Human milk bacteria assembled into functionally distinct synthetic communities in infant formula differently affect intestinal physiology and microbiota in neonatal mini-piglets

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Abstract

The contribution of Human milk (HM) microbiota to infant gut health was addressed by evaluating the effects of HM bacteria combined in two synthetic communities (SynComs, with an *in vitro* characterized anti-inflammatory (AI) or high immunomodulatory (HI) profile) on systemic and ileal immune cell functions, gut epithelial barrier and microbiota. Neonatal mini-piglets were fed either a formula without supplementation (CTRL) or supplemented with AI or HI SynComs and compared to sow milk-fed (SM) piglets. The two HM-derived SynComs differently modulated microbiota and intestinal functions (mainly immunity). Notably, the abundance of more than half of the intestinal taxa that differed between the three formula groups differed between AI and HI piglets. In addition, fewer differently abundant genera were observed between HI and SM piglets than between CTRL and SM piglets, suggesting that SynCom HI supplementation brought the microbiota of HI piglets closer to that of SM piglets. SynCom supplementation affected immune functions at both systemic and mucosal levels. Fecal sIgA level in HI piglets, which was slightly lower than in SM piglets, was markedly higher than in CTRL and AI piglets. Finally, SynCom bacteria were correlated with several genera in ileum and colon microbiota and both were highly correlated with physiological variables, supporting the HM bacteria-driven influence on gut microbiota and on multiple gut functions. Overall, our data show that SynComs with contrasting immunomodulatory properties but similar taxonomic composition can differently modulate the developmental profile of intestinal functions and microbiota in mini-piglets used as a human infant model.

Importance

Early life environmental factors, such as neonatal diet, modulate gut microbiota that is a driving force for the functional development of the gut. However, the role of the human milk (HM) microbiota is not well understood, particularly the importance of the immunomodulatory properties of HM bacteria. This study provides insight into the differential effects of two synthetic communities, with similar taxonomic composition representative of the taxonomic diversity of the HM cultivable microbiota, but with contrasting immunomodulatory properties (previously characterized *in vitro* on an intestinal quadricellular model). Daily supplementation with these two SynComs differently modulated gut microbiota composition and gut physiology, particularly intestinal immune signatures. In conclusion, the functional profile of bacteria within the HM microbiota may induce a different developmental profile of gut physiology in infants.

Introduction

Early nutrition is essential to ensure optimal infant growth and development, especially regarding gut digestive and immunological functions and microbiota, which are immature at birth (1). Human milk (HM) is the optimal source of nutrition for the first few months of infant life (2, 3). Its beneficial properties are mediated by the presence of bioactive compounds particularly promoting the maturation of the gut immune system in interaction with the development of gut microbiota (4, 5). Breast milk has a diverse bacterial community with dozens of genera and over 200 different species identified (6, 7), including prevalent genera such as *Bifidobacterium* and *Lactobacillus*, as well as *Streptococcus, Staphylococcus, Cutibacterium* or *Corynebacterium* (6, 8, 9). Despite the low bacterial load in HM (10^3-10^4 colony forming units (CFU)/mL), these bacteria contribute to the neonatal gut microbiota and

would account for 5 to 33 % of total bacteria present in feces of breastfed infants under 6 months of life (10, 11). It is also noteworthy that maternal milk bacteria are the primary colonisers of the small intestine and colon of piglets, contributing approximately to 90% of the mucosa-associated microbial community in the small intestine throughout the first month of the life. It also contributes to 90% and to less than 20% of the mucosa-associated microbial community in the colon at birth and after the first postnatal week of life, respectively (12). This suggests that milk-derived bacteria are present in the intestine from an early stage of development (12) and can directly impact gut physiological functions, as demonstrated with specific HM-derived probiotics such as *Bifidobacterium* and *Lactobacillus* strains supplied separately in infant diet (13, 14). However, the role of HM-derived bacteria assembled in complex communities that mimic the actual bacterial communities present in HM, on gut functions and microbiota has been insufficiently addressed so far.

To further explore the *in vivo* role of HM bacteria assembled in synthetic bacterial communities (SynComs) on intestinal homeostasis, Yucatan piglets used as a human infant model, were fed dairy-based infant formula supplemented with HM SynComs. Two SynComs of 11 HM strains each, closely related in terms of taxonomy and covering the prevalent taxa of HM microbiota were used. We have previously demonstrated *in vitro* that the intestinal epithelium was differently affected depending on the HM strain assembly, these two SynComs displaying an anti-inflammatory profile *vs.* a high immunostimulatory one (9). It was hypothesized that the two HM-derived bacterial communities added to infant formula at a concentration close to physiological concentrations (~$5.5.10^5$ CFU/mL infant formula) would differentially affect key gut immune and barrier functions *in vivo*, according to their functional characteristics, in association with changes in gut microbiota composition. The gut

immune and barrier functions and microbiota were also examined in sow milk-fed (SM) piglets as a natural physiological control group.

Results

From postnatal day (PND) 2 to PND24, Yucatan piglets were fed dairy-based infant formula (Supplemental Table S1) without supplementation (CTRL) or supplemented with HM SynComs of 11 HM strains each, which exhibited an *in vitro* anti-inflammatory (AI) or immunostimulatory (HI) profile according to a previous *in vitro* characterization on an intestinal quadricellular model (Table 1; (9)). The AI and HI bacterial doses added in formulas corresponded to 5.5 x 10^5 CFU/mL formula, amounting to an average daily intake of 1.28 x 10^8 CFU/kg body weight (BW) for piglets fed supplemented formulas (with a range of daily bacterial intake from 1.09 x 10^8 CFU at PND5 to 3.24 x 10^8 CFU at PND23). During the whole experimental period, formula-fed piglets remained healthy and kept growing with an average daily BW gain of 74 ± 14 g/day. The average daily volume of formula intake was not different between AI, HI and CTRL groups, with an average value of 255 ± 3 mL/kg BW. Supplementing formulas with SynComs had no significant effect on BW gain, compared to the CTRL formula. The average piglet BW was 1.01 ± 0.03 kg at PND 5 up to 2.35 ± 0.06 kg at PND23. As expected, the growth of the fourth group of SM piglets was significantly higher with an average daily BW gain of 146 ± 4 g/day, compared to 74 ± 14 g/day for the formula-fed piglets ($P<0.05$).

SynCom bacterial strains were present in AI and HI intestinal microbiota

To determine the presence of SynCom bacteria in the intestine of piglets, metabarcoding was applied to AI- and HI-supplemented formulas in order to identify

the OTU potentially corresponding to each SynCom bacteria in supplemented formulas and in the ileal and colonic piglet digesta. At the exception of *Cutibacterium* and *Veillonella*, OTUs corresponding to the genera added in SynComs AI and/or HI were identified in AI and/or HI formulas (Table 1). In few cases, multiple OTUs have been considered appropriate for a strain, or conversely, one potential OTU was assigned to two strains of a single genus, considering that the 16S sequencing identification of bacteria was reliable at the genus level (Table 1).

Most SynCom bacteria were traced in the intestinal microbiota of AI and/or HI piglets at PND24. In the ileum, most of them displayed higher relative abundances in the intestinal microbiota of PND24 AI and/or HI piglets compared to that of CTRL piglets (Figure 1, Table 1). In the colon, the abundance of *Bifidobacterium breve* and *Staphylococcus haemolyticus* added in formula AI and that of *Corynebacterium sp.* added in formulas AI and HI were increased whereas the abundances of the other bacteria assembled in Syncoms were not significantly changed, the presence of their corresponding OTUs being very low in some colon digesta. Comparison of the ileal and colonic microbiota of AI and HI piglets with CTRL piglets indicates that most SynCom bacteria were recovered from the ileal microbiota and, to a lesser extent, the colonic microbiota.

SynCom AI and HI differentially impacted the intestinal homeostasis of formula-fed piglets

To analyze the overall effect of SynCom supplementation of formulas on intestinal homeostasis, Partial Least Squares-Discriminant Analysis (PLS-DA) was performed on the complete dataset from the three formula-fed piglet groups in ileum and colon. The most discriminant variables with a Variable Importance in Projection (VIP)

score>1 in the PLS-DA (n = 112 out of a total of 201 variables in the ileum and n = 141 out of a total of 250 variables in the colon) were selected for multifactorial analysis (MFA) after grouping by function (6 functions) or phylum (5 phyla) (Supplemental Figures S1, dataset accessible in the repository https://doi.org/10.57745/ATTHZ3). MFA was able to distinguish the three groups of formula-fed piglets in the ileum on Dimensions 1 and 2 (11.4 and 10.5 % of variance recovered on Dimension 1 and 2, respectively) and in the colon on Dimension 2 (10% of variance recovered on Dimension 2) (Figure 2). In the ileum, the groups of variables contributing to the discrimination between formula-fed piglet groups included immunity, endocrine, tryptophan metabolism and barrier functions, and Pseudomonadota, Actinomycetota and Bacillota phyla. In the colon, the discrimination of formula-fed piglet groups was mainly explained by Bacillota, Pseudomonadota and Actinomycetota phyla, and endocrine and immunity functions. These results indicate that the two SynComs differentially modulate the intestinal homeostasis, both on the ileal and colonic microbiota and on physiological functions.

SynCom supplementation modified microbiota composition and activity

Microbiota sequencing was performed on ileal digesta, colonic digesta and feces from formula-fed and SM-fed piglets at PND24. In addition, fecal microbiota was also analyzed at PND8 to detect early dietary effects on fecal microbiota composition. Moderate differences occurred in the structure of ileal, colonic and fecal bacterial ecosystems between the three formula-fed groups. At PND24, α-diversity was lower in the ileum of CTRL than in that of HI group, AI group value being intermediate (Supplemental Figure S2). No difference in β-diversity occurred between the three formula-fed groups (Supplemental Figure S2). However, consistent with the MFA

results, differential abundance analysis revealed several differences in the composition of ileal and colonic microbiota between the formula-fed groups that go beyond the presence of the SynCom bacteria in the microbiota of SynCom-supplemented piglets (Table 2; Supplemental Table S2). At PND24, a lower abundance of Pseudomonadota, Bacteroidota and Campylobacterota phyla was observed in the ileum of AI group than in that of CTRL group, and that of HI group for the latter two phyla (Table 2). Although not visible at the phylum level, the abundance of several genera within Bacillota was also affected by SynCom supplementation. The abundance of *Butyricimonas* (in the 2 sites), *Parasutterella* (in colon) and *Actinomyces* (in ileum) was higher in AI and HI groups than in CRTL groups (Table 2). HI microbiota was enriched with *Turicibacter*, *Eubacterium* and *Romboutsia* (in ileum) compared to CTRL, and with *Clostridium* and *Prevotellaceae UCG-003* (in ileum) and *Anaerococcus* (in colon) compared to AI and CTRL microbiota. Finally, AI microbiota was enriched with *Alistipes* (in ileum) and with *Staphylococcus* (in both sites) compared to CTRL and HI microbiota (Table 2). In the feces, there were also differences in the abundance of several genera belonging mainly to Bacillota and to a lesser extent to the Bacteroidota and Actinomycetota, between the formula-fed groups at both PND8 (Supplemental Table S3) and PND24 (Table 2).

The ileal, colonic and fecal microbiota composition of the formula-fed groups was compared with that of SM piglets. Of note, the bacterial composition of the sow milk was also determined (546 OTUs identified), revealing that several genera predominant in sow milk microbiota are commonly found in HM microbiota and were also present in the SynComs, including *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Rothia* and *Corynebacterium* (Table *'OTUs identified in sow milk'* accessible in the repository https://doi.org/10.57745/ATTHZ3). However, a few

differences occurred between sow milk and HM microbiota (15, 16) or SynComs, including the low abundance of *Bifidobacterium* (corresponding to less than 0.1 % of the total OTUs) or the presence of *Terrisporobacter* in sow milk.

Overall, both □-and □-diversities differed in ileal and colonic microbiota of SM-fed piglets compared to the three groups of formula-fed piglets (Supplemental Figure S2). The α-diversity of SM piglet microbiota was lower than that of formula-fed groups in the ileum at PND24 and this was already observed at PND8 in the feces (Supplemental Figure S2). As expected, the relative abundance of numerous bacterial phyla, families and genera in the ileum, colon and feces differed between SM-fed and formula-fed groups (Supplemental Table S4). Some differences in genera abundance between CTRL and SM groups were not observed between piglets fed AI and/or HI formulas and SM piglets, and vice-versa. As a result, piglets fed supplemented formulas, especially with SynCom HI, had a lower total number of differentially abundant genera than CTRL piglets compared to SM piglets.

To determine whether SynCom supplementation affected microbiota activity, SCFA concentrations were also analysed at PND8 (feces) and PND24 (feces and colonic digesta). The concentration of acetate was higher in HI feces than in CTRL and AI ones at PND8, whereas difference no longer occurred at PND24 (Supplemental Figure S3). Similarly, fecal SCFA concentrations in SM piglets differed from that of formula-fed piglets mainly at PND8 (Supplemental Figure S3). In the colon, propionate, valerate, butyrate, isovalerate, isobutyrate and total SCFA concentrations were higher in SM-fed than in formula-fed groups and proportions between SCFAs also differed (Table 3). Overall, adding HM-derived functionally distinct SynComs to the formulas specifically altered the microbiota composition. These changes mainly observed with HI SynCom reduced, but did not eliminate, the differences observed

between CTRL and SM piglets. The effect of SynComs on fermentative activity was mainly observed in the first days of supplementation.

