Universitat Autònoma de Barcelona

Development of intestinal microbiota in the commercial pig and early intervention strategies to improve this proce

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Chapter 6

Potential of two *Bacillus* probiotic strains to improve performance of breeding sows, microbial colonization, and the response of suckling piglets

Potential of two *Bacillus* probiotic strains to improve performance of breeding sows, microbial colonization, and the response of suckling piglets

6.1 Abstract

The effect of long-term administration of two *Bacillus* strains was tested on 98 breeding sows and their litters allotted into three treatments: a control group (CON); supplemented with $5x10^8$ cfu/kg *B. subtilis* – 541 (BSU); or with $5x10^8$ cfu/kg *B. amyloliquefaciens* – 516 (BAM). Reproductive and performance variables were recorded over three cycles with 56 dams remaining through the third lactation. Blood and fecal samples were taken longitudinally from 12 sows per treatment on days 8 and 21 of the third lactation and milk samples were taken on day 21. Feces from one piglet per litter was sampled on days 21 and 33 and jejunal gene expression was assessed in two piglets on day 21. Changes in fecal microbiota were assessed by 16S rRNA gene sequencing (Illumina MiSeq) and gene expression by Open-Array technology. Metabolomic responses were analyzed in milk by NMR and Ig-G and Ig-A specific antibodies were determined by ELISA.

No significant differences were observed on feed intake, body weight, or fat mobilization of the sows. However, a significant increase in the total number of piglets born was observed in supplemented sows. Whereas the increase was seen from the first cycle with BAM, improvements were not seen with BSU until the third cycle. BAM also increased the number of born-alive and weaned piglets. NMR analysis showed an impact of BAM on milk composition. No differences were found in milk or blood immunoglobulins. A different structure of the fecal microbiota was found in supplemented sows, with changes across phylum, family, and genus. These changes were greater at day 8, suggesting a relevant role of probiotics establishing a new intestinal balance after labor. Shifts in the microbiota were also seen in the piglets, with a clearer impact post-weaning than in suckling. In this regard, correlations

between microbial groups of sows and piglets showed a higher link with weaned (d33) than with suckling pigs (d21) reinforcing the idea of an early maternal carry-over. No changes due to treatment in jejunal gene expression were detected, however, piglet size had a clear impact on different genes.

In summary, the addition of both probiotics, and particularly *Bacillus amyloliquefaciens*, demonstrated potential benefits on the prolificacy of sows. Daily feeding of *Bacillus amyloliquefaciens* resulted in an increase in the number of weaned piglets. The high correlations between the compositions of the microbiota of sows and their piglets is evidence of maternal imprinting, with effects lasting beyond weaning.

Keywords: probiotic, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, sow, piglet, microbiota.

6.2. Introduction

Modern intensive production systems have the constant challenge of achieving high rates of reproductive success from their sows. The use of probiotics has emerged as a promising strategy to improve the reproductive performance of sows by increasing feed consumption along with lactation, reducing fat mobilization, promoting milk production, and increasing litter weight (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Zhang *et al.*, 2020). Moreover, several studies have also shown that when probiotics are administered to sows, positive effects can be also seen in the performance of piglets, with increases in rates of growth (Kritas *et al.*, 2015; Betancur *et al.*, 2021; Crespo-Piazuelo *et al.*, 2021) and reduction in the clinical signs of post-weaning diarrhea (Alexopoulos *et al.*, 2004; Taras *et al.*, 2005, 2006; Betancur *et al.*, 2021). Although the mechanisms of action have not yet been fully elucidated, these benefits could have been derived from a beneficial modulation of the intestinal microbiota of nursing piglets by their mothers. In fact, probiotics have been demonstrated to be transferred from the mother to the piglet through contact with maternal feces (Jadamus, Vahjen and Simon, 2001; Kenny *et al.*, 2011). Moreover, modulation of the maternal microbiota with probiotics could also have an impact on the health of her piglets. The initial

development of the microbiota of piglets is fundamentally dependent on their intimate contact with their sow (Konstantinov *et al.*, 2006; Thompson, Wang and Holmes, 2008; Mach *et al.*, 2015) and this process plays a crucial role in the development of the neonatal immune system with implications throughout the life of the pigletss (Hansen *et al.*, 2012; Everaert *et al.*, 2017; Ferret-Bernard and Le Huërou-Luron, 2019; Jiang *et al.*, 2019).

Although the potential benefits of supplementing the diets of sows with probiotics is well documented in the literature, the relevance of commercial husbandry conditions and long-term administration of probiotics are unreported. Therefore, the present study aimed to evaluate the effect of supplying $5x10^8$ cfu/kg feed of viable spores of one of two *Bacillus* probiotic strains: *Bacillus subtilis* – 541 or *Bacillus amyloliquefaciens* – 516 during three consecutive cycles, on the performance of sows and their litters The impact of supplementation on the fecal microbiota of sows and piglets, the composition of milk during lactation, maternal transfer of passive immunity, and jejunal gene expression of the piglets were assessed.

6.3. Materials and methods

6.3.1 Animals and housing

The present study was carried out in a commercial pig farm with an average herd size of 1150 sows in the province of Lleida, Spain. A total of 98 Danbred (Landrace x Yorkshire) hyperprolific sows started the first cycle and were fed the experimental diets during three complete reproductive cycles. The sows were allocated to three treatments in such a way that sows in all groups were similar in terms of parity (2.8 $\pm$ 0.14) and dam body weight (211.8 $\pm$ 1.10 kg).

Breeding dams were allocated to individual crates in the service barn where they were inseminated, and pregnancy was confirmed at ca. 30-35 days of gestation. Pregnant sows were then moved to the gestation barn, where they were group-housed (pens of 10 dams/pen) until ca. 110 days gestation when dams were moved to individual farrowing crates in farrowing rooms (5 rooms of 10 pens). Within 24 hours after farrowing, all stillborn, dead, splay-legged, and moribund piglets were removed from the study, leaving only healthy

piglets suckling the sow. Cross-fostering to equalize litter size was carried out within 24-48 h after farrowing and further movements were accepted if required due to the common farm management, but only within the same treatment groups. After piglets were weaned, dams were kept in individual crates until estrus. Each farrowing pen had a farrowing crate on a partially slatted floor with a heated floor pad for piglets. Water was provided ad libitum from nipple drinkers. Each unit was lit by daylight (via windows) and artificial light (non-programmable). Ventilation was via single, variable-speed fans linked to temperature sensors. The temperature inside the buildings was automatically controlled.

6.3.2. Diets and experimental treatments

Sows were fed standard gestation and lactation feeds. All nutrients were supplied at normal concentrations, not exceeding EU maximum permitted content of trace minerals or vitamins. Diets were calculated to be iso-nutritive, meeting NRC nutrient requirements recommended for sows and suckling piglets (NRC, 2012). Sow and piglet feed formulae and calculated analyses are presented in **Annex 2: Tables S6.1. and S6.2.**

For the entire study period, sows were offered pelleted feeds. At service, dams were fed 1.8 to 2.0 kg/d. From service to day 35 of gestation, dams were fed 2.9 to 3.0 kg/d. From day 35 to 114 of gestation dams were fed 2.6 to 2.8 kg/d. In lactation, sows were not fed on the day of farrowing. Sows were fed 1, 1.7, 2.4, 3.2, and 4 kg/d from 1-5 days post-farrow, and then *ad libitum* to appetite. Daily feed intake was adjusted according to body condition, assessed via back fat, measured every 3 weeks by ultrasound scanner (AV-3000V Digital Handheld Electronic B Ultrasound Scanner, AMBISEA Technology Corp., Ltd; Hong Kong, China). Backfat thickness was measured 6 cm from the midline at the height of the last rib, always by the same person. Daily feed was then decreased for dams considered too fat and increased for dams considered too thin. Dams were fed twice daily in service, once daily in gestation, twice daily for the first 5 days of lactation, and then ad libitum to appetite. Top dressings were added to service/gestation feeds of the experimental treatments at the first daily feeding, added to the automatic feeder doser. For individual feed intake monitoring, each gestating pen was equipped with enough mechanical

free access self-closing semi-cage without pneumatic actuators (Rotecna, Spain), as previously reported by Reyes-Camacho *et al.* (2020). Suckling piglets were offered creep mash feed from ca. 7 days of age to weaning at ca. 23 days of age, minimum 21 days.

Two experimental treatments were tested (BSU and BAM) in which different probiotic strains were added to the control diet (CON). Probiotic supplemented diets were given to corresponding sows throughout gestation and lactation of three consecutive cycles. Piglets from the BSU and BAM groups received the appropriate probiotics in the creep-feed. All sow and piglet control diets were formulated with no added antibiotics, organic acids, polysaccharides, or probiotics. For the BSU treatment, the diet was supplemented with $5x10^8$ cfu/kg feed of viable spores of *Bacillus subtilis* – 541, and for the BAM treatment, the diet was supplemented with $5x10^8$ cfu/kg feed of viable spores of *Bacillus amyloliquefaciens* – 516. The addition of probiotic strains in the gestation diets was done by top-dressing (150 g on top of every kg feed) and for lactation diets, probiotics were included in the final diets. The intended dosage and the periods of administration of top-dressings are specified in **Annex 2: Table S6.3.** Each ton of gestation top-dressing was produced by adding 3.1 kg of *B. subtilis* or *B. amyloliquefaciens* base premix to a 50 kg aliquot of cornmeal, mixing, and then adding to 946.9 kg basal gestation feed, and then mixing to ensure homogeneity. Top-dressings were then pelleted at 65°C and packed in 25 kg bags. Lactation feeds were mixed, pelleted at 65°C, trucked in bulk, and stored on-farm in separate silos. Basal gestation feeds were delivered daily by automatic feeders. Lactation feeds were delivered manually from bulk silos using barrows with scales (three different barrows for CON, BSU, and BAM).

Piglet creep feed was mixed into mash as a single lot then split into three aliquots (CON, BSU, and BAM). *Bacillus subtilis* and *Bacillus amyloliquefaciens* base premix was added to ca. 50 kg of each BSU or BAM aliquot and remixed to homogeneous dispersion. No probiotic was supplemented for the 3rd cycle in the creep feed. Piglet creep feeds were packed in 40 kg bags.
Feeds and top-dressings were made and stored cool and dry until required for feeding. Lactation and gestation diets, piglet creep feeds and sow gestation top-dressings were analyzed before use to confirm viability of the probiotics.

6.3.3. Experimental procedure

The study was started with 98 dams in the first cycle and finished with 56 in the third cycle. Reproductive performance of the sows was recorded during each of the three cycles, documenting the total number of piglets born (alive or dead), the number of piglets born alive, the number of stillborn and mummified piglets, the cross-fostering between litters, the number of piglets weaned, and mortality for both sows and piglets. Performance of the piglets, i.e., birth weight, weight after cross-fostering, weaning weight, and average daily gain (ADG) were collected during the first and second cycles of the farm trial. The performance of the sow including the evolution in body weight (BW), the average daily feed intake (ADFI), and the back-fat thickness were recorded throughout the first two cycles. From the 98 dams that initially started the study (33 in CON, 32 in BSU, and 33 in BAM) from wean/service and during gestation, 76 of them continued for the second cycle (27 in CON, 25 in BSU, and 24 in BAM) from wean/service and during gestation. For the third and final productive, cycle only 56 dams (21 in CON, 17 in BSU, and 18 in BAM) from wean/service and during gestation remained in the study. The main reasons for sow removal (presented in **Annex 2: Table S6.4.**) were exclusion due to repetition (most frequent), culling due to claw lesion, abortion, or death.

Samples from milk, feces, and blood from the sows, and feces, blood, and jejunum tissue from the piglets were taken from 12 sows per treatment and their litters during the third cycle. Eight and 21 days after parturition, sows from each treatment (n=12/treatment) were sampled for blood and feces. On day 21 after parturition, milk samples were collected following the usual procedure (with oxytocin) shortly after a basic udder cleaning procedure to remove leftover feces (if necessary). From each sow, one 15mL tube was collected and stored at -20°C. Blood samples were collected from the tail. The tubes containing blood samples were centrifuged (2500 x g, 15 minutes) and serum collected was stored at -20°C until analysis. Feces were collected by stimulating the defecation into small bags and stored at -20°C.

Feces from one random piglet from each of the sampled sows (n=12) were collected on days 21 (before weaning) and 33 of life (12 days after weaning) (not necessarily the same pig). Feces were obtained by digital stimulation and

stored in small bags at -20°C. Moreover, for tissue sampling, two piglets from 8 sows per treatment (n=16) of medium- and small-size, were humanly euthanized by intravenous injection of sodium pentobarbital (140 mg/kg, Euthasol, Ecuphar, Belgium) on day 21. Jejunum samples (ca. 1 cm^2) were collected into tubes with RNAlater (Deltalab, Rubí, Spain), which were left overnight in the refrigerator and put in the freezer (-20°C) the next day.

6.3.4. Analytical procedures

6.3.4.1. Immune response

The assessment of the possible impact of the experimental treatments on the immune response was performed by quantification of specific immunoglobulin concentrations in serum and milk samples collected from the sows. Concentrations of IgG and IgA antibodies specific for Aujeszky and PRRS were determined by enzyme-linked immunosorbent assays (ELISA). Commercial pig ELISA quantitation kits were used (INgezim PRRS and ADV ELISA Kits from INGENASA, Madrid, Spain) following the manufacturer's recommendations.

6.3.4.2. Metabolomic analysis of the milk

Milk samples were processed as detailed previously (Gómez-Gallego *et al.*, 2018). Milk samples were thawed, carefully mixed by inversion, and then centrifuged at 14000 rpm for 20 min at 4°C. The fat layer was removed, and whey milk was transferred to a clean Falcon tube and centrifuged again; this procedure was repeated twice until a clear supernatant was obtained.

For Proton Nuclear Magnetic Resonance (NMR) analysis, whey milk samples (455 µl) were mixed with 45 µl of sodium-3′-trimethylsilylpropionate-2,2,3,3-d4 (TSP) dissolved in deuterium oxide and placed in a 5 mm NMR tube. The final concentration of TSP in each sample was 2.5 mM. All spectra were recorded in a Bruker Avance DRX 600 spectrometer (Bruker GmbH, Rheinstetten, Germany) operating at a ^1H frequency of 600.13 MHz.

Metabolite spin systems and resonances were identified by using literature data and the commercial resonances database Chenomx NMR Suite Profiler (Chenomx NMR Suite 8.1, Alberta, Canada). The spectra were manually phase corrected and baseline adjusted, referenced to TSP, and normalized to the total aliphatic spectral area (0.50 and 4.40 ppm) to eliminate differences in metabolite total concentration. Signals belonging to identified metabolites were integrated and quantified using semi-automated ^{1}H-NMR signal deconvolution routines in MestReNova 8.1. Concentrations of final metabolites were calculated in arbitrary units as the area under the peak.

6.3.4.3. Fecal microbiota

The fecal DNA was extracted (250 mg of each fecal sample) using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions following the optimization steps. Concentration and purity of DNA were checked with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For 16S rRNA gene high-throughput sequencing, amplicon libraries were prepared using Nextera XT Index Kits 16S V3–V4 Amplicon-Seq Kit (Illumina, San Diego, CA, USA). For sequencing on the MiSeq® instrument, the generated libraries were placed in the reagent cartridge and loaded on the instrument along with the flow cell. The MiSeq® Reagent Kit V2 (500-cycle) (Illumina, San Diego, CA, USA) was used. All subsequent steps were performed on the MiSeq® Illumina instrument, including cluster generation and paired-end sequencing.

6.3.4.4. 16S rRNA gene sequencing bioinformatics

The sequence reads generated by the 16S rRNA were processed, aligned, and categorized independently using the Divisive Amplicon Denoising Algorithm 2 or DADA2 (Callahan *et al*., 2016), which was run as an R script (in R v.4.0.2) using its R package (dada2 v.1.16.0).

When reads were de-duplicated, amplicon sequence variants (ASV) were inferred. After building the ASV table ("makeSequenceTable" function) and removing chimeras ("removeBimeraDenovo" command), taxonomy was

assigned using the SILVA reference database (v138) provided by the SILVA web service (Quast *et al.*, 2013).

6.3.4.5. Jejunal gene expression

Gene expression was quantified by RT-qPCR to study the expression of 56 genes in piglet jejunum samples by a customized Open Array Real-Time PCR Platform (OpenArray® plate) on QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, United States) as described by González-Solé *et al.* (2020). For that total RNA was extracted using the Ambion RiboPure™ Kit (Life Technologies, Carlsbad, United States), according to the manufacturer's protocol. RNA was analyzed using a NanoDrop 1000A spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States) to determine if it satisfied the minimum purity and integrity standards for total RNA quality. Ten µl of total RNA (100 ng/ul) were used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States). The resulting cDNA was subjected to a PCR amplification followed by a real-time q-PCR reaction using the manufacturer's TaqMan® PreAmp Master Mix Kit Protocol (Life Technologies, Foster City, CA).

6.3.5. Statistical methods

Data are presented as means and standard deviations. The experimental unit for statistical purposes was the dam and its litter. Significant differences were declared at $P \leq 0.05$, while $0.05 < P \leq 0.10$ was considered near significant trends.

Performance: The statistical analysis of sow performance was performed using the GLM, MIXED and GENMOD procedures of the statistical package SAS® (SAS Institute Inc., Cary, NC) with the following model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where Y_{ij} was the parameter for the observations; μ was the general mean of all observations; α_i was the effect of the experimental treatments (CON, BSU, BAM); β_j was the reproductive cycle effect: $\alpha\beta_{ij}$ was the

interaction between the experimental treatments and the cycle number; and $\varepsilon \sim N(0, \sigma^2\varepsilon)$ was the unexplained random error.

Immune response: The analysis of the immunomodulatory effects (Igs in serum and milk samples) was performed using statistical package R (R Core Team, 2020). The following model was used: $Y_i = \mu + \alpha_i + \varepsilon_i$, where Y_i was the variable for the observations; μ was the general mean of all observations; α_i was the effect of the experimental treatments (CON, BSU, BAM); and $\varepsilon \sim N(0, \sigma^2\varepsilon)$ was the unexplained random error. When treatment effects were established, the mean comparison was adjusted with the Tukey-Kramer test.

Microbiota: The patterns of fecal microbial diversity within the ASV table were analyzed using a custom bioinformatics pipeline implemented in R 4.0.2 (http://www.r-project.org). Support for DADA2 in R was achieved through the *phyloseq* package (v.1.32.0; available at https://joey711.github.io/phyloseq/) (McMurdie and Holmes, 2013). Alpha diversity metrics were calculated using the phyloseq "estimate_richness" function from the rarefied ASV tables and using the *microbiome* package (v.1.10.0) (Lahti *et al.*, 2017). The observed species, the Chao1 index, the Simpson and inverse Simpson metrics, and the Shannon diversity measures were estimated. For beta diversity, measurements were calculated using the Whittaker index (Whittaker, 1960) and the betadisper () function of the vegan package (v.2.5.6) (Oksanen *et al.*, 2013) using relative abundances. To compare any differential effects, an ANOVA analysis was performed for alpha richness and diversity with R stats package using the following model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where Y_{ij} was the parameter for the observations; μ was the general mean of all observations; α_i was the effect of the experimental treatments (CON, BSU, BAM); β_j was the sampling day (d8 or d21 for sows and d21 or d33 for piglets); $\alpha\beta_{ij}$ was the interaction between the experimental treatments and sampling day; and $\varepsilon \sim N(0, \sigma^2\varepsilon)$ was the unexplained random error. Non-metric multidimensional scaling (NMDS), analysis of similarities (ANOSIM), permutational analysis of variance (PERMANOVA), and unweighted pair-wise grouping method with hierarchical arithmetic mean grouping (UPGMA), all based on the distance of Bray-Curtis, were carried out for the ordering and analysis of beta diversity. The normalization of the raw counts was performed using cumulative sum scaling (CSS) (Paulson, Stine, *et al.*, 2013) and the differential abundance analysis was performed following the metagenomeSeq package (v.1.30.0)

(Paulson, Talukder, *et al.*, 2013). Taxa were aggregated at phylum, family, and genus levels and expressed as compositional data. Relative abundances were used to plot taxon abundances whereas raw family and genera counts were used to correlate sow-piglet microbiota. A correlation was performed in R 4.0.2 through the stats package. Mother-piglet samples were correlated by sampling day as follows: day 8 post-partum with suckling piglets (day 21), day 8 post-partum with weaned piglets (day 33); day 21 post-partum with suckling piglets (day 21), and day 21 post-partum with weaned piglets (day 33). Significant differences were declared at $P \leq 0.05$ (the adjusted P for differential abundance analysis).

Metabolomics: Chemometrics statistical analysis for the metabolomic approach of the milk was performed using in-house MATLAB scripts and the PLS_Toolbox 8.0.2 (Eigenvector Research, Inc., Wenatchee, WA, USA) statistical multivariate analysis library. Principal component analysis (PCA) was applied to NMR spectra data sets. Principal components were chosen to explain at least 70% of the variance. The loading plots of the corresponding principal components were used to detect the positions of most discriminative variables in the NMR spectra. To maximize the separation between samples, partial least-squares discriminant analysis (PLS-DA), was applied with SIMCA 14.1 software. A permutation test was performed to check the overfitting of the PLS-DA models. The multivariate chemometric models were cross-validated with 10-fold Leave-one-out cross-validation; in each run, 10% of the data were left out of the training and used to test the model. The whole cross-validation process was run 10 times. The spectral regions responsible for the classification of the models were identified using the variable importance in projections (VIP) coefficients obtained during PLS-DA (Spectral regions with high VIP coefficients are more important in providing class separation during analysis, while those with very small VIP coefficients provide little contribution to classification.

Gene expression: The statistical analysis of gene expression was performed in open-source R (R Core Team, 2020) using the DCrt data matrix. Data was previously normalized with the reference genes. Firstly, and for each gene, normality tests were performed with *shapiro.test* (R stats package). Genes with normal distributions were analyzed with an ANOVA, while the genes with non-normal distributions were analyzed with a Kruskal-Wallis test. For

ANOVA, the following model was used: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where Y_{ij} was the parameter for the observations; μ was the general mean of all observations; α_i was the effect of the experimental treatments (CON, BSU, BAM); β_j was weight block effect (medium or small size); $\alpha\beta_{ij}$ was the interaction between the experimental treatments and block of weight; and $\varepsilon \sim N(0, \sigma^2\varepsilon)$ was the unexplained random error. Finally, the p-values were adjusted by the Benjamini-Hochberg FDR method and Tukey tests were performed for each gene if significance was observed.

6.4. Results

6.4.1. Sow and litter performance

During the two first cycles, the average BW of sows prior to farrowing and at weaning were 269.6 kg ± 38.67 kg (expressed as mean ± standard deviation) and 231.3 kg ± 35.14 kg, respectively. The average back-fat thickness was 17.6 mm ± 3.95 mm prior to farrowing and 14.1 mm ± 3.62 mm at weaning, and the bodyweight loss during lactation was 38.3 kg ± 17.40 kg. The average daily feed intake was 2.6 kg ± 0.02 kg per day during gestation and was 5.8 kg ± 1.16 kg per day during lactation. Days weaning to estrus were 4.1 ± 0.58 days. No differences were observed between treatments.

The effects of the experimental treatments on farrowing performance during the three consecutive cycles are presented in **Table 6.1.** Regarding differences between reproductive cycles, a significant increase in the number of weaned piglets at the third cycle ($P = 0.038$) and also in weaning weight along time ($P = 0.004$) were observed. Regarding probiotic supplementation, *Bacillus amyloliquefaciens* (BAM) significantly increased the number of total piglets per sow compared to CTR ($P = 0.008$) and BSU showed intermediate values. The number of piglets born alive and the number of piglets weaned were also increased by BAM compared to CTR ($P = 0.029$ and $P = 0.025$ respectively). No significant interaction between cycle and treatments was observed.

Table 6.1. Effect of *Bacillus subtilis* (BSU) and *Bacillus amyloliquefaciens* (BAM) on sows' farrowing performance during the three complete productive cycles.

Parameter[1]	Productive cycle					Treatment[2]				
	1st	2nd	3rd	SEM	P-value	CON	BSU	BAM	SEM	P-value
N° total piglets	18.7	19.7	20.3	0.33	0.125	18.3^{a}	19.5^{ab}	20.7^{b}	0.33	**0.009**
N° piglets born alive	15.8	16.4	16.7	0.27	0.405	15.7^{a}	15.7^{a}	17.4^{b}	0.27	**0.009**
N° stillborn piglets	1.9	2.1	2.4	0.15	0.418	1.8	2.5	2.1	0.15	0.129
N° mummified piglets	1.0	1.2	1.2	0.11	0.558	0.9	1.3	1.2	0.11	0.215
N° piglets weaned	13.9^{xy}	13.8^{x}	14.3^{y}	0.09	**0.038**	13.9^{a}	13.6^{a}	14.4^{b}	0.09	**0.001**

$^{a-b, x-y}$ Means within a row with different superscripts differ ($P < 0.05$).
[1] Cycle 1: 98 dams (33 in CON, 32 in BSU and 33 in BAM) from wean/service and during gestation and 78 dams (27 in CON, 25 in BSU and 26 in BAM) during lactation.
Cycle 2: 76 dams (27 in CON, 25 in BSU and 24 in BAM) from wean/service and during gestation and 56 dams (21 in CON, 17 in BSU and 18 in BAM) during lactation.
Cycle 3: 56 dams (21 in CON, 17 in BSU and 18 in BAM) from wean/service and during gestation and 45 dams (17 in CON, 12 in BSU and 16 in BAM) during lactation.
[2] Treatments: CON = Control (no supplementation); BSU = $5x10^{8}$ CFU/kg feed of *Bacillus subtilis*; BAM = $5x10^{8}$ CFU/kg feed of *Bacillus amyloliquefaciens*.
No interaction effect (Productive cycle x treatment) was found significant.

Piglet performance data was monitored during the first two cycles and is presented in **Table 6.2.** A significant increase in weaning BW, ADG, and consumption of creep feed was observed in the second productive cycle concomitant with a trend towards a lower BW at birth. No significant changes related to the treatments were found in piglet BW at birth, and any possible differences in litter weight were balanced after cross-fostering. During the studied cycles, *Bacillus subtilis* (BSU) was associated with a lower weight of piglets at weaning compared to CON ($P = 0.015$) and numerical differences in average daily gain (ADG) although differences did not reach statistical significance ($P = 0.138$). Estimated amounts of average daily creep feed intake (ADFI) were not different among treatments. Supplementation of sows with *Bacillus amyloliquefaciens* (BAM) tended to reduce the mortality rate of piglets compared to CON ($P = 0.082$) and significantly decreased the rate of loss of piglets when compared to BSU ($P = 0.024$). No significant interaction between cycles and treatments was found for the performance of piglets.

Table 6.2. Effect of *Bacillus subtilis* (BSU) and *Bacillus amyloliquefaciens* (BAM) on piglet performance during the first two productive cycles.

Parameter[1]	Productive cycle		SEM	P-value	Treatment			SEM	P-value
	1st	2nd			CON	BSU	BAM		
BW birth (all piglets), g	1300^{x}	1222^{y}	20.01	0.060	1290	1299	1210	37.5	0.145
BW after cross-fostering, g	1371	1319	20.22	0.228	1372	1384	1325	37.1	0.450
BW weaning, g	4863^{a}	5739^{b}	96.7	**<0.001**	5621^{a}	5085^{b}	5360^{ab}	163.2	**0.044**
ADG, g/d	150	172	3.27	**0.001**	169	153	164	0.04	0.138
Creep feed FI, g/d/litter	29.0	34.1	0.57	**<0.001**	31.4	31.5	32.0	0.03	0.917
Mortality rate, %	3.65	2.77	0.432	0.348	3.09^{x}	3.46^{x}	1.66^{y}	0.327	**0.085**
Pig loss rate, %	5.02	4.35	0.548	0.655	4.07^{ab}	6.38^{a}	3.03^{b}	0.268	**0.038**

Notes: CON=Control; BSU=*Bacillus subtilis*; BAM=*Bacillus amyloliquefaciens*; BW=body weight; ADG=daily gain; FI=feed intake.
[1] Cycle 1: 98 dams (33 in CON, 32 in BSU and 33 in BAM) from wean/service and during gestation and 78 dams (27 in CON, 25 in BSU and 26 in BAM) during lactation.
Cycle 2: 76 dams (27 in CON, 25 in BSU and 24 in BAM) from wean/service and during gestation and 56 dams (21 in CON, 17 in BSU and 18 in BAM) during lactation.
Different superscripts in same row are significant or trending (a/b: $P \leq 0.05$; x/y $0.05 < P \leq 0.10$).
No interaction effect (productive cycle x treatment) was found significant.

6.4.2. Immune response

Specific concentrations of IgG and IgA for Aujeszky and concentrations of IgG for PRRS in serum and milk samples from the sows at days 8 and 21 are presented in **Table 6.3.** Compared to CON, dietary supplementation with BAM significantly decreased the serological titers of IgG specific for Aujeszky at day 21 ($P = 0.009$) and tended to decrease serological titers of IgG and IgA specific for Aujeszky at day 8 after farrowing ($P = 0.089$ and $P = 0.097$, respectively). No other trend or a significant difference was found in concentrations of IgG specific for PRRS or any of the immunoglobulins determined in milk.

Table 6.3. IgG and IgA specific for Aujeszky and PRRS determined by ELISA in serum samples and sows' milk on days 8 and 21 after farrowing.

Parameter, in AU[2]	Treatment[1]			RSE	P-value
	CON	BSU	BAM		
Serum d8					
IgG Aujeszky	2.15	2.02	1.91	0.322	0.074
IgA Aujeszky	0.30	0.24	0.20	0.134	0.082
IgG PRRS	0.39	0.47	0.35	0.196	0.760
Serum d21					
IgG Aujeszky	2.26[a]	2.19[a]	1.95[b]	0.256	0.003
IgA Aujeszky	0.31	0.48	0.18	0.198	0.233
IgG PRRS	0.42	0.38	0.38	0.201	0.559
Milk d8					
IgG Aujeszky	0.85	0.81	0.88	0.304	0.911
IgA Aujeszky	0.47	0.61	0.44	0.253	0.914
IgG PRRS	0.07	0.08	0.07	0.013	0.767
Milk d21					
IgG Aujeszky	0.47	0.60	0.50	0.154	0.551
IgA Aujeszky	0.36	0.36	0.24	0.204	0.146
IgG PRRS	0.06	0.06	0.06	0.004	0.894

[a-b] Means within a row with different superscripts differ ($P < 0.05$).
[1]Treatment: CON=Control; BSU=*Bacillus subtilis*; BAM=*Bacillus amyloliquefaciens*.
[2]AU=Absorbance units

6.4.3. Differences in milk metabolites among interventions

The global metabolic profile of a total of 40 milk samples taken 21 days after parturition were analyzed (n = 15 for CON, n = 11 for BSU, and n = 14 for BAM) by partial least squares discriminant analysis (PLS-DA). As a result, no differences were found in the PLS-DA between groups. Nevertheless, the PLS-DA analysis showed a bigger dispersion in the samples from CON and BSU while samples from BAM seemed more centered. When the analysis was performed by comparing separately each treatment to control (**Figure 6.1.**), two clusters could be identified when comparing BSU to CON.

