 1A) four weeks after weaning. In line with previous studies, only SAM male mice were affected by a severe growth delay comparable to SAM condition in children with a weight-for-length Z score (WL-Z) at –4.96 ± 1.43 (Figure 1C and Figure S1B). This growth delay occurred despite a calorie intake-to-weight gain ratio higher or equivalent in SAM mice compared to Ctrl mice (Figure S1C). The SAM males were also smaller (Figure 1C and S1D), had shorter tails (Figure 1C) and femurs (Figure S1E) than Ctrl males. Henceforth, only male mice were considered for the rest of the study.

Children with SAM suffer from nutrient malabsorption associated with villus atrophy, increased intestinal permeability, and chronic intestinal inflammation in a subclinical condition termed environmental enteropathy. Our SAM mouse model also exhibited significant intestinal morphological changes characterized by a shorter small intestine (SI) (Figure 1D), an atrophy of ileal villi and of Peyer’s patches (PPs) (Figures 1E and 1F), and shorter colonic but not ileal crypts (Figures 1E, and S1F–S1G). SAM mice also showed a significant rise in the concentration of fecal neutrophil gelatinase-associated lipocalin (NGAL), a marker of intestinal inflammation (Figure 1G). This inflammation was accompanied by altered intestinal functions, highlighted by a significant increase in intestinal permeability (Figure 1H) and a decrease of...
the zonula occludens-1 (ZO-1) network at the follicle-associated epithelium of PPs and along the ileal villi (Figure 1I). Finally, the mucus layer in SAM mice was thicker and enriched with carbohydrate motifs recognized by the lectin VVA (α- or β-linked terminal N-acetylgalactosamine) (Figure 1J). Altogether, these data showed that our weanling model of SAM mimics most of the anthropometric and intestinal pathophysiological features of SAM in children suffering from environmental enteropathy.

Reversibility of growth and intestinal barrier defects of malnourished weanling mice

To test whether the effects of two weeks of severe undernutrition during this critical period of growth could be reversed, we carried out nutritional intervention by switching SAM to Ctrl diet (Int mice) (Figure 1A). This resulted in a gradual recovery of zoometric parameters within 2 weeks, with a final weight gain and growth slightly below that of the Ctrl mice (Δ = 1.45 g ± 1.83 g, p-value<0.05) (Figures 1B, and S1A), an increase in the size of the mice (Figures 1C, and S1D) and in the femur length (Figure S1E), and a WL-Z at −1.22 ± 1.02 (Figure 1C). Regarding the intestinal parameters, we observed a total recovery of the SI and ileal villus length (Figures 1D–1F) and of the size of PPs (Figure 1F). The colonic crypt depth (Figures 1E, and S1G) remained slightly below that of Ctrl mice although not significantly different. Fecal NGAL concentration (Figure 1G), intestinal permeability (Figure 1H), and ileal ZO-1 network (Figure 1I) were also restored. Finally, the overall aspect of the mucus layer appeared intermediate between Ctrl and SAM mice (Figure 1J). Therefore, return to a regular diet that met the nutritional requirements of the critical period of growth elicited zoometric and physiological recovery.

SAM alters the spatial organization and composition of the ileal microbiota

Since SAM is associated with an abnormal microbiota,11,12,14,24 we studied its spatial organization in the terminal part of the ileum by fluorescence in situ hybridization (FISH). In Ctrl mice, the microbiota was kept at bay from the epithelium of villi and PPs at the exception of segmented filamentous bacteria (SFB) known to anchor ileal villi and PPs.25 (Figure 2A). Remarkably, in SAM mice, the microbiota was 3–4 times closer to the intestinal epithelium of villi and PPs at the exception of segmented filamentous bacteria (SFB) known to anchor ileal villi by fluorescence in situ (Figure 1J). Therefore, return to a regular diet that met the nutritional requirements of the critical period of growth elicited zoometric and physiological recovery.

Nutritional intervention results in only partial recovery from SAM-induced cecal dysbiosis

We observed a significant decrease in the weight and size of the ceca of SAM and Int mice compared to Ctrl mice (Figure S3A), likely indicative of microbial changes in the cecum.28,29 Indeed, unweighted PCA and
weighted partial least squares discriminant analysis (PLSDA) separated cecal microbiota according to the diet (Figure 3A). Shannon 1 indexes were not significantly different between Ctrl and SAM mice (Figure S3B) but we observed a significant decrease in Bacteroidetes, whereas Firmicutes and Desulfovibacterota, previously classified as proteobacterial classes Delta-proteobacteria, were significantly increased in SAM mice (Figure S3C and Table S3A). A clear separation between Ctrl and SAM mice was also observed at the species level (Figure 3B). Of the 52 ASVs significantly modified in SAM mice compared to Ctrl mice, 33 ASVs belonging to Oscilllospiraceae (Oscillibacter, Colidextribacter, Oscilllospiraceae_g_NK4A214 group), Erysipelotrichaceae (Turicibacter, Faecalibaculum), Lachnospiraceae (Tuzzerela, Lachnospiraceae NK4A136 group, Acetatifactor, Blautia), Clostridiaeae (Clostridium sensu stricto 1), Ruminococcaceae, Anaerovoraceae (Family_XIII_AD3011_group), Desulfovibronaceae, and Eggerthellaceae (Enterorhabdus) families had increased abundances in SAM mice (Figure 3C and Table S3B). Turicibacter, Clostridium sensu stricto 1 are known to be associated with weight loss and are increased during consumption of protein-deficient diets in animals and humans. 14,31,32 SAM and stunting are also associated with enrichment in inflammogenic and opportunistic species such as Acetatifactor and Enterorhabdus or members of Erysipelotrichaceae and Desulfovibronaceae. 13,37 Undernutrition is in general linked to depletion of several taxa from Lachnospiraceae and Ruminococcaceae families whose members are mainly short-chain fatty acids (SCFA) producers. However, some of them were both enriched and depleted in SAM mice compared to Ctrl mice (Figure 3C and Table S3B). Among the 19 ASVs diminished in SAM mice, Alistipes from Rikenellaceae family, Blautia, Lachnospiraceae NK4A136, Lachnoclostridium, and Roseburia from Lachnospiraceae family are also depleted in children with SAM and are butyrate producers associated with good health. 38,43,44 The Christensennellaceae R-7 group was also decreased in SAM mice compared to Ctrl mice (Figure 3C and Table S3B). Consistent with our data, conditions associated with inflammation such as stunting, high fecal calprotectin, or inflammatory bowel diseases appear to deplete Christensennellaceae R-7 group, whose anti-inflammatory and SCFA-producer properties make it one of five taxa considered necessary for a healthy gut depleted in inflammatory bowel diseases. Finally, and in accordance with the ileal data (Figure 2), SAM mice were significantly depleted in Candidatus Arthromitus compared to the Ctrl mice (Figure 3C and Table S3B). Interestingly, Erysipelotrichaceae expansion and notably a Faecalibaculum species are able to displace SFB in the small intestine of malnourished mice.