SynCom supplementation affected systemic and intestinal immune function

MFA indicated that immunity was one of the main function affected by SynCom supplementation. Both the systemic and intestinal immune functions were affected (Figure 3, Table 4, Supplemental Tables S5 and S6). IL-10 production under basal condition and TNF-α production under ConA-stimulated condition tended to be higher in HI PBMC than in CTRL and AI PBMC (Figure 3). In PP cells, SynCom HI and AI supplementation tended to increase basal TNF-α secretion compared to CTRL group, whereas no differences in TNF-α and IL-10 secretion were observed in stimulated conditions (Supplemental Table S5). The effect of SynCom supplementation on intestinal immune functions was also highlighted through changes in the expression of genes involved in pro-inflammatory (IL6, TNFaR1), antioxidant (SOD2) and Treg (FOXP3) pathways in ileal and colonic tissues compared to the CTRL group, although no clear pattern emerged (Table 4 and Supplemental Table S6). Compared to SM, the expression of genes encoding anti-inflammatory proteins (IL-10, SOCS3), pro-inflammatory proteins (CCL20, CX3CL1, TNFaR1, IL1b, IL6), antioxidant (SOD2) and Treg pathways (FOXP3) and cellular signalling (TLR4) differed in CTRL, AI and/or HI piglets in the ileum and/or colon. Finally, the fecal sIgA content of HI piglets was 6- and 11-fold higher than in CTRL and AI piglets, respectively (Figure 3). A similar profile was observed for ileal content, but with non-significant differences. In SM piglets, the concentration of sIgA in ileum, colon and feces was 45-, 5- and 12-fold, respectively, higher than in CTRL group.

Interestingly, the level of fecal sIgA in HI group was only 1.9-fold lower than in SM group.

Overall, the addition of SynCom HI to formula enhanced PBMC cytokine secretion. At the intestinal level, the expression of a few genes was altered with SynCom AI and/or HI supplementation compared to the CTRL group. Supplementation of the formulas with SynCom HI increased the secretion of sIgA, thereby reducing the differences in fecal sIgA levels observed between CTRL and SM piglets.

Intestinal morphology and barrier functions differed between groups at PND24

To determine the effect of formula supplementation with SynComs and of SM feeding on the epithelial barrier, the intestinal morphology, the expression of genes related to cell renewing and barrier function and the *ex vivo* para- and transcellular permeability were evaluated in ileum and colon (Figure 4, Table 5 and Supplemental Table S6). SynCom HI supplementation reduced the ileal villus height (414 ± 61 µm) compared to the CTRL (561 ± 53 µm) and AI (523 ± 62 µm) groups and was closer to the ileal villus height of SM piglets (192 ± 23 µm), although still higher. There was no difference in ileal and colonic crypt depths between groups (mean values: 94 ± 3 µm in ileum and 378 ± 31 µm in colon). The expression of PCNA gene (proliferation) in the ileum and colon, and that of Caspase 3 gene (apoptosis) in the colon of the SM group were higher than those of formula-fed groups, while the opposite change was observed for the latter in the ileum (Table 5). In ileum, goblet cell density of AI and HI groups was higher in villi and lower in crypts than those of SM group (Figure 4A). In the colon, crypt goblet cell density of formula-fed piglets was higher than that of SM piglets (Figure 4B). The expression of MUC2 gene was higher in HI than SM group (Table 5). Finally, the expression of IAP gene (mucosal defense) was higher in the

ileum of HI and CTRL than SM group, while it was lower in the colon of HI than SM group (Table 5).

In the ileum, the paracellular permeability was lower in formula-fed than SM-fed piglets (Figure 4C). Concomitantly, the expression of genes encoding tight junction proteins, CLDN4, MLCK and ZO1, as well as a tendency for CTNNB1 and OCLN, was higher in CTRL and AI than in SM ileum, with intermediate values for HI ileum (Table 5). In the colon, there was no difference in *ex vivo* paracellular permeability between the four groups (Figure 4C), while the expression of MLCK, ZO1 and CTNNB1 (as a tendency) genes was higher and that of CLDN7 was lower in formula-fed than SM piglets (Table 5). The transcellular permeability was higher in AI than in HI ileum, while it was higher in CTRL than in AI, HI and SM colon (Figure 4D).

Overall, these results showed an effect of SynCom supplementation on ileal villus size and goblet cell density, as well as on ileal and colonic transcellular permeability. In addition, the ileal expression of several genes encoding tight junction proteins was closer in the HI group to that in the SM group than in the CTRL or AI groups.

Intestinal nutrient transport, tryptophan metabolism and endocrine functions were moderately affected by the SynCom supplementation of formulas at PND24

The expression of genes encoding proteins involved in nutrient transport, tryptophan metabolism and endocrine functions was evaluated in the ileum and colon of formula-fed and SM-fed piglets (Table 6 and Supplemental Table S6). Only a few genes were differentially expressed between formula-fed piglets: the gene expression of SLC15A1 (peptide transporter) in the ileum and that of GCG (endocrine function) in the colon was higher and that of IDO (tryptophan metabolism) in the ileum was lower

in HI than AI group (Table 6). In addition, the expression of several other genes encoding proteins involved in nutrient transport, tryptophan metabolism and endocrine function in the ileum and colon was different between formula-fed and SM-fed piglets (Table 6).

Correlation analyses

To get a deeper insight into the individual associations between variables, correlation analyses were performed between the SynCom bacteria, and microbiota and physiological variables (Figure 5), and between microbial genera and physiological variables (Figures S4), in the ileum and colon of formula-fed piglets. In the ileum, the abundance of ten (out of thirteen SynCom bacteria that were detected in the piglet microbiota) SynCom bacteria was highly correlated to immune variables, in particular *Bifidobacterium breve* (OTU specific of HI group) was correlated to sIgA level in ileal digesta (Figure 5A). Conversely, the abundance of three SynCom OTUs was only correlated with a few ileal barrier variables (BD2, OCLN) (Figure 5A). It is noteworthy that only three SynCom OTUs, either common to SynComs AI and HI (*Bifidobacterium bifidum* and *Rothia mucilaginosa*) or specific to SynCom HI (*Staphylococcus capitis*), were correlated with physiological variables in the colon (Figure 5B). Regarding correlations between SynCom bacteria and microbiota genera in the ileum and colon, all SynCom OTUs correlated with several genera in the ileum (Figure 5C) while nine of them correlated with several other genera in the colon (Figure 5D). Finally, the individual associations between microbiota genera and physiological variables were analysed separately in the ileum (Supplemental Figure S4A) and the colon (Supplemental Figure S4B) of formula-fed piglets. The abundances of microbiota genera were positively or negatively correlated ($p < 0.05$

and |r| > 0.7) with 30.6 % of total physiological variables analysed in the ileum, and with 77.6 % of total physiological variables analysed in the colon. These results support the HM bacteria-driven influence of gut microbiota composition on multiple gut functions.

Discussion

The aim of our study was to evaluate the effect of two SynComs with similar taxonomic composition, representative of the taxonomic diversity of the HM cultivable bacteria, but with contrasting immunomodulatory properties as characterized *in vitro* on an intestinal quadricellular model (9), on gut physiology and microbiota in piglets used as a human infant model. Our data provide evidence that the majority of the bacteria assembled in the SynComs were present in the intestine of piglets fed SynCom-supplemented formulas and that the two SynComs differentially affected the intestinal immune and barrier functions and microbiota composition. The SynCom HI showed a global effect on intestinal immune markers and, to a lesser extent, barrier function, associated with changes in microbial composition, particularly within Bacillota. In contrast, the immune and barrier effects of the SynCom AI were less pronounced than those of SynCom HI. In addition, correlation studies revealed some correlations between several SynCom bacteria and both microbiota composition and intestinal functions, mainly in the ileum. Additional correlations between intestinal functions and several genera not provided by SynComs were demonstrated in the colon and, to a limited extent, in the ileum, suggesting that the addition of SynComs in formulas also induced physiological changes through changes in microbiota composition.

The HM bacteria assembled in SynComs were safe and did not induce intestinal disorders or growth differences in piglets among the three formula-fed groups. OTUs corresponding to the added SynCom bacteria were present in the intestine, consistent with vertical transfer of HM bacteria to the infant gut and the fecal presence of probiotic bacteria during the supplementation period (10, 17–20). In fact, metabarcoding analysis of the three formulas allowed to specifically match the bacteria assembled in the two SynComs and the corresponding OTUs in the formulas and the intestine. Most SynCom OTUs were identified in ileal and, at a lower abundance, in colonic digesta and feces, allowing for the intestinal presence of all SynCom bacteria, with the exception of *Cutibacterium*, *Lactobacillus gasseri,* and V*eillonella* strains. This may be explained by the use of the V3-V4 regions, which were not optimal for amplification of the 16S rRNA gene for *Cutibacterium* (21). Of note, although most SynCom OTUs were retrieved from intestinal microbiota of SynCom-supplemented piglets, we cannot distinguish between gut colonization by these SynCom bacteria or their simple transit, as piglets were fed SynCom daily throughout the experiment. In addition, the use of a metabarcoding approach does not allow conclusions about the physiological state of the SynCom bacteria (either alive or not). Notably, the relative abundance of these SynCom OTUs varied from 0.0002 to 0.86 % of the total OTUs in the ileum and colon, suggesting that certain bacterial species of SynComs had the ability to grow, colonize and persist in the mucosal environment. It was shown that *Lacticaseibacillus rhamnosus* and *Leuconostoc mesenteroides* isolated from HM and added to the diet, colonized and multiplied in the germ-free mouse intestine and this was also suggested for *Streptococcus, Staphylocccus*, *Corynebacterium* and *Cutibacterium* (22, 23). Alternatively, the differences in the relative abundance of SynCom bacteria in the

ileum and colon may be related to the survival rate of SynCom strains in the intestinal environment, as previously reported (24–26).

The observed direct effect of SynCom bacteria on intestinal immune and barrier functions highlights the modulatory role of HM-derived bacteria on intestinal physiology. Indeed, ten and four of the thirteen SynCom OTUs that were detected in the piglet microbiota, were correlated with ileal markers of immune and epithelial barrier functions, respectively. Such functional roles of SynCom bacteria confirmed their immunomodulatory profiles as characterized *in vitro* (9). Correlations between the SynCom OTUs and ileal immune variables concerned OTUs common to the SynComs AI and HI or specific to one of them, and belonging to *Rothia mucilaginosa, Bifidobacterium breve, Corynebacterium sp.*, *Staphylococcus haemolyticus, Winkia neuii* and *Micrococcus luteus*. Our results are consistent with the recognized immunomodulatory role of *Bifidobacterium* strains as probiotics (27–31). They also reinforce the scarce information available on HM genera such as *Corynebacterium, Staphylococcus, Rothia, Micrococcus, Winkia*, confirming their potential interest in terms of probiotic use when assembled in SynComs.

In addition to the presence of SynCom bacteria in the intestine, supplementation of formulas with SynComs AI and HI induced changes in the composition of the microbiota. At PND24, microbial α-diversity was higher in the HI than in the CTRL formula-fed ileum. Several taxa were differentially abundant among the three formula-fed groups, regardless of intestinal compartment. More than half of the taxa that were differentially abundant among the three formula-fed groups were differentially abundant between AI and HI piglets. In addition, microbial α-diversity in PND8 feces and PND24 ileal content was lower in SM than in CTRL piglets, consistent with results reported in 0- to 6-month-old breastfed infants and in HM-fed

piglets compared to formula-fed piglets (5, 32, 33). Interestingly, the number of taxa with differential abundance between HI and SM groups in the ileum and colon was lower than between CTRL and SM. It highlights that SynCom HI may drive changes in microbiota composition towards that of SM-fed piglets, although no differences in β-diversity were observed between formula-fed piglets.

The formula supplementation with SynCom HI induced specific changes in the abundance of several genera belonging to the Bacillota and Bacteroidota phyla compared to the formula supplementation with SynCom AI. The abundance of *Clostridium sensu stricto 1*, *Eubacterium*, *Romboutsia* and *Turicibacter* belonging to Bacillota and *Prevotellaceae UCG-003* (Bacteroidota) was higher in HI than in CTRL ileum. Several of these genera (*Eubacterium*, *Prevotellaceae UCG-003*, *Clostridium sensu stricto 1* and *Turicibacter*) were positively correlated with the TNFα response of ileal PP cells. Likewise, in the colon, *Anaerococcus*, a member of the Clostridiales vadimBB6 group family and *Sutterella* whose abundances were higher in HI and/or AI compared to CTRL, correlated with the expression of colonic genes involved in mucosal immunity. These correlations suggest the involvement of these genera affected by SynCom supplementation in the maturation of the intestinal immune system. Early immune boost reported in breastfed infants and HM-fed piglets was associated with the composition of the microbiota (5, 34–37). The specific importance of Bacillota in the induction of immune ontogeny, essential for regulating susceptibility to immunopathologies later in life, has been demonstrated in rodents (38). The genera *Clostridium* and *Prevotellaceae UCG-003* are known to be important genera of the preweaning piglet microbiota (39–41). An increase in the abundance of *Clostridium*, among others, has been shown to have beneficial long-term effects on post-weaning health (42, 43). *Turicibacter*, and *Clostridium* are

hallmarks of immune status. Indeed, the abundance of *Turicibacter* was decreased in immunodeficient mice (44, 45) and negatively correlated with immune-related adverse events (46). That of *Clostridium* was positively correlated with fecal calprotectin concentration, an indicator of immune maturation, in preterm infants (36). It is noteworthy that other genera, such as *Romboutsia*, *Eubacterium oxidoreducens* group, which did not differ between groups, also correlated with immune genes, as previously shown (47). In addition, at PND8 the early increase in acetate in HI feces compared to CTRL and AI feces may be involved in immune maturation, SCFAs having immunogenic activity locally within the intestine and at systemic level (48). Interestingly, a direct effect of the HI SynCom *Bifidobacterium breve* (OTU 205) on sIgA was suggested by its positive correlation with ileal sIgA levels, as well as an indirect effect illustrated by its positive correlation with the genera *Bavaricoccus, Blautia* and *Clostridiales vadinBB60 group* (Bacillota) and *Sutterella* (Pseudomonadota), which were also associated with ileal sIgA levels. sIgA plays an important role in the intestinal defense against bacterial pathogens, by limiting the adhesion of potentially harmful antigens to mucosal tissues and neutralizing their toxins and virulence factors. Thus, sIgA is a relevant marker for the development of the immune system in infants. Compared to breastfed infants, fecal sIgA concentrations were decreased in formula-fed infants and restored with probiotic supplementation (49–51). Taken together, our data suggest a beneficial effect of Syncom HI-induced changes in microbiota composition on immune markers.