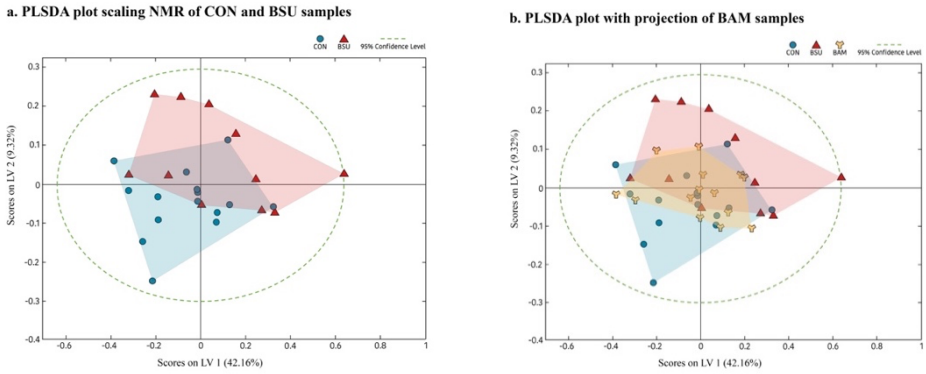


Figure 6.1. Partial least squares discriminant analysis (PLS-DA) scores plot scaling NMR data from CON and BSU (**a**); and projection of samples from BAM (**b**). Samples are indicated as blue dots (CON), red triangles (BSU), and yellow three-pointed stars (BAM). CON=Control; BSU=*Bacillus subtilis*; BAM=*Bacillus amyloliquefaciens*.

In addition to the PLS-DA, the possible impact of experimental treatments on particular metabolites was evaluated. **Annex 2: Table S6.5.** shows the list of milk metabolites that were identified in sow milk samples and were selected due to their relevance in the VIP coefficients. Among them, there were identified amino acids and derivatives, sugars and derivatives, and fatty acid-associated metabolites. The most abundant metabolite was lactose, followed by UDP-N-acetylglucosamine, creatine phosphate, UDP-galactose, and glycoprotein.

6.4.4. Sow fecal microbiota

The global structure, dynamics, and functionality of sow fecal microbial populations were analyzed on days 8 and 21 after parturition by high-throughput sequencing. As a result, the NMDS based on the Bray-Curtis distance of relative abundance of ASV showed a distinct microbial structure related to treatments on day 8 post-farrowing (PERMANOVA: $P = 0.026$; ANOSIM: $P = 0.018$), reaching a statistical trend on day 21 post-farrowing (PERMANOVA: $P = 0.058$; ANOSIM: $P = 0.074$). As for the different time points, the NMDS showed a clear clustering of samples by day (PERM-

ANOVA: P < 0.001; ANOSIM: P = 0.001) with more dispersed samples at day 8 after parturition (**Figure 6.2.**).

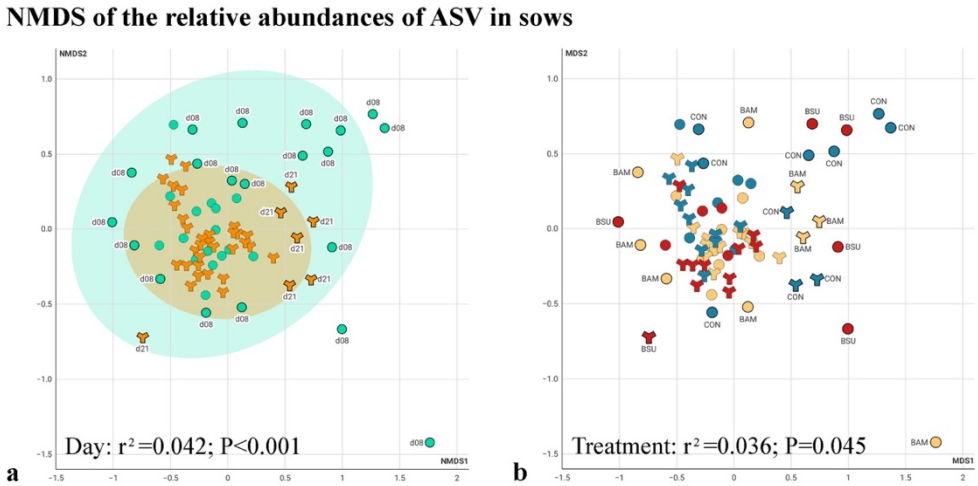


Figure 6.2. NMDS of the relative abundances of ASV in sow fecal content based on Bray-Curtis distance (stress = 0.157) and grouped by sampling day (d8 after farrowing (green) vs d21 after farrowing (orange)). In order to facilitate the distinction between experimental treatments from figure **a**, the same NMDS figure has been placed in parallel as figure **b** with the three diets highlighted in color.

The alpha diversity indexes of sow fecal samples are presented in **Table 6.4.** In general terms, there was a significant increase in the species richness (P = 0.046) and Chao1 index (P = 0.046) from d8 to d21 after farrowing. Concerning the dietary treatments, BSU and BAM treatments showed a significantly lower alpha diversity on d8 postpartum when compared to CON sows. However, on d21 only BSU treatment showed a lower alpha diversity compared to CON. Regarding beta diversity, no difference was detected with the Whittaker's index between sampling days (0.525 and 0.499, for d8 and d21 after farrowing, respectively, P = 0.135) nor treatments (0.489, 0.523 and 0.522, for CON, BSU and BAM, respectively, P = 0.177).

Table 6.4. Alpha diversity values obtained in each sampling day both on sows and their offspring. The Observed species, Chao1, Shannon and Simpson indices are presented. The values obtained in each sampling day are presented separately, differentiating between treatments and with their corresponding P-value.

	Index	d8			SEM	P-value	d21			SEM	P-value
		CON	BSU	BAM			CON	BSU	BAM		
Sows	Observed species	2180^{a}	1340^{b}	1455^{b}	145.26	0.032	2219	1568	2492	176.61	0.100
	Chao1	2195^{a}	1353^{b}	1466^{b}	146.11	0.033	2235	1585	2509	177.40	0.102
	Shannon	7.09^{a}	6.54^{b}	6.47^{b}	0.115	0.038	6.89	6.60	6.91	0.082	0.250
	Simpson	0.999^{a}	0.997^{b}	0.996^{b}	0.001	0.286	0.997	0.997	0.998	0.000	0.670
	Index	**d21**			**SEM**	**P-value**	**d33**			**SEM**	**P-value**
		CON	**BSU**	**BAM**			**CON**	**BSU**	**BAM**		
Piglets	Observed species	1321^{x}	787^{y}	1475^{x}	125.98	0.081	834	1047	1004	72.79	0.438
	Chao1	1324^{x}	789^{y}	1478^{x}	125.96	0.081	838	1052	1008	72.72	0.429
	Shannon	6.39^{a}	6.02^{b}	6.42^{a}	0.072	0.049	5.90	6.28	6.29	0.152	0.457
	Simpson	0.997	0.996	0.997	0.000	0.180	0.993	0.995	0.997	0.001	0.556

Different superscripts in same row are significant or trending (a/b: $P \leq 0.05$; x/y $0.05 < P \leq 0.10$).

In the analysis of the abundance of differential taxa, 42 different phyla were detected. In general terms, the most abundant phyla in all samples were Firmicutes (68.51%) and Bacteroidetes (21.44%), followed by Spirochaetes, Proteobacteria, and Actinobacteria, whose percentages were 3.03%, 2.82%, and 1.24% respectively. The rest of the phyla were presented with lower abundances (<1%). Concerning the families (**Annex 2: Table S6.6.**), a total of 197 were detected. At this level, *Erysipelotrichaceae* represented the main family found in all samples (12.95%), followed by *Clostridiaceae* (9.67%), *Prevotellaceae* (9.36%), *Peptostreptococcaceae* (7.98%), *Oscillospiraceae* (7.14%), *Lachnospiraceae* (6.33%), *Lactobacillaceae* (5.57%) and *Ruminococcaceae* (5.34%). Five families were found representing between 1 and 5% of the relative abundance (*Christensenellaceae*, *Bacteroidaceae*, *Spirochaetaceae*, *Rikenellaceae*, and *Muribaculaceae*, in decreasing order of abundance, respectively) and the rest of the families obtained a relative abundance of less than 1%. Finally, at the genus level, a total of 462 genera were identified. However, an average of 15.33% of the relative abundance could not be assigned to any bacterial genus in particular. Only 16 genera were presented with a relative abundance greater than 1%. The most abundant genera were *Turicibacter* (12.21%), *Clostridium sensu stricto 1* (9.22%), *Lactobacillus* (5.57%), *Terrisporobacter* (4.96%), and *Prevotella* (4.54%), followed by *Bacteroides* (3.94%) and *Christensenellaceae* R-7 group (3.20%).

Regarding differences in taxonomic groups between sampling days (**Figure 6.3.** and **Annex 2: Table S6.6.**), a greater relative abundance of the *Erysipelotrichaceae* and *Peptostreptococcaceae* families was observed on day 21 postpartum. There was also a greater abundance of *Muribaculaceae* and a decrease in the abundance of *Enterobacteriaceae* and *Bifidobacteriaceae* when compared to day 8 after farrowing. Moreover, some statistical differences were observed in families with a lower magnitude of representation, such as p-2534-18B5 or *Selenomonadaceae*, which showed higher values on day 21. At the genus level, some butyrate- and methane-producing microorganisms were found in significantly greater abundance at day 21 postpartum, such as *Lachnospiraceae* (group NK3A20), *Coprococcus*, *Methanosphaera*, *Prevotellaceae* (group UCG-004), or *Butyricicoccus*.

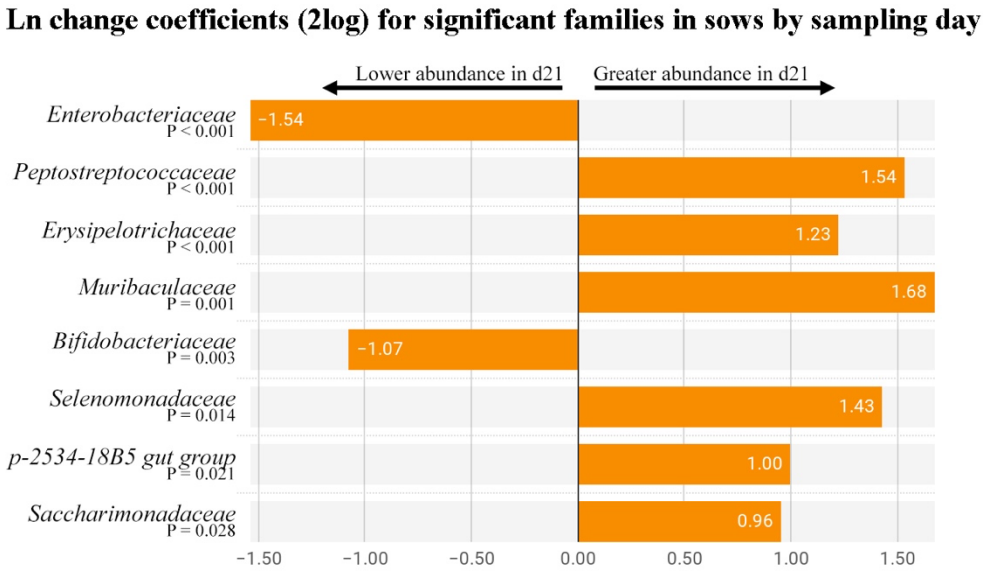


Figure 6.3. Differentially abundant taxa at family level from sow fecal content (ln change coefficients (2log) and FDR-adjusted p<0.05) between d08 and d21 samplings. Only significant taxa with greater relative abundance than 0.05% are presented; positive values and negative values indicate greater and lower abundance, respectively, in d21 animals; taxa are sorted by level of significance (from higher to lower).

The impact of the experimental treatments on particular taxonomic groups was analyzed by sampling day since significant effects between days post farrowing were observed. The impact of experimental treatments was higher on day 8 than on day 21. On day 8 BSU and BAM showed lower abundances of *Prevotellaceae*, (P = 0.007), *Lachnospiraceae* (P = 0.037), *Ruminococcaceae* (P = 0.002), and *Bacteroidaceae* (P = 0.001) than CON (**Figure 6.4a**). Regarding particular genera (**Figure 6.4b**), BSU and BAM promoted lower abundances of *Bacteroides* (P= 0.001), *Faecalibacterium* (P = 0.002), *Phascolarctobacterium* (P = 0.012), *Prevotella* (P = 0.003), *Blautia* (P < 0.001), *Dorea* (P = 0.005) and *Roseburia* (P = 0.003) compared to CON and higher relative abundances of the genus *Sarcina* (P = 0.041). On day 21 after farrowing, differences were only observed for the *Enterococcaceae* family (P < 0.001), with lower relative abundances in BSU and BAM groups, and three minor genera.

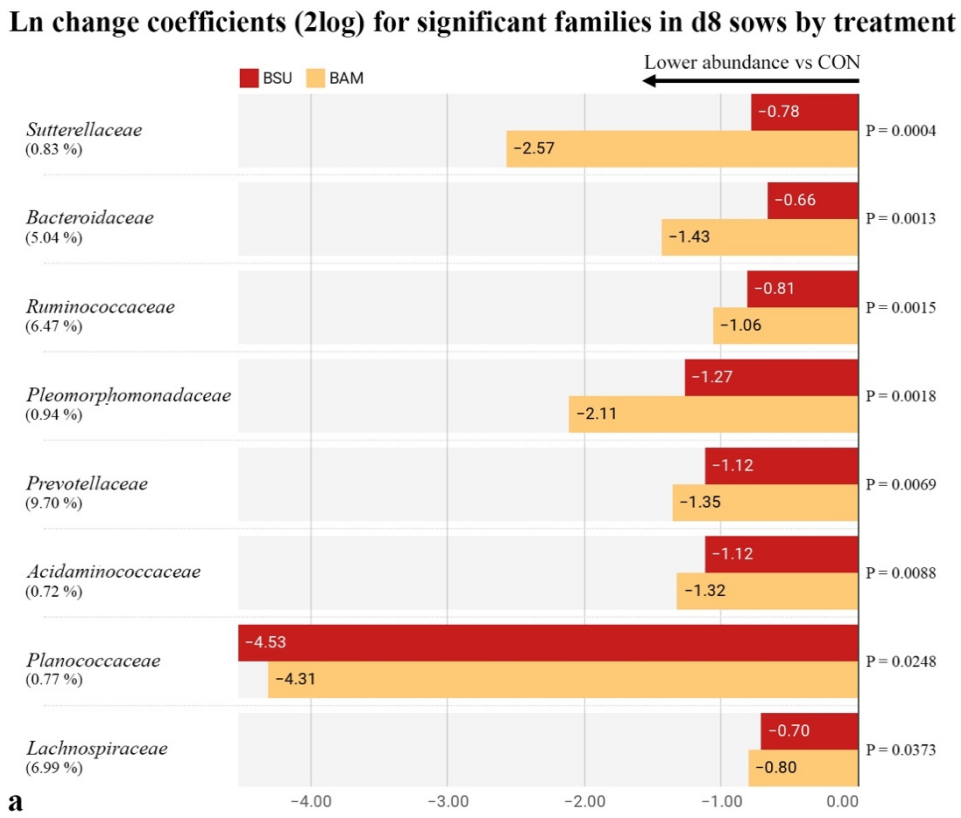
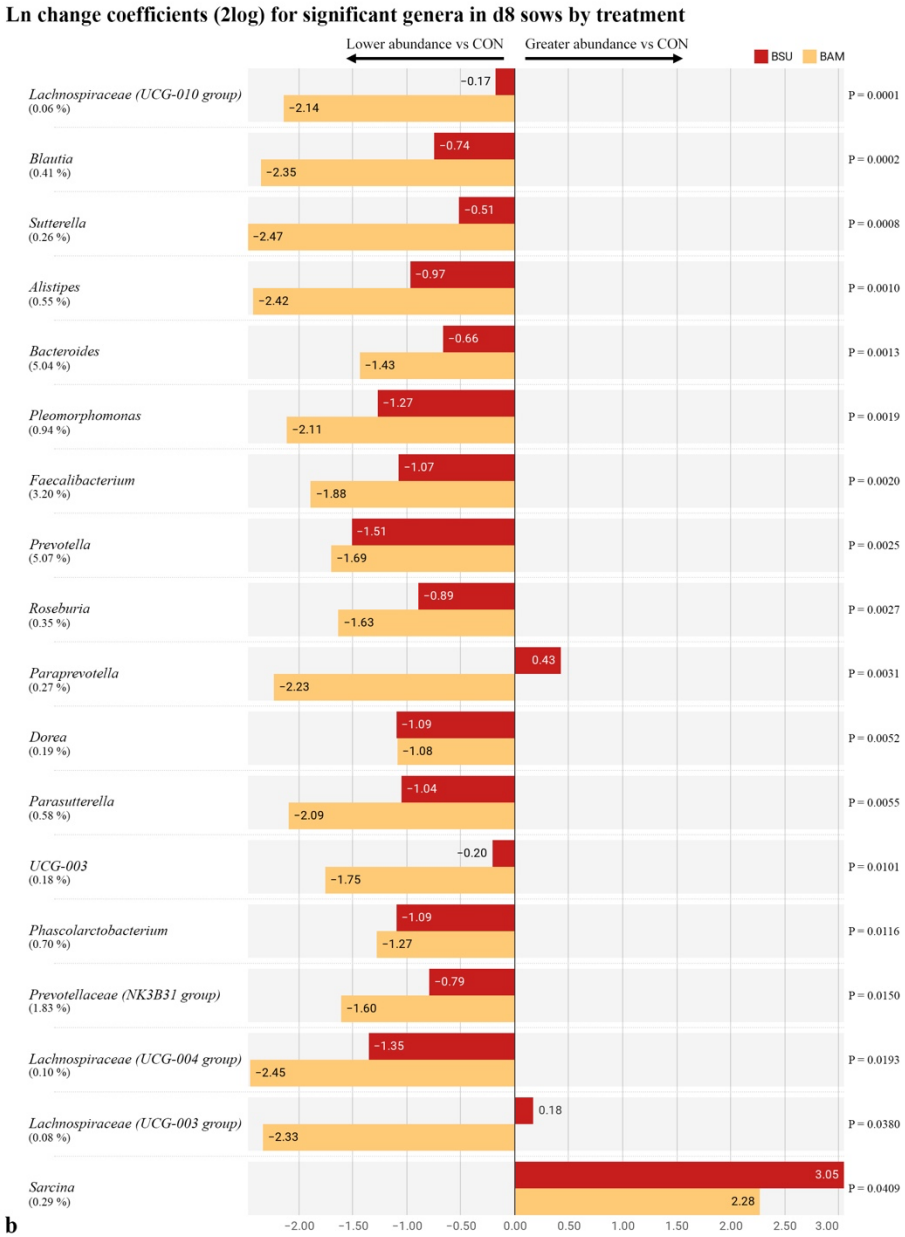


Figure 6.4a. Differentially abundant taxa from fecal content (ln change and FDR-adjusted $p < 0.05$) on day 8 after farrowing between: BSU vs. CON (red), and BAM vs. CON (yellow) at family level. Only significant taxa with greater relative abundance than 0.05% are presented; positive values and negative values indicate greater and lower abundance, respectively; the average relative abundance of each taxa is expressed in % below the family name; taxa are sorted by level of significance (from higher to lower).



b

Figure 6.4b. Differentially abundant taxa from fecal content (ln change and FDR-adjusted p < 0.05) on day 8 after farrowing between: BSU vs. CON (red), and BAM vs. CON (yellow) at genus level. Only significant taxa with greater relative abundance than 0.05% are presented; positive values and negative values indicate greater and lower abundance, respectively; the average relative abundance of each taxa is expressed in % below the genus name; taxa are sorted by level of significance (from higher to lower).

6.4.5. Piglet fecal microbiota

The analysis of the piglets' fecal microbiota on days 21 and 33 of life showed that weaning promoted an evident change in the ecosystem with significant differences between suckling (d21) and weaned (d33) piglets (ENVFIT: $P < 0.001$; PERMANOVA: $P < 0.001$; ANOSIM: $P = 0.001$) as shows the NMDS of the relative abundances of ASV based on Bray-Curtis distance in **Figure 6.5.** The administration of probiotic supplemented diets to their mothers was not associated to structural changes in piglets' fecal community during suckling (ENVFIT: $P = 0.470$; PERMANOVA: $P = 0.209$; ANOSIM: $P = 0.388$) or after weaning (ENVFIT: $P = 0.886$; PERMANOVA: $P = 0.882$; ANOSIM: $P = 0.999$).

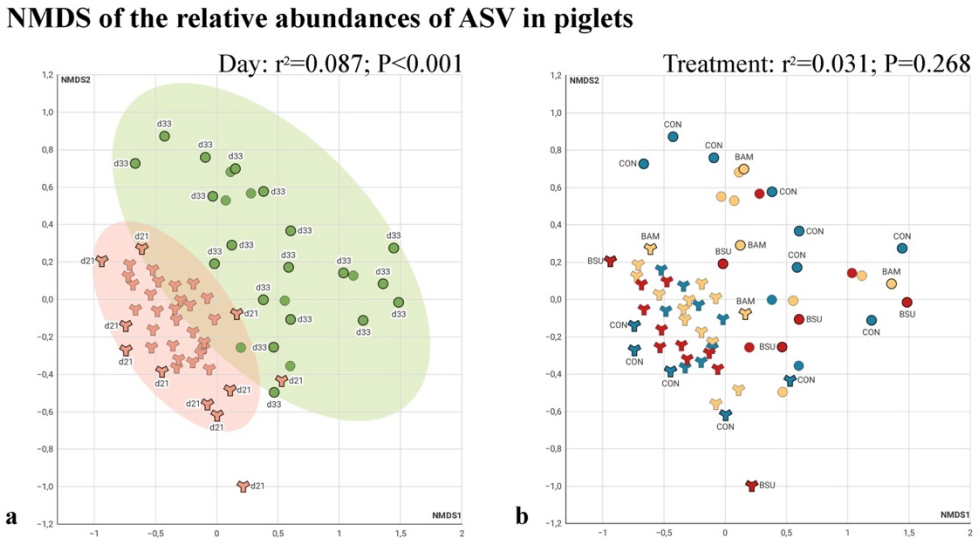


Figure 6.5. NMDS of the relative abundances of ASV in piglet fecal content based on Bray-Curtis distance (stress = 0.169) during lactation (pink, d21 of life) and after weaning (green, d33 of life and d12 after weaning). In order to facilitate the distinction between experimental treatments in figure **a**, the same NMDS figure has been placed in parallel as figure **b** with the three diets highlighted in color.

Concerning alpha diversity (**Table 6.4.**), weaning promoted a trend for a lower species richness at d33 (1224 vs. 951 for observed species, $P = 0.090$; and 1226 vs. 955 for Chao1, $P = 0.092$; for d21 and d33 respectively) and a sig-

nificant lower Simpson index (0.997 vs. 0.994 for d21 and d33, respectively, P = 0.027). Regarding treatments, a tendency to lower species richness (observed species and Chao1 indexes) and a significantly decreased Shannon index alpha diversity were observed with BSU compared to CON and BAM at d21. No significant changes were detected at d33. Regarding beta diversity, distances increased significantly after weaning compared to suckling piglets (0.539 and 0.595, for suckling and weaned piglets, respectively, P = 0.006), however, no significant changes were observed between treatments during lactation (P = 0.916) or after weaning (P = 0.351).

In the analysis of the abundance of differential taxa, 22 different phyla were detected. In general terms, the most abundant phyla in all samples were Firmicutes (50.70%) and Bacteroidetes (25.74%), followed by Proteobacteria, Actinobacteria, and Spirochaetes, whose percentages were 10.59%, 2.26%, and 1.94% respectively. Concerning the families, a total of 126 were detected. At this level, *Bacteroidaceae* represented the main family found in all samples (8.30%), followed by Enterobacteriaceae (7.19%), *Erysipelotrichaceae* (7.18%), and *Lachnospiraceae* (6.75%). *Oscillospiraceae* (6.08%), *Prevotellaceae* (5.78%), *Lactobacillaceae* (5.63%), and *Ruminococcaceae* (5.25%) were the rest of the families with a relative abundance greater than 5%. At the genus level, a total of 335 genera were identified. A 16.3% of the sequences could not be assigned to any bacterial genus, and only 21 genera were presented with a relative abundance greater than 1%. The most abundant genera were *Bacteroides* (8.30%), *Escherichia-Shigella* (7.16%), *Lactobacillus* (5.63%), *Turicibacter* (4.93%), *Clostridium sensu stricto 1* (3.28%), and UCG-002 (2.92%), followed by *Christensenellaceae group R-7* (2.71%) and *Phascolarctobacterium* (2.58%).

The weaning process promoted significant changes in several taxonomic groups (phylum, family, and genus, **Annex 2: Tables S6.7. and S6.8.**). As seen in **Figure 6.6.**, the increase of families such as *Prevotellaceae*, *Spirochaetaceae*, and *Enterobacteriaceae* was observed after weaning, whereas families like *Lactobacillaceae*, *Lachnospiraceae*, *Bacteroidaceae*, and *Clostridiaceae* decreased.

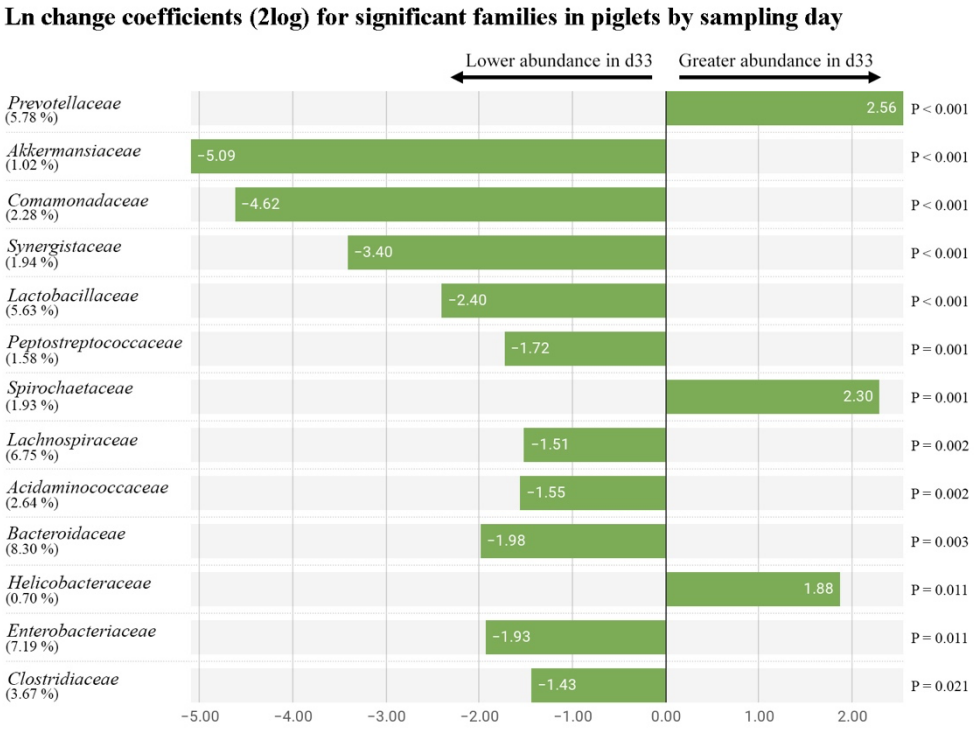


Figure 6.6. Differentially abundant taxa from fecal content (ln change and FDR-adjusted p < 0.05) between d21 and d33 samplings. Only significant taxa with greater relative abundance than 1.5% are presented; positive values and negative values indicate greater and lower abundance, respectively, in d33 animals; the mean average relative abundance of each taxa is expressed in % between brackets; taxa are sorted by level of significance (from higher to lower).

Regarding the impact of supplementing probiotics to the sow on particular microbial taxa of piglets, **Annex 2: Figure S6.1.** shows the bar plots for relative abundances of the main families of each experimental treatment on both sampling days. Most of the changes produced by the treatments were observed at minor taxa (<0.5%) and a greater effect was observed after weaning. During lactation (d21), only a higher relative abundance of *Campylobacteraceae* (P = 0.043) and its respective genus, *Campylobacter* was observed in both groups supplemented with the probiotic (0.19, 0.84, and 0.81%, for CON, BSU, and BAM, respectively, P = 0.0345). After weaning (d33), however, BSU and BAM piglets presented lower abundances of *p-2534-18B5* than CON (2.38, 1.19, and 1.87%, for CON, BSU, and BAM,

respectively, P = 0.041) and greater abundances of *Ruminococcaceae* (2.51, 4.25 and 5.40%, for CON, BSU and BAM, respectively, P = 0.019). Finally, BAM piglets showed greater abundances of *Bacteroidales BS11 gut group* (0.00, 0.00 and 0.64%, for CON, BSU and BAM, respectively, P = 0.003) and *F082* (0.01, 0.001 and 0.57%, for CON, BSU and BAM, respectively, P = 0.019). The ln change coefficients in those families significantly modified by the treatments can be seen in **Figure 6.7.** At the genus level, no significant differences were observed except for minor taxa.

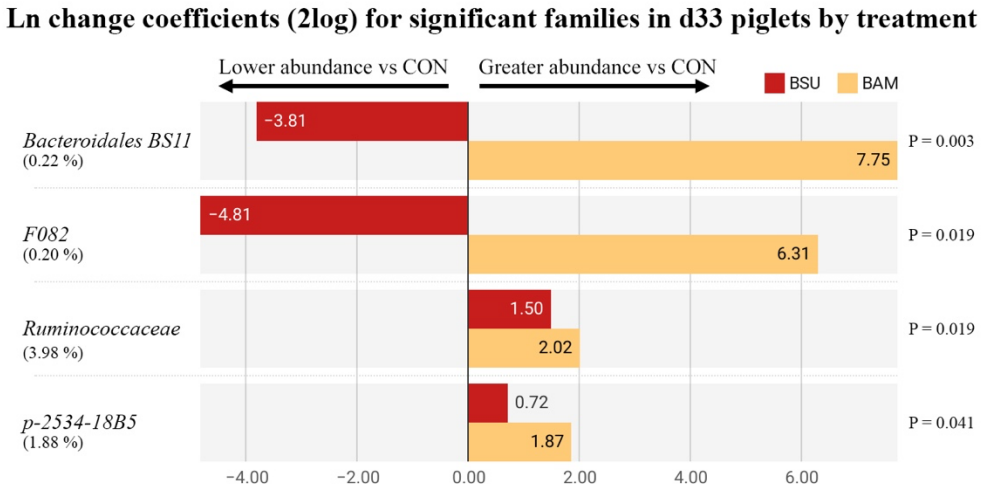


Figure 6.7. Differentially abundant taxa from fecal content (ln change and FDR-adjusted p < 0.05) of weaned piglets (d33) between: BSU vs. CON (red), and BAM vs. CON (yellow) at family level. Only significant taxa with greater relative abundance than 0.05% are presented; positive values and negative values indicate greater and lower abundance, respectively, in d33 animals; the mean average relative abundance (d33 only) of each family is expressed in % below the family name; taxa are sorted by level of significance (from higher to lower).

To study the hypothesis of maternal transfer and the role of the mother in the early gut colonization of the piglets, sow family and genus microbiota were correlated with those of their piglets. As a result, a high number of significant positive correlations were observed between the microbiota of the dams and the microbiota of the weaned piglets whereas no moderate nor high negative correlations were found at family nor genus level. **Table 6.5.** shows those

significant positive correlations (families and genera) with correlation sizes from 0.7 to 1.0.