Nutritional intervention resulted in a microbiota profile closer to Ctrl mice than to SAM mice (Figure 3A, S3C–S3D, and Table S3D), but with specific signatures such as increase in members of Oscilllospiraceae, Ruminococcaceae, Defluviitaleaceae, Anaerovoraceae, and Eggerthellaceae families, and decrease in Blautia and Candidatus Arthromitus compared to Ctrl mice (Figure 3D and Table S3C). Altogether, these results reveal the onset of a cecal dysbiosis in SAM mice that cannot be totally solved by this nutritional intervention.

**Nutritional intervention results in only partial recovery from SAM-induced metabolic alterations**

A wide variety of metabolites including vitamins and SCFAs are produced by the intestinal microbiota and drastic changes in microbiota induced by SAM may alter the profile of produced metabolites. Cecal contents of Ctrl, SAM, and Int groups were analyzed for changes in metabolite abundance using untargeted metabolomic analysis by UPLC-HRMS. The metabolomic profile of SAM mice was indeed distinct from the Ctrl mice as shown by PCA and PLS-DA (Figure 4A). Among 50 identified metabolites, 5 were significantly increased and 23 decreased in SAM mice compared to Ctrl and Int mice (Figures 4B–4C, S4A and Table S4), as expected, from the higher protein content of the Ctrl diet, there was an overrepresentation of amino acid (AA) metabolism in Ctrl mice (Figure S4A). The significant reduction in L-Lysine, cadaverine (Lysine decarboxylation product), and 5-aminopentanoate (Lysine and cadaverine degradation product) converged to a shift toward the lysine degradation pathway in SAM mice, highlighting the crucial need for this essential AA (Figure S4A). The bile acid biosynthesis pathway (12-Ketolithocholic acid and deoxycholate) was also underrepresented in SAM mice (Figure 4C). The increase in 4,6-dihydroxyquinoline and
3-hydroxyanthranilate and decrease in kynurenic acid in SAM mice demonstrated a shift in the tryptophan metabolic pathway compared to the two other groups (Figure 4C). The increase in pyridoxamine (water soluble vitamin B6 form) in SAM mice pointed out a higher vitamin B6 concentration than in Ctrl mice (Figure S4A), consistent with the rise in the main phyla producing specific B-vitamins.50 Bacteroidetes, Actinobacteria, and Proteobacteria (Figure 2C). Since some butyrate producers were depleted in SAM mice (Figure 3C), we analyzed cecal SCFA concentrations by gas chromatography-mass spectrometry. There was a significant decrease in the production of SCFAs, especially in butyrate, in SAM mice (Figures 4D and S4B). Nutritional intervention resulted in a metabolic profile closer to Ctrl mice than to SAM mice (Figures 4A, and S4A). SCFAs production and the ratio between acetate, butyrate, and propionate were completely restored (Figures 4D and S4B). Nevertheless, it induced specific signatures, including an increase in kynurenic acid and phenylacetic acid (Figures 4C, and S4A). Spearman’s rank correlation coefficients between bacterial relative abundance and metabolite concentrations showed strong positive correlation between the ASVs enriched in SAM mice and the increase of choline, 4,6-dihydroxyquinoline, 3-hydroxyanthranilic acid, caffeate, phenylacetic acid, and pyridoxamine (Figure 4E), and strong negative correlation between the ASVs enriched in SAM mice and the increase of choline, 4,6-dihydroxyquinoline, 3-hydroxyanthranilic acid, caffeate, phenylacetic acid, and pyridoxamine (Figure 4E), and strong negative correlation between the ASVs and SCFAs at the exception of isovalerate (Figure 4F). Altogether, these data demonstrate that SAM mice had an unusual metabolic profile that cannot be fully restored by this nutritional intervention, except for SCFAs.

Alteration of the IgA response in Peyer’s patches and villi of SAM mice

Continuously exposed to food-derived antigens, microbiota, and pathogens, PPs are primary immune response initiation sites of the SI, notably inducing the generation of IgA-secreting plasma cells, which play a key role in controlling pathogen invasion and ensuring microbiota regulation.51–53 Yet, the impact of malnutrition on PPs is understudied.54 Due to PP atrophy, the numbers of total B cells, IgA-expressing B cells (IgA⁺ B cells), germinal center B cells (GL-7⁺ B cells), and naive B cells (IgD⁺ B cells) were significantly reduced in SAM mice compared to Ctrl mice (Figure S5A and S5A). However, there was also a significant decrease in frequency and proportion among B cells of IgA⁺ B cells in SAM mice (Figures 5A, S5A, and S5A). IgA-secreting cells generated in PPs home into villus lamina propria of the SI. In PPs, the majority of IgA⁺ cells are B220⁺CD19⁺ cells (Figure S5B) whereas the IgA⁺ cells lose the B220 marker in the SI (Figure S5C). We found in the SI of SAM mice a significant decrease in the percentage of both total B and IgA⁺ cells, although their absolute numbers were not significantly modified (Figure S5C). Accordingly, the ratio between IgA⁺ and IgM⁺ cells showed a decrease in IgA⁺ cells (Figures 5D, and S5D). By contrast, the fecal concentration of total sIgA was significantly higher in SAM mice than in Ctrl mice (Figure 5E). Nutritional intervention tended to restore the number of total B cells and IgA⁺ cells in the villi, but without reaching the percentages observed in Ctrl mice (Figures S5A–S5D, and S5A–SSD). However, the fecal concentration of sIgA of Int mice dropped to the level of Ctrl mice (Figure 5E). These data reveal that SAM diet impacts the intestinal immune system by limiting the expansion of IgA⁺ cells in PPs and villi although fecal sIgA increase.