Formula supplementation with Syncom AI modulated the abundance of genera, increasing that of *Staphylococcus* (ileum, colon, and feces), *Alistipes* (colon and feces), and *Parasuterella* (colon), and decreasing that of *Peptococcus* and *Parasutterella* (ileum) compared to the CTRL and HI groups. *Alistipes*, considered to

be a human gut commensal involved in protein fermentation, has been associated with the immune system, but with conflicting evidence on its effects (52, 53), and *Alistipes onderdonkii* has shown interest as an anti-inflammatory probiotic in transplant recipients (54). *Parasutterella*, a member of the human core gut microbiota, has diverse roles, including a role in tryptophan and bile acid metabolism, and protective effects against metabolic disease (55, 56) and in maintaining host immunity during late pregnancy in mice (57). Regarding *Peptococcus*, some studies have suggested that it may be pathogenic, and it has been shown to be associated with diarrhea in piglets and ulcerative colitis in humans (58, 59). In addition, niacin-induced relief of intestinal inflammatory symptoms was associated with a decrease in *Peptococcus* abundance (60). Therefore, the effect of SynCom AI-induced changes in the composition of the intestinal microbiota in terms of immune consequences was not entirely clear from the literature.

The correlations between several genera, whose abundance was altered or not with the SynCom supplementation, and colonic barrier variables (genes encoding tight junction proteins and mucins) illustrate the relationship between the microbiota and intestinal barrier functions, although no difference in *ex vivo* paracellular permeability was observed between the formula-fed groups in the ileum and colon. It is noteworthy that the higher paracellular permeability in the SM ileum was consistent with the higher permeability reported in some studies in breastfed infants (61) and HM-fed piglets (5) compared to formula-fed piglets. In agreement, the expression of some genes encoding tight junction proteins was hardly modulated between formula-fed piglets, but decreased in SM piglets, with sometimes, an intermediate situation for HI piglets. It has been reported that high intestinal permeability, which facilitates the passage of molecules across the epithelium, influences the maturation of the

immune system and the acquisition of tolerance to indigenous bacterial and dietary antigens (62). Additional changes in the expression of genes involved in other functions (nutrient transport, endocrine function and tryptophan metabolism) were significantly correlated with numerous genera in the ileum and colon microbiota, reinforcing the evidence for an effect of SynComs AI and HI on intestinal functions through changes in the composition of the intestinal microbiota.

In conclusion, HM-derived bacteria can reach the intestinal compartments and influence the intestinal epithelium, its immune function and the microbiota. Consistent with our *in vitro* study on a quadricellular model mimicking the intestinal epithelium, we confirmed that SynComs with contrasting immunomodulatory properties but similar taxonomic composition can differentially modulate the developmental profile of intestinal functions. The immunostimulatory profile of SynCom HI induced deeper changes in intestinal immune properties than the anti-inflammatory profile of SynCom AI. A major limitation of our study was that the SynComs, with 11 strains, did not fully represent the complexity of the HM microbiota. Furthermore, the strains were assembled in equal proportions, whereas dominant taxa are observed in the HM microbiota (4, 6). Despite its limitations, this study highlights that the continuous supply of HM-like SynComs in the diet, at physiological doses - 5.5 x 10^5 CFU/mL, which is in the upper mid-range of HM bacterial load - was sufficient to induce short-term physiological effects in a SynCom-dependent manner. Further investigation is needed to more fully characterize long-term health effects. In addition, such apparently beneficial effects, mainly observed with SynCom HI in healthy infants, should be evaluated in unhealthy situations where the immune system is challenged, such as in allergic situations, which are common in infants.

Materials & Methods

Animal study

The study was designed and conducted at INRAE experimental facilities (Pig Physiology and Phenotyping Experimental Facility, Saint-Gilles, France, doi 10.15454/1.5573932732039927E12; Agreement No. D35-275-32) in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), and the French legislation on animal experimentation and ethics. The ethics committees of CREEA (Rennes Committee of Ethics in Animal Experimentation) and of France's Ministry of Higher Education and Research approved (authorization #35727-2022030411197283) the entire procedure described in this protocol. Animal welfare was ensured by daily observation throughout the experiment. The piglets did not receive any medication or antibiotic treatment.

Thirty female and male Yucatan piglets were separated from their dam at postnatal day (PND) 2 and housed in individual stainless-steel metabolic cages. Room temperature was maintained at 30°C for the first week and then lowered at 28°C until the end of the experiment. Piglets were fed one of the three experimental formulas with an automatic milk feeder, as previously described (63). To account for litter-to-litter variation, three piglets with a body weight (BW) close to the mean birth weight of the litter were selected from each litter and assigned to one of the three formulas. Mean birth weight of selected piglets was 0.849 ± 0.023 kg. Allocation to formulas was balanced between groups for birth weight, BW at PND2 and gender. Formulas were daily rehydrated to 20 % dry matter extract in water prior to distribution. Formula supply was divided into ten meals that were automatically distributed throughout the day. BW was measured twice a week and feeding amounts were adjusted accordingly. The daily net energy offered was 1450 kJ/kg per $BW^{0.75}$.

Formula intake was automatically recorded for each meal. In addition, six piglets selected from the same litters as the formula-fed piglets were allowed to suckle the sow naturally (SM) to provide benchmarks for gut development and microbiota. All piglets were euthanized at PND24 (± 2.6 days) and tissues were collected.

Diets and synthetic bacterial communities

Formulas were manufactured at a semi-industrial pilot scale at Bionov (Rennes, France). The three formulas had the same nutritional content (Supplemental Table S1). They differed by the bacterial supplementation: two HM-derived SynComs with anti-inflammatory (AI) *vs.* high immunomodulatory (HI) properties (9) as described in Table 1, or no bacterial supplementation for the control (CTRL) formula.

The AI and HI SynComs were each composed of 11 strains in equal proportions. Three of the strains were common to both SynComs and a further eight strains were specific to each SynCom. Firstly, the two SynComs were designed to mimic HM microbiota with representatives of genera frequently found in HM and with at least, one representative of the 4 most widespread genera of the cultivable HM microbiota: *Staphylococcus*, *Cutibacterium*, *Streptococcus* and *Corynebacterium* (6). Secondly, to consider the wide variability in the immunomodulatory capacities of HM bacteria, the 2 communities were assembled to have theoretically contrasting immunomodulatory effects, depending on their individual properties, as previously described (9). The SynCom AI was designed to display mainly anti-inflammatory properties and the SynCom HI to display both anti- and pro-inflammatory properties. Each bacterial strain was cultured separately on its optimal growth medium for 1 to 5 days as previously described (9) and its concentration was estimated with an OD measurement. Bacterial cells were harvested by centrifugation (6000 g, 10 min, 4°C),

washed with 0.9 % (wt /vol) saline solution, pooled and resuspended in the formula. Daily doses equivalent to a 500-fold concentrated bacterial suspension (total bacterial concentration of 2.75 x 10^8 Colony Forming Units (CFU)/mL) were prepared and stored at -80°C until used. After thawing in a water bath at 37°C for a few minutes, AI and HI bacterial doses were added in the AI and HI diets, respectively, at a total bacterial concentration of 5.5 x 10^5 CFU/mL of formula (corresponding to a concentration of 5 x10^4 CFU/mL of formula for each strain).

Sample collection

Diet

Fifteen mL milk samples were collected from sows nursing the SM piglets on day 20 postpartum. Injection of oxytocin (2 mL, Laboratoires Biové, Arques, France) was carried out to facilitate milking. Before sampling, the sow teats were thoroughly washed with water and soap and rinsed with sterile physiological water (0.9 % NaCl) before cleaning with ethanol. Samples of formulas CTRL, AI and HI were also randomly collected at the time of rehydration of formula powders during the third week of the experimental period. Sow milk and formula samples were stored at -80°C until microbiota analysis.

Piglets

During the experimental period, feces were collected at PND8 and stored at -80°C for microbiota, short-chain fatty acid (SCFA) and sIgA analyses. On the last day of the experiment, piglets were euthanized 1 h after their last meal by electrical stunning immediately followed by exsanguination. Blood was collected in 2 BD Vacutainer CPT (BDBiosciences, NJ, USA) and stored at room temperature until PBMC extraction. Ileal digesta and tissue were collected from an 80-cm segment anterior to

the ileocecal junction, and colonic digesta and tissue were collected from the first third part of the colon. Samples of 100 mg ileal and colonic digesta and feces were immediately frozen in liquid nitrogen and stored at -80°C for microbiota and sIgA analyses. In addition, 0.5 to 2 g of ileal and colonic digesta and feces were collected for SCFA analysis. A 10-cm segment of distal ileum (containing the ileal Peyer Patch (PP)) was collected, rinsed with ice-cold Hank's balanced saline solution supplemented with 50 mg/mL gentamicin, 200 UI/mL penicillin, 200 mg/mL streptomycin and 10 mM hepes for isolation of PP mononuclear cells. The remaining ileal segment and the proximal colon were rinsed with cold phosphate buffered saline (PBS). A 10-cm segment was kept in ice-cold Dulbecco's Minimum Essential Medium (Gibco, Thermo Fisher, France) for immediate Ussing chamber analysis. About 100 mg of ileal (without PP) and colonic tissues were kept in a RNA later solution for 24 h at 4°C and stored at −20°C until RNA extraction and gene expression analysis. Finally, adjacent segments (10-cm) were fixed in 4 % paraformaldehyde for 48 h until further dehydration in ethanol and embedding in paraffin, for morphometry analysis and goblet cell counting.

Microbiota analysis

Extraction of total bacterial DNA from ileal and colonic digesta and feces was performed as described in the instruction guide of the Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZYMO Research, Irvine, USA). Sow milk and formula samples were pre-treated before total bacterial DNA extraction as described (64). The V3-V4 region of 16S rRNA gene was amplified using the following primers: CTTTCCCTACACGACGCTCTTCCGATCTACTCCTACGGGAGGCAGCAG (V3F) and GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCC (V4R),

Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Évry-Courcouronnes, France) and dNTP (New England Biolabs) for 25 cycles (10 s to 98°C, 30 s at 62°C, and 30 s at 72°C). Agarose gel electrophoresis was performed to verify amplicon purity prior to sequencing using Illumina MiSeq technology, performed at the Genotoul GeT-PlaGe platform (Toulouse, France).

SCFA analysis

After collection, ileal and colonic digesta and feces were weighed, mixed with 1 mL 0.5 % orthophosphoric acid solution per g of digesta and centrifuged at 1700 g for 15 min at 4°C. Supernatants were then stored at -20°C until SCFA quantification. SCFAs were analyzed by High Performance Liquid Chromatography (HPLC, Ultimate 3000, Thermo Fisher Scientific, Courtaboeuf, France). Acid separation was performed using a Rezek ROA organic acid H+ column (300*7.8mm Phenomenex, California) with H2SO4 0.005M as mobile phase at a flow rate of 0.4ml/min at 60°C. Two detectors were used: a UV detector (Dionex UVD 170U) operating at 210nm and a refractometer (RI 2031 Plus Jasco). Quantification was performed with an external calibration using acetic acid (PanReac, Lyon, France), propanoic, 2-methylpropanoic, butanoic, 3-methylbutanoic and pentanoic acids (Merck, St. Quentin Fallavier, France) as standards (5).

***Ex vivo* permeability measurement**

Ileal and colonic permeability measurements were performed using Ussing chambers (Physiological Instruments, San Diego, CA, USA) as described (5). Permeability was determined using tracer molecules, fluorescein sodium salt (Na-FITC) for paracellular permeability and peroxidase from horseradish type VI (HRP) for transcellular

permeability. The tracer molecules were added into the apical compartment and those transferred through the epithelium were analyzed in the serosal compartment. Concentration of Na-FITC in the samples collected after 120-min incubation from the serosal buffer was measured by fluorimetry (Varioskan™ LUX multimode microplate reader, ThermoFisher Scientific, Saint-Herblain, France) whereas concentration of HRP was determined using spectrophotometry (Varioskan™ LUX multimode microplate reader, ThermoFisher Scientific, France) after enzymatic reaction using o-Dianisidine dihydrochloride as substrate (Merck, Molsheim, France).

Histomorphometry analysis

Histomorphometric analysis was performed after alcian blue and periodic acid Schiff staining on 7 µm sections of formalin-fixed and paraffin-embedded ileal and colonic tissues. Sections were analysed using a light microscope (Nikon Eclipse E400, Nikon Instruments, France) with image processing software (NIS-Elements AR 3.0, Nikon Instruments), as described (65). Villus and crypt sizes and goblet cell density were measured in at least 15-20 crypt-villus units per piglet.