Interestingly no high correlations were found between the sow microbiota one week after farrowing (d8) and the piglets at the end of lactation (d21). However, some microbial groups of the sow at d8 showed to be correlated with the microbiota of piglets at d33. The highest correlated families in the weaned piglets (d33) with mother microbiota early after birth (d8) belonged to the Firmicutes and Proteobacteria phyla including families such as *Enterobacteriaceae*, *Pasteurellaceae*, *Selenomonadaceae*, *Veillonellaceae*, and *Peptostreptococcaceae*. The minoritary *Atopobiaceae* family from Actinobacteria phylum also showed to be correlated to sow's microbiota.

Table 6.5. Significant high correlations (from 0.7 to 1.0) obtained from the comparison among sows' (d08 and 21 after farrowing) and piglets' (d21 and d33 of life) fecal microbiota (families and genera).

		Sow taxa	Piglet taxa	cor value	P-value
d8 sow vs d21 piglet		No high correlation values found neither at family nor genus level.			
d8 sow vs d33 piglet	**Family**	*Muribaculaceae*	*Atopobiaceae*	0.767	<0.001
		Selenomonadaceae	*Atopobiaceae*	0.794	<0.001
		Veillonellaceae	*Atopobiaceae*	0.832	<0.001
		Peptostreptococcaceae	*Enterobacteriaceae*	0.716	<0.001
		Peptostreptococcaceae	*Pasteurellaceae*	0.724	<0.001
		Veillonellaceae	*Selenomonadaceae*	0.729	<0.001
		Coriobacteriaceae	*Veillonellaceae*	0.743	<0.001
		Muribaculaceae	*Veillonellaceae*	0.755	<0.001
		Selenomonadaceae	*Veillonellaceae*	0.815	<0.001
		Veillonellaceae	*Veillonellaceae*	0.773	<0.001
	Genus	*CAG-873*	*Bacteroides*	0.743	<0.001
		Alloprevotella	*Escherichia/Shigella*	0.807	<0.001
		Terrisporobacter	*Escherichia/Shigella*	0.766	<0.001
		Megasphaera	*Megasphaera*	0.858	<0.001

d21 sow vs d21 piglet	**Family**	*Akkermansiaceae*	*Campylobacteraceae*	0.742	<0.001
		Streptococcaceae	*Campylobacteraceae*	0.776	<0.001
	Genus	*Akkermansia*	*Campylobacter*	0.742	<0.001
		Streptococcus	*Campylobacter*	0.774	<0.001
d21 sow vs d33 piglet	**Family**	*p-251-o5*	*Selenomonadaceae*	0.720	<0.001
		Akkermansiaceae	*Succinivibrionaceae*	0.740	<0.001
		Anaerovoracaceae	*Succinivibrionaceae*	0.706	<0.001
		Bacteroidales BS11 gut group	*Succinivibrionaceae*	0.744	<0.001
		Oligosphaeraceae	*Succinivibrionaceae*	0.749	<0.001
		Peptococcaceae	*Succinivibrionaceae*	0.809	<0.001
		Spirochaetaceae	*Succinivibrionaceae*	0.726	<0.001
		Paludibacteraceae	*Veillonellaceae*	0.758	<0.001
	Genus	*Akkermansia*	*CAG-873*	0.763	<0.001
		Lachnospiraceae NK4A136	*CAG-873*	0.764	<0.001
		Treponema	*CAG-873*	0.712	<0.001
		Actinomyces	*Megasphaera*	0.845	<0.001
		Fusobacterium	*Megasphaera*	0.780	<0.001
		Akkermansia	*Succinivibrio*	0.745	<0.001
		Family XIII AD3011	*Succinivibrio*	0.704	<0.001
		Treponema	*Succinivibrio*	0.727	<0.001

Microbiota of sows at day 21 postpartum, also showed significant high correlations (>0.7) with those of weaned piglets (d33). In this case, *Selenomonadaceae* and *Veillonellaceae* families showed also to be correlated with different microbial families in the sows and particularly *Succinivibrionaceae* family showed to be correlated to *Akkermansiaceae*, *Anaerovoracaceae*, *Oligosphaeraceae*, *Peptococcaceae*, and *Spirochaetaceae* families in the mothers. Only two high positive correlations were found when comparing microbiota of sows and piglets at d21, involving *Akkermansiaceae* and *Streptococcaceae* families in the sow that correlated to the piglets' *Campylobacteraceae* family.

At the genus level, and in a similar way to the previous level, a greater number of correlations were found between the dams (both at day 8 and 21 postpartum) and the weaned piglets. On day 8 postpartum, a high correlation was observed between the maternal genera *Alloprevotella* and *Terrisporobacter* and the genus *Escherichia-Shigella* of the piglet and also between the *Megasphaera* genera of the sows and their piglets. Likewise, several moderate positive correlations were observed between *Lactobacillus* and various maternal butyric fermentation genera such as *Butyricimonas*, *Blautia*, *Megasphaera*, *Prevotella*, with other butyric fermentation genera in piglets, such as *Coprococcus*, *Megasphaera*, *Prevotellaceae* (NK3B31 group), and *Ruminococcaceae UCG-002* and *UCG-008.* Sow's microbial genera at day 21 postpartum also showed similar significant high correlations with piglet's genera at days 8 and 33. Because of the relevance of the genera, it should be remarked the significant high correlations between *Akkermansia* in the mothers and *Campylobacter* (d21) and *CAG-873* and *Succinivibrio* (d33) genera in the piglets.

6.4.6. Intestinal gene expression

Detailed results of jejunal gene expression of medium- and small-sized piglets can be found in **Annex 2: Table S6.9.** for the 51 genes that could be quantitatively determined. Despite some numerical differences in some genes between treatments, there was no significant effect associated with the sows' dietary treatments, as shown in **Figure 6.8.** However, significant differences were observed when comparing gene expressions according to piglet size

(medium or small-sized) regardless of the treatment. Small-sized piglets showed up-regulated expression of *IGF1R* (Insulin-like growth factor 1 receptor; P = 0.052); *HSP27* (Heat shock protein 27; P = 0.038); and *CLDN15* (Claudin-15; P = 0.052) genes compared to medium-sized piglets. No interaction was found between sow's dietary treatment and piglet size.

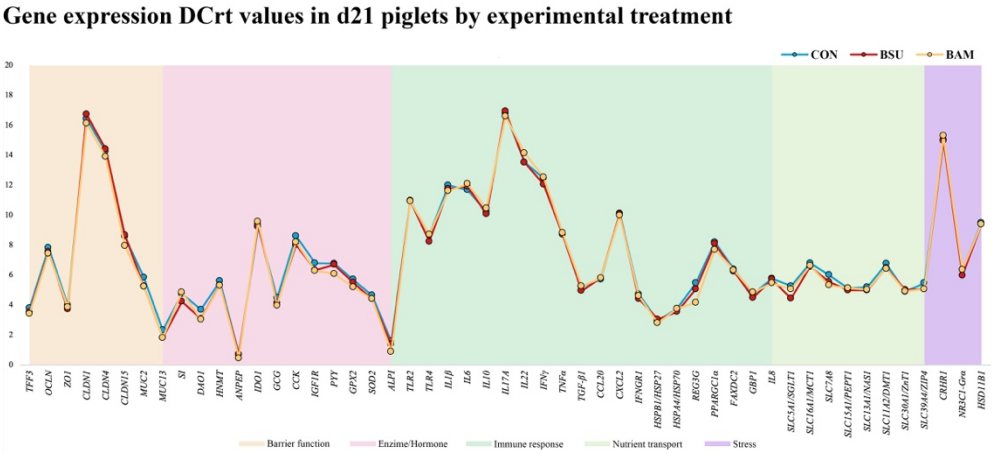


Figure 6.8. Mean DCrt expression of all the genes analyzed sorted by dietary treatment. Genes have been grouped by function with different background colors. CON=Control; BSU=*Bacillus subtilis*; BAM=*Bacillus amyloliquefaciens*.

6.5. Discussion

In recent years, dietary supplementation of sows with probiotics has gained considerable attention due to their potential to improve reproductive performance (Barba-Vidal, Martín-Orúe and Castillejos, 2019). Particularly, different strains of *Bacillus* spp. have been shown to increase feed consumption in lactation, reduce fat mobilization, promote milk production, increase litter weight, promote digestive health, and inhibit pathogenic bacteria (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Stamati *et al.*, 2006; Larsen *et al.*, 2014; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016). While higher milk production or improved economy of fat reserves of the sow could be behind these effects, other modes of action, related to differential early events in the life of the piglets, could also be involved. In

this regard, modulation of the maternal intestinal microbiota by probiotics could determine changes in the process of early microbial colonization of the gastrointestinal tract of piglets with beneficial implications throughout their lives. Currently, the crucial role of early events in the development of the neonatal immune system is largely recognized (Hansen *et al.*, 2012) and appropriate development of the intestinal microbiota is considered as a key point with potential benefits throughout the productive life of the pig (Nowland *et al.*, 2019). In this work, we assess the potential benefits of two probiotic Bacillus strains, when supplemented to sows, trying to give some light on those mechanisms that could explain the improvements reported in the progeny.

6.5.1. Impact of probiotics on sow performance

Several studies in the literature have pointed out that supplementation of sows with *Bacillus spp.* probiotics during gestation and lactation may increase feed consumption, promote milk production and reduce the mobilization of reserves, improving body condition at the end of lactation (Jeong *et al.*, 2015; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Menegat *et al.*, 2019). Moreover, a reduction in the weaning-estrus interval has also been reported (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016). In the present study, however, we were not able to find such improvements. This is consistent with the findings of other authors (Zhang *et al.*, 2020; Hu, Kim and Kim, 2021). Variability in the response between studies could be due to differences in the probiotic strains used but could also be due to differences in the management of the animals, age, or breeds of the sows, the health status of the farm, or the environmental conditions.

Despite not finding improvements in feed intake or mobilization of reserves, these results clearly show an increase in prolificacy, in terms of total number of piglets per sow, particularly when supplementing BAM. This treatment was also related to a significant increase in the number of piglets born alive, with almost two more piglets per litter (17.4 vs. 15.7, $P = 0.017$), which, in turn, resulted in a greater number of weaned piglets. The enhancement of litter size with *Bacillus spp.* probiotics has been also described by many other authors

(Alexopoulos *et al.*, 2004; Taras *et al.*, 2005, 2006; Stamati *et al.*, 2006; Baker *et al.*, 2013; Apic *et al.*, 2014; Jeong *et al.*, 2015). This could be due to an improvement in the rates of ovulation and conception, and/or early embryonic maturation. Therefore, based on maternal performance, our results suggest that it should be enough to supplement the probiotics from mating to confirmed gestation (1st third of gestation) since the only impact on performance was the increased prolificity. Moreover, this outcome might be more important in conventional genetic lines than in hyperprolific genetic lines. Interestingly, relationships between intestinal microbiota and reproductive success have been described by some authors in zoo animals, even identifying some potentially probiotic bacteria species (Antwis *et al.*, 2019). Nasiri *et al.* (2018) also demonstrated that supplementing lactating dairy cows with live yeast culture had a positive impact on the hormonal profile, promoting the development of larger ovulatory follicles. The potential of probiotics to improve fecundity had been also previously evidenced by Gioacchini *et al.* (2010) in a zebrafish model in which the implementation with a *Lactobacillus plantarum* strain was demonstrated to increase oocyte maturation, modifying the transcription of some relevant genes. These improvements in fertility could have been mediated by a modulation of the immune response. In this regard Bhandari *et al.* (2016) described in a mouse model how a probiotic strain of *Lactobacillus plantarum* could ameliorate the inflammatory induced infertility associated with an LPS challenge.

Few authors have focused their studies on evaluating the potential additional effects of long-term administration of probiotics on the reproductive performance of sows. Although in our study the interaction (treatment x cycle) did not show any significant effect on any of the measured variables, it is true that, the beneficial impact of the treatments on the number of born piglets showed a differential numerical evolution across cycles. Whereas with BAM the increase in the number of total and born alive piglets was improved from the first cycle, for the BSU treatment differences were only observed from the third cycle (21.4 vs 18.2 total piglets, $P = 0.034$) suggesting that for a positive impact of this probiotic on prolificacy, long-term administration of at least three cycles would be necessary.

6.5.2. Impact of probiotics on sow fecal microbiota and maternal milk

In the present study, the global structure, dynamics, and functionality of sow fecal microbial populations were analyzed on days 8 and 21 after parturition by high-throughput sequencing. In general terms, the impact of the probiotic treatment on sow microbiota was observed from day 8 post-farrowing with reductions in biodiversity and significant changes in particular microbial groups with both treatments, although changes were more evident with BAM. PERMANOVA analysis also showed that the impact of treatments was clearer on day 8 than on day 21. The apparent higher impact of probiotics on the microbial ecosystem on d8 could have been due to the higher dispersion of mothers' microbiota shortly after labor. During gestation, the microbiota undergoes many changes (Liu *et al*., 2019), and after farrowing probably needs to establish a new equilibrium. It's in this process that probiotics could have a relevant role in speeding up this transition and preventing transient dysbiosis. In consonance with other authors (Zhang *et al*., 2020), α-diversity was decreased by both probiotics on day 8, and only by BSU on day 21. Although in general terms, an increase in biodiversity is regarded as a positive sign of a more robust and resilient ecosystem (Sommer *et al*., 2017), the supplementation with probiotics is not necessarily associated with an increase in biodiversity. Grazul *et al.* (2016) showed in mice how in a disturbed microbiota, following antibiotic treatment, the administration of probiotics did not alleviate the loss of diversity and even was associated with a lower number of microbial species in the recovery phase. It is reasonable to think that probiotic intervention can be related to a reduction in the complexity of the microbiota ecosystem, at least transitionally, due to the constant arrival of high numbers of such particular microorganisms. This could be particularly true in a scenario of transient disequilibrium which occurs post-partum. From this scenario, a transient reduction in biodiversity could be regarded as a positive sign, if the ecosystem is effectively driven by the probiotic to a new beneficial equilibrium, thereby preventing dysbiosis.

Regarding taxonomic changes promoted by probiotics on the sow fecal microbiota, one of the most reported effects of *Bacillus spp*. probiotics has been an increase in numbers of *Lactobacillus* and a decrease in numbers of *Escherichia coli* (Baker *et al*., 2013; Kritas *et al*., 2015; Hayakawa *et al*., 2016; Hu, Kim and Kim, 2021), however, no significant changes in these groups were

observed in our study. It is important to consider here, the methodological differences between studies. High-throughput sequencing methods are not thought to be able to elucidate changes in particular microbial groups, despite their ability to give semi-quantitative data for taxonomic groups. To assess particular effects on specific groups, like *Lactobacillus* or *E. coli*, other methods like traditional culturing of specific qPCR would be preferred.

Despite limitations in the method, results of sequencing showed significant changes in particular taxonomic groups. The changes observed were somehow similar to those described by Zhang *et al.* (2020) in reproductive sows supplemented with a *Bacillus subtilis* strain. Differences were found on *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae* families that were decreased with probiotic supplementation on d8 after farrowing. The genera belonging to *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* families are adapted to metabolize a wide range of complex oligosaccharides and polysaccharides while producing short-chain fatty acids. Indeed, *Roseburia* is a major contributor to the metabolic network of carbohydrate utilization and production of butyrate (Duncan, Louis and Flint, 2004). From this point of view, the lower abundance of *Roseburia*, *Ruminococcus*, *Faecalibacterium*, *Dorea*, *Blautia*, and *Phascolarctobacterium* genera observed in BSU and BAM sows, would suggest a lower capability on these animals to cope with diets rich in complex carbohydrates, although this is likely an over-simplified conclusion considering the complexity of microbiota.

Another important aspect of the impact of probiotics on the mothers' microbiota is that although BAM and BSU did modify the same microbial groups, BAM changes were of greater magnitude than those reported for BSU and they fundamentally occurred on day 8 postpartum. As described above, this could be related to a better modulation of the digestive balance of the dams during the transition process after farrowing that could have led to an improvement in the early colonization process of the piglets during the first days after delivery. The transition of animals to an improved microbial environment, driven by their mothers, could be behind the lower mortality and pig loss rate documented in the BAM group.

Some probiotics have also been reported to modulate the immune response of the sow herd (Medina *et al.*, 2007) or even litter immunity (Scharek-Tedin

et al., 2015; Hayakawa *et al.*, 2016). The inclusion of *Bacillus subtilis* in lactating sows has been reported to be beneficial for milk production and increase the concentration of IgG (Ayala *et al.*, 2016). Moreover, in fecal samples, probiotic administration has been reported to slightly increase the total IgA concentration (Hayakawa *et al.*, 2016). Considering this, in the present study we assessed the possible immunomodulatory effects of the tested probiotic on the sows and the subsequent transfer of passive immunity by the quantification of specific IgG and IgA for Aujeszky and PRRS in blood and milk samples. Nonetheless, we were not able to demonstrate any improvement. The absence of significant effects does not eliminate a possible impact of the probiotics on the immune response of the sows, given the potential inadequacy of the selected methodology to detect those changes.

Probiotic strains could have also benefited the composition of milk. In this regard, the supplementation with probiotics during gestation and lactation has been reported to induce beneficial effects on the milk composition of rats (Azagra-Boronat *et al.*, 2020). Moreover, in that study, the authors demonstrated that although the microbiota of the milk was not modified, the probiotic was able to reach the milk. Although the microbiota of the milk was not analyzed in the present study, a metabolomic analysis was performed. The dietary supplementation with *Bacillus amyloliquefaciens* (BAM) was associated with a more similar milk composition between animals compared to CON and BSU. Changes in milk composition could be mediated by changes in the metabolic response of the sow induced by the changes promoted by probiotics in their gut microbiota. Actually, the more stable composition of BAM sows' milk shows some parallelism with the closer clustering of the gut microbiota of BAM mothers on day 8 postpartum. These results are consistent with the potential of probiotics to promote changes in the metabolomic profile of mother's milk.

From the metabolite profile identified in milk samples, several metabolites were consistent with the existing literature. Choline, creatine, creatinine, lactose, sn-glycerophosphocholine, taurine, and UDP-galactose have all been detected by different authors in the analysis of the metabolomic profile of sow milk (Curtasu, Theil and Hedemann, 2016; Picone *et al.*, 2018; Tan *et al.*, 2018). Choline is essential for membrane and neural development as it is a precursor for the biosynthesis of the components of membranes (Blusztajn,

1998). Creatine functions as a high-energy phosphate buffer, being essential in tissues with a high energy demand such as the muscle and the brain (Brosnan and Brosnan, 2007). Taurine plays a critical role in neonatal development and represents an important factor in dietary fat absorption (Picone *et al.*, 2018). The presence of creatine phosphate and UDP-N-acetyl-glucosamine in sow milk has also been reported by Picone *et al.* (2018). Moreover, betaine, acetylcarnitine, and phosphocholine were also identified in sow milk by Curtasu, Theil and Hedemann, (2016). Betaine is known for minimizing stress-induced cell damage and has been used previously as a feed additive to enhance growth performance in pigs (Eklund *et al.*, 2005). Phosphocholine and glycerophosphocholine are important storage forms for choline and their level in swine milk is usually higher than that of free choline (Curtasu, Theil and Hedemann, 2016).

6.5.3. Maternal microbial imprinting

The natural exposure of piglets to sow's feces, together with the possibility of an entero-mammary route for microbial transfer (Jost *et al.*, 2014; Xue Chen *et al.*, 2018; Jiang *et al.*, 2019; Liu, Zeng, *et al.*, 2019), opens the possibility of gut microbiota modulation in the piglet through probiotic supplementation of the sow. Furthermore, the mother's imprinting on the piglet could occur even before its birth. In a recent study, microbial colonization of the spiral colon occurred in stillborn pigs, suggesting microbial exposure before birth (Nowland, Kirkwood, *et al.*, 2021). After birth, milk consumption is essential for the formation of the piglet's gut microbiota. As demonstrated by (Liu, Zeng, *et al.*, 2019), maternal milk microbes were primarily responsible for the colonization of the small intestine, contributing approximately 90% of the bacteria found there throughout the first 35 days of neonatal life. Moreover, this study also shows how this initial impact of sow milk on the piglet is gradually replaced by maternal fecal microbes. In this context, the addition of a novel mixed probiotic culture in pregnant sows has been reported to influence the piglets' gut colonization with beneficial bacteria and reduce the number of *Enterobacteriaceae* (Veljović *et al.*, 2017). Supplementing sows with *E. faecium* and *Bacillus*-based probiotics during the previous month to labor has been reported to modify the fecal microbiota of the mother with some translated impact on their litters (Baker *et al.*, 2013; Starke *et al.*, 2013; Kritas

et al., 2015). Moreover, *B. subtilis* probiotic-fed sow progenies have been reported to show a similar fecal microbial population than their mothers (Menegat *et al.*, 2019). Different probiotic bacteria appear to have different abilities to transfer from the mother to their offspring, thereby having different effects on their progeny (Jiang *et al.*, 2019). Therefore, one of the main purposes of this study was to evaluate the impact of probiotics fed to sows on the establishment of the microbiota of their piglets.

The modulation of the gut microbiota in the piglets was analyzed on days 21 and 33 of life (12 days after weaning) by high-throughput sequencing (HTS). Results showed that the diversity and community structure of fecal microbiota were in consonance with the predominant taxa described previously for healthy piglets (Holman *et al.*, 2017; Xue Chen *et al.*, 2018; Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021; Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021). Bacteroidetes, Firmicutes and Proteobacteria constituted the three predominant phyla, both pre- and post-weaning, as reported in several studies (Hu *et al.*, 2016; Chen *et al.*, 2017; Holman *et al.*, 2017; Y. Li, Guo, *et al.*, 2018; Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021). Moreover, and in agreement with previous studies (Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021), the weaning process promoted significant changes in considerable taxonomic groups.

Regarding the impact of supplementing probiotics to the sows, although we were not able to detect significant structural changes in piglets' fecal community, we were able to show changes in some particular microbial groups, particularly after weaning. After weaning (d33), both probiotic strains were associated with significant increases in *Ruminococcaceae* and also *p-2534-18B5* families. Interestingly, opposite effects were found for each probiotic on other microbial groups. Whereas *Bacteroidales BS11* and *F082* families were decreased in BSU pigs, BAM showed remarkable increases of more than 6 log units (**Figure 6.7.**). These results would suggest a differential impact of experimental treatments on the gut microbiota of weaned piglets. It is also interesting to note that most of the changes were detected after weaning. During lactation (d21), only a higher relative abundance of *Campylobacteraceae* was observed in BSU and BAM piglets. These results would suggest that the changes induced on weaning piglets would not be mediated by a direct impact of the sow's probiotic-modulated microbiota, but

by a differential response of the animals to the post-weaning stressors due to a different sequence of colonization along the first days of life with their mothers. As we did not analyze microbiota of the piglet up to day 21 of life, we cannot confirm this hypothesis, however, it should be said here that the biggest changes induced by the probiotic treatments on the sow's microbiota were observed 8 days after delivery, with a clearer impact of BAM supplemented diets.

Considering the hypothesis that a change in the mother's microbiota during the first days postpartum may have a greater impact on the piglet's microbiota in later stages, the correlation between sow-litter microbiota was analyzed. Similarly, to the higher impact of probiotics in the microbiota of piglets after weaning, a greater number of significant positive correlations were observed between the microbiota of the dams (d8 and 21) and the microbiota of the weaned piglets (d33). All of the significant high correlations obtained were positive and between taxonomic groups which shared similar functionalities. For example, maternal butyric fermentation genera such as *Blautia*, *Megasphaera*, or *Prevotella* correlated highly with other butyric fermentation genera in piglets, such as *Coprococcus*, or the same *Megasphaera* or *Prevotella*. Similarly, genera considered negative for intestinal health such as *Terrisporobacter* correlated positively with *Escherichia-Shigella* in piglets. Also, it is interesting to remark the significant correlations found between the genera *Akkermansia* in the sows at d21 and genera *Succinivibrio* and *Prevotella sp.-CAG-873* in the piglets at d33. The genera *Akkermansia* has been reported to be universally distributed in the gut of the animal kingdom and has been considered to contribute to a healthy mucus-associated microbiota composition (Belzer and de Vos, 2012). Moreover, it has recently been shown beneficial to the host by restoring gut barrier function and reducing adiposity in pigs (Everard *et al.*, 2013; H. Yang, Xiang, *et al.*, 2018). In addition to these benefits, changes in the *Akkermansia* genus in the dams could also affect the development of microbial groups of interest in the piglets. *Succinivibrio* can metabolize various carbohydrate sources, resulting in fermentation products such as acetate and succinate (Hippe *et al.*, 1999), whereas *Prevotella* can break down the plant cell wall through enzymes such as xylanases, mannanases, and β-glucanases (Flint and Bayer, 2008). Both genera are associated with the fermentation of complex carbohydrates and are likely important contributors towards the establishment of a more mature

microbiota, contributing to the alteration of the overall function of gut microbiota.

Although there are very few studies in this area, there are authors who highlight the impact of early events on the immune system and the resilience of the adult animal microbiota (Nowland *et al.*, 2019). For instance, some evidence has been published defining differences in the fecal microbiota of piglets of as early as 7 days of life determining their susceptibility to suffering post-weaning diarrhea four weeks later (Dou *et al.*, 2017), emphasizing the potential of the early microbiota establishment on the development of the immune response. Moreover, some authors have also been able to establish relationships among specific taxonomic groups and the health status of the piglets. For example, an increased abundance of *Actinobacteria* before weaning has been found as a marker of piglets predisposed for diarrhea (Karasova *et al.*, 2021).
The sow represents the main and first donor of fecal microbiota to the piglet with a relevant role in this early process of microbiota establishment. In this sense, recent studies administering maternal fecal microbiota to neonatal piglets have demonstrated that this early intervention can improve the growth performance of piglets, decrease intestinal permeability and stimulate IgA secretion modulating gut microbiota composition (C. S. Cheng *et al.*, 2019). The importance of the mother-effect defining a particular microbiota composition in the nursing piglet was also evidenced by Mu *et al.* (2019) analyzing the early-life microbiota succession in pigs using a cross-fostering piglet model. Therefore, maternal environmental factors (diet composition, probiotic treatment, etc.), that induce changes in maternal microbiota, may have huge effects on offspring gut physiology (Kelly and Conway, 2005).

The possible effect of *Bacillus subtilis* (BSU) and *Bacillus amyloliquefaciens* (BAM) on the jejunal gene expression of piglets was analyzed. Although no significant effect was associated with the sows' dietary treatments, statistically significant differences were observed when comparing the genetic expressions of the piglets according to their size (medium or small-sized within the same litter). Small-sized piglets showed up-regulated expressions of *IGF1R*, *HSP27*, and *CLDN15*. The *IGF1R* gene (Insulin-like growth factor 1 receptor) is a cytokine receptor. *IGF1R* is an important regulator of intestinal cell growth and differentiation. It has been shown to be up-regulated by ETEC

(Liu *et al.*, 2014) and by high dietary ZnO (Li *et al.*, 2006), but down-regulated by age. The *HSPB1* gene (Heat shock protein 27) is a stress protein involved in protection against stress in general, and specifically against toxic stress. It has been described to be up-regulated by weaning (David, Grongnet and Lallès, 2002). The *CLDN15* gene (Claudin-15) codifies for a transmembrane protein of the tight junction (barrier function). Its downregulation decreases the permeability of the epithelial monolayer. It is important for the normal-sized morphogenesis of the small intestine and mucosal differentiation. Therefore, a higher expression of these three genes may be an indication of a greater genetic effort necessary in smaller piglets to increase their gut maturity and robustness and their intestinal differentiation. Very few studies have been devoted to analyzing the effect of piglet size within the same litter on their intestinal gene expression. Recently, Villagómez-Estrada *et al.* (2021) reported a downregulation of several genes involved in barrier, immune, and digestive functions in light piglets compared with their average littermates. Moreover, gene expression studies have been carried out in piglets with low birth weight (LBW) and intrauterine growth restricted (IUGR) piglets. As a result, no differences were found in small intestinal IFG1R expression neither in LWB nor 21-day-old IUGR piglets (Chen *et al.*, 2011; De Vos *et al.*, 2013). On the other hand, and contrarily to our findings, Ayuso *et al.* (2021), found lower expression of genes involved in nutrient digestion and barrier function in LBW piglets. Moreover, lower protein IGF1R abundance in the small intestine of LBW piglets has also been described by Michiels *et al.* (2013). The discrepancy among all findings exhibits that gene expression has a different response depending on weight, age and tissue analyzed.

6.5.4. Piglet performance during lactation

The impact of sow probiotic supplementation on litter performance is variable in the literature. Despite many studies reporting improvements in growth rates, the number of weaned piglets, and reduction of clinical signs of diarrhea when supplementing *Bacillus spp.* probiotics (Alexopoulos *et al.*, 2001, 2004; Taras *et al.*, 2005; Stamati *et al.*, 2006; Baker *et al.*, 2013; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Hu, Kim and Kim, 2021) results are not always positive and some others did not find significant changes in the piglet's performance (Böhmer, Kramer and Roth-Maier, 2006; Menegat *et al.*, 2019, 2020; Davis *et*

al., 2020). In our study, results suggest that the administration of any of the probiotic strains was not able to increase weight gain along with lactation, with similar weaning weights for BAM compared to CON and even lower weights with BSU. The lower weaning weights registered with *Bacillus subtilis* (BSU) could initially be associated with the observed increased litter size, although these adverse impact on body weight was not in BAM piglets. Different studies have described a negative linear correlation between litter size and piglet weight (Zhang *et al*., 2020) due to the higher competition between embryos for uterine resources and that could have an impact on piglet thriving along with lactation. However, in our study, despite larger litters, BSU piglets showed similar weights at birth compared to CON piglets. Lower gains during lactation could also be due to higher competition for the udders and a lower intake of milk, however, this should be discarded since litters were balanced through cross-fostering. Lower weaning weights registered with the BSU treatment would seem therefore associated with a lower ability of these piglets to cope with the challenges of the lactation period. Actually, with BSU treatment, pig loss rate showed the highest values, and the mortality rate was also significantly higher compared to BAM. We could hypothesize that the lower maternal carry-over reported for this probiotic, compared to BAM, would not have equal benefit on the intestinal health and immunocompetence of piglets to compensate for the challenge of larger litters. Contrary, the supplementation with *Bacillus amyloliquefaciens* (BAM) could have improved the health status of piglets considering the lower mortality rate (trend) and the similar weaning weight compared to CON despite the highest litter sizes. It is also fair to note that with BAM the number of weaned piglets was also significantly increased with almost one more piglet per litter. It is difficult to give a clear explanation for these evident positive effects of BAM on the performance of piglets but, as stated above, we could hypothesize that a better modulation of the microbiota of the mothers, especially during the first days after delivery (d8 post-partum), when the sows' microbiota is still reestablishing, could have had a benefit on the intestinal colonization of the piglet promoting a better training of the immune system.