Imbalance of the Th17/Treg ratio in the intestinal mucosa of SAM mice

As for B cells, the absolute numbers of T cells and their subsets significantly diminished in PPs of SAM mice compared to those of Ctrl mice, except for the RORγt⁺ Foxp3⁺ cells (Figures 6A–6C and S6A). However, a significant increase in the frequency of regulatory T cells (Treg, CD4⁺CD44⁺Foxp3⁺RORγt⁻ cells) and a trend to decrease for Th17 cells (CD4⁺CD44⁺RORγt⁺Foxp3⁺ cells) were observed in SAM mice (Figures 6A–6C, and S6A–S6B). This generated an imbalance in the Th17/Treg ratio in SAM mice compared to Ctrl mice (Figure 6D). Nutritional intervention did not fully restore the numbers of T cells and their subsets (Figures 6A–6C, and S6A), although the Th17/Treg ratio returned to normal (Figure 6D). In villi, absolute numbers and frequencies of CD3⁺, CD4⁺, CD8⁺, activated CD4⁺CD44⁺, RORγt⁺Foxp3⁺, CD8⁺CD44⁺
Figure 5. Alteration of the IgA response in Peyer’s patches and villi of SAM mice
(A) Absolute numbers of total and IgA+ B cells, and frequencies of total, IgA+, IgD+, and IgM+ B cells in PPs of Ctrl, SAM, and Int mice.
(B) Ratio (%) between IgA+, IgM+, and IgD+ among total B cells in PPs.
T cells (Figure 6C), and Th17 cells (Figures 6E and 6F) and the balance between CD4+/CD8+ cells were not altered by the diet (Figure 6D). Nonetheless, the frequency of Treg rose in the villi of SAM mice compared to Ctrl mice (Figures 6E and 6G). Consequently, the SAM diet induced an imbalance in the Th17/Treg ratio in the villi but to a lesser extent than in PPs (Figure 6H). Nutritional intervention increased the Th17/Treg ratio in villi as compared to Ctrl mice (Figures 6E–6H). These data show that SAM diet favors Treg at the expense of Th17 cells in both PPs and villi with a capacity for nutritional intervention to restore Th17/Treg ratio in PPs and to favor Th17 cell population in villi.

Alteration of Peyer’s patch phagocyte distribution in SAM mice

We have previously characterized the PP mononuclear phagocyte system (Figure S7A) pinpointing notably the pivotal roles of the lysozyme-expressing dendritic cells termed LysoDC, in bacterial and particulate antigen sampling, innate defense against pathogens as well as Th cell priming. Noteworthily, SAM diet induced a 3.2-fold diminution in absolute number and a reduction by half in the frequency of LysoDC among MCHII hiCD11c+ cells when compared to Ctrl mice (Figure 7A); in contrast, other PP phagocytes, i.e. macrophages (LysoMac) and conventional dendritic cells (cDC1 and cDC2), were not significantly affected, either in absolute numbers or frequencies (Figure 7A). This resulted in an imbalance of the CD11c+ phagocyte distribution within PPs mainly favoring cDC2 at the expense of LysoDC (Figures 7B, and S7B). By microscopy, we also noticed in SAM mice that although LysoDC were still present at the top of the domes formed by the follicles, they were less frequent on their side (Figure 7C). Nutritional intervention allowed a recovery of LysoDC number, frequency, and location on the side of domes (Figure 7A and S7B). However, an imbalance of phagocyte distribution was still observed after two weeks of nutritional intervention (Figures 7B–7C and S7B). In conclusion, SAM diet significantly alters the composition and distribution of phagocytes in PPs, especially limiting the number of LysoDC. This defect is mitigated by nutritional intervention.

DISCUSSION

Despite nutritional therapeutic treatments, the mortality among SAM children remains high, emphasizing the need to unravel the complexity of SAM pathology and improve the current treatments. We have developed a physiological weanling mouse model of SAM that mimics key SAM features in children, including villus atrophy rarely observed in other animal models of undernutrition in the absence of infection or damage to the intestinal barrier. Therefore, type II nutrient deficiency at the time of weaning may be critical in SAM onset, in accordance with piglet experiments showing that a similar diet induces severe stunting accompanied by villus atrophy.

Our model displays impaired ileal ZO-1 network, enhanced intestinal permeability, and NGAL production, which have been positively correlated with intestinal inflammation in undernourished children. In malnutrition situations, this inflammation can be triggered by an increase of tissue-adherent bacteria, which we have indeed observed in our model. Mucus structure is also dependent on microbiota composition and we indeed observed a thicker layer of ileal mucus in SAM mice. A sticky mucus as well as protein- and iron-limiting conditions promote the expansion of Bacteroidetes in the ileum. Accordingly, we observed a significant rise in Bacteroidetes but also in Proteobacteria in the ileum of SAM mice, mimicking their increase in children with SAM, whose growth synergy is associated with inflammatory-driving properties causing enteropathy and contributing to undernutrition. At the genus level, we observed a significant decrease of the mucin degrader Akkermansia. Although poorly characterized, the role in intestinal homeostasis of Akkermansia-like spp. in the ileum may be similar to that in colon, i.e. competition with other bacteria, strengthening of the gut epithelial barrier, and immune signaling. Their decrease may therefore contribute to the intestinal barrier integrity loss observed in our model.