Cell isolation and *in vitro* culture

PBMC extraction was performed within 2 h after collection following the manufacturer instructions of the BD Vacutainer CPT (BDBiosciences, NJ, USA). After centrifuging the BD Vacutainer CPT tube at 1500 g for 20 min, the cell ring was recovered. The PBMC cells obtained were resuspended with 10 mL of HBSS supplemented with 10 % Foetal Calf Serum (FCS) and centrifuged 10 min at 600 g and 4°C. The supernatant was discarded. The pellet containing PBMC was resuspended with 7 mL of Ammonium-Chloride-Potassium (ACK) lysis buffer, and with 7 mL of RPMI

complemented with 10 % FCS and 1 % Penicillin/Streptomycin (complete RPMI). The suspension was centrifuged 5 min at 600 g and 4°C. The pellet was resuspended with 14 mL of complete RPMI and centrifuged 10 min at 600 g and 4°C. Finally, PBMC were suspended in complete RPMI to achieve cell concentration of 4.10^6 cells/mL. Mononuclear cells from freshly-removed ileal PP were isolated as previously described (65). PP cells were suspended in complete RPMI to achieve cell concentration of 8.10^6 cells/mL.

PBMC and PP mononuclear cells were cultured in RPMI complete medium for 48 h at 37°C in a 5% CO_2 water-saturated atmosphere. Culture was performed in unstimulated conditions and stimulated conditions with ConA (5 µg/ml of Concanavalin A from Canavalia ensiformis Merck, Molsheim, France) or LPS (10 µg/ml of LPS-EB ultrapure from Escherichia coli 0111:B4 strain, InvivoGen, San Diego, USA). In addition, PP mononuclear cells were cultured at 37°C in a 5 % CO_2 water-saturated atmosphere for 7 days to assess sIgA production. Supernatants of PBMC and PP cells were collected and following the addition of antiprotease cocktail 1X (SigmaFast, Merck Sigma, Saint Quentin Fallavier, France) stored at −20 °C until cytokine and sIgA analyses.

Cytokine and sIgA analyses

Concentrations of IL-10 and TNF-α in culture supernatants of PBMC and PP cells were measured by ELISA (R&D Systems, USA: DY693B and DY985, respectively).

For sIgA analysis, ileal and colonic digesta and fecal samples were 10-fold diluted with PBS-EDTA (0.5 M) buffer supplemented with a protease inhibitor cocktail (250 µg/L; P2714-1BTL). After a vigorous shaking, suspensions were incubated 30 min at room temperature on a Rotary laboratory shaker and centrifuged at 18 000 g, 30 min

and 4°C. Supernatants were stored at -20°C until ELISA analysis. Concentration of sIgA in 7 days-cultured PP cell supernatants as well as in ileal, colonic and fecal supernatants was assessed as previously described (65).

Gene expression analysis

Total RNA extraction from ileal and colonic tissues was performed using the "NucleoSpin® RNA" kit (Macherey Nagel). Extracted RNA was quantified using a DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA). RNA quality and RNA Integrity Number (RIN) were assayed using the Agilent RNA 6000 Nano kit in combination with an Agilent 2100 Bioanalyzer (Agilent Technologies France, Massy, France). Reverse transcription was then carried out on 1 µg of extracted RNAs using the High Capacity Complementary DNA Reverse Transcription Kit, as previously described (66).

Quantitative real-time PCR was performed on a 384-well plates real-time PCR machine using a PowerSYBR green PCR Master Mix kit (4368708, ThermoFisher Scientific, France) for detection (67). The housekeeping genes selected were YWHAZ and PGK1 for ileum, and HTPR1 and RPL4 for colon. Relative expressions of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method for group comparisons.

Statistical analysis

Data are presented as means ± SEM (standard error of the mean). For microbiota data, raw sequences were analyzed using the bioinformatic pipeline FROGS (Find Rapidly OTU with Galaxy Solution) software (68). Data were treated with the following steps: pre-processing, clustering with swarm algorithms, remove of chimera, OTU filters, and finally taxonomic affiliation. The descriptive analysis of the

structure (α- and β-diversity) of the microbiota was conducted with the Phyloseq function (EdgeR package, Bioconductor). The α-diversity index used were Chao1 representing the bacterial richness. Phylogenetic β-diversity was studied using the Bray-Curtis distances and group differences were evaluated with principal co-ordinate analysis (PCoA) and permutational multivariate analysis (PERMANOVA) of variance using distance matrices. Differences in phyla, families, genera and OTUs were assessed with pairwise comparisons on OTUs or after aggregation at the desired taxonomic rank (phyloseq tax_glom function) using DESeq2 as described in (5).

Statistical analysis of the other variables was performed using R software, version 4.3.2. Differences between groups (SM, CTRL, AI and HI) were assessed using a three-way analysis of variance (ANOVA), testing diet, gender, litter, and interactions between diet and gender, and diet and litter, followed by Tukey *post hoc* test for parametric variables. When gender or litter effects or their interactions with diet were non-significant ($p > 0.05$), these factors were removed from the model. The normal distribution and the homoscedasticity of the residuals of each linear model were tested using Shapiro-Wilk and Levene's tests, respectively. When the raw data did not fulfil these model assumptions, a natural logarithmic transformation of the data was performed prior to running the linear model. If the assumptions were still not satisfied, data were tested with a non-parametric Kruskal Wallis test associated with Dunn test. Differences were considered as statistically significant for $p < 0.05$ and a trend for difference at $0.05 < p < 0.1$.

For the multidimensional analysis of the three formula-fed groups, Partial Least Squares Discriminant Analyses (PLS-DA; mixOmics package (69)) were performed on all the variables measured in the ileum (n=201) and colon (n=250) to calculate

their Variable Importance in Projection (VIP) score (70) on the first and the second components of the PLS-DA that discriminated the three formula groups. The variables with a VIP score greater than 1 on components 1 or 2 were selected, corresponding to 112 variables for the ileum and 141 variables for the colon. Variables were then divided into eleven groups as follows: Barrier, Immunity, Microbiota activity, Nutrient transporter, Endocrine and Tryptophan metabolism, plus five microbiota phyla: Actinomycetota, Bacillota, Bacteroidota, Pseudomonadota, and 'Other Phyla' (that gathered the Campylobacterota, Deferribacterota, Desulfobacterota, Euryarchaeota, Fusobacteriota, Spirochaetota, Synergistota, Verrucomicrobiota and Patescibacteria phyla), for the Multifactorial analysis (MFA; FACTOMINE R package (71, 72)).

Figure legends

Figure 1. Ileal (A) and colonic (B) relative abundances of the OTUs potentially corresponding to the SynCom strains added in AI and/or HI formulas.

Each individual is represented with one dot; Median with interquartile range; [a,b,c] non-common letters indicate that piglet groups differed significantly, $P < 0.05$

Figure 2. Multifactorial analysis (MFA) using microbiota and intestinal variables of formula-fed piglets (CRTL, AI, HI), and grouped by function or phylum (n = 11 groups of quantitative variables).

Variables used in the MFA were the most discriminating variables preselected by Partial Least Squares-Discriminant Analysis (PLS-DA) with a Variable Importance in Projection (VIP) score > 1 for components 1 and 2 (n=112 and 141 variables in the ileum and colon, respectively; Supplemental Figure S1 and Table '*Table Individual ileal and colonic data used for PLS-DA and MFA*' accessible in the repository https://doi.org/10.57745/ATTHZ3). Red dot line, level of statistical significance corresponding to the inverse of the number of groups of variables used in the analyses.

Dim, Dimension

Figure 3. PBMC **(A)** IL-10 and **(B)** TNFα production in unstimulated (basal) and stimulated (Concanavalin A, ConA) conditions; **(C)** Secretory IgA (sIgA) concentration in ileal and colonic digesta and feces of formula-fed (CTRL, AI, HI) and sow milk-fed (SM) piglets at PND24.

[a,b,c,d] non-common letters indicate that piglet groups differed significantly, $P < 0.05$; # indicates that piglet groups tended to differ, $P < 0.1$.

PBMC, Peripheral Blood Mononuclear Cells; PND, postnatal days

Figure 4. Effects of diets on intestinal barrier. Goblet cell density in **(A)** ileal villi, and **(B)** ileal and colonic crypts; *ex vivo* (Ussing chamber) epithelial barrier **(C)** paracellular and **(D)** transcellular permeability in formula-fed (CTRL, AI, HI) and sow milk-fed (SM) piglets at PND24.

[a,b,c] non-common letters indicate that piglet groups differed significantly, $P < 0.05$.

PND, postnatal days

Figure 5. Correlation matrix between the relative abundance of the OTUs potentially corresponding to AI and/or HI SynCom strains and **(A and B)** physiological variables, **(C and D)** microbial genera abundances in **(A and C)** ileum and **(B and D)** colon of formula-fed piglets ($|r| > 0.7$, $P < 0.05$).

* indicates genera that may partly correspond to the SynCom bacteria added in the formula.

TNFα Basal PEYER, TNFα secretion by Peyer Patch cells in unstimulated condition; IL10 LPS PEYER, IL-10 secretion by Peyer Patch cells in LPS-stimulated condition; isovalerate, isovalerate concentration; the variables in capital letters correspond to the expression of genes listed in Tables 4 and 5, and Supplemental Table 6.

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Conflict of interest

The authors declare no competing financial interests. The funders had no role in study design, data collection or the interpretation of the work.

Data Availability Statement

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets presented in this study can be found in online repositories (four Tables named ‘Relative abundance of the OTUs that did not correspond to AI and HI bacteria in formulas’, ‘Individual ileal and colonic data used for PLS-DA and MFA’, ‘OTUs identified in sow milk’, ‘Cytokine production by Peyer Patch and PBMC cells in formula-fed piglets’)

https://doi.org/10.57745/ATTHZ3. In addition, the raw sequences of microbiota analysis are available on NCBI repositories (PRJNA1197167).

Contributions

SE, ILHL, YLL and SB conceived the project that led to the submission of this work. SE and ILHL supervised research; CLB, SE and ILHL designed the experiment; CLB, GR, AC, PD, SG, RJ, VR, LR, MBM, SE and ILHL acquired data; CLB, SE and ILHL played an important role in interpreting the results; AB and LR realised the human milk collection; CLB, SE and ILHL drafted the manuscript and had primary responsibility for final content. All authors revised and approved the final manuscript.

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Table 1. Relative abundances of the Syncom OTUs in formulas, and in the feces at PND8 and ileum, colon and feces of formula-fed (CRTL, AI and HI) piglets at PND24. OTUs potentially corresponding to the strains added in SynCom AI and/or HI formulas are presented together with their relative abundance (in % of total OTUs) in feces, ileon and colon microbiota.