6.6. Conclusion

In conclusion, both tested probiotic strains supplemented to reproductive sows were demonstrated a significant impact on prolificacy. Whereas with *Bacillus amyloliquefaciens* – 516 (BAM) the benefits were observed from the first reproductive cycle, with *Bacillus subtilis* – 541 (BSU) the improvements were not seen until the third complete productive cycle. Moreover, *Bacillus amyloliquefaciens* (BAM) also increased the survival of piglets at birth and the number of piglets at weaning. Positive effects could be associated with the ability of the tested probiotics, and particularly the BAM strain, to modify the structure of the mothers' intestinal microbiota with significant changes in several microbial groups. The most relevant microbiota changes were observed a few days after delivery (d8 postpartum), suggesting the relevant role of probiotics on the establishment of a new intestinal balance after pregnancy and labor. Microbial shifts were also observed in the piglets, with a clearer impact during the post-weaning than in the lactation period, confirming the relevance of the early process of gut colonization shaping the gut microbiota of the growing pig. In this regard, correlations between the microbial groups of the mothers and the piglets were higher with the microbiota of the weaned piglet (d33) compared to the suckling pig (d21) reinforcing the idea of an early maternal carry-over. Tested probiotic strains were also shown some impact on milk composition, although no improvements could be demonstrated in the transfer of passive immunity or in jejunal gene expression of the piglets. In summary, results demonstrate the potential benefits of supplementing probiotics, and particularly a strain of *Bacillus amyloliquefaciens*, to improve prolificacy, re-establish mother gut microbiota after labor, reinforce maternal imprinting and improve the performance of piglets during lactation.

6.7. Declarations

6.7.1. Ethics declarations

The housing, management, husbandry, and slaughtering conditions of the animals used in the present study conformed to the European Union Guidelines (Directive 2010/63/EU). All experimental procedures were approved beforehand by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (permit n° CEEAH 3817).

Chapter 7

Early socialization and environmental enrichment of lactating piglets affects the caecal microbiota and metabolomic response after weaning

Early socialization and environmental enrichment of lactating piglets affects the caecal microbiota and metabolomic response after weaning

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7.1. Abstract

The aim of this study was to determine the possible impact of early socialization and an enriched neonatal environment to improve the adaptation of piglets to weaning. We hypothesized that changes in the microbiota colonization process and their metabolic response and intestinal functionality could help the animals face weaning stress. A total of 48 sows and their litters were allotted into a control (CTR) or an enriched treatment (ENR), in which piglets from two adjacent pens were combined and enriched with toys. The pattern of caecal microbial colonization, the jejunal gene expression, the serum metabolome, and the intestinal physiology of the piglets were assessed before (-2 d) and after weaning (+ 3d). A differential ordination of caecal microbiota was observed after weaning. Serum metabolome suggested a reduced energetic metabolism in ENR animals, as evidenced by shifts in triglycerides and fatty acids, VLDL/LDL and creatine regions. The *TLR2* gene showed to be downregulated in the jejunum of ENR pigs after weaning. The integration of gene expression, metabolome, and microbiota datasets confirmed that differences between barren and enriched neonatal

environments were evident only after weaning. Our results suggest that improvements in adaptation to weaning could be mediated by a better response to the post-weaning stress.

7.2. Introduction

In intensive pig farming, the process of weaning is a multifactorial stressor in the piglet's life affected by physiological, social, environmental, and nutritional challenges. In the current production systems, piglets are housed with their mothers in farrowing pens, separated from other sows and their progenies. After weaning, usually, at around 28 days of life, suckling pigs are moved prematurely from their mothers and mixed with new pen mates with whom they need to establish new hierarchies (Fels, Hartung and Hoy, 2014). Moreover, piglets experience an abrupt change to a solid diet and are suddenly exposed to a different microbiological environment with a digestive and immune system still immature. In this scenario, weaning is frequently associated with alterations in intestinal function (Lallès *et al.*, 2004, 2007b). Dysbiosis, alteration of the intestinal barrier function, and diarrhoea are common due to the overgrowth of opportunistic pathogens such as E. coli (Lallès *et al.*, 2004).

To improve the adaptation of piglets to weaning, alternative neonatal environments during the lactation period have been proposed. Among them, allowing sows and piglets from different litters to interact from the first day, has been proposed as a novel mean to facilitate the establishment of ubiquitous intestinal microbiota and reduce social stress after weaning (Hessel, Reiners and Van den Weghe, 2006; Ledergerber *et al.*, 2015; Camerlink *et al.*, 2018). In this regard, the existence of a relationship between the housing system and the microbiota of the sows has been demonstrated (Kubasova *et al.*, 2017). Keeping the sows and their litters individualized during the suckling period, might limit the microbiota exchange between adult sows and lead to a poorer microbial exposure for their piglets. This is particularly relevant considering that the intestinal microbiota of newborn animals has been demonstrated to play a fundamental role in the development of intestinal function and the innate immune system (Collado *et al.*, 2012). In humans, the reduced microbial exposure during early childhood

has been associated with the appearance of immune deficiencies and health conditions (Vo *et al.*, 2017).

An enriched environment during the early life of piglets is known to positively influence behavioural development and stress adaptation later in life (Oostindjer *et al.*, 2011), by providing piglets with the appropriate social skills and stress coping capabilities (Brunson *et al.*, 2003). Moreover, favouring social interaction between litters during lactation can improve the social adaptation of the piglet at the time of weaning (Morgan *et al.*, 2014; de Ruyter *et al.*, 2017; Salazar *et al.*, 2018), with a clear decrease in agonistic behaviour between piglets (Hessel, Reiners and Van den Weghe, 2006; Ledergerber *et al.*, 2015; Martin, Ison and Baxter, 2015). The combination of both physical and social enrichment has been reported to have a substantial impact on piglets' socio-cognitive development (Martin, Ison and Baxter, 2015), improving their ability to cope with routine stressors. However, the underlying mechanisms that explain this reduction of stress response remain unknown. It was hypothesized that combining early socialization and environmental enrichment could improve the early intestinal colonization of suckling piglets and also their adaptation to the stress of weaning contributing altogether to reducing its negative impact on intestinal health. Thus, the aim of the present study was to determine the combined effects of early socialization and neonatal enriched environment during lactation on the pattern of caecal microbial colonization, the jejunal gene expression, the serum metabolome, and the intestinal physiology of the piglets before and after weaning and investigate the potential association with the adaptive response at weaning.

7.3. Methods

7.3.1 Animals and study design

This study was performed at an intensive commercial farm, located in Puiggròs, Lleida (Spain). Housing, husbandry, and slaughtering conditions conformed to the European Union Guidelines (Directive 2010/63/EU). Experimental procedures were approved by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (UAB;

permit code CEEAH 1406) and designed in compliance with the ARRIVE guidelines.

A total of 48 Danbred sows were selected and randomly allotted into two groups with a similar distribution of parity times (24 sows per group, 10 primiparous, and 14 multiparous). The sows were confined in farrowing crates from 7 days before the expected parturition date until weaning. They were distributed across six rooms (3 for multiparous and 3 for primiparous), with ten pens per room and a balanced distribution of treatments by pen. Farrowing was synchronized and cross-fostering was performed within 24 hours after parturition in order to standardize the litter size at 13 to 14 piglets. A differential management was carried out between groups, including a control treatment (CTR), with the usual management, and an enriched treatment (ENR) in which two adjacent farrowing pens from the same parity (primiparous or multiparous) were opened to allow piglet socialization 14 days after birth by removing the separation fences. Three different types of enrichment objects (Ko *et al*., 2020) (two hearty chew dog toys, two squid-shaped toys, and two natural ropes per pen) were also placed around the farrowing pens in the ENR groups from birth. Sows were fed twice a day with ad libitum commercial feed and water; piglets were provided with creep feed from two weeks of age and ad libitum water. Piglets were weaned on average at 25 days of age and regrouped randomly based on the treatment group and their body weight into 16 pens (40 piglets/pen (ca. 0.20 m^2/animal); 8 pens per group). Regrouped pens from the ENR treatment had more familiar pen mates (3.9±0.1 familiar pen mates representing 10.3±0.3%) than from the CON (1.7±0.1 familiar pen mates representing 4.7±0.2%). Same management conditions were applied to all piglets after weaning. Weaners were offered ad libitum commercial feed and water.

7.3.2. Blood and intestinal sampling

Two samplings were performed throughout the study, two days before weaning (-2 d), and three days after weaning (+3 d). Fourteen litters (7 litters per treatment) were randomly selected considering a balanced parity within and between treatment groups. From these litters, one medium-weight male piglet per litter was selected for each sampling. The piglets were sedated with

an intramuscular injection containing 20 mg/kg of ketamine (Ketamidor) and 2 mg/kg of xylazine (Xilagesic), and humanely euthanized with an overdose of pentobarbital (Euthasol). Blood samples were collected after opening the abdominal cavity directly from the caudal vena cava and serum was obtained by centrifugation during 15 minutes at 3500 rpm and stored at -80°C. Jejunum tissue samples (1 cm^2) were collected from mid-jejunum (1 m after duodenum), washed thoroughly with PBS, and immediately preserved frozen in 1 mL of RNAlater (Deltalab, Rubí, Spain). Caecal content was also collected directly from the cecum and immediately frozen in dry ice. Tissue and caecal samples were kept at -20°C until further analysis.

For functional studies, ten additional male piglets per experimental group were selected (balanced for parity) and transported to UAB facilities 2 days after weaning. Transport was carried out under sedation by means of xylazine (2.2 mg/kg BW) and Zolazepam-Tiletamine (Zoletil; 8 mg/kg) given intramuscularly. Once in the UAB, piglets were group-housed and offered free access to water and the same commercial feed as were receiving on the farm. One day after (+3d), euthanasia was performed by means of an overdose of pentobarbital, and fresh colon samples were collected and placed in carbogenated Krebs buffer in order to perform functional studies of the intestine. Each functional experiment was conducted with one animal of each group and sampling order was alternated between groups in each experiment. Although at this age, it is not expected that sex had a relevant impact, sampling and functional studies were only performed in male pigs to minimize residual variability.

7.3.3. DNA extraction and 16S rRNA gene sequencing

DNA was extracted from 250 mg of each caecal sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions following the optimization steps. DNA concentration and purity were checked with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For high-throughput sequencing of caecal microbiota, the MiSeq Reagent Kit V2 (500-cycle) (Illumina, San Diego, CA, USA) was used and the V3-V4 region of 16S rRNA

was targeted. All subsequent steps were performed on the MiSeq Illumina instrument.

7.3.4. Sequencing data bioinformatics

The sequence reads generated were processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 software. The paired-end reads were merged using join_paired_ends.py using the fastqjoin.py tool. Quality filtering of reads was performed using split_libraries_fastq.py allowing the maximum unacceptable Phred quality score of Q20. The remaining reads were clustered into OTU using UCLUST by subsampling open-reference OTU picking at 97% identity with bacterial 16S GreenGenes (v. 13_8) reference database. The percent of failure sequences to include in the subsample to cluster de novo was set at 0.1. Sequence alignment and phylogenetic tree building were obtained through UCLUST and FastTree. Chimeric sequences were removed via identify_chimeric_seqs.py with ChimeraSlayer as default. Further filtering was performed using filter_otus_from_otu_table.py setting the minimum total OTU observation count at 0.005% as recommended by Bokulich *et al.* (2013).

7.3.5. RNA extraction and cDNA preparation

Total RNA was obtained from 100 mg of frozen jejunum tissue with the RiboPure kit (Ambion, Foster City, CA, USA) following the manufacturer's protocol. The subsequent steps of RNA extraction and cDNA preparation procedures were carried out as described previously by Reyes-Camacho *et al.* (2020).

7.3.6. Plate design and gene expression study by qPCR

A custom Open-Array plate (Applied Biosystems, Foster City, CA, USA) was designed with a total of 56 selected genes related to intestinal health (Supplementary Table S4). Details regarding genes and primers can be found in previous published work (Reyes-Camacho *et al.*, 2020). Multiplex real-

time qPCRs were performed in a QuantStudio 12K Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA) using TaqMan Open-Array Real-Time PCR Custom Assays. A final cDNA volume of 6 µl from each sample was transferred to 384-well plates and analysed per duplicate. One sample was used as an inter-plate control to check the replication of results from different plates.

Gene expression data analysis was performed as specified by Reyes-Camacho *et al.* (2020).

7.3.7. Nuclear Magnetic Resonance spectroscopy

NMR samples were prepared by mixing 400 µL of serum with 200 µL of a saline buffer 0.9% NaCl (wt/vol) in D_2O directly in the 5 mm NMR tube (Beckonert *et al.*, 2007). NMR experiments were carried out on a Bruker AVANCE II 600 spectrometer operating at 14.1 T (600.13 MHz frequency for ^1H), equipped with a z-axis pulsed-field gradient 5 mm triple channel probe (TBI), BACS 60 automatic sample changer, and a BCU-Xtreme unit for temperature control. The probe temperature was maintained at 300.0 K for all experiments.

Data were collected using the presat PROJECT experiment (Le Guennec, Tayyari and Edison, 2017), a T2-filtered experiment with water signal suppression that attenuates broad signals from high molecular weight. The experiment minimizes J-modulation by using perfect echoes (Aguilar *et al.*, 2012) instead of the standard spin-echoes used in the standard CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958). The overall experimental time for each spectrum was 15 min 17 s; acquired using 256 transients with a recovery delay of 2s and a T2-filter time of 128 ms. Data were collected into 32 K data points and setting a spectral width of 12019.23 Hz which results in an acquisition time of 1.36 s.

7.3.8. ^{1}H-NMR data pre-processing

Spectra were pre-processed prior to statistical analysis using TOPSPIN 3.6 (Bruker BioSpin, Germany). An exponential Fourier Transform using a line broadening factor of 0.3 was used. Lactate signal was used for calibration (1.33 ppm), automatic phase and baseline correction were applied with manual refinement when necessary. Then, spectra were transferred to AMIX 3.9 software where the water region from 4.78 to 4.66 ppm was removed, and normalization to the total area was applied. Finally, a bucket table, containing 250 area regions of 0.04 ppm wide, was extracted to perform statistical analysis on it.

7.3.9. Ussing Chamber experiments

Colon mucosa was stripped from the muscle layers and myenteric plexus, opened along the mesenteric border, and divided into 1.5 cm^2 flat segments, excluding Peyer's patches. The pieces were mounted in Ussing chambers (World Precision Instruments, Aston, UK) as described by Fernández-Blanco *et al.* (2011), with minor changes described below. Strips were bilaterally bathed with 5 mL of carbogenated (95% 02 and 5% C02) and warmed (37±1 °C) Krebs buffer. A voltage step of 1 mV was applied every 30 min and the change in Isc was used to calculate tissue conductance (G) and its reciprocal, transepithelial resistance (TEER), by Ohm's law. Tissues were allowed to stabilize for 30 - 40 min before baseline values of PD, Isc, and G were recorded. Basolateral samples (250 µL, replaced by 250 µL of Krebs buffer) were taken at 30-min intervals during the following 120 min experimental time.

7.3.10. Statistical analysis

The statistical analysis of caecal microbiota was performed in open-source software R v3.5.3. (R Foundation for Statistical Computing, Vienna, Austria). Support for QIIME in R was achieved through the *phyloseq* package (McMurdie and Holmes, 2013). Alpha diversity analysis was performed using *vegan* (Oksanen *et al.*, 2013) and *microbiome* (Lahti *et al.*, 2017) packages

from raw counts (OTU level), including observed species, Chao1, Shannon, and Simpson indices. For beta diversity, measurements were calculated using the Whittaker index (Whittaker, 1960) and the betadisper function of the vegan package using relative abundances. To compare any differential effects an ANOVA analysis was performed for richness and alpha diversity. A non-metric multidimensional scaling (NMDS), an analysis of similarities (ANOSIM), a permutational analysis of variance (PERMANOVA), and unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering, all based on Bray-Curtis distance, were also performed for ordination and beta diversity analysis. Cumulative sum scaling (CSS) (Paulson, Stine, *et al.*, 2013) normalization of raw counts and differential abundance analysis were performed following the *metagenomeSeq* package pipeline (Paulson, Talukder, *et al.*, 2013). Taxa were aggregated at phylum, family and genus level and expressed as compositional data. Relative abundances were used to plot taxon abundances. Statistical significance was assumed at $P<0.05$. The parity number (primiparous/multiparous) was initially included in the different statistical approaches but did not show any significant impact on the data.

For gene expression statistical analysis, RQ values were checked for normalization with R 3.5.3 software, and log2 transformation was applied. Two-way ANOVA was performed, and Benjamini-Hochberg false discovery rate (FDR) was used to adjust P-values. Statistical significance was assumed at $FDR<0.05$.

Concerning NMR statistical analysis, integral data from the bucket table was introduced to SIMCA 14.1 software for multivariate analysis. PCA was applied to the pareto-scaled data. OPLS-DA was performed to identify potential metabolites differences between pre-defined groups. The validity and the degree of overfitting for the OPLS-DA model were made by 100 permutation tests and by cross-validation. To analyse the performance of classification and discrimination of the OPLS-DA model, a ROC plot was performed. NMR spectra area regions (0.04 ppm) contributing to separation between classes in the OPLS-DA model were identified by VIP-plot and S-plot, bucket regions with VIP values ≥ 0.75 and which its spots were located high up or low to the left corner of the S-plot, were chosen.

The integration of gene expression, metagenomics, and metabolites was performed by using the open-source software R v3.6.1 and the LinkHD package (Zingaretti *et al.*, 2019), which was designed to integrate multiple heterogeneous datasets. For this, three data matrices were prepared with raw OTU counts, gene expression, and NMR results. The pipeline established by the program was followed and samples were stratified into clusters. The sample cluster classification derived from the compromised structure was employed to perform the variable selection based on the regression biplot and differential abundance testing.

In Ussing chamber experiments, 2 to 4 colonic strips were studied for each animal and a mean was calculated for each animal. Electrophysiological parameters and FD4 slope were analysed through a t-test (Mann-Whitney test). FD4 kinetics was compared between groups using a two-way ANOVA. Data are expressed as mean ± SEM. Data were considered significant when $P<0.05$. n values represent different experimental animals. Statistical analysis was performed with GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA).

7.4. Results

This work was part of a larger behavioural study that has been published (Ko *et al.*, 2020) and is recommended for complementary information. That study included behavioural observations, registers of skin and ear-biting lesions as indicators of aggression, and salivary stress biomarkers. In that work, it was shown a lasting positive effect of the ENR treatment on piglets' behaviour with an increase in object exploration before weaning and a mitigated weaning stress with reduced aggression from post-weaning until slaughter.

From the forty-eight sows initially included in the study, one control sow and its litter were discarded due to lameness prior to parturition. The average litter size was 14.1 ± 0.1 piglets for both CTR and ENR groups.

The impact of the treatments on the performance of these animals has been also previously reported (Ko *et al.*, 2021). It was found a higher average daily gain (ADG) in ENR piglets during the first 5 days after weaning (23-27d; P =

0.030) compared to CTR piglets. Moreover, a trend for an increased ADG was also observed in ENR piglets during the nursery to the fattening period (d69-79; P= 0.060). When analysing ADG from birth until market weight (90 kg), no differences were found between CTR or ENR piglets although the slaughter age for ENR piglets was lower than for CTR piglets (194.4 ± 1.0 vs. 197.7 ± 1.3 days (P = 0.080)) suggesting a potentially improved long-term growth performance due to enrichment.

7.4.1. Caecal microbiota (16S rRNA gene sequencing)

7.4.1.1. Microbiota structure and biodiversity

On average, 78562 ± 24539 sequences per sample with an average length of 460 bp were obtained from 28 caecal content samples, with no differences between treatments or sampling day (P = 0.742 and 0.424, respectively), despite variability ranging from 40061 to 132201 sequences per sample. The rarefaction curves reached the plateau phase, proving that almost all bacterial species were detected. The sequences were assigned to 976 Operational Taxonomic Units (OTU) based on a 97% sequence similarity. The number of OTU that were common in groups as well as within the groups was evidenced using the Venn diagram, which showed there were 11 and 37 unique OTU in suckling piglets (-2 d) and weaned piglets (+3 d), respectively.

The indexes of Chao1, observed species, Shannon, and Simpson were calculated to estimate alpha diversity. No significant differences were observed between control or enriched piglets (P > 0.1), either when measured for the whole study period or separated by sampling day. However, differences were found as expected related to the weaning process between suckling and weaned piglets, with a significant increase in richness after weaning (P = 0.015, P = 0.017, P = 0.013, P = 0.080; for Chao1, observed species, Shannon and Simpson indices, respectively). Regarding beta diversity, no difference was found related to differential management (P = 0.538), and a tendency was detected between nursing and weaned piglets (P = 0.062) for a higher diversity as animals grow.

The microbial structure of the caecal content and differences in overall beta-diversity were calculated using Anosim, Adonis, and Envfit tests, all of them based on Bray-Curtis distance. For the whole study, no significant differences were detected due to neonatal conditions (CON vs. ENR) ($P = 0.387$, $P = 0.523$ and $P = 0.445$, for Envfit, Anosim and Adonis tests, respectively). However, when analysing differences due to the experimental treatments by sampling day, although no differences were found during the suckling period, a statistical trend for an increased beta-diversity in the control piglets was found after weaning ($P = 0.033$, $P = 0.053$, and $P = 0.058$, for Envfit, Anosim and Adonis tests, respectively). As expected, weaning was associated with a change in the microbiota structure, and significant differences between suckling and weaned piglets were found ($P = 0.0001$, $P = 0.001$, and $P = 0.0001$, for Envfit, Anosim, and Adonis tests, respectively). At last, a cluster dendrogram was constructed using the UPGMA method (**Figure 7.1.**). As a result, a clear clustering is observed between suckling and weaned piglets. However, it is also interesting to note that enriched weaned piglets assimilated more to suckling animals than to the other control weaned piglets.

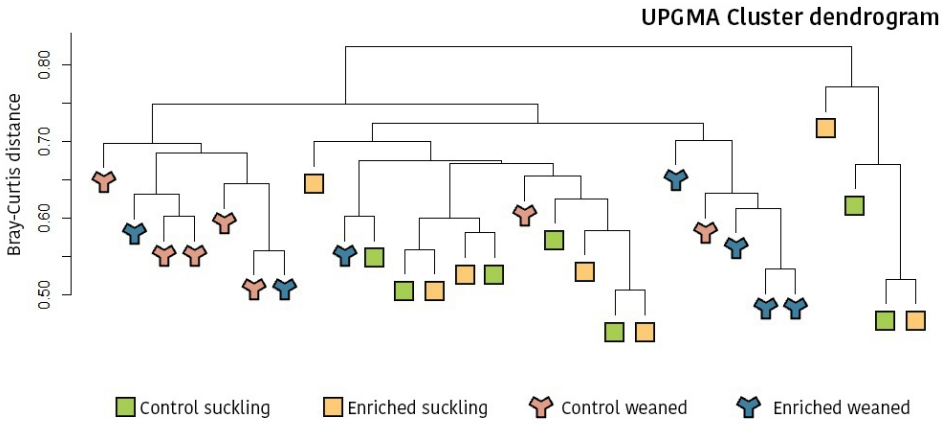


Figure 7.1. Hierarchical grouping dendrogram by UPGMA (average method) based on Bray-Curtis distances and relative OTU counts. A clear clustering is observed between suckling and weaned piglets. Likewise, enriched weaned piglets assimilate more to suckling animals than to the other control weaned piglets. Figure created by using open-source software R v3.5.3. (https://www.r-project.org/foundation/).

7.4.1.2. Taxonomy of caecal microbiota

Firmicutes and Bacteroidetes constituted the two predominant phyla in the caecal microbiota of both suckling and weaned piglets, contributing with 44.6% Firmicutes and 38.2% Bacteroidetes of the relative abundance. Proteobacteria (5.75 and 9.76%, for CTR and ENR, respectively), Spirochaetes (2.90 and 4.08%), and Fusobacteria (3.42 and 2.29%) were considered as predominant phyla as well. Other phyla were represented in less than 1% of relative abundance. Phylum relative counts and their respective P-values for suckling and weaned piglets can be found in **Annex 2: Table S7.1.**

No significant differences were found in any phyla related to the neonatal environment, neither in the lactation period nor after weaning. At the genus level, 69 genera were detected, among which there were 16 genera with a relative abundance higher than 1%, although only 11 of them were above this value in both groups. *Prevotella* was the most predominant genus both in suckling and weaned piglets, with an average relative abundance of 15.4% in the control group and 11.9% in the enriched group. Genus relative counts and the differences observed before and after weaning are shown in **Annex 2: Table S7.2.** *Fusobacterium* and *Bacteroides* showed a decrease in the percentage of total sequences observed between suckling and weaned piglets. Similarly, whereas *Lactobacillus* and *Megasphaera* represented around 2 % in suckling pigs, they did not reach 1% after weaning. Again, concerning the neonatal environment, although some minor differences were seen, they were not statistically relevant in neither suckling nor weaned piglets.

7.4.2. Jejunal gene expression

Jejunum samples from the piglets were collected to analyse the expression of genes related to intestinal health and functionality by using the Open-Array technology. Results are shown in **Table 7.1.**

Table 7.1. Mean DCrt results obtained for the 51 genes that could be quantitatively determined, both during lactation and after weaning for CTR and ENR piglets. Details for the different genes can be found in **Annex 2: Table S7.4.** (BF: Barrier function related genes / EH: Enzymes/Hormones related genes / IR: Immune system related genes / NT: Nutrient Transport related genes / ST: Stress-related gene).

		LACT				WEAN			
Function	**Gene**	**CTR**	**ENR**	**SEM**	**P-value**	**CTR**	**ENR**	**SEM**	**P-value**
BF	*TFF3*	3.69	3.74	0.151	0.9254	3.17	3.21	0.197	0.9799
BF	*OCLN*	7.89	7.67	0.125	0.7494	6.97	7.13	0.075	0.8255
BF	*ZO1*	4.28	4.11	0.132	0.8836	4.06	3.83	0.090	0.7439
BF	*CLDN1*	17.51	16.07	0.550	0.7494	16.48	15.56	0.397	0.7466
BF	*CLDN4*	15.63	15.28	0.188	0.7494	14.64	14.83	0.204	0.8644
BF	*CLDN15*	9.00	8.58	0.234	0.7494	9.49	9.36	0.112	0.8255
BF	*MUC2*	5.37	5.61	0.211	0.8836	4.87	4.64	0.199	0.8255
BF	*MUC13*	2.43	2.30	0.294	0.9194	1.22	1.07	0.092	0.8255
EH	*SI*	3.07	2.74	0.323	0.8836	1.40	1.54	0.199	0.9888
EH	*DAO1*	2.95	2.74	0.373	0.9491	2.37	2.54	0.138	0.8255
EH	*HNMT*	5.49	5.16	0.157	0.9062	4.63	4.64	0.099	0.9799
EH	*ANPEP*	1.43	0.48	0.378	0.7788	0.37	0.33	0.120	0.9799
EH	*IDO1*	9.19	8.65	0.443	0.9062	8.21	7.35	0.318	0.7439
EH	*GCG*	4.95	4.17	0.220	0.6220	4.19	4.01	0.122	0.8255
EH	*CCK*	7.36	7.36	0.165	0.9925	9.15	9.77	0.303	0.8255
EH	*IGF1R*	6.39	6.15	0.171	0.8836	7.93	7.11	0.199	0.7439
EH	*PYY*	7.16	6.61	0.233	0.7494	6.83	7.04	0.194	0.8255
EH	*GPX2*	4.88	5.21	0.327	0.8836	5.65	4.45	0.395	0.7439
EH	*SOD2.m*	4.62	4.63	0.170	0.9925	5.00	4.49	0.133	0.7439
EH	*ALPI*	2.28	1.17	0.545	0.9194	0.54	1.23	0.196	0.7439
IR	*TLR2*	13.57	13.15	0.234	0.7494	13.94	11.69	0.391	**0.0315**
IR	*TLR4*	7.63	7.55	0.179	0.9254	8.07	7.22	0.291	0.7439
IR	*IL1B*	10.79	9.57	0.354	0.6220	9.61	9.00	0.342	0.8255
IR	*IL6*	13.62	12.70	0.373	0.7494	13.20	12.67	0.270	0.8255
IR	*IL10*	10.24	9.91	0.171	0.7494	9.55	9.58	0.173	0.9799
IR	*IL17A*	17.86	17.43	0.469	0.8836	16.58	16.67	0.470	0.9799

IR	*IL22*	12.57	12.10	0.583	0.8836	11.84	11.91	0.324	0.9799
IR	*IFN-γ*	9.86	9.05	0.448	0.7494	9.13	8.85	0.261	0.8255
IR	*TNF-α*	9.62	8.97	0.231	0.7494	9.08	8.56	0.186	0.7439
IR	*TGF-β1*	5.29	5.33	0.101	0.9254	5.22	5.01	0.148	0.8255
IR	*CCL20*	5.88	4.77	0.561	0.7494	4.64	4.79	0.392	0.9799
IR	*CXCL2*	10.23	9.10	0.373	0.7494	10.15	9.65	0.309	0.8255
IR	*IFNGR1*	4.78	4.58	0.214	0.8813	3.29	3.36	0.094	0.9115
IR	*HSP27*	3.35	2.83	0.228	0.7494	2.97	3.35	0.128	0.7439
IR	*HSP70*	3.51	3.24	0.186	0.8698	3.24	3.24	0.075	0.9964
IR	*REG3G*	6.38	6.58	0.502	0.9254	7.02	3.74	0.970	0.7439
IR	*PPARGC1α*	7.28	7.28	0.147	0.9925	7.90	8.09	0.159	0.8255
IR	*FAXDC2*	6.23	4.52	0.490	0.6825	4.05	4.76	0.298	0.7164
IR	*GBP1*	3.19	2.59	0.329	0.8797	2.77	2.63	0.131	0.8255
IR	*IL8*	4.67	4.39	0.245	0.8836	4.78	4.53	0.200	0.8255
NT	*SLC5A1*	2.38	1.97	0.530	0.9491	1.35	1.46	0.252	0.8225
NT	*SLC16A1*	6.44	6.78	0.196	0.9062	7.86	7.65	0.166	0.8255
NT	*SLC7A8*	8.03	5.84	0.621	0.6220	7.73	7.18	0.432	0.8255
NT	*SLC15A1*	5.18	4.00	0.463	0.7788	3.14	3.80	0.264	0.7907
NT	*SLC13A1*	7.55	5.07	0.576	0.6825	4.15	4.62	0.181	0.7798
NT	*SLC11A2*	6.63	6.58	0.092	0.9254	6.99	6.84	0.117	0.8255
NT	*SLC30A1*	5.07	4.48	0.171	0.7326	3.59	3.77	0.136	0.8255
NT	*SLC39A4*	4.54	4.70	0.202	0.8836	5.91	6.29	0.152	0.7439
ST	*CRHR1*	15.00	15.68	0.362	0.7494	15.20	15.14	0.333	0.9799
ST	*NR3C1-Grα*	6.59	6.11	0.123	0.7326	6.51	6.41	0.089	0.8255
ST	*HSD11B1*	8.56	8.98	0.184	0.8486	10.24	9.42	0.270	0.7439

No differences in expression were observed between experimental groups in any of the jejunal genes during lactation. However, the effect of the differential neonatal environment of piglets was observed after weaning for the *TLR2* gene, which showed a higher expression in the control group (13.94 vs. 11.69, P = 0.0315).