We further reported a cecal dysbiosis in SAM mice associated with an unusual metabolomic profile. SAM mice harbor specific microbiota signatures characterized by enrichment in taxa associated with leaness,
Figure 6. Imbalance of the Th17/Treg ratio in the intestinal mucosa of SAM mice

(A–H) Representative dot plots (A), absolute numbers and frequencies of (B) Th17 (CD4+CD44+RORγt+Foxp3− cells), and (C) Treg (CD4+CD44+Foxp3+RORγt− cells), and (D) ratio (%) between Th17 and Treg cells in villi of Ctrl, SAM, and Int mice. Representative dot plots (E), absolute numbers and frequencies of (F) Th17 (CD4+CD44+RORγt+Foxp3− cells) cells and (G) Treg (CD4+CD44+Foxp3+RORγt− cells), and (H) ratio (%) between Th17 and Treg cells in villi of Ctrl, SAM, and Int mice. All values are represented as mean ± SD, with 5 independent experiments. For PPs analyses, Ctrl n = 5, SAM n = 5, and Int n = 5. For villi analyses, Ctrl n = 9, SAM n = 12, and Int n = 9. Eight thousand of cells of the parent population, CD4+CD44+ cells, were included to generate each representative dot plots for the three groups. Dunn’s multiple comparisons test, *p-value<0.05, **p-value<0.01. NS: non-significant. See also Figure S6.

An unbalanced exposure to the intestinal microbiota as well as an inadequate education of the immune system as are set in undernourished children. Those with low DC numbers, impaired DC maturation, and T cell priming have a bad prognosis. In our model, the composition of phagocytes is altered in PPs with a decrease of LysDC number, which is with LysoMac, the main phagocytes in the subepithelial region of PPs, playing pivotal roles in innate antiviral and antibacterial defenses. Among PP phagocytes, LysoDC and LysoMac express the highest levels of Clec4e encoding Mincle, a C-type lectin involved in the detection of commensals in PPs and in the differentiation of Th17 cells. In addition to SFB depletion, the well-known inducers of Th17 cells, the low numbers of LysDC could therefore contribute to the Th17/Treg ratio imbalance of our model. Here, the mechanism by which Treg cells are favored remains to be fully explored, but we hypothesize that the immunosuppressive response through Treg cells may be beneficial to the host in the context of the inflammation present in SAM. The decrease of IgA+ cells in PPs and villi might be also caused by phagocyte distribution alteration in addition to Tfh-reduced numbers and frequencies of (F) Th17 (CD4+CD44+RORγt+Foxp3− cells) cells and (G) Treg (CD4+CD44+Foxp3+RORγt− cells), and (H) ratio (%) between Th17 and Treg cells in villi of Ctrl, SAM, and Int mice. All values are represented as mean ± SD, with 5 independent experiments. For PPs analyses, Ctrl n = 5, SAM n = 5, and Int n = 5. For villi analyses, Ctrl n = 9, SAM n = 12, and Int n = 9. Eight thousand of cells of the parent population, CD4+CD44+ cells, were included to generate each representative dot plots for the three groups. Dunn’s multiple comparisons test, *p-value<0.05, **p-value<0.01. NS: non-significant. See also Figure S6.

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protein-deficient and low-fat content diets, inflammation, and opportunism (Turicibacter, Acetatifactor, Enterorhabdus, Faecalibaculum, and members of Desulfovibrionaceae family), whereas anti-inflammatory and SCFA-producing bacteria (Blautia, Lachnospiraceae NK4A136, Christensenellaceae R-7 group, Lachnolocomiidium, and Roseburia) and butyrate production are decreased in SAM mice. These data mimic those of undernourished children, where butyrate producers are discriminatory taxa between stunted and non-stunted children, and a low fecal butyrate concentration is predictive of non-recovery and death. In our model, the combined effect of food iron deficiency (8.8-fold reduction) and iron sequestration by NGAL might explain the decrease of Roseburia-related butyrate producers sensitive to iron availability and the promotion of bacteria with minimal iron requirements such as Proteobacteria, lactic acid bacteria, and Turicibacter. Consistently with iron depletion/repletion studies, the return to a regular diet leads to a full recovery of butyrate-producing bacteria and SCFAs production. The SAM diet also produced other striking changes in host metabolism such as alterations in essential AA (lysine and tryptophan) and bile acid pathways, which are major metabolic changes observed during protein starvation and contribute to stunting. As observed in undernourished children and in animal models following nutritional rehabilitation, AA and bile acid metabolism were improved in Int group during the re-nutrition phase. However, despite catch-up growth, theecal metabolome and associated microbiota of the Int mice were partially restored, suggesting that these alterations could be either time-dependent or long-lasting, even after adequate feeding due to epigenetic alterations that may occur during weaning to adapt to nutritional deficiencies.

An unbalanced exposure to the intestinal microbiota as well as an inadequate education of the immune system as are set in undernourished children. Those with low DC numbers, impaired DC maturation, and T cell priming have a bad prognosis. In our model, the composition of phagocytes is altered in PPs with a decrease of LysDC number, which is with LysoMac, the main phagocytes in the subepithelial region of PPs, playing pivotal roles in innate antiviral and antibacterial defenses. Among PP phagocytes, LysoDC and LysoMac express the highest levels of Clec4e encoding Mincle, a C-type lectin involved in the detection of commensals in PPs and in the differentiation of Th17 cells. In addition to SFB depletion, the well-known inducers of Th17 cells, the low numbers of LysDC could therefore contribute to the Th17/Treg ratio imbalance of our model. Here, the mechanism by which Treg cells are favored remains to be fully explored, but we hypothesize that the immunosuppressive response through Treg cells may be beneficial to the host in the context of the inflammation present in SAM. The decrease of IgA+ cells in PPs and villi might be also caused by phagocyte distribution alteration in addition to Tfh-reduced numbers and frequencies of Th17 (CD4+CD44+RORγt+Foxp3− cells) cells and (G) Treg (CD4+CD44+Foxp3+RORγt− cells), and (H) ratio (%) between Th17 and Treg cells in villi of Ctrl, SAM, and Int mice. All values are represented as mean ± SD, with 5 independent experiments. For PPs analyses, Ctrl n = 5, SAM n = 5, and Int n = 5. For villi analyses, Ctrl n = 9, SAM n = 12, and Int n = 9. Eight thousand of cells of the parent population, CD4+CD44+ cells, were included to generate each representative dot plots for the three groups. Dunn’s multiple comparisons test, *p-value<0.05, **p-value<0.01. NS: non-significant. See also Figure S6.