Strain	added in AI	added in HI	OTU		OTU relative abundance (%) in formulas			OTU relative abundance (%) in feces PND8[2]			OTU relative abundance (%) in feces PND24[2]			OTU relative abundance (%) in ileum[2]			OTU relative abundance (%) in colon[2]		
			ID[1]	Identification	CTRL	AI	HI	CTRL	AI	HI	CTRL	AI	HI	CTRL	AI	HI	CTRL	AI	HI
Bifidobacterium bifidum (CIRM BIA 2840)	x	x	172	*Bifidobacterium bifidum*	0.002	1.428	1.286	0.0003		0.0003		0.0003		0.0003	**0.0206**	0.0170		0.0002	0.0004
Bifidobacterium breve (CIRM BIA 2844)	x		99	*Bifidobacterium breve*		5.341		0.0003	**0.0584**	0.0012	0.0002	***0.0144***	*0.0002*	0.0006	***0.1159***	*0.0008*		***0.0103***	*0.0002*
			184	*Bifidobacterium breve*		2.489			0.0209		0.0004	0.0049		0.0003	**0.0485**	0.0009		0.0044	0.0003
			2283	*Bifidobacterium breve*		0.084			0.0009						0.0012				
Bifidobacterium breve (CIRM BIA 2845)		x	205	*Bifidobacterium breve*			0.893	0.0002	0.0002	0.0011	0.0002		0.0113	0.0006	*0.0003*	***0.2889***	0.0003		0.0148
Corynebacterium simulans (CIRM BIA 2853)[3]	x		78	*Corynebacterium sp.*	0.002	7.205	1.575	0.0005	**0.0180**	0.0043	0.0002	**0.0288**	**0.0119**		**0.3388**	**0.0709**	0.0002	**0.0147**	**0.0065**
Corynebacterium kroppenstedtii (CIRM BIA 2852)[3]		x	78	*Corynebacterium sp.*	0.002	7.205	1.575	0.0005	**0.0180**	0.0043	0.0002	**0.0288**	**0.0119**		**0.3388**	**0.0709**	0.0002	**0.0147**	**0.0065**
Lactobacillus gasseri[4] (CIRM BIA 2841)	x		10	*Lactobacillus sp.*	0.025	3.212	0.022	0.0967	0.1923	0.1277	0.3195	0.3337	0.2538	8.4725	12.053	10.764	0.5893	0.7879	0.4547
			1622	*Lactobacillus sp.*		0.018			0.0007					0.0357	0.0347	0.0466	0.0010	0.0005	
Lactobacillus jensenii (CIRM BIA 2839)		x	203	*Lactobacillus jensenii*			1.566			0.0006			0.0009		0.0006	0.0127	0.0005	0.0002	0.0008
Micrococcus luteus (CIRM BIA 2843)		x	204	*Micrococcus sp.*	0.002		1.676		0.0002		0.0002		0.0005	0.0003		0.0181		0.0006	0.0023
Rothia mucilaginosa (CIRM BIA 2847)	x	x	209	*Rothia mucilaginosa*		1.283	0.629	0.0002	0.0002	0.0002	0.0002	0.0008			**0.0225**	0.0117		0.0002	0.0002
Staphylococcus haemolyticus (CIRMBP-1635)	x		94	*Staphylococcus haemolyticus*	0.004	13.97	0.002	0.0040	0.0166	0.0071	0.0019	***0.0150***	*0.0018*	0.0512	***0.8563***	*0.0184*	0.0036	**0.0245**	*0.0026*
			3443	*Staphylococcus sp.*		0.088								0.0007	0.0102				
			3980	*Staphylococcus sp.*		0.043					0.0002			0.0003	0.0015	0.0002			
			5808	*Staphylococcus sp.*		0.018								0.0013	0.0221	0.0007			
Staphylococcus capitis (CIRMBP-1634)		x	41	*Staphylococcus capitis*	0.009	0.191	43.10	0.0175	0.0036	0.0035	0.0029	*0.0030*	***0.0175***	0.0415	0.0311	0.4045	0.0036	0.0032	0.0186
			2175	*Staphylococcus sp.*			0.043	0.0003			0.0002			0.0005		0.0022			
			2556	*Staphylococcus sp.*			0.061									0.0010			
			2648	*Staphylococcus sp.*		0.002	0.051					0.0002		0.0008	0.0008	0.0097			
			3748	*Staphylococcus sp.*			0.038							0.0002	0.0003	0.0026	0.0003		
		x	2175	*Staphylococcus sp.*			0.043												
Staphylococcus epidermidis (CIRMBP-1633)			2556	*Staphylococcus sp.*			0.061									0.0010			
			2648	*Staphylococcus sp.*		0.002	0.051					0.0002		0.0008	0.0008	0.0097			
			3748	*Staphylococcus sp.*			0.038							0.0002	0.0003	0.0026	0.0003		
Streptococcus infantis (CIRMBP-1636)	x		1690	*Streptococcus sp.*		0.032	0.097					0.0002		0.0011	0.0006				
			1091	*Streptococcus sp.*	0.002	0.084	0.025								0.0003				
Streptococcus salivarius (CIRM BIA 2846)		x	1690	*Streptococcus sp.*		0.032	0.097												
			1091	*Streptococcus sp.*	0.002	0.084	0.025												
Winkia neuii (CIRM BIA 2850)	x		127	*Winkia neuii*		4.899		0.0002	0.0067			**0.0125**		0.0003	***0.1470***	*0.0002*	0.0003	***0.0075***	*0.0005*
Veillonella dispar (CIRM BIA 2842)	x	x	No corresponding OTU																
Cutibacterium granulosum (CIRM BIA 2851)	x		No corresponding OTU																
Cutibacterium granulosum (CIRM BIA 2848)		x	No corresponding OTU																
Cutibacterium acnes (CIRM BIA 2849)	x		No corresponding OTU																

[1] ID of OTUs identified in formulas

[2] In bold, significative differential abundances in AI or HI formula-fed piglets compared to CTRL formula-fed piglets; in italic, significative differential abundances between HI and AI formula-fed piglets (Deseq analysis)

[3] *Corynebacterium simulans* CIRM BIA 2853 and *Corynebacterium kroppenstedtii* CIRM BIA 2852 were associated to a single OTU (78)

[4] For *Lactobacillus gasseri*, two OTUs of *Lactobacillus sp.* (10 and 1622) were identified in the intestinal compartments without any possibility to discriminate between the strain added in SynCom AI and those of the piglet intestine microbiota, knowing that *Lactobacillus* was highly predominant in the ileum and colon of the three formula-fed groups.

It is noteworthy that additional OTUs were also identified in the three prepared formulas (Table *'Relative abundance of the OTUs that did not correspond to the AI and HI bacteria in formulas'* available on the online dataverse https://doi.org/10.57745/ATTHZ3), as early as the rehydration of formula powders. These additional OTUs were not differentially abundant between the three rehydrated formulas, suggesting that this environmental contamination probably occurred during the process of formula manufacturing and/or were present in dairy ingredients.

PND, postnatal day

Table 2. Impact of SynComs AI and HI on relative abundances of bacterial phyla, families and genera in ileum, colon and feces of formula-fed (CRTL, AI and HI) piglets at PND24.

			CTRL *vs.* AI		CTRL *vs.* HI		AI *vs.* HI	
Phylum	**Family**	**Genus**	**p-adjusted**	**log_2 (Fold Change)**	**p-adjusted**	**log_2 (Fold Change)**	**p-adjusted**	**log_2 (Fold Change)**
ILEUM								
Bacteroidota			0.014	1.54	NS		0.034	-1.40
Campylobacterota			<0.001	15.58	NS		<0.001	-15.89
Pseudomonadota			0.045	1.90	NS		NS	
Actinomycetota	Actinomycetaceae		0.001	-2.55	0.036	-1.92	NS	
Bacteroidota	Bacteroidaceae		NS		NS		0.003	-2.02
	Marinifilaceae		<0.001	-11.41	<0.001	-13.02	NS	
Bacillota	Anaerovoracaceae		NS		0.022	-2.36	0.004	-2.50
	Lachnospiraceae		NS		NS		0.049	-1.07
	Lactobacillaceae		0.002	1.81	NS		NS	
	Peptococcaceae		<0.001	18.81	NS		<0.001	-19.10
	Peptostreptococcaceae		NS		0.006	-3.30	NS	
	Ruminococcaceae		NS		NS		0.041	-1.29
	Staphylococcaceae		<0.001	-7.29	NS		<0.001	8.46
	Turicibacteraceae		NS		0.020	-3.08	0.012	-3.08
	UCG-010		NS		<0.001	17.77	<0.001	16.94
Actinomycetota	Actinomycetaceae	*Actinomyces*	<0.001	-3.27	0.050	-2.17	NS	
Bacteroidota	Marinifilaceae	*Butyricimonas*	<0.001	-11.78	<0.001	-12.91	NS	
	Prevotellaceae	*Prevotellaceae UCG-003*	NS		<0.001	-12.89	<0.001	-42.53
Bacillota	Clostridiaceae	*Clostridium sensu stricto 1*	NS		0.038	-3.09	NS	
	Eubacteriales incertae sedis	*Evtepia*	NS		NS		0.008	-2.28
	Family XI	*Anaerococcus*	0.006	15.81	NS		<0.001	-19.50
	Lachnospiraceae	*Eubacterium fissicatena grp*	NS		0.030	-2.60	NS	
	Peptococcaceae	*Peptococcus*	<0.001	17.37	NS		<0.001	-17.96
	Peptostreptococcaceae	*Romboutsia*	NS		0.011	-3.66	NS	
	Staphylococcaceae	*Staphylococcus*	<0.001	-8.65	NS		<0.001	9.57
	Streptococcaceae	*Lactococcus*	0.030	3.40	NS		NS	
	Turicibacteraceae	*Turicibacter*	NS		0.038	-3.27	NS	
	UCG-10	*unknown genus*	NS		<0.001	15.94	<0.001	15.41
Pseudomonadota	Sutterellaceae	*Parasutterella*	<0.001	16.46	NS		<0.001	-16.34
COLON								
Bacteroidota	Marinifilaceae		<0.001	-41.87	<0.001	-33.32	0.036	8.55
Bacillota	Butyricicoccaceae		<0.001	2.77	NS		NS	
	Clostridia vadinBB6 group		NS		0.015	-5.11	0.028	-4.50
	Family XI		<0.001	-17.32	<0.001	-22.49	0.007	-5.18
	Staphylococcaceae		<0.001	-4.38	NS		<0.001	5.37
Bacteroidota	Marinifilaceae	*Butyricimonas*	<0.001	-42.10	<0.001	-33.50	NS	
	Rikenellaceae	*Alistipes*	<0.001	-6.59	NS		<0.001	7.14
Bacillota	Butyricicoccaceae	*Butyricicoccus*	0.003	2.81	NS		NS	
	Clostridia vadinBB6 group	*unknown genus*	0.016	-5.11	NS		NS	
	Family XI	*Anaerococcus*	NS		<0.001	-23.78	<0.001	-24.17
	Lactobacillaceae	*Leuconostoc*	0.017	2.77	NS		NS	
	Staphylococcaceae	*Staphylococcus*	0.001	-4.67	NS		<0.001	5.36
	Streptococcaceae	*Lactococcus*	NS		NS		0.003	-4.24
Pseudomonadota	Sutterellaceae	*Parasutterella*	<0.001	-16.04	<0.001	-23.51	NS	
FECES								
Actinomycetota	Actinomycetaceae		0.023	-2.28	0.001	-2.75	NS	
	Atopobiaceae		NS		0.016	3.03	NS	
Bacteroidota	Marinifilaceae		0.046	-8.12	<0.001	38.06	<0.001	-46.18
Bacillota	Carnobacteriaceae		0.023	4.16	0.023	3.95	NS	
	Family XI		NS		<0.001	-7.90	<0.001	7.95
	Staphylococcaceae		<0.001	-5.88	NS		<0.001	6.20
Actinomycetota	Actinomycetaceae	*Actinomyces*	0.003	-2.91	0.007	-2.90	NS	
	Eggerthellaceae	*Eggerthella*	<0.001	-45.80	<0.001	-43.93	NS	
Bacteroidota	Marinifilaceae	*Butyricimonas*	0.036	-8.30	<0.001	-35.57	<0.001	43.87
	Muribaculaceae	*Muribaculum*	NS		<0.001	11.01	<0.001	8.06
	Rikenellaceae	*Alistipes*	<0.001	-8.15	NS		<0.001	7.83
Bacillota	Carnobacteriaceae	*Granulicatella*	0.027	4.11	NS		NS	
	Eubacteriaceae	*Eubacterium*	0.001	-9.82	0.003	-9.25	NS	
	Family XI	*Anaerococcus*	0.003	-16.95	<0.001	-24.23	NS	
	Lactobacillaceae	*Leuconostoc*	0.005	2.85	NS		NS	
	Peptostreptococcaceae	*Peptostreptococcus*	0.044	-3.40	NS		NS	
	Staphylococcaceae	*Staphylococcus*	<0.001	-6.16	NS		<0.001	6.38
	Streptococcaceae	*Lactococcus*	0.003	4.15	NS		NS	

Log_2 Fold Change, the Fold Change indicates the significant differential abundance of bacteria (at phylum, family or genus level) in the first cited group compared to the second cited group (Deseq analysis). For example, Bacteroidota abundance is higher in CTRL ileum than in AI one.
PND, postnatal days.

Table 3. SCFA concentration and proportion in colonic digesta of formula-fed (CTRL, AI, HI) and sow milk-fed (SM) piglets at PND24.

		CTRL	AI	HI	SM	p-value
Concentration (mmol/kg dry matter)[1]	Acetate	23.64 ± 4.50	26.61 ± 6.80	24.45 ± 7.30	47.81 ± 12.20	0.255
	Propionate	**6.85 ± 1.1[a]**	**8.20 ± 1.9[a]**	**6.98 ± 1.7[a]**	**19.80 ± 5.2[b]**	**0.007**
	Butyrate	**2.68 ± 0.4[a]**	**3.12 ± 0.6[a]**	**3.07 ± 0.9[a]**	**7.43 ± 1.5[b]**	**0.017**
	Valerate	**0.95 ± 0.2[a]**	**0.77 ± 0.1[a]**	**0.97 ± 0.2[a]**	**3.06 ± 0.8[b]**	**0.004**
	Isobutyrate	**0.34 ± 0.1[a]**	**0.31 ± 0.1[a]**	**0.40 ± 0.1[a]**	**2.14 ± 0.4[b]**	**0.002**
	Isovalerate	**0.96 ± 0.2[a]**	**1.20 ± 0.2[a]**	**1.69 ± 0.7[a]**	**3.91 ± 0.6[b]**	**0.001**
	Total SCFA	**35.42 ± 6.2[a]**	**40.20 ± 9.4[a]**	**37.56 ± 10.8[a]**	**84.16 ± 20.4[b]**	**0.034**
Proportion (%)	**Acetate**	**64.39 ± 2.4[b]**	**63.68 ± 1.5[b]**	**63.53 ± 2.1[b]**	**54.84 ± 3.5[a]**	**0.016**
	Propionate	**20.18 ± 1.1[a]**	**20.63 ± 0.6[b]**	**19.89 ± 1[a]**	**22.83 ± 1[c]**	**0.002**
	Butyrate	8.72 ± 1.20	8.86 ± 0.80	8.32 ± 0.60	9.82 ± 1.30	0.152
	Valerate	**2.93 ± 0.3[ab]**	**2.43 ± 0.3[a]**	**2.89 ± 0.3[ab]**	**3.84 ± 0.5[b]**	**0.018**
	Isobutyrate	**0.92 ± 0.1[a]**	**0.83 ± 0.1[a]**	**1.05 ± 0.1[b]**	**2.86 ± 0.3[c]**	**0.001**
	Isovalerate	**2.85 ± 0.30[a]**	**3.57 ± 0.60[a]**	**4.32 ± 0.90[ab]**	**5.81 ± 1.3[b]**	**0.031**

Data are presented as means ± SEM

[1] SCFA concentration are expressed as mmol / kg dry matter of colonic digesta.

[a,b,c] non-common letters indicate that piglet groups differed significantly ($P < 0.05$).

SCFA, short chain fatty acids.