7.4.3. Metabolomic response

The representative proton nuclear magnetic resonance (^{1}H-NMR) profiles of serum samples were obtained from the enriched and control groups both during lactation and after weaning (**Annex 2: Figure S7.1.**), and an ampliation of one of them is shown in **Annex 2: Figure S7.2.** A number of endogenous metabolites were assigned from the 1H-NMR spectra, such as LDL/VLDL, leucine, valine, isoleucine, lactate, alanine, adipate, acetate, N-acetyl glycoproteins, O-acetyl glycoproteins, glutamine/glutamate, pyruvate, glutamate, creatine, choline, trimethylamine-N-oxide (TMAO), glucose, creatinine, tyrosine and phenylalanine based on comparing chemical shifts and multiplicities of peaks to public access databases like Human Metabolome Data Base (HMDB) (Wishart *et al.*, 2007) and Biological Magnetic Resonance Data Bank (BMRB) and published studies (Nicholson *et al.*, 1995; Clausen *et al.*, 2011; He *et al.*, 2012).

With the purpose of investigating potential differences in the ^{1}H-NMR metabolites profiles between enriched and control piglets during lactation and after weaning, a non-targeted metabolomics approach was made. Previously, in order to reduce the number of variables, filtering of ^{1}H-NMR bucket table was done by significant differences on Student's t-test between the integrated buck regions of enriched and control piglets (**Annex 2: Table S7.3.**). During lactation, principal components analysis (PCA) was made to evaluate the global metabolic profile of the two groups but did not show a clear clustering. Additionally, an orthogonal projection to latent structures discriminant analysis (OPLS-DA) model was constructed but the model did not show either an acceptable predictive ability (**Annex 2: Table S7.3.**). However, when the same multivariate analysis was made after weaning, a trend of separation between enriched weaned piglets and control weaned piglets along PC1 could be observed indicating that both groups were metabolically differenced. This can be seen in **Figure 7.2a**, which shows a biplot of PCA [$R^2x_{(cum)}$=0.95, $Q^2_{(cum)}$=0.83] from the reduced data where each spot represents the metabolic serum profile for each sample. A supervised OPLS-DA model was constructed to identify any subtle change in serum metabolites due to enrichment, a model with accepted fitness R^2 and predictive ability Q^2 parameters was obtained [$R^2x_{(cum)}$=0.91, $R^2y_{(cum)}$=0.68, $Q^2_{(cum)}$=0.53], that produced good separation into the two clusters along PC1

(**Figure 7.2b**). Moreover, both the cross-model validation (**Annex 2: Figure S7.3b**) and the 100 times permutation test (**Annex 2: Figure S7.3c**) indicated that the constructed OPLS-DA model was positive and valid and confirmed the distinction among enriched and control weaned piglets. Furthermore, the area under the curve (AUC) for the receiver operating characteristic (ROC) plot (**Annex 2: Figure S7.4a**) with a value of 0.92 indicated a robust discrimination power (high sensitivity and specificity) for the OPLS-DA classifier model. An S-plot was constructed to identify the ^{1}H-NMR regions that contributed significantly to the differentiation of enriched and control weaned piglets (**Annex 2: Figure S7.4b**) and the detected regions were screened according to their corresponding variable importance in the projection (VIP) values of the OPLS-DA model. The metabolites corresponding to each one of these shifts were identified as explained previously and were triglycerides and fatty acids, VLDL, unsaturated lipids, LDL, and creatine. All metabolites were significantly higher in the control piglets when compared to the enriched piglets (**Table 7.2.**).

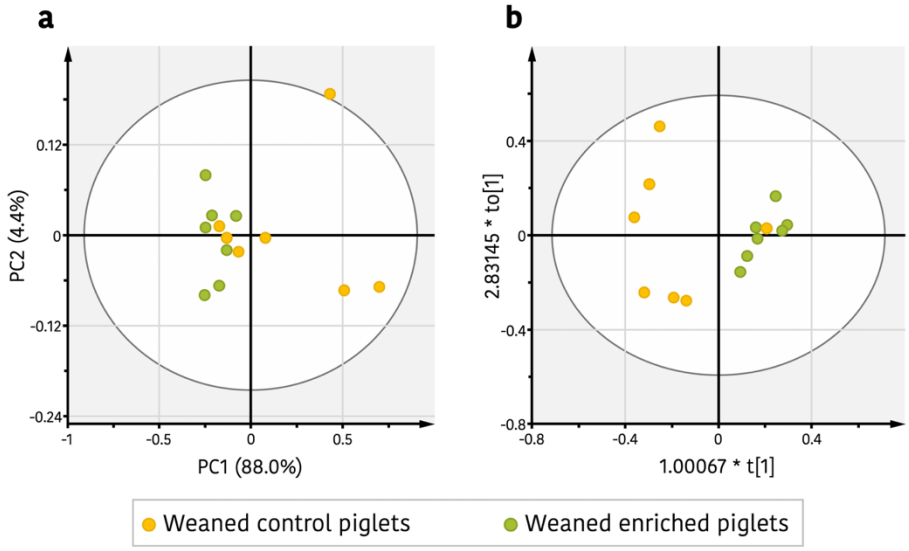


Figure 7.2. Effect of environmental and social enrichment on the serum metabolic profiles of piglets. (**a**) Principal components analysis (PCA) score plot of serum data set from weaned enriched (blue) and weaned control piglets (red). (**b**) Orthogonal partial least squares discrimination analysis (OPLS-DA) score plot between weaned enriched piglets (blue) and control group (red). Figure created by using open-source software R v3.5.3. (https://www.r-project.org/foundation/)

Table 7.2. Key metabolites that differentiate serum of enriched piglets (ENR) from control (CTR) piglets at post-weaning period. P-values were derived from Student's t-test. Variable importance in the projection (VIP) value was derived from OPLS-DA with a threshold of 0.75.

^{1}H Chemical shift ppm (Central bucket point)	Metabolite	Moieties	KEEG IDs	ENR *vs* CTR		
				Fold change CTR/ENR	P-value	VIP
1.30	Lipids[a]	$-(CH_2)_n-$	NA	2.7	0.021	2.09
1.26	Lipids[a]	$-(CH_2)_n-$	NA	2.9	0.024	1.97
0.90	VLDL	$CH_3^*CH_2CH_2C=$	NA	2.6	0.014	1.25
5.30	Unsaturated lipids	$-CH=CH-$	NA	3.6	0.027	1.17
2.02	Unsaturated lipids	$-CH_2^*-CH=CH-$	NA	1.7	0.039	1.12
0.86	LDL	$CH_3^*(CH_2)_n-$	NA	1.9	0.018	1.09
3.94	Creatine	$-CH_2-$	C00300	1.5	0.006	0.82
1.58	Lipids[a]	$-CH_2^*CH_2CO-$	C06104	4.7	0.033	0.77

[a] Triglycerides and fatty acids

VLDL: very low-density lipoprotein; LDL: low-density lipoprotein.

7.4.4. Integration of the omics technologies

Gene expression, caecal microbiota, and metabolomics were integrated by using the open-source software R v3.6.1 and the LinkHD package. As a result, samples were stratified into clusters. The relationship between clusters and the variables responsible for the attained structure was obtained.

During lactation, clusters were not related to the experimental treatments (**Figure 7.3a**), while after weaning, the samples were gathered in two differentiated clusters (**Figure 7.3b**). After implementing variable selection based on regression biplot, those variables that were most associated with the common structure of the data (i.e.: a compromise that maximizes the relationship between the different omics layers) were those related to the microbiota of the caecal content. Although no differential abundance was

observed at the taxonomic level after weaning, LinkHD separated the samples into two differentiated clusters, that were characterized mainly by a greater abundance of *Lactobacillaceae*, *Fusobacteriaceae*, *Alcaligenaceae*, *Bacteroidaceae*, and *Campylobacteraceae*, and a less abundance of *Erysipelotrichaceae* and *Clostridiaceae* in the enriched piglets after weaning compared with the control group.

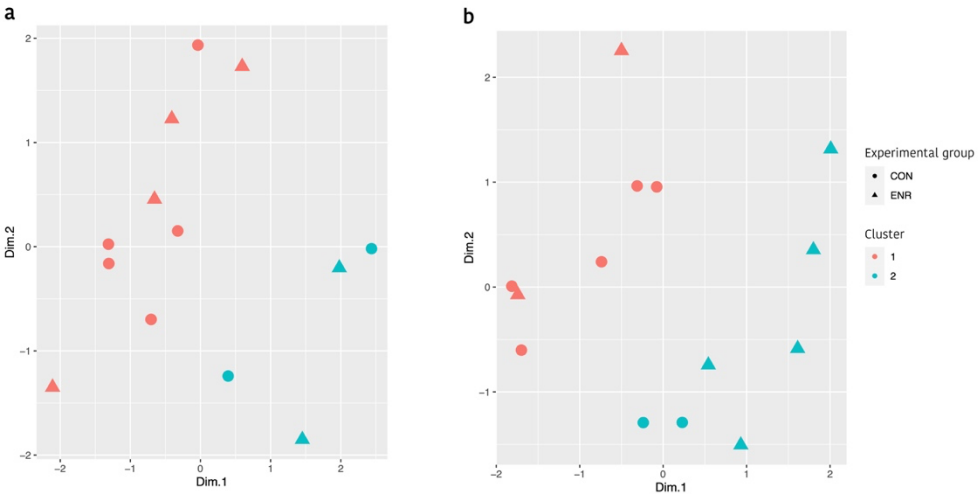


Figure 7.3. Scatterplot of cluster stratification according to LinkHD blind analysis. Figure **a** shows the clustering of the samples during lactation, whereas **b** shows the clustering of the samples after weaning. A similar cluster distribution was observed with the hierarchical grouping dendrogram by using the UPGMA (average method) based on Bray-Curtis distances and relative OTU counts (**Figure 7.1.**). Figures created by using open-source software R v3.5.3. (https://www.r-project.org/foundation/)

Regarding the impact of weaning itself, **Figure 7.1.** shows three clusters that clearly separated piglets in lactation or after weaning. Again, this differential clustering was mostly explained by the changes in the piglet gut microbiota. Confirming the previous approach, the disparity between suckling and weaned piglets was found to be due to reductions in *Fusobacteriaceae*, *Bacteroidaceae*, *Enterobacteriaceae*, and *Lactobacillaceae*; and increases in *Lachnospiraceae* and *Erysipelotrichaceae* after weaning.

7.4.5. Functionality of the large intestine

Different assays with Ussing chambers and colon mucosa were done to evaluate the possible impact of the experimental treatments in intestinal physiology. Accordingly, **Figure 7.4.** shows the assessment of the electrolyte transport across the intestinal epithelium as well as of the barrier integrity in both experimental groups.

At day 3 post-weaning (+3 d), an increase in basal colonic short-circuit current (I_{sc}) and potential difference (PD) was observed in the CTR group compared to the ENR one (**Figure 7.4a and 7.4b**), suggesting a higher level of ion transport across the colonic tissue of control animals (P= 0.029 and P = 0.050, respectively). Basal colonic TEER did not show however differences between groups (control group: 47.4 ± 3.0 $\Omega \cdot cm^2$, enriched group: 48.7 ± 2.7 $\Omega \cdot cm^2$) (**Figure 7.4c**).

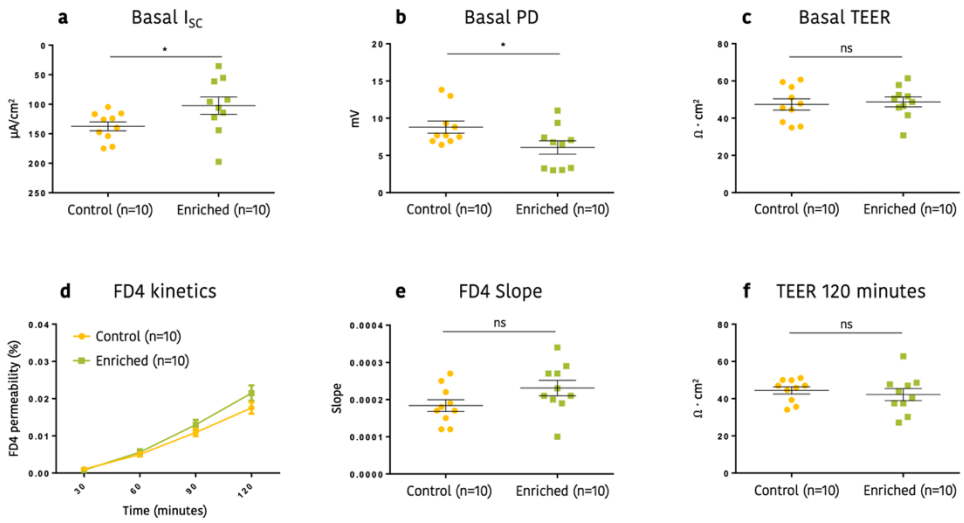


Figure 7.4. Effect of early socialization and environmental enrichment on transepithelial ion transport paracellular permeability to fluorescent tracers at day 3 post-weaning. (**a**) Basal colonic I_{sc}, (**b**) Basal colonic PD, (**c**) Basal colonic TEER, (**d**) FD4 flux, (**e**) FD4 slope, and (**f**) TEER at 120 minutes.

Regarding changes in the paracellular permeability to fluorescent tracers, mucosal to basolateral passage of FD4 across the colon was measured every 30 minutes. Fluorescent tracer passage was time-dependent in both groups (**Figure 7.4d**) but differences between treatments observed after 120 minutes were not significant, being 0.018 ± 0.0016% in the control group and 0.022 ± 0.002% in the enriched group (P = 0.220) (**Figure 7.4d**). In the same line, both groups showed a tendency to a similar slope of the FD4 linear regression (**Figure 7.4e**; P = 0.0900). In order to have another measurement of the colonic barrier integrity, TEER was also measured every 30 minutes, with almost negligible changes between experimental time and treatments. At the last time point, 120 minutes, TEER was around 43 $\Omega \cdot cm^2$ in both groups (control group: 44.44 ± 1.925 $\Omega \cdot cm^2$, 42.23 ± 3.229 $\Omega \cdot cm^2$ enriched group) (**Figure 7.4f**).

7.5. Discussion

The early life is a critical period for the development of intestinal microbiota and immune system in pigs (Zhang, 2014). Differences in the way piglets are reared at the beginning of their lives are therefore expected to affect the gut microbial colonization and the intestinal immune development (Schokker *et al.*, 2014). Moreover, differences in the way piglets are socially exposed and cognitively stimulated during their first days of life, could also determine differences in their abilities to cope with social and environmental challenges at weaning (Ko *et al.*, 2020). In this study, we assessed the potential benefits of a combined early socialization and an enriched environment during lactation on the pattern of caecal microbial colonization, the intestinal functionality, and the metabolomic response of the piglets in order to improve their adaptive response to weaning stress.

In this study, the impact of socializing litters on the intestinal microbial colonization process during lactation appeared to be scarce and we were not able to find differences in the microbiota structure between groups along the suckling period. During this time Bacteroidetes, Firmicutes and Proteobacteria constituted the three predominant phyla in the caecal microbiota of suckling piglets, which is in accordance with previous studies (H. B. Kim *et al.*, 2012; Hu *et al.*, 2016; Chen *et al.*, 2017; Holman *et al.*, 2017; Y. Li, Guo, *et al.*, 2018),

followed by Fusobacteria that also has been described as one predominant phylum during lactation (Pajarillo *et al.*, 2014; Niu *et al.*, 2015; Hu *et al.*, 2016; Chen *et al.*, 2017). At the genus level, although a high individual variability was observed, *Bacteroides* and *Lactobacillus* showed a higher relative abundance, in consonance with similar studies (Frese *et al.*, 2015; Mach *et al.*, 2015; Chen *et al.*, 2017; Gresse *et al.*, 2017), which can be correlated with a milk-oriented microbiome (Frese *et al.*, 2015). Other genera, such as *Fusobacteria* and *Megasphaera* were also abundant in suckling piglets, as stated by Chen *et al.* (2017). However, no significant differences were found for particular taxonomic groups between experimental treatments during this period. According to our results, despite the ENR treatment demonstrated to have an impact on the behaviour of piglets, giving piglets the opportunity to socialize with other litters does not have a remarkable impact on the microbial colonization process. During this period, we neither found significant dissimilarities in gene expression nor the metabolic profiles. However, the piglets in the ENR group spent more time engaging in pen and object exploration and also showed an increased number of aggressions before combining litters (Ko *et al.*, 2020). These results would suggest that observed behavioural changes during lactation do not seem to have a remarkable impact on the metabolomic or genomic response of the animals.

Bian *et al.* (2016) reported that the nursing mother and the breed do not influence gut microbiota as much as the introduction of solid feed and subsequent weaning, which dominated the succession of gut microbiota. Moreover, some studies have reported that the mothers do not represent the most important source of colonization during the early life of piglets (Kubasova *et al.*, 2017). In fact, the composition of the microbiota after birth tended to be similar to microbes present on the slatted floor, sows' milk, and nipple surface, although this composition did not have a long stay during lactation (Xue Chen *et al.*, 2018). Therefore, our results could not confirm our initial hypothesis about the possible impact of early socialization in the gut colonization process but suggest that the changes observed in the microbial community ($P = 0.033$, $P = 0.053$, and $P = 0.058$, for Envfit, Anosim, and Adonis tests, respectively) after weaning are more likely due to the decrease of aggression and stress response registered after weaning in the ENR group (Ko *et al.*, 2020). In this regard, the usual increase of lesions after weaning was more than 3 times greater in CTR compared to ENR pigs. On the other hand,

the post-weaning increase of stress-related markers such as salivary cortisol and chromogranin A was only significant in CTR piglets (Ko *et al.*, 2020) evidencing the clear potential of this enrichment strategy to mitigate weaning stress.

A reduced stress could have led to changes in metabolic response. In this regard, the serum metabolome analysis of piglets by ^{1}H-NMR showed changes that could be compatible with a decrease in the amounts of triglycerides, fatty acids, VLDL/LDL, and creatine in the ENR pigs. Interestingly, these metabolites are directly related to lipid and energy metabolism. The increased concentration of creatine and VLDL suggests an increased energy demand in CTR piglets after weaning, as higher VLDL/LDL may be an adaptive response of the liver to provide energy to peripheral tissues (Wu *et al.*, 2014) and the increase in creatine concentration may suggest an extensive glycogenolysis and glycolysis (de Jonge *et al.*, 2001). Creatine plays a major role in energy metabolism by converting adenosine diphosphate (ADP) and phosphocreatine into adenosine triphosphate (ATP) (Brosnan and Brosnan, 2007). Although there are very few studies in this field, Peeters *et al.* (2006) observed lower levels of creatine-kinase in pigs given straw bedding when compared to control pigs. Straw-enriched pigs also showed a decreased pen interaction that could be thought to require lower energy expenditure. These metabolic changes could be related to the reduced stress evidenced by the lower cortisol salivary levels and the fewer fights registered in the ENR pigs (Ko *et al.*, 2020). An improved metabolic response could be also due to a better adaptation to dry-food intake in the ENR group as suggested by the higher ADG registered in the ENR piglets along with the first 5 days post-weaning ($P = 0.030$). However, as stated by Mkwanazi *et al.* (2019), there is a large gap in research, especially according to the role of environmental enrichment and early socialization on changes in blood metabolites, and further comprehension on this matter is needed.

Regarding the possible impact of early-socialization and environmental enrichment on the microbial colonization after weaning, the high throughput sequencing (HTS) results showed that, in general terms, the diversity and community structure of caecal microbiota were in consonance with the predominant taxa described previously for healthy piglets (Holman *et al.*, 2017). The species richness and diversity of caecal microbiota were increased

in piglets during weaning transition as reported by other studies (Pajarillo *et al.*, 2014; Mach *et al.*, 2015; Niu *et al.*, 2015; Chen *et al.*, 2017). A higher diversity in the gut microbiota has been related to a more mature gut microbiota and is in agreement with the concept of functional redundancy, which supports that additional taxa add redundancy to specific functions, helping the ecosystem to preserve its resilience and stability after environmental stresses (Naeem, Kawabata and Loreau, 1998; Konopka, 2009). The succession of microbial colonization observed in both CTR and ENR piglets also fitted perfectly with the existing literature, and as reported by Bian *et al.* (2016) was caused majorly by the impact of weaning. The abrupt change to a solid cereal-based diet and the withdrawal of milk explain the decrease of genera like *Bacteroides* and *Lactobacillus* and the increase of butyrate-producing genera including *Roseburia*, *Ruminococcus*, and *Lachnospira*, among others, as reported by several other authors (Mach *et al.*, 2015; Chen *et al.*, 2017; Zhao *et al.*, 2018). Altogether, the higher abundance of *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea*, and *Lachnospira* genera in weaned piglets show the microbial evolution of the piglets' gut microbiota to cope with diets rich in complex carbohydrates.

Although no changes in the relative abundance of particular taxonomic groups after weaning related to the neonatal environment were identified, we observed changes in the global structure of caecal microbiota suggesting that early socialization of piglets, and an enriched neonatal environment during lactation, can influence the development of the intestinal microbiota even if we were not able to evidence changes along the suckling period. A similar outcome was obtained by D'Eath (2005), who also studied the effect of early socialization of piglets between 10 and 30 days of age by removing the barriers between two adjacent pens. Their results in piglets also became especially evident after weaning but not during lactation. Therefore, the combined effects of early socialization and environmental enrichment could exert their effects on piglets' microbiota by improving their adaptability to stress and consequently, stress-related intestinal dysfunction.

To assess the impact of physical and social enrichment on intestinal functionality, gene expression analysis was performed. Fifty-six genes, related to gut health, were analysed from jejunum samples by using the Open-Array technology. As previously observed, no differences could be

detected between CTR and ENR piglets during lactation, but only a down-regulation of the *TLR2* gene in the ENR group after weaning. The *TLR2* gene encodes the toll-like receptor 2 (TLR2) protein, a transmembrane receptor that plays a fundamental role in pathogen recognition and activation of innate immunity (Takeda, Kaisho and Akira, 2003). *TLR2* has been shown to recognize conserved molecules derived from microorganisms known as pathogen-associated molecular patterns (PAMPs), activating the signalling pathways to modulate the host's inflammatory response. Many factors have been reported to trigger an upregulation of the *TLR2* gene, such as the presence of pathogenic bacteria such as *Salmonella* and ETEC, weaning or dietary probiotic administration, among others (Meurens *et al.*, 2009; Zhang *et al.*, 2011; Liu *et al.*, 2014; Tao, Xu and Wan, 2015). These weaning-associated factors may disrupt the intestinal barrier, which enables toxins, bacteria, or feed-associated antigens to cross the epithelium (Tao, Xu and Wan, 2015). Although the results of this study confirmed that the integrity of the intestinal barrier was not affected by the experimental treatments, since neither occludin expression nor FD4 permeability was altered, down-regulation of *TLR2* expression in the ENR group and the significant reduction found ion transport across the colonic tissue (Ussing chambers) could suggest a reduction in pathogenic insults in this experimental group.

Ultimately, the integration of gene expression, metabolome and metagenome datasets with LinkHD program was not able to demonstrate any difference between experimental groups in the suckling period. However, after weaning, a differential response between CTR and ENR piglets was evidenced, as samples were distributed into two clusters mostly driven by the experimental treatment. LinkHD package was able to discriminate both clusters (CTR vs ENR) based on differential abundances patterns in particular taxonomic groups. Particularly, greater abundances of *Fusobacteriaceae*, *Alcaligenaceae*, *Bacteroidaceae*, and *Campylobacteraceae* were pointed out by LinkHD in the cluster including most of enriched piglets, and *Lactobacillaceae*, *Erysipelotrichaceae*, and *Clostridiaceae* were found as remarkably lower in this cluster. In general terms, *Lactobacillaceae* and *Bacteroidaceae* families can be classified as favourable bacteria and genera belonging to *Fusobacteriaceae*, *Clostridiaceae*, and *Campylobacteraceae* are commonly associated with intestinal diseases (Songer and Uzal, 2005; Allen-Vercoe and Jobin, 2014; Hermann-Bank *et al.*, 2015; Liu *et al.*, 2015). It is therefore difficult to extract

conclusions from these results, as we cannot objectively associate these changes to a more or less beneficial microbiota. Moreover, to analyse these changes, we need to keep in mind that faecal samples were collected just 3 days after weaning when probably the microbial ecosystem was undergoing an intense evolution from a milk-based diet to a dry feed. From this point of view, conventional microbial indicators for a more or less robust ecosystem should be regarded with precaution considering the complexity of the ecological interactions within the gut microbiota. Further research would be needed to see whether these changes can be associated with a differential disease sensitivity.

7.6. Conclusion

Rearing suckling piglets in an enriched environment and an early piglet socialization program do not seem to have a relevant impact on the microbial colonization pattern during the lactation period and neither on the metabolomic response of the animals. However, this differential neonatal environment results in a divergent response after weaning with differences in the microbial structure and a reduced jejunal expression of the *TLR2* gene in ENR piglets. Changes detected in metabolites like triglycerides, fatty acids, VLDL/LDL, or creatine also suggest an impact on energy metabolism consistent with the previously reported reductions of aggressions in these animals. These results suggest that creating a physically and socially enriched environment in early life can modify caecal microbiota structure and animal response after weaning probably by means of diminishing social stress response.

7.7. Declarations

7.7.1. Data availability

The raw sequencing data employed in this article has been submitted to the NCBI's sequence read archive (https://www.ncbi.nlm.nih.gov/sra); BioProject: PRJNA767391.

The rest of the datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

7.7.2. Ethics declarations

Housing, husbandry, and slaughtering conditions conformed to the European Union Guidelines (Directive 2010/63/EU). Experimental procedures were approved by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (UAB; permit code CEEAH 1406) and designed in compliance with the ARRIVE guidelines.

Chapter 8

General discussion

General discussion

The relevance of perinatal microbial colonization of piglets in the development of the digestive and immune function and in the response capacity after weaning was investigated in the present thesis dissertation. It is well known that the gut microbiota of pigs undergoes extensive shifts between birth and weaning. A "developmental window" of approximately one month (Thompson, Wang and Holmes, 2008) has also been described, during which the host-microbiome is more susceptible to external influences, including the environment (Thompson, Wang and Holmes, 2008; Zhou *et al.*, 2016; Tsai *et al.*, 2018), host diet (Bian *et al.*, 2016; Salcedo *et al.*, 2016; Choudhury *et al.*, 2020), and management strategies (Wen *et al.*, 2021). Moreover, several factors such as age, breed, genetics, and the use of antimicrobials can affect the microbial population in the gut affecting the health and growth of the pigs (Crespo-Piazuelo *et al.*, 2019; X. Wang *et al.*, 2019). Therefore, the modulation of intestinal microbiota towards a more beneficial microbial community in the earliest stages of life can be a key factor in enhancing intestinal health and therefore increasing the growth performance of nursery pigs (Duarte and Kim, 2021).

Several nutritional approaches have been examined to reduce the incidence of health problems around weaning during the last decades (Lallès *et al.*, 2007a). Altogether, the effect of the numerous feed additives promoting health and growth response in pigs can be associated with changes in the intestinal microbiota. However, in the present thesis dissertation, in addition to investigating the intestinal microbial colonization pattern during the first days of the piglet and the possible effect of environmental variation ("farm" effect), we focused on two intervention strategies that play a major role in modulating the gut microbiota of suckling piglets: the management practices (environmental enrichment and early socialization) and the use of probiotics in the sows during gestation and lactation periods.

In order to provide greater clarity, the general discussion has been structured in the following four sections:

1. Gut microbial colonization from birth to weaning and factors capable of modifying this pattern.
2. Weaning has a remarkable impact on the piglet gut ecosystem, intestinal function, and metabolic response.
3. What happens during the first days of life can reshape the future development of the animal.
4. It is possible to modulate the development of piglet microbiota by early intervention strategies.

8.1. Gut microbial colonization from birth to weaning and factors capable of modifying this pattern

The process of microbial colonization of the intestine after birth plays a crucial role in the development of the neonatal immune system of mammals with implications throughout their lives (Hansen *et al.*, 2012). Adequate colonization maintains the homeostasis of the immune system and directly influences the probability of the development of pathologies in the future, such as, for example, diarrhea from the post-weaning syndrome. Therefore, abnormal microbial exposures, such as decreased diversity or delayed colonization, can negatively affect the development of a robust and mature intestine (Houghteling and Walker, 2014). In the first case, insufficient diversity can weaken the beneficial immunomodulatory signals produced by the activation of the immune system by bacteria. On the other hand, the timing of colonization is important because the immune system receives its microbial programming during the early neonatal period. Delayed colonization implies a longer period after birth with fewer microbes and less diversity so that the establishment of intestinal anaerobes associated with a mature intestine occurs later.

As reviewed in **Chapter 2**, immediately after birth, the piglet's gastrointestinal tract is colonized by bacteria present in the environment (Konstantinov *et al.*,

2006; Jost *et al.*, 2014; Xue Chen *et al.*, 2018). During the first hours of life, the piglet comes into contact with the birth canal, the maternal feces and nipples, the sow's milk, the farrowing box, its littermates, and, if applicable, with possible toys or environmental enrichment items. Therefore, all those bacteria present in each of these niches are potential colonizers of the newborn's intestine. However, it is worth mentioning again those studies that have recently shown that intestinal bacterial colonization of animals does not begin at birth, but that there is probably already some previous colonization in the maternal uterus or by placental transfer (Jiménez *et al.*, 2008; Mshvildadze *et al.*, 2010; Aagaard *et al.*, 2014).

Regardless of the origin of the initial colonization, there is some unanimity in the bacterial groups that have been identified as the first intestinal colonizers. Some studies have identified the facultative anaerobic bacteria, such as *Enterobacteriaceae*, *Enterococcocaceae*, and *Streptococcaceae*, as the first gut colonizers, followed by a gradual replacement by obligate anaerobic bacteria, such as *Clostridiaceae* (Inoue *et al.*, 2005; Patil, Gooneratne and Ju, 2020). Likewise, throughout this succession of organisms, the microbiota increases in diversity (Koenig *et al.*, 2011; Jakobsson *et al.*, 2014). Although the periods in which these changes occur do not coincide between studies, as some establish periods of 6 hours, 2 days, or 5 days, they are in agreement with the highest abundances after birth, belonging to the *Enterobacteriaceae* and *Clostridiaceae* families (Inoue *et al.*, 2005; Petri, Hill and Van Kessel, 2010; Patil, Gooneratne and Ju, 2020). Similarly, an increase in the *Lactobacillaceae* family has also been observed from three days of life (Inoue *et al.*, 2005; Konstantinov *et al.*, 2006; Petri, Hill and Van Kessel, 2010), associated in some cases with a decrease in *Streptococcaceae* bacteria (Petri, Hill and Van Kessel, 2010). However, it is well known that this initial colonization sequence is highly variable between individuals during the first two weeks of life, indicating that there is considerable randomness to the process of acquiring microbes (Thompson, Wang and Holmes, 2008).

In this project, microbiota analyzes of feces and cecal content of piglets were carried out at different ages, which has allowed us to analyze the temporal development of the microbiota under commercial conditions in a whole set of farms. Although some studies focused on the period around weaning (**Chapters 5, 6, and 7**), in **Chapter 4** an intensive sampling was carried out on

days 2, 7, 14, and 21 of the piglets' life, offering us an image of this colonization sequence in commercial practice on two different farms. Likewise, in the second trial of **Chapter 4**, feces were sampled from two-day-old piglets in 4 different farms, once again offering a broader vision of the possible "farm" effect in the first microbial gut colonization. **Tables 8.1. and 8.2.** show the results obtained in the six farms from the two-day-old piglets at phylum and family and genus level, respectively.