Strikingly, our model exhibits a delayed recovery of the immunological, metabolic, and microbiota profiles after nutritional intervention despite positive zootomic changes, mirroring malnourished food-complemented children who harbor a sustained alteration of their fecal microbiota despite anthropometric improvement. In this regard, our model allowed the identification of critical biomarkers to target for nutritional intervention, including SFB, Akkermansia, and SCFA-producing bacteria as well as LysDC and Th17 cells. In conclusion, our model not only led to the identification of the multiple defects induced by SAM but also provides a preclinical model, which allows an integrated view of the contribution of gut physiology, immunity, and microbiota in malnutrition.

Limitations of the study

We report a new preclinical model of diet-induced severe chronic undernutrition in conventional mice. In children, SAM is often associated with infections that worsen malnutrition. Our model recapitulates without any bias introduced by infection many aspects of SAM with environmental enteropathy in
A

**Control diet**

**SAM diet**

**Nutritional intervention**

B

Relative proportion in PPs (%)
children. However, the physiology and gut microbiota of the mouse undoubtedly differ from those of humans, some of which may be important. As such, the impact of a burst of enteropathogens (Enterobacteriaceae) or infectious agents will be one of the next steps to investigate. Although colonization of a human microbiota at the species and strain levels is not complete in rodents, humanized mice with the microbiota of children with SAM have proven to be a powerful tool in identifying biomarkers and/or microbial targets in humans. In our study, SAM mice are not humanized; yet, they mimic many of the microbial characteristics of SAM in children and may pave the way to the identification of crucial microbial targets. In our system, like in many institutes in the world, normo-nourished mice harbor SFB, a highly immunogenic and diet-sensitive commensal, unlike the SAM mice. Therefore, the straightforward implementation of the SAM model in various institutes should take into consideration the initial composition of the in-house microbiota. Our study was focused on the early stages of nutritional intervention (2 weeks). Nutritional intervention resulted in a fast recovery of zoometry and intestinal physiology, but in a partial restoration of the intestinal microbiota, metabolism, and immunity. To determine whether the effects of SAM on microbiota and intestinal immunity at weaning are reversible, longer duration and/or different types of intervention may be required. Nevertheless, our study presents a new animal model that phenocopies most features of a complex human disease, which we believe will be useful to the research community for investigating the underlying mechanisms and testing sustainable alternative therapies.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106910.
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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


## STAR★METHODS

### KEY RESOURCES TABLE

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julie Tomas (tomas@ciml.univ-mrs.fr).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**

**Data**
Raw fastq files 16s rRNA sequencing data have been deposited at Zenodo and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

Workflow data on the parameters used for the microbiome profiling of the terminal ileum and caecal contents have been deposited at Zenodo and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

**Code**

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**REAGENT or RESOURCE**

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EXPERIMENTAL MODEL DETAILS

Animals
Animal experiments were approved by the French Ethics Committee and the French Research Ministry (APAFIS #31437). Mice were maintained in our animal house facility in ventilated cages under animal biosafety level 2 conditions at an ambient temperature of 22°C with a 12h light/dark cycle. At weaning (21 days), C57BL6/J male mice separated from normo-nourished mothers (Janvier Labs, Saint-Berthevin, France) were fed during one month either with regular diet (A03; Safe, France), or with a SAM diet based on a porcine model of infant malnutrition18 (MAIS +1800 mg/kg Sodium, SAFE R8960A01R 00128; Safe, France). The detailed composition is shown in Table S1. Nutritional intervention was performed on weaning mice fed with SAM diet for two weeks by switching to the regular diet for another two weeks. Weight and food consumption of mice were followed once a week. Mice were euthanized at 49 days old and length of the mice, small intestine (SI) and femur length were measured. Weight/Length was calculated for each mouse (W/L). The length without the tail was used to calculate the Weight-for-Length Z score (WL-Z). The Z score is calculated by deducting the mean of the referent population (μ_referent) from the given variable (X) and then dividing the result by the standard deviation of the referent population (SD_referent) leading to the following formulation: Z = (X - μ_referent)/SD_referent. In the case of the WL-Z, the variable X = W/L, and the control group (Ctrl, n = 27) chosen as the referent group to calculate the μ_referent (W/L_referent) and the SD_referent leading to the formulation: WL-Z = (W/L - W/L_referent)/SD_referent. Caecum (N = 10/group of diet), terminal ileal content (N = 8 for Ctrl, N = 13 for SAM) and feces were weighted and immediately stored at -80°C for metagenomic, metabolomic and ELISA studies. Peyer’s patches and small intestine were collected for flow cytometry and histology studies. Due to the limited number of male mice generated from our own breeding, the smallest cohorts were generally used to assess physiological parameters, while the largest cohorts were dedicated to immune studies. A minimum of 3 mice per group is indeed required to analyze Peyer’s patches phagocyte populations. Furthermore, we first compared the two groups Ctrl and SAM, and only later included the third group, Int. Therefore, the number of mice and the number of replicates differ between the groups. For instance, 5–10 independent experiments means that five independent experiments were performed including all groups, while the other five included only the Ctrl and SAM groups. Therefore, the legend indicates 5 to 10 independent experiments.

METHOD DETAILS

Peyer’s patch and villus cell extraction for flow cytometry analyses
Peyer’s patches (PPs) were digested for 40 min at room temperature with collagenase/DNase as previously described.35 CD11c+ cells were enriched using anti-CD11c microbeads and an autoMACS magnetic cell separator according to manufacturer’s instructions (Miltenyi Biotec, France).