Table 4. Ileal and colonic immune-related genes, which were differentially expressed between formula-fed (AI, HI and CTRL) and sow milk-fed (SM) piglets at PND24.

Site	Function	Protein name	Gene	CTRL	AI	HI	SM	p value
Ileum	Anti-inflammatory	*Suppressor Of Cytokine Signalling 3*	*SOCS3*	*1.04 ± 0.12*	*1.33 ± 0.21*	*1.12 ± 0.11*	*0.92 ± 0.17*	*0.069*
	Pro-inflammatory	**C-C Motif Chemokine Ligand 2**	**CCL2**	**1.11 ± 0.22^{ab}**	**1.59 ± 0.16^{b}**	**1.38 ± 0.12^{ab}**	**0.97 ± 0.10^{a}**	**0.046**
		C-C Motif Chemokine Ligand 20	**CCL20**	**1.78 ± 0.24^{b}**	**1.51 ± 0.19^{ab}**	**1.21 ± 0.18^{ab}**	**0.99 ± 0.1^{a}**	**0.048**
		C-X3-C Motif chemokine Ligand 1	**CX3CL1**	**1.08 ± 0.14^{b}**	**1.25 ± 0.1^{b}**	**1.21 ± 0.13^{b}**	**0.67 ± 0.07^{a}**	**0.017**
		Interferon gamma	*IFNg*	*1.23 ± 0.31*	*1.35 ± 0.18*	*1.78 ± 0.41*	*0.77 ± 0.23*	*0.067*
		Interleukin 4	*IL4*	*1.17 ± 0.21*	*2.09 ± 0.36*	*1.48 ± 0.31*	*0.93 ± 0.19*	*0.089*
		Interleukin 6	**IL6**	**1.28 ± 0.19^{a}**	**2.18 ± 0.27^{b}**	**1.41 ± 0.19^{ab}**	**1.57 ± 0.28^{ab}**	**0.034**
		Interleukin 8	*IL8*	*1.08 ± 0.20*	*1.14 ± 0.23*	*0.95 ± 0.21*	*0.56 ± 0.09*	*0.094*
		Tumor necrosis factor alpha	*TNFa*	*1.15 ± 0.21*	*1.56 ± 0.18*	*1.06 ± 0.15*	*1.05 ± 0.14*	*0.088*
		Tumor necrosis factor alpha rcpt 1	**TNFaR1**	**1.07 ± 0.16^{b}**	**1.06 ± 0.11^{b}**	**1.32 ± 0.15^{c}**	**0.61 ± 0.09^{a}**	**0.009**
	Antioxidant activity	**Superoxide dismutase 2**	**SOD2**	**1.07 ± 0.13^{b}**	**1.00 ± 0.07^{b}**	**1.28 ± 0.14^{c}**	**0.75 ± 0.09^{a}**	**0.009**
	Treg pathway	**Forkhead box P3**	**FOXP3**	**1.10 ± 0.16^{a}**	**1.48 ± 0.14^{b}**	**0.97 ± 0.11^{a}**	**1.24 ± 0.08^{ab}**	**0.008**
Colon	Anti-inflammatory	**Interleukin 10**	**IL10**	**1.11 ± 0.18^{a}**	**1.15 ± 0.22^{a}**	**0.90 ± 0.19^{a}**	**2.26 ± 0.55^{b}**	**0.013**
		Suppressor Of Cytokine Signalling 3	**SOCS3**	**1.04 ± 0.14^{a}**	**1.31 ± 0.4^{a}**	**1.43 ± 0.39^{ab}**	**2.48 ± 0.17^{b}**	**0.047**
	Pro-inflammatory	**C-C Motif Chemokine Ligand 20**	**CCL20**	**1.21 ± 0.21^{ab}**	**1.34 ± 0.26^{ab}**	**1.74 ± 0.32^{b}**	**0.89 ± 0.25^{a}**	**0.027**
		C-X3-C Motif chemokine Ligand 1	*CX3CL1*	*1.01 ± 0.06*	*0.94 ± 0.09*	*0.96 ± 0.08*	*1.26 ± 0.10*	*0.080*
		Interleukin 1-Beta	**IL1b**	**1.20 ± 0.31^{a}**	**2.16 ± 0.83^{ab}**	**2.17 ± 0.74^{ab}**	**4.19 ± 0.75^{b}**	**0.030**
		Interleukin 4	*IL4*	*1.14 ± 0.2*	*1.69 ± 0.33*	*0.87 ± 0.13*	*1.17 ± 0.20*	*0.067*
		Interleukin 6	**IL6**	**1.11 ± 0.19^{a}**	**1.12 ± 0.12^{a}**	**1.64 ± 0.26^{ab}**	**2.30 ± 0.52^{b}**	**0.006**
		Interleukin 8	*IL8*	*1.05 ± 0.14*	*1.47 ± 0.31*	*1.67 ± 0.36*	*1.72 ± 0.17*	*0.052*
		Tumor necrosis factor alpha rcpt 1	*TNFaR1*	*0.99 ± 0.07*	*0.98 ± 0.06*	*1.04 ± 0.07*	*1.23 ± 0.06*	*0.094*
	Receptor/cellular signalling	*Myeloid Differentiation Primary Response Gene*	*MYD88*	*1.00 ± 0.08*	*0.99 ± 0.08*	*1.01 ± 0.06*	*1.27 ± 0.09*	*0.067*
		Toll-like receptor 2	*TLR2*	*1.01 ± 0.08*	*1.11 ± 0.13*	*1.59 ± 0.32*	*0.91 ± 0.06*	*0.099*
		Toll-like receptor 4	**TLR4**	**1.03 ± 0.1^{a}**	**1.07 ± 0.1^{ab}**	**1.14 ± 0.13^{ab}**	**1.50 ± 0.15^{b}**	**0.042**
	Antioxidant activity	**Superoxide dismutase 2**	**SOD2**	**1.10 ± 0.17^{a}**	**1.29 ± 0.17^{b}**	**1.38 ± 0.09^{c}**	**1.36 ± 0.1^{c}**	**0.003**
	Treg pathway	**Forkhead box P3**	**FOXP3**	**1.08 ± 0.18^{b}**	**0.91 ± 0.14^{ab}**	**0.67 ± 0.10^{a}**	**2.02 ± 0.36^{c}**	**0.001**

Data are presented as means ± SEM ; ^{a,b,c} non-common letters indicate that piglet groups differed significantly ($P < 0.05$) ; bold indicates p-values inferior to 0.05 ; italic indicates a tendency with p-values between 0.05 and 0.1.

PND, postnatal days

Table 5. Ileal and colonic genes related to epithelial barrier functions, which were differentially expressed between formula-fed (AI, HI and CTRL) and sow milk-fed (SM) piglets at PND24.

Site	Function	Protein name	Gene	CTRL[1]	AI[1]	HI[1]	SM[1]	p value
Ileum	Permeability	**Claudin 4**	**CLDN4**	**1.27 ± 0.1[bc]**	**1.56 ± 0.2[c]**	**0.98 ± 0.09[ab]**	**0.71 ± 0.08[a]**	**<0.001**
		β-catenin	*CTNNB1*	*1.05 ± 0.17*	*1.00 ± 0.12*	*0.79 ± 0.08*	*0.58 ± 0.06*	*0.098*
		Myosin light-chain kinase	**MLCK**	**1.21 ± 0.18[b]**	**1.07 ± 0.09[b]**	**1.00 ± 0.2[ab]**	**0.52 ± 0.04[a]**	**0.014**
		Occludin	*OCLN*	*1.17 ± 0.14*	*0.96 ± 0.09*	*1.00 ± 0.14*	*0.61 ± 0.12*	*0.064*
		Tight junction protein 1	**ZO1**	**1.02 ± 0.11[b]**	**0.98 ± 0.11[b]**	**0.88 ± 0.14[ab]**	**0.56 ± 0.06[a]**	**0.022**
	Defense	**Intestinal Alkaline Phosphatase**	**IAP**	**1.29 ± 0.18[b]**	**1.18 ± 0.18[ab]**	**1.62 ± 0.23[b]**	**0.74 ± 0.2[a]**	**0.008**
	Proliferation	**Proliferating Cell Nuclear Antigen**	**PCNA**	**1.10 ± 0.19[a]**	**1.09 ± 0.17[a]**	**0.93 ± 0.16[a]**	**1.87 ± 0.27[b]**	**0.019**
	Apoptose	**Caspase 3**	**CASP3**	**1.02 ± 0.1[b]**	**1.02 ± 0.08[b]**	**1.23 ± 0.19[b]**	**0.63 ± 0.1[a]**	**0.037**
Colon	Permeability	*Claudin 3*	*CLDN3*	*1.05 ± 0.11*	*1.10 ± 0.11*	*1.36 ± 0.16*	*1.21 ± 0.17*	*0.057*
		Claudin 7	**CLDN7**	**0.76 ± 0.13[a]**	**0.94 ± 0.2[a]**	**0.84 ± 0.1[a]**	**1.19 ± 0.21[b]**	**0.007**
		β-catenin	*CTNNB1*	*1.17 ± 0.22*	*1.08 ± 0.09*	*1.21 ± 0.19*	*0.81 ± 0.04*	*0.098*
		Junctional adhesion molecule-A (JAM-A)	*F11R*	*0.96 ± 0.05*	*1.02 ± 0.06*	*1.16 ± 0.07*	*0.97 ± 0.06*	*0.085*
		Myosin light-chain kinase	**MLCK**	**1.05 ± 0.1[b]**	**1.11 ± 0.15[b]**	**1.13 ± 0.19[b]**	**0.63 ± 0.07[a]**	**0.001**
		Tight junction protein 1	**ZO1**	**1.00 ± 0.05[b]**	**0.93 ± 0.06[ab]**	**1.05 ± 0.1[b]**	**0.74 ± 0.07[a]**	**0.033**
	Defense	**Intestinal Alkaline Phosphatase**	**IAP**	**2.12 ± 0.6[ab]**	**1.67 ± 0.054[ab]**	**0.92 ± 0.26[a]**	**3.33 ± 0.76[b]**	**0.047**
		Mucin 2	**MUC2**	**1.33 ± 0.33[ab]**	**1.04 ± 0.22[ab]**	**1.98 ± 0.47[b]**	**0.65 ± 0.15[a]**	**0.009**
	Proliferation	**Proliferating Cell Nuclear Antigen**	**PCNA**	**1.03 ± 0.11[a]**	**1.05 ± 0.11[a]**	**0.97 ± 0.09[a]**	**1.36 ± 0.11[b]**	**0.002**
		Trefoil factor 3	*TFF3*	*1.21 ± 0.25*	*1.22 ± 0.24*	*1.56 ± 0.40*	*1.51 ± 0.45*	*0.051*
	Apoptose	**Caspase 3**	**CASP3**	**1.02 ± 0.1[a]**	**1.19 ± 0.16[a]**	**1.30 ± 0.15[ab]**	**1.61 ± 0.14[b]**	**0.002**

[1]Data are presented as means ± SEM ; [a,b,c] non-common letters indicate that piglet groups differed significantly ($P < 0.05$) ; bold indicates that p-values were inferior to 0.05 ; italic indicates a tendency with p-values between 0.05 and 0.1.

PND, Postnatal days.

Table 6. Ileal and colonic genes encoding proteins involved in nutrient transport, tryptophan metabolism and endocrine functions, which were differentially expressed between formula-fed (AI, HI and CTRL) and sow milk-fed (SM) piglets at PND24.