The gastrointestinal microbiome of the pig is highly diverse (Isaacson and Kim, 2012). However, the porcine gut microbiota has been found to contain at least 7 identifiable bacterial phyla and at least 171 genera of bacteria (Kim *et al.*, 2011; Isaacson and Kim, 2012), although much of the diversity found at the genus level still remains unclassified. Firmicutes and Bacteroidetes are known to be the predominant phyla, regardless of age (Kim *et al.*, 2011; Chen *et al.*, 2017). Moreover, aging has been associated with the increased abundance of Firmicutes and the decreased abundance of Proteobacteria, Fusobacteria, and Actinobacteria (Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017).

Table 8.1. Relative abundances (RAB) of the main phyla (RAB greater than 0.1%) and families (RAB greater than 0.5%) in the two-day-old piglets from **Chapter 4**, ordered from highest to lowest abundance in relation to the mean. The mean of each taxonomic group with its standard deviation is also indicated in the last column of the table. The relative abundance of the rest of the taxonomic groups not included in the table have been grouped and are shown in the label "Other" with its respective percentage.

	Alpha	Bravo	Charlie	Delta	Echo	Foxtrot	Global mean
Phylum							
Proteobacteria	24.8	34.5	49.1	51.0	56.0	76.0	48.6 ± 17.76
Firmicutes	39.7	34.3	35.4	39.8	26.9	20.3	32.7 ± 7.73
Bacteroidetes	12.1	16.5	7.24	3.26	15.2	3.02	9.55 ± 5.886
Fusobacteria	22.5	14.2	6.33	3.18	0.92	0.37	7.91 ± 8.745
Actinobacteria	0.36	0.15	0.94	0.87	0.51	0.20	0.51 ± 0.336
Other (< 0.1%)	0.02	0.22	0.16	0.19	0.06	0.01	0.11 ± 0.091
Family							
Enterobacteriaceae	17.9	31.3	37.3	36.0	43.2	71.8	39.6 ± 17.93
Clostridiaceae	22.4	14.7	24.9	24.8	13.6	16.3	19.4 ± 5.16
Fusobacteriaceae	22.5	14.2	6.30	3.16	0.92	0.37	7.89 ± 8.735
Bacteroidaceae	7.43	13.8	4.03	1.73	14.3	2.83	7.36 ± 5.541
Lachnospiraceae	7.59	8.14	1.67	4.51	3.92	1.33	4.53 ± 2.872
Streptococcaceae	4.74	2.08	3.22	4.40	3.19	1.49	3.19 ± 1.264
Pasteurellaceae	6.07	2.94	1.03	2.33	3.36	2.55	3.05 ± 1.678
Alcaligenaceae	0.08	0.02	6.93	7.57	2.49	0.56	2.94 ± 3.462
Prevotellaceae	4.23	2.02	1.55	0.67	0.16	0.04	1.45 ± 1.568
Veillonellaceae	1.24	2.69	0.56	1.27	1.82	0.19	1.29 ± 0.895
Burkholderiaceae	0.09	0.00	0.23	1.14	4.47	0.54	1.08 ± 1.713
Lactobacillaceae	1.00	2.94	0.79	0.61	0.45	0.23	1.00 ± 0.985
Moraxellaceae	0.17	0.06	1.14	2.84	0.90	0.11	0.87 ± 1.064
Peptostreptococcaceae	0.42	0.26	0.67	0.40	1.47	0.16	0.56 ± 0.479
Enterococcaceae	0.32	1.39	0.35	0.80	0.14	0.10	0.52 ± 0.494
Sutterellaceae	0.36	0.13	1.47	0.35	0.07	0.21	0.43 ± 0.52
Oscillospiraceae	0.33	0.31	0.53	0.72	0.30	0.03	0.37 ± 0.233
Acidaminococcaceae	0.18	0.77	0.34	0.21	0.05	0.07	0.27 ± 0.267
Butyricicoccaceae	0.87	0.19	0.10	0.08	0.14	0.07	0.24 ± 0.311
Other (< 0.5%)	2.08	2.03	6.86	6.45	5.06	1.00	3.92 ± 2.523

Table 8.2. Relative abundances of the main genera (RAB greater than 1%) in the two-day-old piglets from **Chapter 4**, ordered from highest to lowest abundance in relation to the mean. The mean of each taxonomic group with its standard deviation is also indicated in the last column of the table. The relative abundance of the rest of the taxonomic groups not included in the table have been grouped and are shown in the label "Other" with its respective percentage.

	Alpha	Bravo	Charlie	Delta	Echo	Foxtrot	Global mean
Escherichia-Shigella	10.4	10.2	37.1	35.6	42.5	71.7	34.6±22.91
Clostridium sensu stricto 1	5.45	2.64	24.7	24.6	13.2	16.1	14.4±9.30
Bacteroides	11.3	12.5	4.03	1.73	14.3	2.83	7.78±5.517
Fusobacterium	9.76	10.3	6.26	3.13	0.91	0.37	5.13±4.340
Alcaligenes	0.00	0.02	6.92	7.56	2.47	0.55	2.92±3.474
Streptococcus	1.13	1.64	3.20	4.35	3.18	1.48	2.49±1.269
Lactobacillus	1.66	6.76	0.79	0.61	0.45	0.23	1.75±2.504
Actinobacillus	0.84	1.60	0.85	1.76	2.54	2.33	1.65±0.715
Prevotella	0.63	5.05	1.02	0.31	0.03	0.02	1.18±1.934
UCG-002	4.75	1.26	0.22	0.28	0.07	0.01	1.10±1.848
Ralstonia	0.09	0.00	0.23	1.14	4.47	0.54	1.08±1.712
Lachnospiraceae UCG-004	3.56	0.98	0.16	0.29	0.17	0.06	0.87±1.361
Veillonella	0.37	1.06	0.43	1.25	1.78	0.19	0.84±0.620
Phascolarctobacterium	1.71	2.58	0.34	0.20	0.05	0.07	0.83±1.066
Enterococcus	2.70	0.63	0.35	0.80	0.14	0.10	0.79±0.977
UCG-005	2.71	1.41	0.15	0.12	0.12	0.01	0.75±1.093
Dorea	0.97	1.11	0.20	0.65	0.97	0.07	0.66±0.434
Lachnoclostridium	0.67	1.98	0.16	0.50	0.52	0.15	0.66±0.678
Rikenellaceae RC9 gut group	1.98	1.35	0.22	0.22	0.04	0.01	0.64±0.826
Acinetobacter	0.00	0.01	0.79	2.62	0.21	0.03	0.61±1.032
Campylobacter	2.32	1.07	0.01	0.00	0.01	0.00	0.57±0.957
Christensenellaceae R-7 group	2.04	0.59	0.15	0.20	0.06	0.01	0.51±0.777
Sutterella	0.58	0.25	1.47	0.35	0.05	0.21	0.48±0.513
Treponema	0.94	1.38	0.08	0.06	0.04	0.00	0.42±0.595
Lachnospiraceae NK4A136 group	2.04	0.19	0.02	0.04	0.01	0.00	0.38±0.813

NK4A214 group	0.83	1.12	0.07	0.13	0.08	0.00	0.37±0.478
Subdoligranulum	0.28	1.40	0.09	0.19	0.04	0.00	0.33±0.532
Prevotellaceae NK3B31 group	0.15	1.17	0.20	0.12	0.03	0.01	0.28±0.443
Sphaerochaeta	1.13	0.18	0.01	0.03	0.01	0.00	0.23±0.446
Other (< 1%)	29.0	29.6	9.8	11.1	11.6	2.94	15.7±11.010

In our experimental trials, and in general terms, the most abundant phyla in two-day-old piglets in all samples were Proteobacteria (48.6%) and Firmicutes (32.7%), followed by Bacteroidetes (9.55%) and Fusobacteria (7.91%). The rest of the phyla were presented with lower abundance (<1%). However, during the first days of life, great changes are observed in the percentages that these taxonomic groups represent. As shown in **Tables 8.1. and 8.2.**, and in line with the high variability expected at this early age (Thompson, Wang and Holmes, 2008), there is great variability among the six farms noticeable even from the phylum level. Thus, in Alpha and Bravo farms there are large initial abundances of Fusobacteria, while this difference seems to be occupied by Proteobacteria in the rest of the farms. Similarly, the phylum Firmicutes even doubles its relative abundance in the Alpha and Delta farms in comparison with Foxtrot farm and drastic changes are also observed in the Bacteroidetes group. Similar results are observed at the family level, with large differences among farms and piglets of the same age. While in Charlie, Delta, Echo, and Foxtrot the predominant family is *Enterobacteriaceae*, in Alpha and Bravo the *Fusobacteriaceae* family also plays an important role, becoming the predominant family in the case of the Alpha farm. Concerning clostridia, *Clostridicaeae* is observed in all farms with an average relative abundance of around 20%, with quite a similarity between different farms. Therefore, it is true that the predominant groups in two-day-old piglets are Enterobacteriaceae and *Clostridiaceae*, however, there is some controversy with the *Fusobacteriaceae* family. The high abundance of Fusobacteria observed in Alpha and Bravo farms (Trial 1) during the first days of life has also been reported by several other studies (Pajarillo *et al.*, 2014; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Ke *et al.*, 2019; Choudhury *et al.*, 2020). However, some studies have not reported the presence of this bacterial group at all (Frese *et al.*, 2015; Guevarra *et al.*, 2018). This aligns with the results observed in Trial 2, where much lower abundances

than those previously reported in Trial 1 were detected. Such differences in taxonomic abundance could, to some extent, be due to various factors such as the study design and conditions, pig genetics, environmental conditions, not only between farms but also the time of the year in which sampling was performed, the sampling procedures, sample processing, and sequence analysis methods, etc. It should be noted that the samplings were carried out using the same technique (rectal swab) and analyzed with the same DNA extraction kit and Illumina MiSeq laboratory, with the farm and the sampling date being the main distinguishing factors among samples. Therefore, the different environments and sampling dates among experiments could explain this disparity.

At the genus level (**Table 8.2.**), a curious distribution is observed. In those farms that obtained higher abundances of Fusobacteria (Alpha and Bravo), there seems to be a greater abundance of genera that represent less than 1% of the relative abundance (close to 30%), while in the rest of the farms the predominant genera (> 1%) seem to have greater importance and leave less margin for the rest of the less abundant genera (an average of 8.9%). From this observation, a greater abundance of the *Fusobacterium* genus in the early days could be an indicator of a more diverse early colonization, as it would, in turn, allow the presence of a greater variety of genera, although in smaller relative abundances. This greater evenness between minor bacterial groups could be considered beneficial since it could represent an initial ecosystem with an increased capacity for adaptation. However, despite these results, *Fusobacterium* has been typically associated with diarrhea and gut inflammation (Hermann-Bank *et al.*, 2015; Huang *et al.*, 2019; Tan *et al.*, 2019) and, therefore, large abundances of this genus are not associated with beneficial effects on piglets. Nevertheless, it should be noted that the lower relative abundances of *Fusobacterium* in the Echo and Foxtrot farms could also be due to the antibiotic treatment that the mothers receive in their feed. As a matter of fact, *Fusobacterium* has been reported to reduce significantly after an antibiotic treatment (Hermann-Bank *et al.*, 2015). As for the other predominant genera, on the Charlie, Delta, Echo, and Foxtrot farms a very high abundance of *Escherichia-Shigella* is observed (around 40%, although reaching up to 71% on the Foxtrot farm). In the Alpha and Bravo farms, the *Escherichia-Shigella*, *Bacteroides*, and *Fusobacterium* genera share similar abundances, around 10%, without any of them standing out excessively. The

rest of the genera vary considerably between farms, again demonstrating the great individual variability. Therefore, the results of the experimental trials in **Chapter 4** pinpointed the early intestinal colonizers belonging to *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1*, and *Fusobacterium* genera. This is in accordance with Petri, Hill and Van Kessel (2010), who reported the genera *Escherichia*, *Clostridium*, *Fusobacterium*, *Streptococcus*, and *Enterococcus* to be the earliest colonizers of the pig gut, between birth and 2 days.

Regarding the temporal evolution of the microbial colonization sequence, the same 20 piglets belonging to two different farms (Alpha and Bravo) were monitored during four time-points at days 2, 7, 14, and 21 of life (**Figure 8.1.**). Species richness and microbiota diversity gradually increased in piglets with age in accordance with several previous studies (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017; Ke *et al.*, 2019; X. Wang *et al.*, 2019; Choudhury *et al.*, 2020), which describe a continuous increase in the alpha diversity of the intestinal microbiota from birth to weaning. Greater diversity in the gut microbiota has been related to more mature gut microbiota and is in accordance with the concept of functional redundancy, which supports that additional taxa add redundancy to specific functions, helping the ecosystem to preserve its resilience and stability after an environmental stress (Naeem, Kawabata and Loreau, 1998; Konopka, 2009; Holman and Chénier, 2014; Chen *et al.*, 2017).

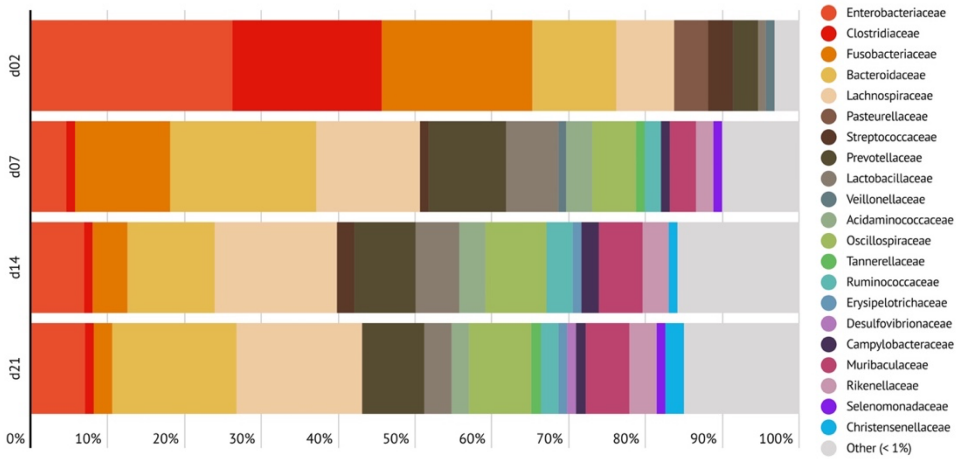


Figure 8.1. Stacked bar plot of the relative abundances of the main bacterial families (>1%) present in the feces of the same piglets (n=20) sampled along different time-points during lactation.

As previously exposed, *Fusobacteriaceae*, *Clostridiaceae*, and *Enterobacteriaceae* are the most predominant families during the first days of life (50-60% of the RAB in 2-day-old piglets). Over the weeks a progressive decrease in the relative abundances of *Clostridiaceae*, *Enterobacteriaceae*, *Fusobacteriaceae*, *Pasteurellaceae*, and *Streptococcaceae* was observed (**Figure 8.1.**). In return, families such as *Campylobacteraceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, and *Prevotellaceae* gradually increased with age. Other families such as *Lachnospiraceae*, *Lactobacillaceae*, and *Veillonellaceae*, showed greater variability, increasing during the first weeks of life to descend again before weaning. Similar initial abundances, as well as their drastic decrease with the age of the piglets, was also described by other authors (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Niu *et al.*, 2015; Hu *et al.*, 2016; Chen *et al.*, 2017; Xue Chen *et al.*, 2018). For instance, in line with other authors, it was observed that by day 7 of life the *Lactobacillaceae* family had increased considerably (Inoue *et al.*, 2005; Konstantinov *et al.*, 2006; Petri, Hill and Van Kessel, 2010), while the *Streptococcaceae* family had descended (Petri, Hill and Van Kessel, 2010). Moreover, during the first week of life, there is a drastic decline of *Clostridicaceae*, in particular, *Clostridium* and *Escherichia-Shigella*. Decreases in the abundances of *Clostridium*, *Fusobacterium*, and *Escherichia-Shigella* with the age of the piglets have also been observed by several other authors (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Mach *et al.*, 2015; Chen *et al.*, 2017; Luise, Le Sciellour, *et al.*, 2021). This decrease has been associated with the increasing activity of IgA (Inoue *et al.*, 2005). Additionally, and in line with other studies, families such as *Ruminococcaceae* and *Lachnospiraceae* increased significantly with age (Frese *et al.*, 2015; Chen *et al.*, 2017; Y. Li, Guo, *et al.*, 2018), taking advantage of the ecological niche left by the previous families. Actually, microorganisms belonging to the *Lachospiraceae* genera, such as *Lachnospira*, *Coprococcus*, and *Dorea*, have been reported to begin to emerge after weaning (Y. Li, Guo, *et al.*, 2018), although a decreased abundance of *Lachnospira* after weaning was reported by Frese *et al.* (2015). The genera belonging to *Lachnospiraceae* and *Ruminococcaceae* families are adapted to metabolize a wide range of complex oligosaccharides and polysaccharides while producing short-chain fatty acids. Altogether, the higher abundance of propionate- and butyrate-producing genera in older piglets, adapted to digest resistant starches and dietary fibres, reflect the evolution and adaptation of the intestinal ecosystem towards a solid diet.

During lactation and the weaning transition, the intestinal microbiome of the piglet rapidly undergoes a remarkable shift from the initial microbial groups which are present during the first days of life to the establishment of an adult-like microbial community, experiencing in between a period of changing microbial successions (Isaacson and Kim, 2012; Pajarillo *et al.*, 2014; Guevarra *et al.*, 2019). During early lactation, *Bacteroides* and *Lactobacillus* genera also acquire greater relative importance. Both genera have been correlated with a milk-oriented microbiome (Frese *et al.*, 2015). *Bacteroides* have been reported to use a wide range of both milk oligosaccharides and host-derived glycans (Marcobal *et al.*, 2010), whereas *Lactobacillus* is a well-known lactate producer by consuming simple milk sugars such as lactose (Schwab and Gänzle, 2011) and has been labeled as a major player in the establishment and the maintenance of the bacterial homeostasis after birth (Konstantinov *et al.*, 2006). Therefore, the outcome obtained in the present thesis dissertation is in agreement with those observations previously described: a dynamic, age-related microbiota maturation with a variety of microbial groups associated with different time-points, demonstrating that age, as well as weaning, are the driving factors in influencing microbiota development.

When considering the development of the gut microbiome in mammals, an understanding of the perinatal environmental factors is imperative. Mammals are routinely inoculated as they pass through the birth canal (Houghteling and Walker, 2014), along which they also encounter maternal intestinal bacteria (Makino *et al.*, 2013). Piglets also receive microbiota from the maternity boxes, their mothers' nipples, and breast milk, sometimes with a greater impact on the development of the newborn's intestinal microbiota than the maternal feces itself (Xue Chen *et al.*, 2018). Therefore, it is likely that the microbiome of a newborn commercial piglet is largely dependent on the sow and the farm environment. However, relatively little is known about the impact of general farm practices.

The process of the gut microbial colonization of piglets followed a similar evolution pattern between different farms, both in terms of species richness and microbiota diversity, which gradually increased in piglets with age, and in relation to the taxonomic groups involved in this process, despite the high individual variability observed at the earlier stages. This indicates that there is a pattern in the establishment of the initial microbiota, which evolves from

a microbiome oriented to the degradation of milk carbohydrates towards a more complex one, oriented to the fermentation of complex carbohydrates, reflecting the evolution and adaptation of the intestinal ecosystem towards a solid diet. Moreover, throughout this thesis, experimental tests have been carried out with many factors that have given us some knowledge about the impact of the environment and rearing conditions of the piglet on its intestinal microbiota. In this way, we have observed how the use of antibiotics in the sows' diet, the injection of antibiotics after birth in "antibiotic-free" programs, the use of rehydrating-acidifying solutions in piglets, and the sanitary level of the exploitation, among others, can affect the sequence of colonization from birth to weaning. Therefore, the intestinal microbiota despite the common general pattern has been found to be susceptible to external changes.

In particular, the possible "farm" or environmental effect was investigated, in order to identify possible beneficial management practices for the intestinal bacterial colonization process. In our first trial, Alpha was considered a high-standard farm, involved in an antibiotic reduction program, with a low incidence of pathologies, whereas Bravo frequently coursed episodes of pleuropneumonia (*Actinobacillus pleuropneumonia*, APP) and swine dysentery (*Brachyspira hyodysenteriae*). In Alpha, piglets received an intramuscular dose of amoxicillin and an oral rehydrating and acidifying solution the first week, whereas in Bravo piglets only received an oral rehydrating solution. Despite these differences and what was initially expected, the piglets from the Alpha farm suffered from post-weaning diarrhea, while the piglets from the Bravo farm did not. The weaning transition is characterized by a shift in the microbial population where pathogenic bacteria increase in numbers (Gresse *et al*., 2017). In this particular case, the lactating piglets from the Bravo farm showed higher abundances of *Lactobacillus*, *Prevotella*, *Roseburia*, and lower abundances of *Fusobacterium* and *Campylobacter* compared to the Alpha farm. Therefore, the outcome obtained can be explained by the microbial shifts observed. For example, among the "negative" bacteria, we can highlight *Fusobacterium*, positively correlated with neonatal diarrhea in piglets (Cheng *et al*., 2018). Moreover, higher relative abundances of *Sutterella*, *Campylobacter*, and *Fusobacterium* have been associated with increased diarrhea incidence (Q. Yang *et al*., 2017; Cheng *et al*., 2018). On the other hand, among the "positive" bacteria, it is worth highlighting the genera *Bacillus*, *Bifidobacterium*, *Lactobacillus*,

Prevotella, and *Roseburia*, all of them related to better growth performances (Mach *et al*., 2015; McCormack *et al*., 2017; Wang *et al*., 2018; Zhang *et al*., 2019; Gaukroger *et al*., 2020; Karasova *et al*., 2021). Some, such as *Lactobacillus*, also have anti-inflammatory and antipathogenic activity against pathogenic bacteria such as *E. coli*.

In our second trial, up to four different farms were selected. A study of the environmental effect or "farm" factor was carried out in which the same piglets were analyzed on days 2 and 21 of life. In this study, the information provided by the initial management of the piglet is essential. In this sense, all the farms except Foxtrot supplied an oral rehydrating solution in the water during the first week of the life of the piglets. Interestingly, a decreased alpha diversity at day 2 was observed in Foxtrot, with marked increased abundances of *Enterobacteriaceae* both at 2 and 21 days of life. Moreover, at 21 days of life, significant changes in other microbial groups were also observed in this farm, such as a greater abundance of *Enterococcaceae* and a lower relative abundance of *Lachnospiraceae* and *Fusobacteriaceae*. Unfortunately, a subsequent follow-up of the piglets was not carried out and these changes could not be related to the subsequent performance or the incidence of post-weaning diarrhea. However, these results indicate, again, that small management changes during the first days of life are capable of generating later changes in the intestinal microbiota of piglets.

The studies of the impact of the environment or rearing farm on the modulation of the intestinal microbiota are scarce and a deeper interpretation of the differences among farms, animals, and production times is still needed. Recently, Lührmann *et al.* (2021) studied the fecal microbiota in 20 different commercial pig farms under practical conditions. In accordance with our results, the shift in microbiota composition in sows and piglets followed the general trend that has been observed in other microbiota studies on this topic (Mach *et al*., 2015; Holman *et al*., 2017; X. Wang *et al*., 2019). Moreover, the animal microbiota of the different farms showed some degree of variability, as practical conditions such as environment, antibiotic use, feeding, and management have an impact on the microbiota. However, they concluded that the comparability of microbiome studies is known to be very low and few distinguishing aspects could be obtained from the study (Poussin *et al*., 2018; Lührmann *et al*., 2021). In another study, H. Yang, Xiao, *et al*., (2018) also

assessed the impact of environmental factors on gut microbial composition among pigs raised in 3 different farms. As a result, little influence was observed of the rearing environment on the structure of the gut microbiota. Other authors have evaluated the effect of other environmental factors, such as excessive hygiene conditions (Schmidt *et al.*, 2011), with a subtle impact on gut microbiota. Therefore, although external factors such as the rearing farm can affect the early colonization of intestinal microbiota and the development of the immune system in neonates (Inman *et al.*, 2010; Bian *et al.*, 2016), it is really the dietary intervention and the administration of antibiotics the external factors that produce the most changes in the microbiota of the piglet. For instance, the early-life antibiotic treatment (day 4 after birth) and routine animal handling has been reported to produce long-lasting effects on the gut system, both in gene expression as well as on microbiota composition (Schokker *et al.*, 2015). Moreover, several authors have stated that maternal antibiotic treatment and early antibiotic administration affect the development of intestinal microbiota of the piglets, along with piglet mucosal tissue gene expression (Janczyk *et al.*, 2007; Bosi *et al.*, 2011; Looft *et al.*, 2012, 2014; Schokker *et al.*, 2014; Holman and Chénier, 2015; de Greeff *et al.*, 2020; Xu *et al.*, 2020). Therefore, these findings reinforce the approach that the early phase of life is critical for the development of intestinal microbiota and the immune system.

8.2. Weaning has a remarkable impact on the piglet gut ecosystem, intestinal function, and metabolic response

In commercial pig husbandry, weaning is an abrupt event comprising significant social, environmental, and nutritional changes. As a consequence of the high stress suffered by the pig, intestinal and immune system dysfunctions are frequent during the weaning transition, resulting in reduced pig health, growth, and feed intake, particularly during the first week after weaning. One of the main stressors is the dietary shift from sow milk to solid-feed-based diets, which poses a challenge to piglets and their intestinal microbiota during early-life development. This shift generally results in a critical period of low voluntary feed intake which leads to the alteration of gut integrity and the appearance of gut-associated disorders (Lallès *et al.*, 2004).

Piglets show an adaptive response to solid plant-based diets approximately after 1–2-weeks post-weaning.

The impact of age and weaning on piglet gut microbiota has been widely reported in the literature, as previously reviewed in **Chapter 2**. All in all, the same pattern has been observed with age and weaning, consisting of dynamic changes towards a more stable and mature microbiota, with a variety of microbial groups associated with different time points. During the pre-weaning phase, microbiome composition is dominated by a milk-oriented microbiome composed of families like *Bacteroidaceae* and *Lactobacillaceae* (Frese *et al.*, 2015), which rapidly changes after weaning when a solid cereal-based diet is introduced. For instance, butyrate-producing genera such as *Prevotella*, having a very low abundance in suckling piglets, dramatically increase post-weaning due to the availability of complex oligosaccharides and polysaccharides in the feed (Frese *et al.*, 2015; Mach *et al.*, 2015; Zhao *et al.*, 2018). The rapidly changing microbiome of the young piglets seems to increase in diversity and richness along with the suckling phase and gradually stabilize after weaning (Kim *et al.*, 2011; Frese *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017).

In the present thesis dissertation, a total of four experimental tests were carried out. In three of them, the changes that occurred in the intestinal microbiota of piglets were studied both before and after weaning, while in one of them the sampling was carried out just before this abrupt event, but not after (**Table 8.3.**).

Table 8.3. General information and alpha diversity values of the main studies carried out around weaning in this thesis dissertation. Samples taken before and after weaning are shown, indicating the age at which the sampling and weaning were carried out, the type of sample, and the bioinformatic analysis performed.

	LACTATION								AFTER WEANING			
	Alpha	Bravo	Charlie	Delta	Echo	Foxtrot	PRO[a]	ENR[b]	Alpha	ENR[b]	PRO[a]	Bravo
Days of life	d21	d21	d21	d21	d21	d21	d21	d23	d28	d28	d33	d36
Weaning age	21	21	21	21	21	21	23	25	21	25	23	21
Sample type	Fecal	Fecal	Fecal	Fecal	Fecal	Fecal	Fecal	Cecal	Fecal	Cecal	Fecal	Fecal
Alpha diversity												
Chao1	1210.7 ± 775.11	1886.2 ± 1030.31	1979.5 ± 338.89	1561.9 ± 550.39	1999.5 ± 691.72	1801.7 ± 485.08	1166.2 ± 739.68	594.5 ± 126.31	1032.4 ± 359.78	699.3 ± 70.25	880.6 ± 330.51	3008.2 ± 1270.81
Shannon	6.46 ± 0.228	6.83 ± 0.490	7.12 ± 7.117	6.81 ± 6.808	6.98 ± 6.982	6.87 ± 6.874	6.24 ± 0.438	4.38 ± 0.486	6.50 ± 0.364	4.79 ± 0.295	6.07 ± 0.752	7.51 ± 0.317

[a] Study of probiotic supplementation in sows and their piglets (**Chapter 6**). The mean of all the piglets included in the experimental test, both treated and control (mean of CON+BSU+BAM).

[b] Study of the effect of environmental enrichment and early socialization (**Chapter 7**). The mean of all the piglets included in the experimental test is presented, both the enriched and the control (mean of CON + ENR).

While a gradual and constant increase in alpha diversity was observed during lactation, as observed by other authors (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017; Ke *et al.*, 2019; X. Wang *et al.*, 2019; Choudhury *et al.*, 2020), after weaning, contradictory results were obtained. A continuous increase in the species richness and diversity of gut microbiota during weaning transition has been reported by several authors (Pajarillo *et al.*, 2014; Mach *et al.*, 2015; Niu *et al.*, 2015; Zhao *et al.*, 2015; Chen *et al.*, 2017). This higher diversity in the gut microbiota has been related to more mature gut microbiota and agrees with the concept of functional redundancy (Naeem, Kawabata and Loreau, 1998; Konopka, 2009). These results are, however, contradictory with other studies that have reported a decreased alpha diversity during the early period after weaning (Hu *et al.*, 2016; Han *et al.*, 2018; Y. Li, Guo, *et al.*, 2018), with a later increase from weaning to adulthood. In the present thesis dissertation, both outcomes have been obtained. Increases in alpha diversity were observed after weaning in the case of the Bravo farm or the study of environmental enrichment, while in the Alpha farm or in the study of the effect of supplementation with probiotics a decreased diversity was observed. This controversy could be due to differences between studies in the day samples were collected but also to differences in other factors like differences in the diet composition, management of the animals during weaning transition, and of how quickly the animals adapt to the solid feeding and the new facilities. In this sense, there may be also great differences between results obtained in controlled studies in experimental facilities and those carried out in conventional farms, where the stress to which the animals are subjected can be very different. In the case of the Alpha and Bravo farms, it could be assumed that this outcome could be due to the physiological changes produced by post-weaning gut dysfunction since Alpha was sampled a week before Bravo due to the appearance of diarrhea, so the decrease in alpha diversity would therefore be explained. Unfortunately, we do not have data on fecal consistency in our studies, but it is likely that decreases in alpha diversity are indicative of improper colonization and might be an early indicator of alteration of the gut microbiota.

The development of the gut microbiota produced by the weaning transition in the main taxonomic groups at the family and genus level can be observed in **Tables 8.4. and 8.5.**, respectively.

Table 8.4. Relative abundances (RAB) of the main families (RAB greater than 1%) obtained in different studies from this thesis dissertation during the weaning transition, ordered from highest to lowest abundance concerning the mean. The mean of each taxonomic group with its standard deviation is also indicated in the last column of the table. The relative abundance of the rest of the taxonomic groups not included in the table have been grouped and are shown in the label "Other" with its respective percentage.