After removal of adherent adipose tissue and resection of PPs, 14 cm of the SI was cut into representative pieces of each part of the SI (duodenum-jejunum and ileum), opened longitudinally, washed in cold PBS 1X and placed in complete medium (RPMI 5% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamine, 10 mM HEPES and 50 μM β-mercaptoethanol) containing 1 mM dithiothreitol. SI was cut into 2 mm pieces, washed in cold HBSS (Thermo Fisher) containing 2% FCS and filtered with a 100 μm cell strainer. Epithelial cells were removed by incubating tissues in HBSS+2 mM EDTA buffer at 37°C for 20 min at 180 rpm. This step was repeated twice before digestion at 37°C in complete medium with collagenase VIII (1 mg/mL, Sigma) and DNase I (200 μg/mL, Sigma) for 15 min at 180 rpm. Digestion was stopped by adding 20 mL of cold PEF 2% buffer (PBS1X + 5 mM EDTA +2% FCS). Debris were removed by filtration. The cells were washed with cold PEF 2% before staining.

Flow cytometry analyses
After autoMACS separation, CD11c+ (positive fraction) and CD11c− (negative fraction) cells were pre-incubated on ice for 10 min with the 2.4G2 antibody to block Fc receptors. Cell mortality was evaluated using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies). Intracellular staining was performed using eBioscience Intracellular Fixation & Permeabilization Buffer set according to manufacturer’s instructions.
Multiparametric flow cytometry was performed using a FACS LSRII UV. Data were analyzed with FlowJo software. Three panels were designed to study the phagocyte population in PPs, T and B cells in PPs and SI.

**Antibodies used for FACS analyses**

**Phagocyte panel:** XCR1-BV421 (clone ZET), CD172a-FITC (clone P84), CD4-PeCy5.5 (clone RM4-5), CD11c-PeCy7 (clone N418), BST2-APC (clone 927), CMHII-A700 (clone M5/114.15.2) and CD11b-APC/Cy7 (clone M1/70). **T cell panel:** CD45-BUV395 (clone 30-F11), PD-1-BV605 (clone J43), CD62L-BV786 (clone MEL-14), CD8α-AF488 (clone 53-6.7), CXCR5-PE (clone 2G8), CD4-PeCy5.5 (clone RM4-5), RORγt-Pe-eFluor610 (clone B2D), Foxp3-APC (clone FJK-16S) and CD44-A700 (clone IM7). **B cell panel:** CD45-BUV395 (clone 30-F11), CD43-BUV737 (clone S7), CD3-eFluor450 (clone 17A2), Siglec-F-BV421 (clone E50-2440), XCR1-BV421 (clone ZET), CD19-BV605 (clone 6D5), B220-BV786 (clone RA3-6B2), IgM-biotin (clone II/41), IgA-PE (clone mA-6E1), IgD-Pe/Cy7 (clone 11-26c.2a), GL-7-eF660 (GL-7), Streptavidin PE-CF594.

**Immunofluorescence staining and confocal microscopy**

The three last ileal PPs were removed and fixed with Antigenfix (Diapath) for 1h. After washed in 1X PBS, PPs were incubated overnight in 34% sucrose at 4°C. After inclusion in OCT (Invitrogen), PPs were cut and stained as previously described. After immunofluorescence staining, the slides were observed with a Zeiss LSM 780 confocal microscope using the spectral imaging mode. Quantification and images processing were done using Adobe Photoshop CC 2019. The following antibodies were used for phagocyte population: Rat anti-MerTK (clone DS5MMER), australian hamster anti-CD11c (clone N418, Biolegend) and rabbit anti-lysozyme (polyclonal, Dako), SIPRα-AF594 (clone P84, Biolegend), CD4-eFluor450 (clone RM4-5, eBioscience) or CD4-eF570 (clone RM4-5, Invitrogen), EpCAM-eF450 (clone G8.8, eBioscience). Secondary antibodies were used: donkey anti rat-Cy3 (Jackson Immuno-research) or –AF488 (Invitrogen), goat anti hamster-AF594 or Cy3 (Jackson Immuno-research), goat anti-rabbit AF514 (Invitrogen). SYTOX blue for nuclei acid stain (ThermoFisher Scientific).

**Fluorescence in situ hybridization (FISH)**

Unflushed distal PPs and SI were collected in Carnoy (absolute ethanol/chloroform/acetic acid 60:30:10) and incubated overnight at 4°C. Samples were dehydrated, and embedded in paraffin according to the standard protocol for hematoxylin and eosin stain (HE) or immunofluorescence (IF). All the staining was done on dewaxed 8-μm sections. In IF experiments, antigen retrieval was performed in citric acid buffer 2mM pH 6 for 45 min at 96°C and fluorescence-labeled secondary antibodies were used and nuclei stained with DAPI. IF assay was performed with the anti-MUC2 antibody (1/500, sc-15334, Santa Cruz Biotechnology) and anti-ZO-1 (1/100, Invitrogen). Procedure used for FISH experiments are described in Pedron et al., 2012. The pan-bacteria probe Eub338-Alexa 555-GCTGCCTCCCGTAGGAGT-3’ and the specific Segmented Filamentous Bacteria (SFB) 1008-AF594 probe 5’-GCCAGTTCCCTCATTACAAGG-3’ were used in embedded paraffin sections of PPs and ileum. DNA was stained with Sytoxblue revealing both eukaryotic and bacterial cells.

Spectral confocal imaging projections of ileal villi and Peyer’s patches of mice from 49-day old Ctrl, SAM and Int mice stained by FISH for all bacteria (Eub-338 probe) and specific probe to identify SFB (SFB-1004 probe) were used to quantify the distance between the microbiota and the intestinal epithelium. Only Eub-338 specific signal that was not identified as positive for SFB-1004 specific signal was used to measure the proximity of the luminal microbiota to the epithelium (top of villi or follicle-associated epithelium, FAE) in each diet group, using ZEN Lite 2.3 SP1 software line tool (Carl Zeiss Microscopy).

**Intestinal permeability assessment**

The gut permeability was tested using 4-kDa FITC-dextran (Sigma-Aldrich). Mice were fasted 4 h before gavage. Each mouse was gavaged with 100 μL of FITC-dextran solution at 80 mg/mL diluted in sterile 1X PBS. Four hours after gavage, mice were anesthetized with isoflurane and blood was collected retro-orbitally in EDTA tubes. The blood was centrifuged at 5000 rpm during 10 min at 4°C. FITC-dextran fluorescence was read in the microplate reader Infinite M1000 PRO using the i-Control software (Tecan) with excitation of 485 nm and emission of 530 nm (20 nm emission bandwidth). Diluted or not diluted samples were added in 96-well black plate in duplicate when possible. Plasma from ungavaged mice as well as 1X PBS
wells were added for the calculation of the concentration. A standard curve was used to convert values to concentration.