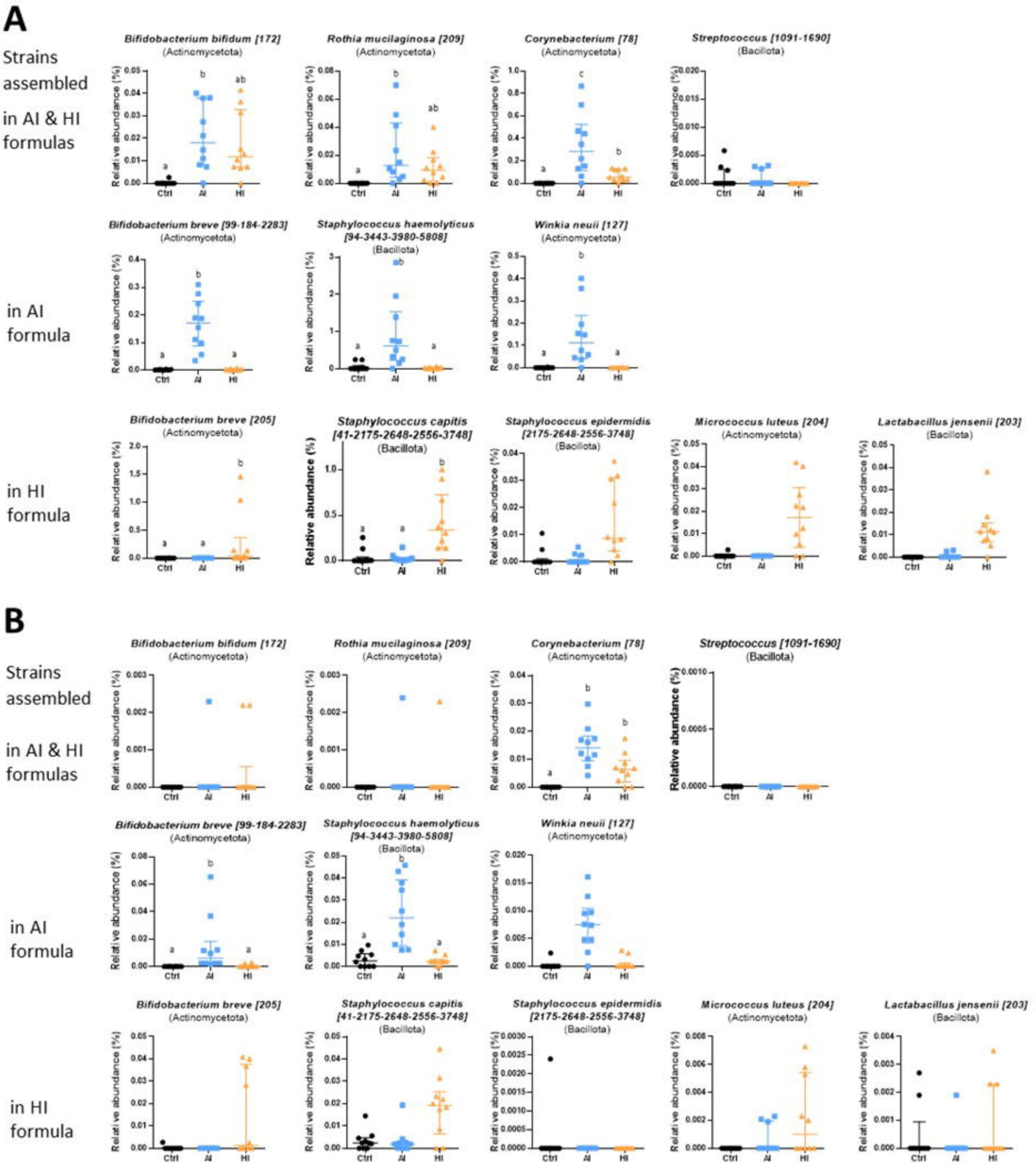
Site	Function	Sub-function	Protein name	Gene	CTRL[1]	AI[1]	HI[1]	SM[1]	p value
Ileum	Nutrient	Amino acid transporter	**Peptide transporter 1**	**SLC15A1**	**1.03 ± 0.14[ab]**	**0.80 ± 0.10[a]**	**1.29 ± 0.18[b]**	**0.82 ± 0.25[a]**	**0.047**
			Monocarboxylate Transporter 1	*MCT1*	*1.03 ± 0.10*	*0.99 ± 0.10*	*1.00 ± 0.11*	*0.73 ± 0.05*	*0.093*
			Monocarboxylate Transporter 2	**MCT2**	**1.07 ± 0.15[b]**	**1.22 ± 0.17[b]**	**0.91 ± 0.08[ab]**	**0.59 ± 0.07[a]**	**0.009**
			Monocarboxylate Transporter 4	**MCT4**	**1.21 ± 0.18[b]**	**0.70 ± 0.12[ab]**	**1.09 ± 0.19[ab]**	**0.56 ± 0.09[a]**	**0.016**
	Tryptophan pathway	Kynurenine pathway	**Indoleamine 2.3-Dioxygenase 1**	**IDO**	**2.18 ± 0.16[ab]**	**2.39 ± 0.18[b]**	**1.57 ± 0.21[a]**	**1.74 ± 0.25[ab]**	**0.013**
			Kynureninase	*KYNU*	*1.05 ± 0.14*	*1.25 ± 0.24*	*0.95 ± 0.11*	*0.74 ± 0.08*	*0.097*
		Tryptophan pathway	**Tryptophan hydroxylase 1**	**TPH1**	**1.15 ± 0.25[b]**	**1.17 ± 0.12[b]**	**1.17 ± 0.15[b]**	**0.54 ± 0.08[a]**	**0.007**
	Endocrine	Endocrine	**Chromogranin A**	**CHGA**	**0.97 ± 0.11[b]**	**0.95 ± 0.19[b]**	**0.90 ± 0.06[b]**	**0.56 ± 0.09[a]**	**0.044**
			Neuropeptide Y Receptor Y1	**NPY1R**	**1.06 ± 0.19[a]**	**1.46 ± 0.27[ab]**	**1.35 ± 0.24[ab]**	**2.11 ± 0.32[b]**	**0.047**
			Peptide Tyrosine Tyrosine	**PYY**	**1.33 ± 0.21[b]**	**0.95 ± 0.22[ab]**	**1.03 ± 0.2[ab]**	**0.54 ± 0.11[a]**	**0.023**
Colon	Nutrient transporter	Amino acid transporter	**Peptide transporter 1**	**SLC15A1**	**1.33 ± 0.27[b]**	**1.28 ± 0.27[b]**	**1.94 ± 0.42[b]**	**0.27 ± 0.06[a]**	**0.001**
		Carbohydrate transporter	**Glucose Transporter Type 1**	**GLUT1**	**1.10 ± 0.16[a]**	**1.09 ± 0.12[a]**	**1.21 ± 0.08[a]**	**1.64 ± 0.14[b]**	**0.001**
			Free fatty acid receptor 3	**FFAR3**	**1.36 ± 0.52[a]**	**1.44 ± 0.50[ab]**	**0.72 ± 0.11[a]**	**3.37 ± 0.81[b]**	**0.011**
			Monocarboxylate Transporter 2	*MCT2*	*1.02 ± 0.09*	*1.11 ± 0.09*	*1.18 ± 0.12*	*0.94 ± 0.12*	*0.061*
			ProGlucagon Precursor	**GCG**	**1.03 ± 0.09[ab]**	**1.06 ± 0.13[a]**	**1.33 ± 0.28[b]**	**0.96 ± 0.19[a]**	**0.025**
			Glucagon Like Peptide 1 receptor	**GLP1R**	**1.02 ± 0.07[ab]**	**1.08 ± 0.13[ab]**	**1.15 ± 0.14[b]**	**0.81 ± 0.14[a]**	**0.043**
			Insulin Receptor Substrate 2	**IRS2**	**1.04 ± 0.11[a]**	**1.31 ± 0.16[ab]**	**1.26 ± 0.19[ab]**	**1.53 ± 0.13[b]**	**0.049**

[1] Data are presented as means ± SEM ; [a,b] non-common letters indicate that piglet groups differed significantly ($P < 0.05$) ; bold indicates that p-values were inferior to 0.05 ; italic indicates a tendency with p-values between 0.05 and 0.1.

PND, postnatal days

1122

Figure 1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Strains assembled in AI & HI formulas
Bifidobacterium bifidum [172] (Actinomycetota)
Rothia mucilaginosa [209] (Actinomycetota)
Corynebacterium [78] (Actinomycetota)
Streptococcus [1091-1690] (Bacillota)
in AI formula
Bifidobacterium breve [99-184-2283] (Actinomycetota)
Staphylococcus haemolyticus [94-3443-3980-5808] (Bacillota)
Winkia neuii [127] (Actinomycetota)
in HI formula
Bifidobacterium breve [205] (Actinomycetota)
Staphylococcus capitis [41-2175-2648-2556-3748] (Bacillota)
Staphylococcus epidermidis [2175-2648-2556-3748] (Bacillota)
Micrococcus luteus [204] (Actinomycetota)
Lactabacillus jensenii [203] (Bacillota)
Relative abundance (%)
Ctrl
AI
HI
B
Strains assembled in AI & HI formulas
Bifidobacterium bifidum [172] (Actinomycetota)
Rothia mucilaginosa [209] (Actinomycetota)
Corynebacterium [78] (Actinomycetota)
Streptococcus [1091-1690] (Bacillota)
in AI formula
Bifidobacterium breve [99-184-2283] (Actinomycetota)
Staphylococcus haemolyticus [94-3443-3980-5808] (Bacillota)
Winkia neuii [127] (Actinomycetota)
in HI formula
Bifidobacterium breve [205] (Actinomycetota)
Staphylococcus capitis [41-2175-2648-2556-3748] (Bacillota)
Staphylococcus epidermidis [2175-2648-2556-3748] (Bacillota)
Micrococcus luteus [204] (Actinomycetota)
Lactabacillus jensenii [203] (Bacillota)
Relative abundance (%)
Ctrl
AI
HI

Figure 2

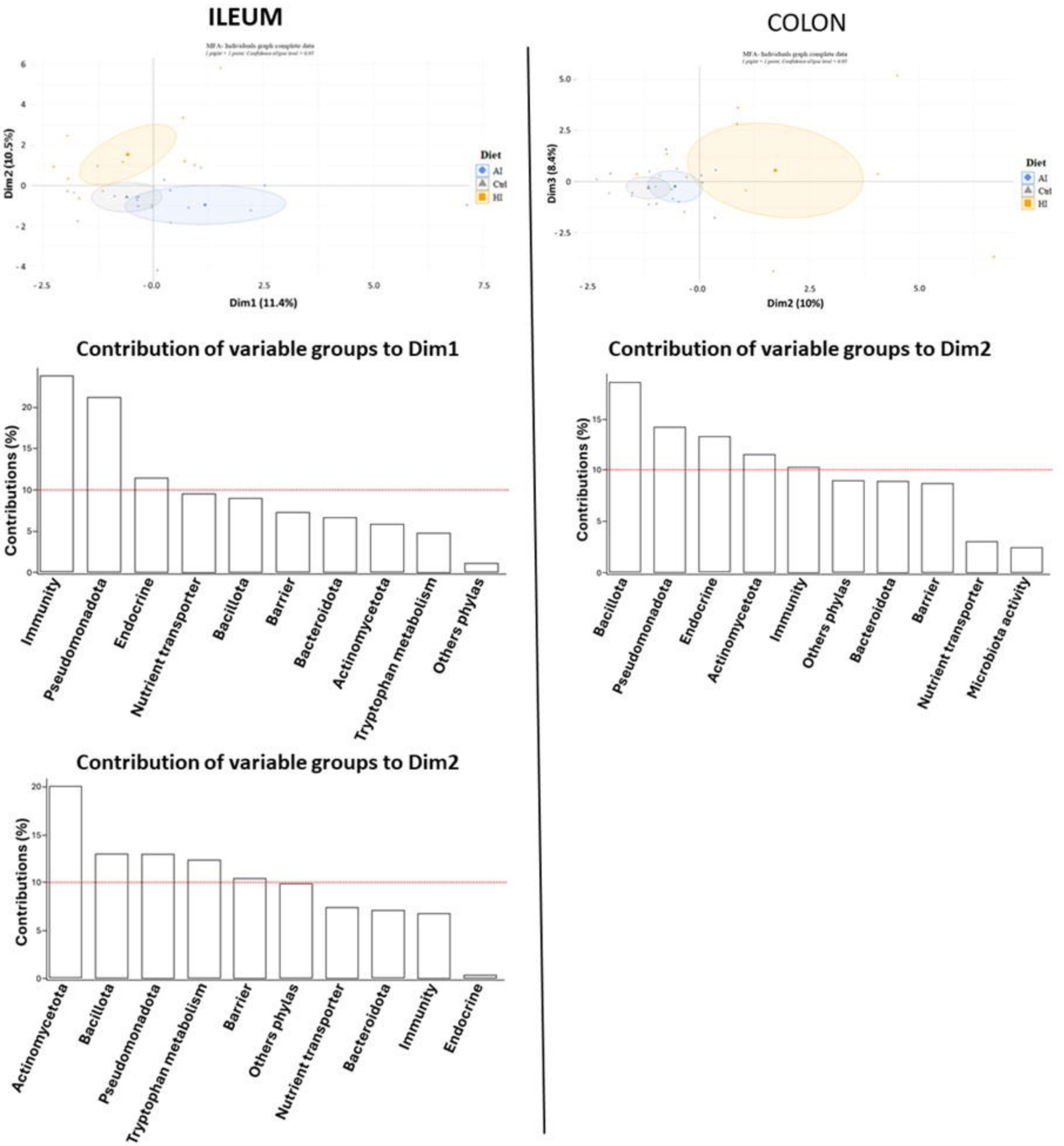
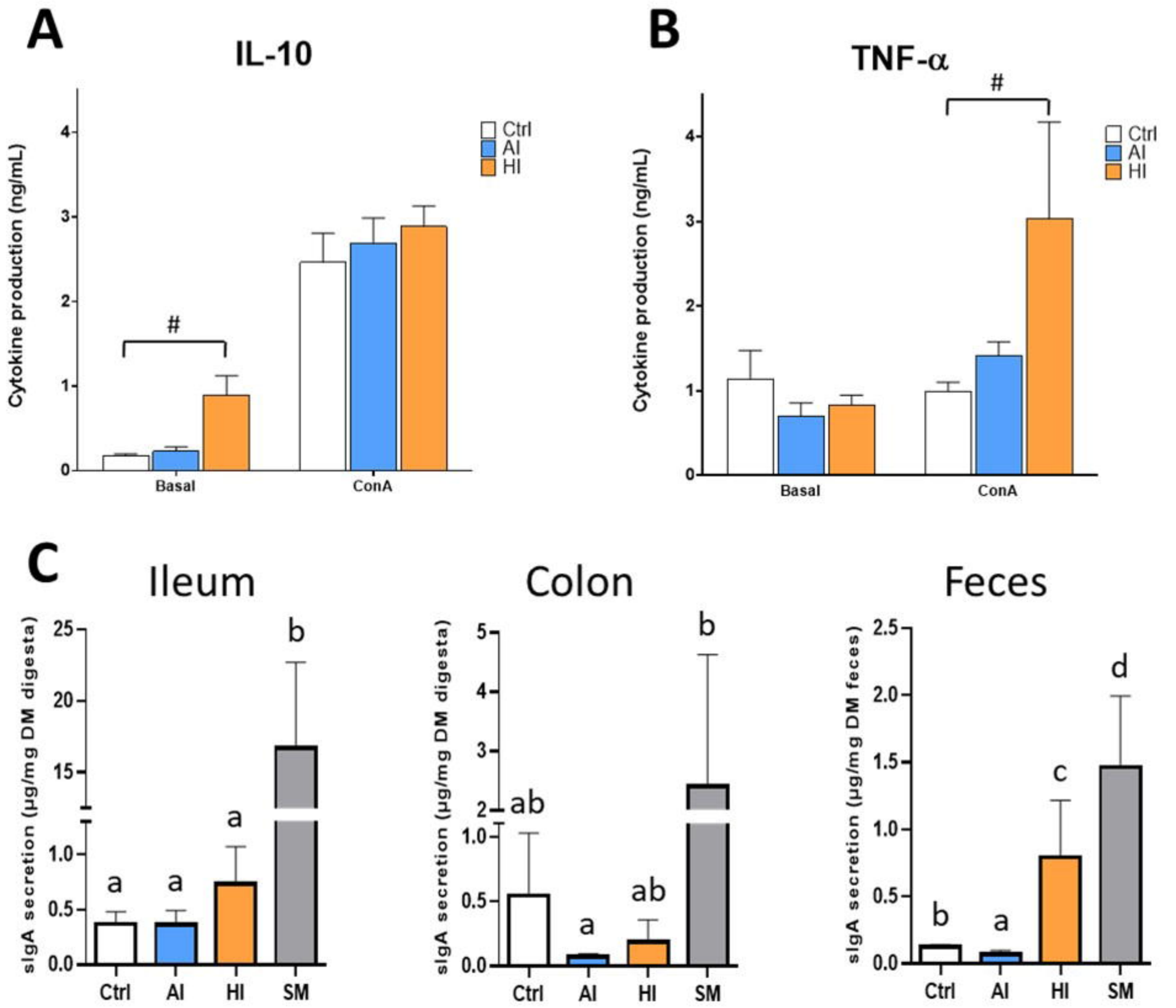