	LACTATION								AFTER WEANING					
	Alpha	Bravo	Charlie	Delta	Echo	Foxtrot	PRO[a]	ENR[b]	Alpha	ENR[b]	PRO[a]	Bravo	**LACT mean**	**WEAN mean**
Bacteroidaceae	16.19	13.75	9.43	13.43	10.10	18.61	11.76	7.00	4.74	2.58	3.24	11.63	12.54 ± 3.768	5.55 ± 4.154
Lachnospiraceae	15.13	14.00	11.07	14.03	12.95	8.41	7.65	5.06	5.88	9.22	5.45	11.75	11.04 ± 3.631	8.07 ± 2.973
Prevotellaceae	2.10	12.49	8.45	8.42	11.81	7.64	2.47	16.21	14.19	10.94	10.62	9.55	8.70 ± 4.841	11.32 ± 2.001
[Paraprevotellaceae]	-	-	-	-	-	-	-	8.64	-	10.16	-	-	8.64 ± 0.000	10.16 ± 0.000
Oscillospiraceae	10.95	4.74	14.44	8.52	9.20	10.69	6.24	-	7.97	-	5.83	9.19	9.25 ± 3.209	7.66 ± 1.698
Ruminococcaceae	2.66	2.84	4.42	4.42	6.98	5.13	6.12	12.92	2.98	18.51	3.98	3.74	5.69 ± 3.273	7.30 ± 7.485
Enterobacteriaceae	8.39	4.92	3.82	5.49	5.38	10.40	6.51	1.66	9.58	0.44	8.17	6.48	5.82 ± 2.686	6.17 ± 4.020
Muribaculaceae	5.84	4.69	8.07	5.49	5.51	3.38	4.22	-	4.04	-	3.60	2.91	5.32 ± 1.488	3.52 ± 0.568
S24-7	-	-	-	-	-	-	-	3.84	-	3.61	-	-	3.84 ± 0.000	3.61 ± 0.000
Lactobacillaceae	1.53	6.44	2.61	5.33	4.03	2.12	7.21	2.40	2.51	0.63	3.32	3.55	3.96 ± 2.144	2.50 ± 1.327
Erysipelotrichaceae	1.80	1.38	1.31	1.92	1.82	2.46	2.95	1.45	2.53	3.81	13.35	1.11	1.89 ± 0.567	5.20 ± 5.546
Rikenellaceae	3.81	4.05	5.35	4.34	3.77	1.92	3.08	0.10	3.41	0.15	2.41	2.85	3.3 ± 1.627	2.20 ± 1.429
Acidaminococcaceae	2.94	2.97	2.28	1.99	1.85	2.74	3.08	-	3.25	-	1.99	2.47	2.55 ± 0.502	2.57 ± 0.636
Christensenellaceae	3.11	0.88	4.44	4.04	2.07	2.95	3.22	0.49	1.36	1.20	2.78	1.82	2.65 ± 1.409	1.79 ± 0.710
Fusobacteriaceae	0.87	3.51	1.07	1.61	1.93	0.05	0.59	4.72	2.86	0.99	1.94	4.17	1.79 ± 1.576	2.49 ± 1.355
Clostridiaceae	1.09	1.36	0.96	1.19	0.44	1.74	4.44	1.80	0.22	3.22	2.55	4.95	1.63 ± 1.218	2.74 ± 1.958
Spirochaetaceae	0.75	0.98	0.92	0.83	0.89	1.27	1.13	1.89	8.15	2.30	3.09	1.31	1.08 ± 0.367	3.72 ± 3.047

Veillonellaceae	0.16	1.35	0.35	0.47	0.69	0.39	0.56	7.98	0.16	6.67	1.37	0.62	1.49 ± 2.645	2.21 ± 3.020
Campylobacteraceae	1.57	1.44	0.61	0.28	1.21	0.44	0.61	1.18	3.36	4.86	1.56	1.50	0.92 ± 0.489	2.82 ± 1.612
Marinifilaceae	2.48	1.99	2.02	0.75	1.40	1.04	1.18	–	1.24	–	0.92	1.01	1.55 ± 0.623	1.06 ± 0.162
Sphaerochaetaceae	–	–	–	–	–	–	–	1.51	–	1.26	–	–	1.51 ± 0.000	1.26 ± 0.000
Tannerellaceae	1.74	0.60	0.73	0.72	1.69	2.37	1.45	–	1.06	–	2.00	1.29	1.33 ± 0.666	1.45 ± 0.491
Desulfovibrionaceae	1.58	0.95	1.62	0.89	1.37	1.10	0.98	0.98	0.86	1.15	0.50	0.60	1.18 ± 0.297	0.78 ± 0.293
p-2534-18B5_gut_group	0.78	0.23	1.97	1.26	0.78	0.68	0.62	0.66	1.57	0.85	1.88	0.63	0.87 ± 0.526	1.23 ± 0.593
Comamonadaceae	0.62	0.00	0.26	0.05	0.66	1.67	3.74	–	0.00	–	0.13	0.00	1.00 ± 1.334	0.05 ± 0.076
Anaerovoracaceae	0.31	0.27	0.46	0.87	0.67	0.49	1.43	–	1.01	–	0.96	0.44	0.64 ± 0.403	0.80 ± 0.316
Enterococcaceae	1.52	0.13	0.35	0.70	0.37	2.00	0.75	0.11	0.00	0.01	0.04	0.64	0.74 ± 0.681	0.17 ± 0.309
Streptococcaceae	0.09	0.83	0.35	0.30	0.46	0.38	0.68	0.17	0.10	0.03	0.21	2.99	0.41 ± 0.248	0.84 ± 1.440
Synergistaceae	1.32	0.09	0.16	0.21	0.30	0.29	2.73	0.15	0.21	0.09	0.78	0.19	0.66 ± 0.929	0.32 ± 0.310
Selenomonadaceae	0.07	3.48	0.09	0.06	0.04	0.12	0.15	–	0.36	–	0.46	0.37	0.57 ± 1.282	0.40 ± 0.054
Pasteurellaceae	0.35	0.94	0.37	0.59	0.38	0.34	0.07	1.28	0.21	0.49	0.20	0.54	0.54 ± 0.390	0.36 ± 0.180
Helicobacteraceae	0.12	0.14	0.27	0.11	0.22	0.24	0.08	0.26	1.04	0.52	1.61	0.10	0.18 ± 0.074	0.82 ± 0.655
Akkermansiaceae	1.29	0.01	0.02	0.02	0.00	0.00	1.68	–	0.07	–	0.05	0.73	0.43 ± 0.729	0.28 ± 0.387
Succinivibrionaceae	0.01	0.74	0.17	0.05	0.35	0.07	0.04	0.33	0.81	0.40	1.01	0.62	0.22 ± 0.249	0.71 ± 0.259
Peptostreptococcaceae	0.13	0.13	0.17	0.12	0.11	0.28	1.95	0.10	0.21	0.06	1.04	0.24	0.37 ± 0.638	0.39 ± 0.442
Actinomycetaceae	0.39	0.02	0.26	0.03	0.05	0.05	1.01	–	0.00	–	0.02	0.05	0.26 ± 0.360	0.02 ± 0.024
Other (< 1%)	8.32	7.65	11.12	11.46	10.51	8.56	9.60	17.10	14.03	15.83	12.95	10.00	10.54 ± 2.985	13.20 ± 2.441

[a] Study of probiotic supplementation in sows and their piglets (**Chapter 6**). The mean of all the piglets included in the experimental test, both treated and control (mean of CON+BSU+BAM).

[b] Study of the effect of environmental enrichment and early socialization (**Chapter 7**). The mean of all the piglets included in the experimental test is presented, both the enriched and the control (mean of CON + ENR).

Table 8.5. Relative abundances (RAB) of the main genera (RAB greater than 1%) obtained in different studies from this thesis dissertation during the weaning transition, ordered from highest to lowest abundance concerning the mean. The mean of each taxonomic group with its standard deviation is also indicated in the last column of the table. The relative abundance of the rest of the taxonomic groups not included in the table have been grouped and are shown in the label "Other" with its respective percentage.

	LACTATION								AFTER WEANING					
	Alpha	Bravo	Charlie	Delta	Echo	Foxtrot	PRO[a]	ENR[b]	Alpha	ENR[b]	PRO[a]	Bravo	**LACT mean**	**WEAN mean**
Bacteroides	15.54	15.06	9.43	13.43	10.10	18.61	11.76	7.01	14.14	2.58	3.24	11.63	12.62 ± 3.775	7.90 ± 5.856
Escherichia/Shigella	9.99	12.92	3.80	5.45	5.33	10.34	6.49	-	4.18	-	8.14	6.44	7.76 ± 3.338	6.25 ± 1.987
Prevotella	2.01	6.01	4.17	3.20	7.15	3.56	0.81	16.22	4.98	10.99	3.00	4.78	5.39 ± 4.824	5.94 ± 3.485
UCG-002	5.48	0.88	9.27	3.74	4.79	4.89	3.24	-	3.34	-	2.45	4.94	4.61 ± 2.552	3.58 ± 1.263
Lactobacillus	1.61	3.44	2.61	5.33	4.03	2.12	7.21	2.40	4.14	0.63	3.31	3.55	3.59 ± 1.883	2.91 ± 1.558
Fusobacterium	6.93	6.03	1.06	1.61	1.91	0.05	0.59	4.72	10.21	0.99	1.92	4.13	2.86 ± 2.640	4.32 ± 4.145
Rikenellaceae (RC9 gut group)	3.64	3.02	4.63	3.68	2.95	1.17	2.23	-	1.92	-	2.06	2.67	3.04 ± 1.113	2.21 ± 0.401
Phascolarctobacterium	2.52	2.36	2.25	1.98	1.81	2.73	2.99	3.31	3.48	4.00	1.97	2.45	2.49 ± 0.505	2.97 ± 0.930
Clostridium sensu stricto 1	3.88	4.96	0.96	1.18	0.44	1.69	4.16	0.45	2.28	0.21	1.98	4.30	2.21 ± 1.826	2.19 ± 1.678
Christensenellaceae (R-7 group)	1.24	0.67	3.75	2.84	1.95	2.71	2.90	-	0.73	-	2.43	1.49	2.29 ± 1.065	1.55 ± 0.849

Lachnospiraceae (UCG-004)	1.03	4.25	1.77	2.30	2.06	0.36	1.93	-	3.76	-	0.07	0.62	1.96 ± 1.213	1.48 ± 1.994
p-75-a5	-	-	-	-	-	-	-	0.60	-	2.93	-	-	0.60 ± 1.113	2.93 ± 1.113
Alloprevotella	1.38	1.01	1.51	3.27	1.49	2.31	0.31	-	1.75	-	3.21	0.94	1.61 ± 0.945	1.97 ± 1.150
Campylobacter	2.21	1.08	0.01	0.01	0.01	0.00	0.61	1.18	1.28	4.87	1.56	1.50	0.64 ± 0.808	2.30 ± 1.715
CAG-873	2.22	1.18	2.50	1.22	0.19	0.30	2.68	-	0.82	-	0.30	0.08	1.47 ± 1.019	0.40 ± 0.377
Butyricimonas	1.90	1.62	1.85	0.64	1.13	0.91	0.99	0.86	1.58	0.37	0.45	0.96	1.24 ± 0.482	0.84 ± 0.561
UCG-005	0.50	0.36	1.91	1.44	0.76	2.28	1.03	-	0.87	-	0.76	1.06	1.18 ± 0.722	0.89 ± 0.152
Parabacteroides	0.33	0.60	0.71	0.71	1.53	2.35	0.96	0.71	1.55	0.51	1.94	1.19	0.99 ± 0.651	1.30 ± 0.608
Dorea	1.39	0.98	0.72	1.28	3.00	1.13	0.71	0.19	1.83	0.40	0.27	1.00	1.17 ± 0.829	0.87 ± 0.710
Turicibacter	0.00	0.02	0.07	0.04	0.14	0.03	0.86	0.06	0.03	0.02	10.88	0.00	0.15 ± 0.288	2.73 ± 5.430
NK4A214 group	0.42	0.52	1.53	1.32	1.20	1.01	0.81	-	0.57	-	0.99	1.12	0.97 ± 0.413	0.89 ± 0.287
Lachnoclostridium	2.14	1.59	0.16	0.50	0.52	0.15	1.46	-	1.49	-	0.26	1.02	0.93 ± 0.790	0.92 ± 0.618
Treponema	0.40	0.22	0.08	0.06	0.04	0.00	0.86	1.90	1.44	2.31	1.96	0.85	0.44 ± 0.652	1.64 ± 0.635
Ruminococcus	0.39	0.68	0.64	0.46	1.21	0.67	2.77	0.31	0.61	0.92	0.62	0.63	0.89 ± 0.807	0.69 ± 0.148
Streptococcus	1.19	0.99	0.35	0.30	0.46	0.38	0.68	0.17	1.19	0.03	0.21	2.97	0.56 ± 0.359	1.10 ± 1.348
Lachnospiraceae (NK4A136 group)	1.08	0.09	1.40	1.76	1.01	0.67	0.21	-	0.07	-	0.33	0.75	0.89 ± 0.606	0.38 ± 0.343

Prevotellaceae (NK3B31 group)	0.20	0.98	0.20	0.12	0.03	0.01	0.32	-	1.64	-	1.21	1.77	0.27 ± 0.333	1.54 ± 0.296
Comamonas	0.44	0.00	0.14	0.01	0.09	0.04	3.71	-	0.69	-	0.12	0.00	0.63 ± 1.367	0.27 ± 0.367
Oscillospira	0.02	0.12	0.00	0.04	0.01	0.00	0.16	2.47	0.08	2.69	0.12	0.14	0.35 ± 0.859	0.76 ± 1.286
Prevotellaceae (UCG-003)	1.70	0.16	0.05	0.04	0.00	0.00	0.08	-	0.62	-	1.50	0.49	0.29 ± 0.625	0.87 ± 0.551
Subdoligranulum	0.04	0.63	0.09	0.19	0.04	0.00	1.64	-	0.55	-	0.60	0.70	0.38 ± 0.597	0.62 ± 0.075
Anaerovibrio	0.00	2.25	0.05	0.05	0.00	0.00	0.12	0.66	0.11	1.37	0.32	0.34	0.39 ± 0.782	0.53 ± 0.566
Actinobacillus	1.03	0.31	0.14	0.21	0.12	0.09	0.03	0.60	1.72	0.45	0.09	0.27	0.32 ± 0.339	0.63 ± 0.737
Megasphaera	0.05	0.31	0.05	0.02	0.00	0.00	0.48	2.27	0.08	0.61	0.91	0.15	0.40 ± 0.775	0.44 ± 0.395
Roseburia	0.33	0.80	0.02	0.02	0.02	0.02	0.10	0.19	0.26	2.12	0.23	0.45	0.19 ± 0.272	0.76 ± 0.908
Enterococcus	0.09	0.56	0.35	0.80	0.14	0.10	0.75	0.11	0.43	0.01	0.04	0.64	0.36 ± 0.301	0.28 ± 0.303
Cloacibacillus	0.11	0.02	0.02	0.02	0.00	0.00	2.43	-	0.09	-	0.33	0.16	0.37 ± 0.907	0.19 ± 0.123
Veillonella	0.45	0.83	0.04	0.20	0.11	0.05	0.01	0.64	0.45	0.04	0.39	0.43	0.29 ± 0.311	0.33 ± 0.194
Akkermansia	0.60	0.00	0.02	0.02	0.00	0.00	1.68	0.03	0.30	0.07	0.05	0.73	0.29 ± 0.597	0.29 ± 0.316
Helicobacter	0.69	0.09	0.00	0.02	0.00	0.00	0.08	0.18	0.08	0.48	1.60	0.09	0.13 ± 0.233	0.56 ± 0.719
Other (< 1%)	24.83	22.40	41.75	40.53	44.26	39.29	29.15	52.77	24.67	60.42	38.14	32.57	36.87 ± 10.450	38.95 ± 15.342

[a] Study of probiotic supplementation in sows and their piglets (**Chapter 6**). The mean of all the piglets included in the experimental test, both treated and control (mean of CON+BSU+BAM); [b] Study of the effect of environmental enrichment and early socialization (**Chapter 7**). The mean of all the piglets included in the experimental test is presented, both the enriched and the control (mean of CON + ENR).

The main families found in the days around weaning were *Bacteroidaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Oscillospiraceae*, *Ruminococcaceae*, and *Enterobacteriaceae*, among many others. In general, each family shows a similar evolution among farms, although there is always an exception in some of the experimental trials, demonstrating the great variability of the intestinal microbiota and the little certainty with which the changes that occur at weaning can be predicted.

In the set of experimental trials carried out in the present thesis dissertation (**Table 8.4.**), a decrease in the *Bacteroidaceae* family can be observed in the weaning transition. Bacteria from the Bacteroidetes phyla has been related to increased abundance in diarrhea-resistant piglets and lighter pigs (Dou *et al.*, 2017; McCormack *et al.*, 2017). Although *Prevotellaceae* is usually associated with large increases after weaning (Chen *et al.*, 2017; Gresse *et al.*, 2017; Guevarra *et al.*, 2018), a decrease in its abundance was observed on the Bravo farm and the enrichment trial. Increased abundance of *Prevotellaceae* in piglets has been associated with the introduction of a plant-based diet (Frese *et al.*, 2015; Guevarra *et al.*, 2018) and with higher average daily gain and healthier outcomes with postweaning diarrhea (Dou *et al.*, 2017). *Ruminococcaceae* and *Enterobacteriaceae* tended to increase after weaning, also with some exceptions. The increased abundance of *Ruminococcaceae* has been associated with improved lactation growth and lower diarrhea incidence (Mach *et al.*, 2015; Dou *et al.*, 2017), whereas an increased abundance of *Enterobacteriaceae* is well known for being associated with the appearance of postweaning diarrhea. It is well known that the reduction of lactic acid-producing bacteria (*Lactobacillus*) during weaning raises intestinal pH, increasing disease susceptibility because low gut pH is bacteriocidal (Lallès *et al.*, 2007a; J. C. Kim *et al.*, 2012). Therefore, post-weaning diarrhea is characterized by reductions in healthy bacteria, including bacteria from the *Lactobacillaceae* family, and increases in pathogenic *Escherichia coli* (Konstantinov *et al.*, 2006; Lallès *et al.*, 2007a). *Lactobacillaceae* declined in abundance after weaning in 3 out of 4 experimental trials. Something similar occurred with *Lachnospiraceae*, which decreased after weaning in all cases except in the study of environmental enrichment. As for *Lachnospiraceae*, increased abundances of this bacteria in piglets have been associated with lower diarrhea incidence (Dou *et al.*, 2017).

Again, at the genus level (**Table 8.5.**), contradictory results were observed. Several studies exemplify *Prevotella* as a prominent microbe in the typical post-weaning microbiota together with species belonging to *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Lachnospira*, *Dorea*, *Blautia*, *Subdoligranulum* (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Mach *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Ramayo-Caldas *et al.*, 2016; Y. Li, Guo, *et al.*, 2018; Guevarra *et al.*, 2018, 2019; Choudhury *et al.*, 2020; Luise, Le Sciellour, *et al.*, 2021). In the present thesis dissertation, while in some farms large increases are observed after weaning of *Prevotella* that coincide with the literature, in others a decrease is observed. *Prevotella* has been related to reduced growth in lactation, but greater outcomes in healthy pigs after weaning and improved ADG and FCR (Mach *et al.*, 2015; Karasova *et al.*, 2021). *Bacteroides*, *Clostridium sense stricto 1*, *Butyricimonas*, and *Lachnoclostridium* decreased in all experimental tests, while *Escherichia-Shigella* decreased dramatically in two of them but increased slightly in one. *Fusobacterium*, *Lactobacillus*, *Campylobacter*, and *Streptococcus* also show contradictory results, decreasing in two experimental trials, but increasing in two others. In this context, Gresse *et al.* (2017) stated that weaning transition is characterized by a decrease in the abundance of bacteria belonging to the *Lactobacillus* group and an increase in the abundance of facultative anaerobes, including bacteria belonging to the *Enterobacteriaceae*, *Proteobacteriaceae*, *Clostridiaceae*, and *Prevotellaceae* families (Chen *et al.*, 2017; Gresse *et al.*, 2017). Although it is true that in some experimental trials these results coincide with what has been described, we can assure that this pattern is not always fulfilled and, therefore, other factors intervene in the establishment of the microbiota after weaning. For instance, *Rikenecalleae* (*RC9 gut group*), *Lachnospiraceae UCG-004* tended to decrease, while *Phascolarctobacterium*, *Dorea*, *Lachnospiraceae NK3B31 group*, *Oscillospira*, *Parabacteroides*, and *Actinobacillus* tended to increase after weaning.

The abrupt change to a solid cereal-based diet and the withdrawal of milk explain the decrease of *Lactobacillus* and *Bacteroides* genera and the increase of propionate- and butyrate-producing genera including *Phascolarctobacterium*, *Dorea*, the genera belonging to *Lachnospiraceae*, and *Oscillospira*, among others (Gophna, Konikoff and Nielsen, 2017; Zhao *et al.*, 2018). However, based on this concept we would also expect to see an increase in the *Rikenellaceae RC9 gut group* and *Lachnospiraceae UCG-004*. Microorganisms

belonging to the *Lachospiraceae* genera, such as *Lachnospira* and *Dorea*, have also been reported to begin to emerge after weaning (Y. Li, Guo, *et al*., 2018). The genera belonging to *Lachnospiraceae* are adapted to metabolize a wide range of complex oligosaccharides and polysaccharides while producing short-chain fatty acids. Altogether, the higher abundance of propionate- and butyrate-producing genera in weaned piglets, adapted to digest resistant starches and dietary fibers to convert them to short-chain fatty acids, show the quick microbial transformation of the piglets' gut microbiota to cope with diets rich in complex carbohydrates, as these abundance shifts occur in a short period of time. Therefore, the porcine microbiota rapidly evolves through time, towards a homogeneous and stable microbiome structure. However, these changes do not always occur in the same magnitude among farms.

Despite the possible "farm" effect already discussed previously, it is also worth noting that differences in the relative abundance of taxonomic groups between experimental trials could also be due to the sample type and the laboratory and bioinformatic analysis of the data. In this way, the main difference among our studies was the great difference obtained in the results based on the type of sample and the bioinformatic analysis performed. For instance, the experimental trial designed to evaluate the effect of environmental enrichment (**Chapters 5 and 7**) was carried out using samples of cecal content that were analyzed by QIIME and with the Greengenes v13_8 database, while the rest of the experimental trials were carried out with fecal samples and using R's DADA2 package and Silva v138 database. Broadly speaking, this translated into lower species richness and alpha diversity in the study of the effect of environmental enrichment (analyzed with QIIME) and some differences in assignment to specific taxonomic groups. In this context, Lima *et al.* (2021) compared two different bioinformatic tools: MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) and Quantitative Insights Into Microbial Ecology 2 (QIIME2). As a result, significant differences between the microbiota profiles were obtained from each pipeline. Similarly, Allali *et al.* (2017) compared three different NGS platforms, obtaining differences in diversity and abundance. However, while there were differences in depth of coverage and phylogenetic diversity, all workflows revealed comparable treatment effects on microbial diversity, leading to similar biological conclusions. Therefore, when performing massive sequencing of the 16s rRNA gene analysis, we should take into account that the relative

abundances can vary depending on the analytical method used for their interpretation.

The changes produced by the weaning transition were studied in depth in **Chapter 5**, where changes in the composition of the cecal content of the piglets were not only studied but their intestinal response was also evaluated by studying gene expression in the jejunum and the impact of weaning on the serum metabolome. In short, in **Chapter 5**, it is not only demonstrated that there is a clear correlation between the microbiota and the metabolome, but also the great impact of weaning on the intestinal health of the piglet is demonstrated, with great changes in the gene expression of several genes. For instance, in the Open-Array analysis, several genes showed significant changes just after weaning, with a decrease in the jejunal gene expression of several barrier function genes (*OCLN*, *CLDN4*, *MUC2*, and *MUC13*) and an increased expression of the nutrient transport gene *SLC16A1*, which could be explained by an increased microbial fermentative activity after weaning with the production of lactate and other SCFAs. The downregulation of *MUC* genes has been associated with the presence of pathogenic bacteria, such as ETEC or Lawsonia intracellularis (Zhou *et al*., 2012; Smith *et al*., 2014), whereas higher expressions of nutrient transport genes carry positive repercussions for gut health and nutrient digestion. In addition, some authors have reported decreases in the expression of *SLC15A1* and *SLC13A1* due to the presence of pathogenic bacteria such as ETEC or *Lawsonia intracellularis* (Trevisi *et al*., 2012, 2018; Smith *et al*., 2014). Consistent with the changes observed in the jejunal gene expression around weaning, ^{1}HNMR results, also evidenced the relevant impact of weaning on the animal metabolomic response. Within 5 days between samplings, animals showed a quite different metabolomic pattern with significant decreases in particular signals attributable to choline, LDL, triglycerides, fatty acids, alanine, and isoleucine and increases in 3-hydroxybutyrate, ethanol, valine, and adipate. The reduced choline, LDL, triglycerides, and fatty acids support the concept that weaning might affect the metabolism of energy substrates. Lower levels of serum alanine in weaned piglets could be a consequence of its consumption during gluconeogenesis in the liver to provide glucose to extrahepatic cells and tissues (Wu, 2009).

Therefore, in response to our hypothesis, weaning is key in the intestinal development of the piglet with great repercussions not only on the

homeostasis of its intestinal microbiota but also with a great impact on the entire response that is triggered at the genetic and physiological level. However, to date, there are still opposite results between different studies, as occurs between the different experimental trials of the present thesis dissertation. For that reason, it is still necessary to go deeper to explain these variations and establish a clear and stable pattern in the development and establishment of the porcine intestinal microbiota.

8.3. What happens during the first days of life can reshape the future development of the animal

The microbial colonization of the gastrointestinal tract as well as the concomitant development of the intestinal immune system in early life are major determinants of the health and performance of animals (Chung *et al.*, 2012; Hooper, Littman and Macpherson, 2012; Schokker *et al.*, 2014; Nowland, Kirkwood and Pluske, 2021). The mother, pen environment, and general husbandry practices such as cross-fostering and antibiotic administration to sows and piglets can influence the intestinal microbiota, impacting long-term piglet health, performance, and survival. Therefore, the maintenance of general health and prevention of disease are critically dependent on intestinal homeostasis and proper immune competence (Schokker *et al.*, 2014).

During the early-life period, the composition and diversity of microbiota are unstable and highly influenced by environmental conditions, including the use of antibiotics, exposure to stress, and nutrition, as observed in several studies using a variety of experimental conditions and models (Palmer *et al.*, 2007; Inman *et al.*, 2010; Mulder *et al.*, 2011; Schmidt *et al.*, 2011; Cho *et al.*, 2012; Schokker *et al.*, 2014). As previously stated, the structural and functional development of the mucosal immune system takes place concomitantly with early-life microbial colonization. Moreover, it is known that the process of immune maturation is influenced by the microbiota that colonizes the gut at the early stages of life (Wagner, 2008; Round and Mazmanian, 2009; Lewis *et al.*, 2012), and that host species-specific microbiota is required for the development of the immune system (Chung *et al.*, 2012). A link has been established between the functionality of the host immune system and the early-life gut microbiota composition (Schokker *et al.*, 2014). Therefore, gut

microbiota colonization during the first days of life can determine the future development of the animal.

Throughout this thesis dissertation, it has been possible to find different evidence regarding how events that occur along the first days of life have an imprint later in life.

To be able to determine the possible maternal transfer of microbiota during lactation, maternal stool samples were analyzed in **Chapter 6** and correlated with samples from their litters before and after weaning. As a result, it was observed that the correlation between the maternal microbiota and that of the piglets was higher after weaning than during lactation. Therefore, the maternal effect seemed to have a late manifestation in the piglet, once the stressor had been overcome. For instance, maternal butyric fermentation genera such as *Blautia*, *Megasphaera*, or *Prevotella* correlated very highly with other butyric fermentation genera in piglets, such as *Coprococcus*, or the same *Megasphaera* or *Prevotella*. Similarly, genera considered negative for intestinal health such as *Terrisporobacter* correlated positively with *Escherichia-Shigella* in piglets. It is well known that genera associated with the fermentation of complex carbohydrates are likely important contributors towards the establishment of a more mature microbiota.

On the other hand, in **Chapter 7**, a similar outcome was obtained. Although no changes could be identified in specific taxonomic groups, an impact on the structure of the gut microbiota of piglets was observed after weaning, but not during lactation. A similar outcome was obtained by D'Eath (2005), who also studied the effect of early socialization of piglets between 10 and 30 days of age by removing the barriers between two adjacent pens. Their results in piglets also became especially evident after weaning but not during lactation. Therefore, the combined effects of early socialization and environmental enrichment could exert their effects on piglets' microbiota by improving their adaptability to stress and consequently, stress-related intestinal dysfunction.

In both studies, the effect of both intervention strategies on intestinal functionality through gene expression in jejunal samples was also analyzed. As a result, in the test with probiotic supplementation, no difference was observed in the expression of genes at the intestinal level, however, a down-

regulation of the *TLR2* gene in the enriched piglets after weaning was observed. This result obtained only after weaning, together with the significant reduction found ion transport across the colonic tissue, could be due to a reduction in the presence of pathogens in the enriched piglets, since *TLR2* has been shown to recognize conserved molecules derived from microorganisms known as pathogen-associated molecular patterns (PAMPs), activating the signaling pathways to modulate the host's inflammatory response. Although there are very few studies in this area, there are authors who highlight the impact of early events on the immune system and the resilience of the adult animal microbiota (Nowland *et al.*, 2019). For instance, some evidence has been published defining differences in the fecal microbiota of piglets of as early as 7 days of life determining their susceptibility to suffering post-weaning diarrhea four weeks later (Dou *et al.*, 2017), emphasizing the potential of the early microbiota establishment on the development of the immune response. Moreover, some authors have also been able to establish relationships among specific taxonomic groups and the health status of the piglets. For example, an increased abundance of Actinobacteria, Chlamydia, or Helicobacter before weaning has been found as a marker of piglets predisposed for diarrhea (Karasova *et al.*, 2021).

8.4. It is possible to modulate the development of piglet microbiota by early intervention strategies

As examined throughout this general discussion, the gut microbiota is known for its fundamental role in moderating host health and phenotype. In this context, the neonatal period can be identified as one of the critical stages in which changes to the microbiota can have long-term consequences on pig health. The “developmental window” of approximately one month after birth during which the host microbiome is more susceptible to external influences, including the environment (Thompson, Wang and Holmes, 2008; Zhou *et al.*, 2016; Tsai *et al.*, 2018), the diet and dietary supplementation (Bian *et al.*, 2016; Salcedo *et al.*, 2016; Choudhury *et al.*, 2020), and management strategies (Wen *et al.*, 2021) is the ideal opportunity to intervene and modulate the intestinal microbiota of the young piglet. During the last decades, several approaches have been examined to increase the health status of piglets around weaning (Lallès *et al.*, 2007a). However, in the present thesis

dissertation, we focused on two specific intervention strategies that play a role in modulating the gut microbiota of suckling pigs: the management practices (environmental enrichment and early socialization) and the use of probiotics.