**Quantification of fecal IgA by ELISA**

Stools were weighted and diluted in an inhibitor cocktail at 100 mg/mL containing PMSF 0.35 mg/mL (Sigma-Aldrich), EDTA-Na2 0.75 mg/mL (Sigma-Aldrich), pepstatine 1 μg/mL (Sigma-Aldrich) and aprotin 2,000 times diluted (Sigma-Aldrich). After mechanical disaggregation, stools were incubated for 20 min in the inhibitor cocktail at 4°C. Stools were then vortexed and centrifuged at 14, 000 rpm for 10 min. Supernatants were collected and conserved at −20°C for IgA quantification. Total IgA detection was determined according to the manufacturer’s protocol (IgA mouse uncoated ELISA kit, Invitrogen). The NUNC MaxiSorp high protein-binding capacity 96 well ELISA plates were used. Colorimetric reaction was measured at OD = 450 nm by microplate reader Infinite M1000 PRO using the i-Control software (Tecan). Corresponding immunoglobulin isotype was used as standard after serial dilutions.

**Quantification of fecal NGAL (neutrophil gelatinase associated lipocalin)**

Stools were weighted and mechanically disaggregated in 300 μL of sample dilution buffer provided by the manufacturer (Mouse NGAL ELISA kit, ELISAGenie, Ozyme). The supernatants were removed after centrifugation (5,000 rpm, 10 min) and diluted for ELISA quantification following manufacturer’s instructions.

**Metagenomics**

**DNA extraction and library preparation**

**Terminal ileal content.** Bacterial DNA was extracted by a manual treatment with acid washed glass beads (G4649-500g Sigma) and 0.5 mm glass beads (Scientific Industries, Inc) using a FastPrep-24 5G Grinder (mpBio) at maximum speed (6.5 m/s) for 90s. To optimize DNA extraction from all the bacteria present in the stools, two protocols were applied: the former using E.Z.N.A Tissue DNA Kit (Omega bio-tek, Norcross, USA) and the latter based on glycan degradation using EZ1 Advanced XL device (Qiagen, Courtaboeuf, France). For each extraction protocol, genomic DNA was amplified for the 16S “V3-V4” regions by PCR for 45 cycles, using the Kapa HiFi Hotstart ReadyMix 2x (Kapa Biosystems Inc,Wilmington, MA U.S.A), and the surrounding conserved region V3_V4 primers with overhang adapters (FwOvAd_341FTCGTCGGCAGCGTCAGATGTATAAGAGACAG CCTACGGGNGGCWGCA G; RevOvAd_785RGT CTCGTGGGCTCGGAGATGTATAAGAGACAGGACTACHVGGGTATCTAATCC). After purification on AMPure XP beads (Beckman Coulter Inc, Fullerton, CA, USA), the concentration was measured using High sensitivity Qubit technology (Beckman Coulter Inc, Fullerton, CA, USA). Library of each extraction (440–470 pb) method were pooled (1:1) at 3.5 ng/μL and Illumina sequencing adapters and dual-index barcodes were added to amplicons. After purification on AMPure XP beads (Beckman Coulter Inc, Fullerton,CA,USA), this library was pooled with 92 others multiplexed samples. The global concentration was quantified by a Qubit assay with the high sensitivity Qubit technology (Beckman Coulter Inc, Fullerton, CA, USA). Purified amplicons were pooled in equimolar concentration to obtain a 7 pm library containing 15% of PhiX control. The samples were then sequenced for 16S rRNA sequencing on MiSeq technology (Illumina, Inc, San Diego CA 92121, USA) with the paired-end 300 bases pair protocol according to the 16S Metagenomic Sequencing Library Preparation (Illumina) at the Institute Hospitalo-Universitaire Méditerranée Infection, Marseille, France. Automated cluster generation and paired-end sequencing with dual index reads were performed in single 39-h run in a 2x250bp.

**Caecum content.** The extraction was performed using the protocol developed and standardized by Genoscreen, as well as the recommendations of the IHMS. An optimized and standardized DNA extraction protocol dedicated to bacterial DNA extraction from stools samples has been used (GenoScreen, Lille, France). Genomic DNA extraction from stools samples was done with the QIAamp Fast DNA stool mini kit (Qiagen, Germany) with optimized protocol for lysis step. After DNA extraction, the concentration was quantified with the SybrGreen assay Kit (Life Technologies, USA).

**16S rRNA gene sequencing and microbiome profiling**

**Terminal ileal content.** Twenty-two samples were sequenced including 9 from the Ctrl group and 13 from the SAM group with PCR mix control. A total of 147,711,748 read pairs were generated (mean 120,045 read pairs per sample with median of 120,401). The reads were first demultiplexed according to their dual barcode by samples. Quality of the raw reads were assessed using FastQC v0.11.9 toolkit.
and low-quality reads were trimmed using Trimmomatic v0.39. V3_V4 primer with overhang adapters (FwOvAd_341F and RevOvAd_785R) were removed using cutadapt v3.2. Amplicon Sequence Variants (ASVs) were produce using DADA2 in QIIME 2. We obtained 6475 ASVs with a length ≥ 400 nt to avoid non v3-v4 ribosomal sequence. A Naive Bayes classifiers obtained on the Silva (release 138) 99% OTUs full-length sequences were applied to obtain the pre-trained taxonomy classifiers used for the taxonomic assignment of the features with the classify-sklearn method. The table was rarefied to 7500 sequences and filtered to exclude samples with less than 7500 sequences. A rooted tree for phylogenetic diversity analysis was generated with SEPP. The resulting phylogenetic tree was further processed to calculate core diversity metrics, including β-diversity based on weighted and unweighted UniFrac distances and α-diversity measures calculated in QIIME 2. To test for significant differences in taxonomic abundances we used the Shannon index (in this case, Faith’s Phylogenetic Diversity) and the non-parametric Kruskal–Wallis test. Differences were considered significant when the false-discovery rate correction is at p < 0.05. The intergroup high similarity and intra group low similarity of microbiota are proven by β-diversity.