ILEUM
Dim2 (10.5%)
Dim1 (11.4%)
Diet
Al
Ctrl
Hl
Contribution of variable groups to Dim1
Contributions (%)
Immunity
Pseudomonadota
Endocrine
Nutrient transporter
Bacillota
Barrier
Bacteroidota
Actinomycetota
Tryptophan metabolism
Others phylas
Contribution of variable groups to Dim2
Contributions (%)
Actinomycetota
Bacillota
Pseudomonadota
Tryptophan metabolism
Barrier
Others phylas
Nutrient transporter
Bacteroidota
Immunity
Endocrine
COLON
Dim3 (8.4%)
Dim2 (10%)
Diet
Al
Ctrl
Hl
Contribution of variable groups to Dim2
Contributions (%)
Bacillota
Pseudomonadota
Endocrine
Actinomycetota
Immunity
Others phylas
Bacteroidota
Barrier
Nutrient transporter
Microbiota activity

Figure 3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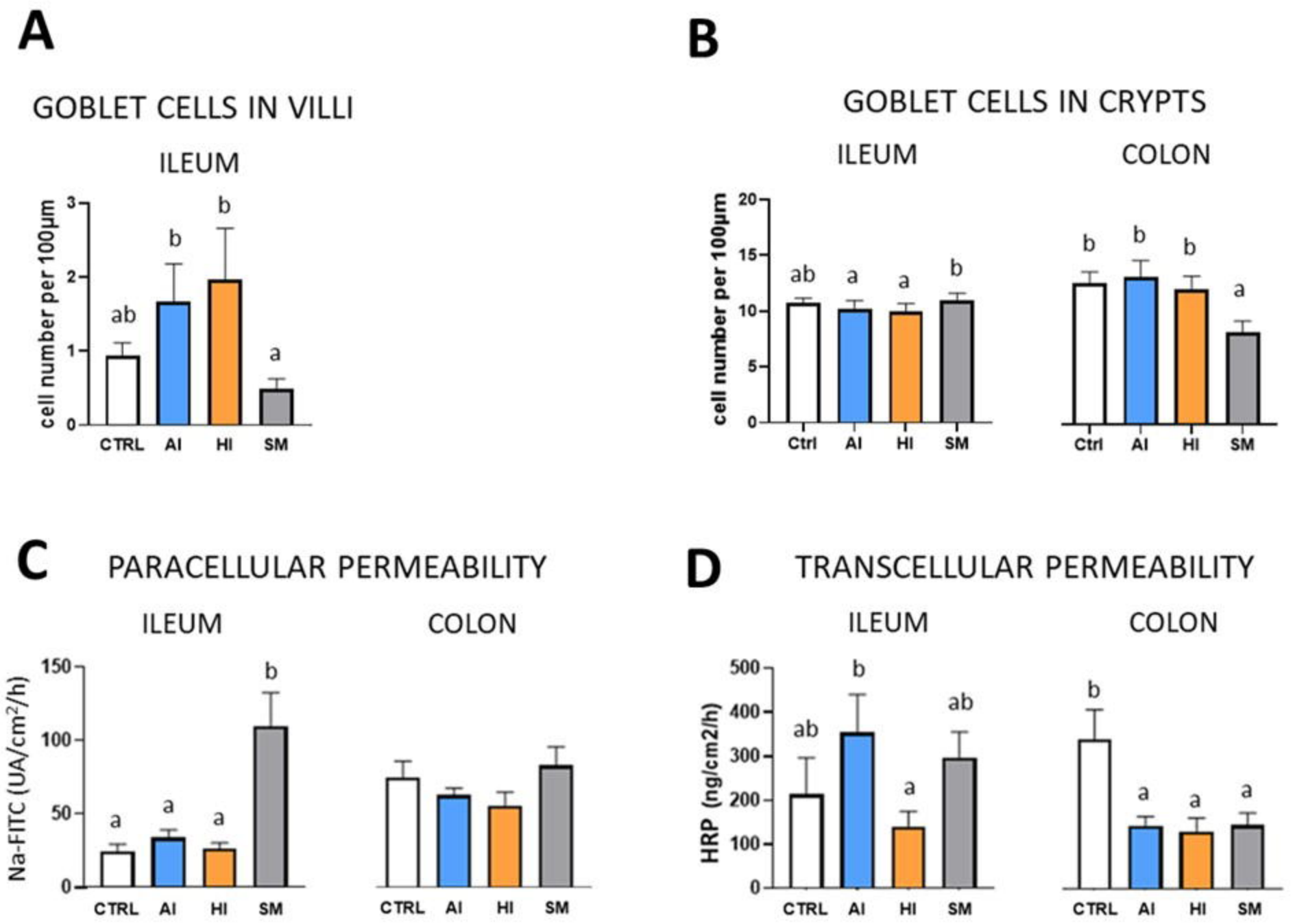
IL-10
Cytokine production (ng/mL)
Ctrl
AI
HI
#
Basal
ConA
B
TNF-α
Cytokine production (ng/mL)
Ctrl
AI
HI
#
Basal
ConA
C
Ileum
sIgA secretion (µg/mg DM digesta)
a
a
a
b
Ctrl
AI
HI
SM
Colon
sIgA secretion (µg/mg DM digesta)
ab
a
ab
b
Ctrl
AI
HI
SM
Feces
sIgA secretion (µg/mg DM feces)
b
a
c
d
Ctrl
AI
HI
SM

Figure 4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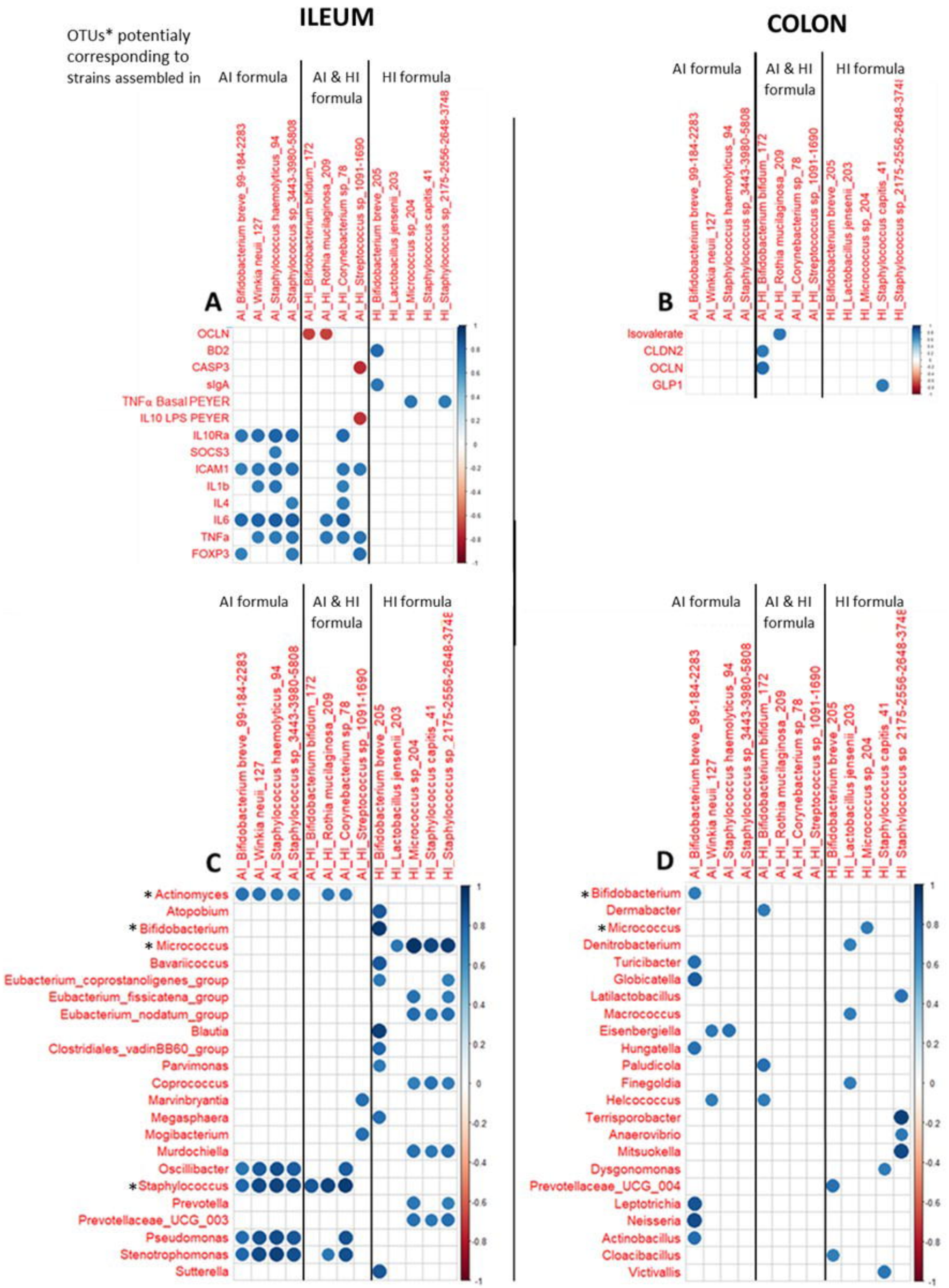
GOBLET CELLS IN VILLI
ILEUM
cell number per 100µm
CTRL
AI
HI
SM
B
GOBLET CELLS IN CRYPTS
ILEUM
COLON
cell number per 100µm
Ctrl
AI
HI
SM
C
PARACELLULAR PERMEABILITY
ILEUM
COLON
Na-FITC (UA/cm²/h)
CTRL
AI
HI
SM
D
TRANSCELLULAR PERMEABILITY
ILEUM
COLON
HRP (ng/cm2/h)
CTRL
AI
HI
SM

Figure 5


ILEUM
COLON
OTUs* potentialy corresponding to strains assembled in
AI formula
AI & HI formula
HI formula
A
B
C
D
AI_Bifidobacterium breve_99-184-2283
AI_Winkia neuii_127
AI_Staphylococcus haemolyticus_94
AI_Staphylococcus sp_3443-3980-5808
AI_HI_Bifidobacterium bifidum_172
AI_HI_Rothia mucilaginosa_209
AI_HI_Corynebacterium sp_78
AI_HI_Streptococcus sp_1091-1690
HI_Bifidobacterium breve_205
HI_Lactobacillus jensenii_203
HI_Micrococcus sp_204
HI_Staphylococcus capitis_41
HI_Staphylococcus sp_2175-2556-2648-3748
OCLN
BD2
CASP3
sIgA
TNFα Basal PEYER
IL10 LPS PEYER
IL10Ra
SOCS3
ICAM1
IL1b
IL4
IL6
TNFa
FOXP3
Isovalerate
CLDN2
OCLN
GLP1
* Actinomyces
Atopobium
* Bifidobacterium
* Micrococcus
Bavariicoccus
Eubacterium_coprostanoligenes_group
Eubacterium_fissicatena_group
Eubacterium_nodatum_group
Blautia
Clostridiales_vadinBB60_group
Parvimonas
Coprococcus
Marvinbryantia
Megasphaera
Mogibacterium
Murdochiella
Oscillibacter
* Staphylococcus
Prevotella
Prevotellaceae_UCG_003
Pseudomonas
Stenotrophomonas
Sutterella
* Bifidobacterium
Dermabacter
* Micrococcus
Denitrobacterium
Turicibacter
Globicatella
Latilactobacillus
Macrococcus
Eisenbergiella
Hungatella
Paludicola
Finegoldia
Helcococcus
Terrisporobacter
Anaerovibrio
Mitsuokella
Dysgonomonas
Prevotellaceae_UCG_004
Leptotrichia
Neisseria
Actinobacillus
Cloacibacillus
Victivallis