**Caecum content.** Thirty samples were sequenced including 10 for each Ctrl, SAM and Int groups. Microbial diversity was determined for each sample by targeting part of ribosomal genes. A 16S RNA gene fragment comprising V3 and V4 hypervariable regions was amplified using an optimized and standardized 16S-amplicon-library preparation protocol (Metabiote, GenoScreen, Lille, France). Briefly, 16S RNA gene PCR was carried out using 5 ng of genomic DNA according to Metabiote protocol (or maximal of DNA volume) instructions using 192 bar-coded primers (Metabiote MiSeq Primers, GenoScreen, Lille, France) at final concentrations of 0.2 μM and an annealing temperature of 50°C for 30 cycles. PCR products were cleaned up with Agencourt AMPure XP-PCR Purification system (Beckman Coulter, Brea, USA), quantified according to the manufacturer’s protocol, and multiplexed at equal concentration. Sequencing was performed using a 250-bp paired-end sequencing protocol on the Illumina MiSeq platform (Illumina, San Diego, USA) at GenoScreen, Lille, France. Raw paired-end reads were subjected to the following process 1) quality filtering with the PRINSEQ-lite PERL script by truncation of bases from the 3’end not with quality < 30 based on the Phred algorithm, 2) paired-end read assembly using FLASH with a minimum length overlap of 30 bases and 97% overlap identity and 3) the search and removal of both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in primers sequences. Assembled sequences for which perfect forward and reverse primers are not found are eliminated. Amplicon Sequence Variants (ASVs) were produce using DADA2 in QIIME 2. We obtained 1549 ASVs with a length ≥ 400 nt to avoid non v3-v4 ribosomal sequence. The following analyses was realized as described for terminal ileum content.

**Untargeted metabolomic approach**

**Short chain fatty acids (SCFAs) analyses**

Acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate were quantified in 35–115 mg of caecal content by gas chromatography-mass spectrometry (GC-MS) following the procedure described in Apper et al., 2020.

**Caecal preparation**

Forty-five mg of each cecal samples were weight in Eppendorff and content was homogenized in cooled methanol (3 μL/mg feces) at –20 °C. Samples were vortexed for 1 min and incubated at –20 °C for 30 min. Samples were then centrifuged for 15 min (11,000 × g, 4 °C). The supernatant recovered from each sample was filtered through 10 kDa filter tubes by centrifuging for 45 min (11,000 × g, 4 °C). The extracts obtained were then dried using a stream of nitrogen. The dry extracts were reconstituted with 125 μL acetonitrile/water (50:50; v/v) and filtered through 0.45 μm filters by centrifugation for 35 min (11,000 × g, 4 °C). Fifty μL of sample extract was transferred into vial for Liquid Chromatography Mass Spectrometry (LCMS) metabolomic analyses. The samples were separated using ultrahigh-performance liquid chromatography (UPLC) ultimate 3000 (Thermo Scientific), coupled to a high-resolution mass spectrometer (HRMS), Q-Exactive Plus quadrupole-orbitrap hybrid equipped with electrospray ionization source (H-ESI II). A reverse-phase C18 Hypersil Gold (100 mm × 2.1 mm × 1.9 μm) (Thermo Scientific, France) column was used for compound separation in the samples. The injection volume was 5 μL. Here, 0.1% formic acid solutions in water and acetonitrile were used as solvents A and B, respectively, as mobile phases. The elution gradient for sample analysis was as follows: Solvent B was maintained at 5% during the first minute. A linear gradient then raised solvent B to 50%, maintained it for 2 min, before raising it again to 97% of B for
the next 6 min. Then, 97% of solvent B was held in isocratic conditions for 2 min, after which initial conditions were restored, and the column re-equilibrated for 4 min. The separated molecules were analyzed in both positive and negative ionization modes in the same run. The repeatability of the analysis was checked by analyzing interspaced (1 out of every 5 samples) quality control samples (QC).

**Data processing and molecule identification**

All the raw data generated by the UPLC-HRMS were converted to mzXML by ProteoWizard (Version 2.0), and then processed by MZmine 2.26. The identification of the metabolites was performed by using an in-house database referencing more than 800 metabolites with their chromatographic retention time and their exact mass and MSMS spectra obtained in positive and negative ionization modes, including their adducts and neutral losses. Statistical analyses were performed using MetaboAnalyst 5.0 software.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was conducted using Prism 9 (GrahPad, San Diego, CA) (Figures 1, 5–7) or using MetaboAnalyst 5.0 software for metabolomic and metagenomic studies (Figures 2–4). The data are expressed as the mean ± the standard deviation (SD). For the analysis in Figures 1, 4D, 5, 6, and 7 we performed a non-parametric one-way ANOVA, followed by Dunn test to assess the significance among pairs of conditions. For the analyses in Figures 2C, 3C and 3D and 4C a non-parametric Kruskal Wallis Test was performed.

For the analysis in Figures 3B and 4B, 4A T-test/Anova was performed. For the analysis in Figure 2D, the non-parametric Wilcoxon rank-sum test was used to generate the volcano plot. The details for each experiment including the number of animals and the number of independent experiments are specified in the figure legends. In the Figures 3C and 3D, Linear discriminant analysis (LDA) effect size (LEfSe) was applied to discern significant differences in the relative abundance of microbial taxa among all the groups, which included the non-parametric Kruskal–Wallis test and LDA2. The groups were compared in pairs. Taxonomical features with p-value <0.05 and LDA effect size >2 were regarded as significant microbial signatures. In the Figures 4E and 4F, Spearman’s rank correlation analyses were performed in the R environment using stats package and cor function. Heatmap where generated with gplots-3.0.1.2 package and heatmap.2 function using dist and hclust arguments to compute the distance and the hierarchical clustering based on pairwise distances calculated from Spearman correlation values.