The natural exposition of the piglet to sow's feces together with the possibility of an entero-mammary route for microbial transfer (Jost *et al.*, 2014; Xue Chen *et al.*, 2018; Jiang *et al.*, 2019; Liu, Zeng, *et al.*, 2019), open the possibility of gut microbiota modulation in the piglet through probiotic supplementation to the sow. Furthermore, the mother's imprinting on the piglet could occur even before its birth. In a recent study, microbial colonization of the spiral colon occurred in stillborn pigs, suggesting microbial exposure prior to birth (Nowland, Kirkwood, *et al.*, 2021). After birth, breastfeeding is essential for the formation of the piglet's gut microbiota.

Dietary strategies and specifically probiotics have gained considerable attention due to their capacity to improve the reproductive both sow and piglet performance (Barba-Vidal, Martín-Orúe and Castillejos, 2019). Moreover, different probiotic strains when administered to sows during gestation and/or lactation have been shown to have positive effects on the performance of piglets. Particularly, different strains of *Bacillus spp*. have been shown to increase feed consumption in lactation, reduce fat mobilization, promote milk production, increase litter weight, promote digestive health, and inhibit pathogenic bacteria (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Stamati *et al.*, 2006; Larsen *et al.*, 2014; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016). While higher milk production or improved economy of fat reserves of the sow could be behind these effects, other modes of action, related to differential early events in the life of the piglets, could also be involved. In this regard, modulation of the maternal intestinal microbiota by probiotics could determine changes in the process of early microbial colonization of the gastrointestinal tract of piglets with beneficial implications throughout their lives. Undoubtedly, the sow represents the main and first donor or fecal microbiota to the piglet with a relevant role in this early process of microbiota establishment. In this sense, recent studies administering maternal fecal microbiota to neonatal piglets have demonstrated that this early intervention can improve the growth performance of piglets, decrease intestinal permeability and stimulate IgA secretion modulating gut microbiota composition (C. S. Cheng *et al.*, 2019). The importance of the

mother-effect defining a particular microbiota composition in the nursing piglet was also evidenced by Mu *et al.* (2019) analyzing the early-life microbiota succession in pigs using a cross-fostering piglet model. Therefore, maternal environmental factors (diet composition, probiotic treatment, etc.), that induce changes in maternal microbiota, may have huge effects on offspring gut physiology (Kelly and Conway, 2005). However, contradictory with these studies, in our experimental trial (**Chapter 6**) few relationships could be established in the gut microbiota of suckling pigs due to the dietary supplementation of their mothers with probiotics. Probably, our lack of significant differences in lactation could be due to the sampling day selected and some differences could have been observed if earlier ages had been sampled.

Switching scope, environmental enrichment arose from the need for pigs to show their natural playing behavior. One of the goals of environmental enrichment is to increase the animal's ability to cope with behavioral and physiological challenges such as environmental variation. Allowing the piglets' playing behavior favors their development and improves their response to weaning stress. It has been suggested that animals reared in an environment that enables the expression of play behavior are better prepared to cope with unfavorable situations at a later stage of life (Spinka, Newberry and Bekoff, 2001). Therefore, an enriched environment during the early life of piglets is known to positively influence behavioral development and stress adaptation later in life (Oostindjer *et al.*, 2011) by providing piglets with the appropriate social skills and stress coping capabilities (Brunson *et al.*, 2003).

Early socialization between piglets allows, on the one hand, contact and mixing between piglets from different litters, favoring their adaptation to new individuals and reducing the impact of weaning mixing; and on the other hand, the acquisition of more diverse microbiota by interaction with a greater number of individuals not belonging to the same litter. Therefore, although in commercial practice individual litters are separated in farrowing crates, previous studies have suggested that housing systems that allow pre-weaning socialization of piglets can reduce aggression after weaning (Morgan *et al.*, 2014; Salazar *et al.*, 2018). In addition, there is direct experimental evidence that the farm environment during the early life of the piglet influences the regulation of immune responses (Lewis *et al.*, 2012).

The effects of environmental enrichment have been studied in piglets before and after weaning. Our results from **Chapter 7** come from the same experimental trial that reported neonatal enrichment to have a lasting positive effect on piglet object exploration pre-weaning, mitigation of weaning stress, and reduced aggression post-weaning until slaughter (Ko *et al.*, 2020). The better post-weaning performance of enriched pigs might reflect an increased adaptability of piglets reared in enriched conditions to stressful processes such as weaning (Oostindjer *et al.*, 2010; C. H. Yang, Ko, *et al.*, 2018). In agreement with our results, Luo *et al.* (2020) found that enriched housed pigs were better able to cope with weaning transition, as they gained more weight and had a higher feed intake during the first days after weaning. Moreover, in another study, enriching the neonatal environment improved the short-term performance after regrouping, benefitting the life-long performance by reducing time to reach market weight (Ko *et al.*, 2021). Therefore, pigs with better social and cognitive skills can improve their ability to cope with routine stressors by improving their well-being and intestinal health.

In addition to improving the social and cognitive skills of the pig to facilitate its adaptation to weaning, managing the stress suffered by the piglet at weaning is essential for its early adaptation and further productive performance. Weaning-induced stress is known to lead to loss of appetite, post-weaning diarrhea, growth retardation, intestinal inflammation, and unbalanced gut microbiota (Pié *et al.*, 2004). Therefore, lower levels of stress at weaning could favor the appearance of appetite in weaned piglets, promoting their growth and, in turn, their health. However, therapies for alleviating weaning stress through modulation of the intestinal microbiota are scarce, and little is known about the relationship between stress and gut microbiota. Nonetheless, relationships between the brain and the intestine have been established through the brain-gut-microbiome axis.

The co-evolution of the intestinal microorganisms with their hosts has led to the acquisition of microbial functions in digestion, utilization of nutrients, elimination of toxins, and protection against pathogens by bacterial competition and interaction (H. Y. Cheng *et al.*, 2019). The gut microbiota contributes to neurophysiological regulation, which subsequently governs neurotransmission, cognition, and behavior, by regulating the immune and endocrine systems through the release of bacterial metabolites (Sandhu *et*

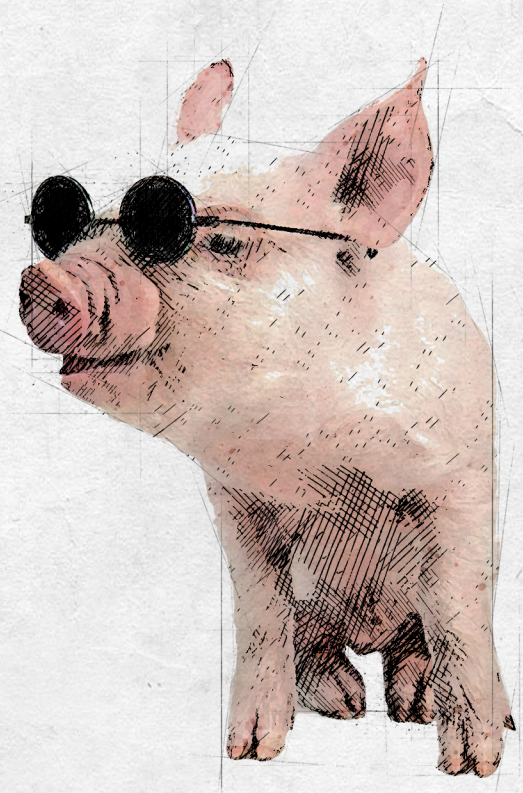
al., 2017). The microbiota and its metabolites, therefore, play an important role in the communication between the gut and the brain, forming the well-known brain-gut-microbiome axis. This axis has been associated with the modulation of behavior and brain processes, including emotional behavior, brain biochemistry, responses to stress and pain, and the functioning of the gastrointestinal tract through changes in intestinal permeability, the immune function of the mucous membranes, and the activity of the enteric nervous system (Mayer, Tillisch and Gupta, 2015; Patil, Gooneratne and Ju, 2020). Therefore, while psychological and physical stressors can affect the composition and metabolic activity of the gut microbiota, experimental changes to the gut microbiome can affect emotional behavior and related brain systems (Mayer *et al*., 2014). These findings have resulted in speculation in the field of human medicine that alterations in the gut microbiome may play a pathophysiological role in brain diseases, including autism spectrum disorder, anxiety, depression, and chronic pain. Moreover, although the brain–gut–microbiome axis has not yet been thoroughly examined in pigs, through analysis of this system in other mammalian species, it could be hypothesized that this axis would also play a key role in pigs (Patil, Gooneratne and Ju, 2020), opening the door to a new field of research yet to be developed.

All in all, a healthy gut microbial community is diverse, stable, and resilient. Piglets with a "more mature" microbiota, that is, with greater species richness, greater diversity, and greater abundance of taxonomic groups capable of degrading the components of a solid cereal-based diet are piglets that show better results in performance and intestinal health, with less probability of developing diarrhea after weaning. Therefore, a greater adaptation to weaning could translate into an advance in intestinal maturation, so that an intestine with a microbiota similar to that shown by a stable adult is more likely to cope with weaning without great negative consequences. Although weaning stress due to separation from the mother, dietary change, handling, transport, and alteration of social and physical environments during the period of weaning (Sutherland, Backus and McGlone, 2014) leads to alteration of the gut microbial community, also known as dysbiosis, there is plenty of evidence that gut microbes and, particularly, probiotics, can help prevent diarrhea (Fouhse, Zijlstra and Willing, 2016; Luise *et al*., 2019; Haupenthal *et al*., 2020), opening the door to the research of new therapies for alleviating weaning stress

through modulation of the intestinal microbiota. As seen in this general discussion, other intervention strategies such as environmental enrichment and early socialization are equally valid methods to increase piglet welfare during this critical phase.

Chapter 9

Conclusions



Conclusions

Based on the results presented in this Ph.D. study, it can be concluded that:

1. The intestinal microbiome rapidly undergoes a remarkable shift as the piglets grow, from the first microbial groups to the establishment of an adult-like microbial community. The initial gut colonization of newborn piglets is characterized by bacteria belonging to the *Clostridiaceace*, *Enterobacteriaceae*, *Fusobacteriaceae,* and *Bacteroidaceae* families, which are progressively replaced by carbohydrate fermenting bacteria, essentially the acetate, propionate, and butyrate-producing micro-organisms. In between, there's a period of changing microbial successions with a variety of microbial groups associated with different time-points.

2. There is a relatively similar pattern among farms in the sequential substitution of microbial groups during the first days of life with a gradual increase in species richness and biodiversity with age. Despite this, modifications in this common pattern can be associated with different management guidelines, such as the use of antimicrobials in lactating sows or the administration of acidifying solutions to newborn piglets.

3. During the weaning transition, the microbial ecosystem evolves from a microbiome oriented to the degradation of milk carbohydrates, composed of families like *Bacteroidaceae* and *Lactobacillaceae*, towards a more intricate one. This shift is oriented to the fermentation of complex carbohydrates and is generally constituted predominantly of butyrate-producing genera such as *Prevotella*, reflecting the evolution and adaptation of the intestinal ecosystem towards a solid diet.

4. Weaning also has a great impact on the jejunal expression of several genes related to immune response and intestinal functionality. Among them, a downregulation of the *Occludin* (OCLN), *Claudin-4* (CLDN4), *Mucin 2* (MUC2), and *Mucin 13* (MUC13) is observed after weaning, evidencing the clear negative impact of weaning on barrier function. Its impact on the animal metabolism is also illustrated by increases in the level of β-hydroxybutyrate, a metabolic stress biomarker, and decreases in choline, LDL, triglycerides, fatty acids, alanine, and isoleucine.

5. Both tested probiotics strains supplemented to reproductive sows (*Bacillus subtilis* strain EB15 and *Bacillus amyloliquefaciens* strain ZM16) have a significant positive impact on prolificacy. Moreover, when they are offered during three reproductive cycles, they can modify the structure of the mothers' intestinal microbiota with significant changes in several microbial groups. Changes were more remarkable with the *B. amyloliquefaciens* strain.

6. Supplementation to sows with these probiotic strains is also capable of producing microbial shifts in the piglets, with a clearer impact on the post-weaning than in the lactation period, confirming the relevance of the early process of gut colonization shaping the gut microbiota of the growing pig.

7. Rearing suckling piglets in an enriched environment and an early piglet socialization program, based on mixing litters, results in a divergent response after weaning but not during lactation, with differences in the microbial structure and a reduced jejunal expression of the *TLR2* gene. These results suggest that creating a physically and socially enriched environment in early life can modify the animal response after weaning probably through diminishing social stress response.

Chapter 10

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Annex 1

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Education

2017 – present	Ph.D. program in Animal Production Universitat Autònoma de Barcelona (UAB), Spain
2012 – 2017	Veterinary Science degree Universitat Autònoma de Barcelona (UAB), Spain
2012	Certificate in Advanced English (CAE C1)
2012	Diplôme d'Études en Langue Française (DELF B1)

PhD activities

Basic training in biosafety. Public Health Agency of Canada Course: Laboratory Biosafety and Biosecurity. October 2017.

Internal course for UAB staff: 'Use of data display screens: ergonomically design your workplace' (code 8855-3). November 2017.

Public Health Day: Antibiotic resistance; organized by the Department of Health of the Generalitat de Catalunya. November 13th, 2017.

Conference 'Initiatives to reduce waste in the agri-food sector'; organized by the Department of Agriculture, Livestock, Fisheries and Food of the Generalitat de Catalunya. November 27th, 2017.

Internal course for UAB staff: 'Initial training in virtual occupational risk prevention' (code 8821-4). December 2017.

Webseminar 'Welfare in production animals', taught by Xavier Manteca and organized by Asís Formación and the Spanish Veterinary College Organization. February 26th, 2018.

Internal course for UAB doctoral students: 'Publish in Open Access'. Faculty of Communication Sciences, UAB. April 16th, 2018.

Internal course for UAB doctoral students: Responsible PhD: Integrating Responsible Research and Innovation in PhD Research. UAB Doctoral School, June 2018.

Feed Ingredient Course, organized by the Polytechnic University of Madrid and the University of Illinois. Madrid, June 2018.

Course for research personnel users of animals for experimentation ('Curso de formación para personal investigador usuario de animales para la experimentación'), accredited by FELASA. UAB, October 15-26th, 2018.

Internal course for UAB Animal Production Doctorate program students: 'Statistics in the field of animal production'. SNiBA-UAB and Servei d'Estadística de la UAB, November – December 2018.

Internal course for UAB doctoral students: Dissemination of Science. Servei de Llengües (UAB), January 2019.

Internal course for UAB Animal Production Doctorate program students: 'Porcine nutrition'. SNiBA-UAB, March – May 2019.

Internal course for UAB doctoral students: 'How to design and defend your PhD with the Idea Puzzle software', UAB Doctoral School, December 11th, 2019.

Conference: 'Management of linguistic diversity and cultural studies in Health Sciences at university level'. UAB Veterinary faculty, December 13th, 2019.

Short stay at the Institute of Agrochemistry and Food Technology (IATA; https://www.iata.csic.es/es/investigacion), under the tutelage of M. C. Collado. January 2020

Training in RNA extraction techniques and rtPCR (in collaboration with CRAG).

Training in DNA extraction techniques (SNiBA-UAB).

Training for the analysis of gene expression in OpenArray technology (in collaboration with CRAG).

Training in the development of a computer application for automated statistical analysis (SNiBA-UAB).

Training in bioinformati6c analysis after DNA sequencing (SNiBA-UAB & IRTA-Torremarimon)

Participation at workshops and congresses

World Veterinary Association Congress 2018 (WVAC)
Barcelona (Spain); 6-8th May 2018
Simple participation

Annual Congress of Young Researchers 2018 (ACYR)
Bellaterra, UAB (Spain); 11-12th July, 2018
Oral presentation (awarded)

X Workshop SEPyP 2019
Las Palmas de Gran Canaria (Spain); 6-8th February, 2019
Oral presentation

XVIII Jornadas sobre Producción Animal (AIDA - ITEA)
Zaragoza (Spain); 7-8th May, 2019
Oral presentation (x2)

ASAS-CSAS Annual Meeting and Trade Show 2019
Austin, Texas (USA); 8-11th July, 2019
Poster presentation (x2)

XI Workshop SEMiPyP 2020
Granada (Spain); 12-14th February, 2020
Poster presentation

ADSA-ASAS Annual Midwest Meeting 2020
Omaha, Nebraska (USA); 2-4th March, 2020
Oral presentation

Scientific publications

Saladrigas-García, M., Solà-Oriol, D., López-Vergé, S., Nielsen, B., Pérez, J. F., Martín-Orúe, S. M. Feeding two Bacillus strains in commercial sows: effects on reproductive performance and gut microbial ecosystem. Abstracts of the 10th Workshop on Probiotics and Prebiotics. *Annals of Nutrition and Metabolism*, 74(suppl 1; 2019):1-31. doi: 10.1159/000496759

Saladrigas-García, M., Solà-Oriol, D., López-Vergé, S., Nielsen, B., Pérez, J. F., Martín-Orúe, S. M. Evaluation of long-term administration of two Bacillus strains in commercial sows on performance and faecal microbiota. *XVIII Jornadas sobre Producción Animal*, Zaragoza, España, 7 y 8 de mayo de 2019 (2019): 116-118.

Saladrigas-García, M., Ko, H.L., Llonch, P., Pérez, J. F., Martín-Orúe, S. M. Environmental enrichment during lactation: an opportunity to modulate piglets' gut health after weaning. *XVIII Jornadas sobre Producción Animal*, Zaragoza, España, 7 y 8 de mayo de 2019 (2019): 113-115.

Saladrigas-García, M., Solà-Oriol, D., López-Vergé, S., Rodriguez, A., Nielsen, B., Pérez, J. F., Martín-Orúe, S. M. Evaluating the impact of two Bacillus probiotic strains in commercial sows and their litters. *Journal of Animal Science* 97, no. Supplement_3 (2019): 203-204.

Saladrigas-García, M., Ko, H.L., Rodriguez, A., Llonch, P., Pérez, J. F., Martín-Orúe, S. M. The combined effects of early-socialization of piglets and neonatal enriched environment on intestinal gene expression and fecal community structure. *Journal of Animal Science* 97, no. Supplement_3 (2019): 204-204.

Saladrigas-García, M., D'Angelo, M., Ko, H.L. et al. Early socialization and environmental enrichment of lactating piglets affects the caecal microbiota and metabolomic response after weaning. *Scientific Reports* 11, 6113 (2021). https://doi.org/10.1038/s41598-021-85460-7

Saladrigas-García, M., D'Angelo, M., Ko, H.L. et al. Understanding host-microbiota interactions in the commercial piglet around weaning. *Scientific Reports* 11, 23488 (2021). https://doi.org/10.1038/s41598-021-02754-6

Montoya, D., D'Angelo, M., Martín-Orúe, S: M., Rodriguez-Sorrento, A., **Saladrigas-Garcia, M.**, Araujo, C., Chabrillat, T., Kerros, S. & Castillejos, L. Effectiveness of two plant-based in-feed additives against an Escherichia coli F4 oral challenge in weaned piglets. *Animals* 11, 2024 (2021). https://doi.org/10.3390/ani11072024

Other publications

Saladrigas-García, M., Martín-Orúe, S. M. El proceso de colonización microbiana intestinal de los lechones durante los primeros días de vida. *NutriNews.* June 2020.

Saladrigas-García, M., Martín-Orúe, S. M. Microbiota intestinal porcina (book chapter). In: Microbiota en el ámbito de la Veterinaria (*in process of edition*).

Curriculum vitae of the author

Annex 2

Supplementary information

Supplementary information

Chapter 4

Table S4.1. Sow standard lactation feed formulas and estimated nutrient content of the experimental basal diets.

Ingredients, %	
Barley (10% CP)	32.07
Corn	17.29
Wheat bran	18.00
Sunflower (28% CP)	10.50
Soybean expeller (44% CP)	11.00
Lard	2.00
Animal fat	4.00
Hydrolysed mucosa	2.00
Calcium carbonate	1.68
Dicalcium phosphate	0.32
Salt	0.27
Lysine sulphate 70%	0.39
Methionine hydroxy analogue	0.03
L-Threonine	0.07
L-Valine 1814	0.02
Vitamin Mineral premix	0.30
Choline chloride 75%	0.04
Liquid 6-phytase	0.02
Antibiotic 220 g/kg premix	None or 0.06[1]
Nutritional composition (as fed basis)	
Metabolizable energy content, kcal/kg	3117
Dry matter, %	89.10
Starch, %	31.43
Neutral detergent fibre, %	20.04
Acid detergent fibre, %	8.96
Fat, %	8.94
CP, %	16.09
Lysine, %	0.93
Methionine + Cystine, %	0.57
Threonine, %	0.64
Tryptophan, %	0.20
Ash, %	5.66
Calcium, %	0.83
Total phosphorus, %	0.57

[1] In the second Trial, sows from Echo and Foxtrot farms received medicated feed with an antibiotic premix (600 ppm, ABF), whereas Charlie and Delta sows did not receive any antimicrobial treatment (non-medicated feed, NMF).

Table S4.2. Impact of age and farm on piglet faecal microbiota biodiversity. (Trial 1). Data are expressed as mean ± standard error. WP = Weaned piglets.

	Observed species	Chao1	Shannon	Simpson	Inverse Simpson
Age					
d2	905 ± 97.47	908 ± 97.42	5.90 ± 0.109	0.995 ± 0.001	243 ± 21.97
d7	1151 ± 120.4	1154 ± 120.91	6.33 ± 0.106	0.997 ± 0	392 ± 41.76
d14	1413 ± 145.71	1419 ± 145.49	6.63 ± 0.09	0.998 ± 0	532 ± 48.07
d21	1543 ± 212.44	1548 ± 213.02	6.64 ± 0.094	0.998 ± 0	543 ± 61.45
WP	2012 ± 304.66	2020 ± 304.43	7.01 ± 0.137	0.999 ± 0	914 ± 107.69
Farm					
Alpha	903 ± 65.89	909 ± 66.33	6.24 ± 0.064	0.997 ± 0	389 ± 23.4
Bravo	1906 ± 139.8	1911 ± 139.89	6.77 ± 0.088	0.998 ± 0	662 ± 61.75
P-value					
Age	<0.001	<0.001	<0.001	<0.001	<0.001
Farm	<0.001	<0.001	<0.001	0.018	<0.001
Age: Farm	0.005	0.005	0.006	0.354	<0.001

Table S4.3. Impact of age, farm and in-feed antibiotic supplementation of sows, on piglet faecal microbiota biodiversity (Trial 2). Data are expressed as mean ± standard error.

	Observed species	Chao1	Shannon	Simpson	Inverse Simpson
Age					
d2	778 ± 49.15	781 ± 49.21	5.35 ± 0.101	0.990 ± 0.001	154 ± 14.90
d21	1834 ± 75.22	1840 ± 75.24	6.95 ± 0.060	0.998 ± 0.000	773 ± 44.98
Farm					
Charlie	1430^{a} ± 121.73	1434^{a} ± 121.79	6.33^{a} ± 0.174	0.995 ± 0.001	524 ± 73.86
Delta	1187^{b} ± 122.69	1192^{b} ± 123.20	6.14^{ab} ± 0.172	0.995 ± 0.001	411 ± 63.42
Echo	1427^{a} ± 159.22	1431^{a} ± 159.21	6.31^{a} ± 0.189	0.995 ± 0.001	495 ± 83.88
Foxtrot	1170^{b} ± 146.6	1176^{b} ± 146.96	5.82^{b} ± 0.239	0.992 ± 0.002	417 ± 87.85
Dietary treatment					
NMF	1318 ± 87.44	1322 ± 87.61	6.24 ± 0.123	0.995 ± 0.001	472 ± 49.54
ABF	1293 ± 108.39	1298 ± 108.51	6.05 ± 0.156	0.993 ± 0.001	454 ± 60.53
P-value					
Age	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Farm	**0.033**	**0.034**	**0.027**	0.086	0.252
Use of AB	0.785	0.791	0.108	0.075	0.718
Age: Farm	0.148	0.150	0.297	0.229	0.501
Age: AB	0.137	0.139	0.231	0.209	0.766

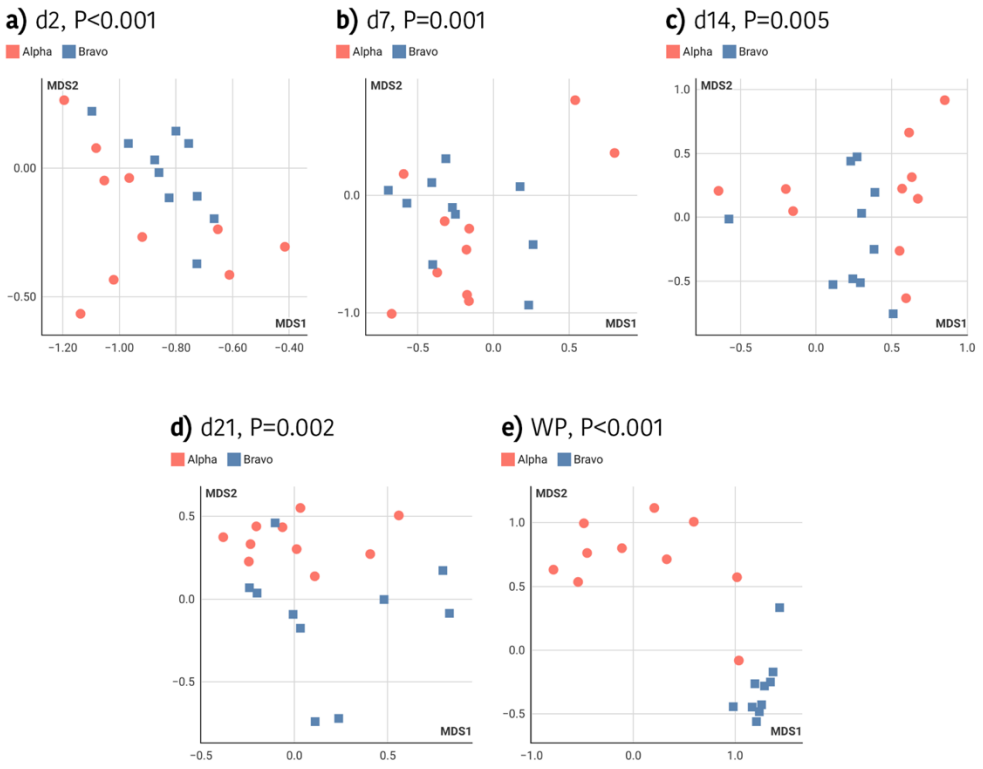


Figure S4.1. NMDS of the relative abundances of ASV during trial 1 for each sampling day. Five additional permutational analysis of variance (PERMANOVA) were performed. All comparisons between Alpha and Bravo farms were significant at each sampling age.

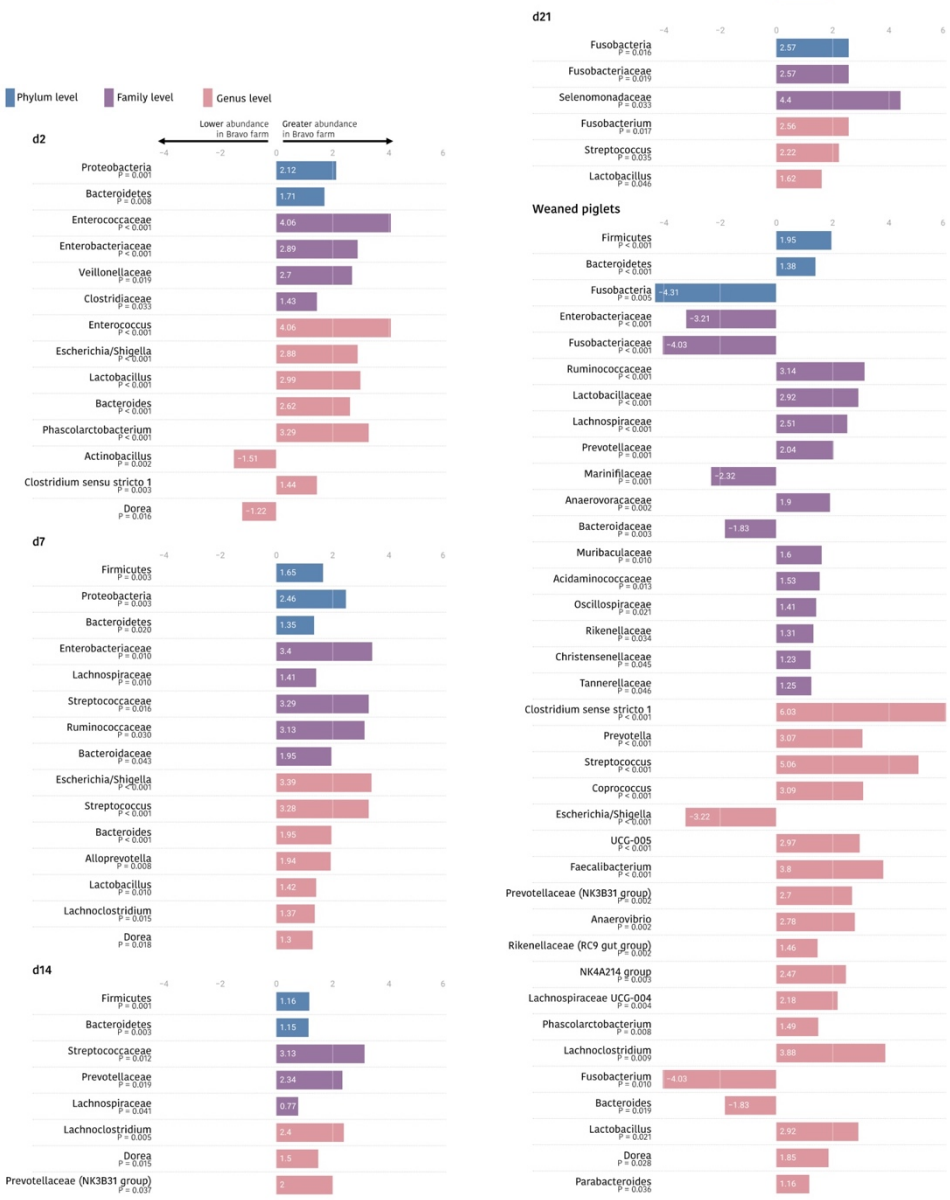


Figure S4.2. Ln changes in taxa promoted by farm origin (Bravo vs Alpha; ln change and P-value<0.05) at phylum, family and genus level in the microbiota of piglets sampled on days 2, 7, 14 and 21 of lactation and 14 days post-weaning (7 days in Alpha farm). Piglets were weaned at 21 days of age. Positive values and negative values indicate higher and lower abundance, respectively, in piglets from Bravo farm. Taxa are sorted by level of significance (from higher to lower). Only taxa with relative abundances higher to 1% are included in the figure. The presented differences are based only on taxa detected in at least half of the samples per sampling.

Chapter 5

Table S5.1. Estimated chemical composition of diets. Pre-starter diet was also offered as creep-feeding.

Lactating diet		Pre-starter diet	
Net Energy (Kcal/kg)	2450	Net Energy (Kcal/kg)	2480
Crude protein (%)	15.5	Crude protein (%)	16.5
Crude Fat (%)	4.5	Crude Fat (%)	4.8
Crude Fiber (%)	5.7	Crude Fiber (%)	4.1
Lys (%)	1.02	Lys (%)	1.33
Ash (%)	6.70	Ash (%)	5.40
Ca (%)	1.05	Ca (%)	0.55
P (%)	0.60	P (%)	0.61
Main ingredients: Barley, Corn, Wheat middlings, Soybean meal, Rapeseed meal, Corn flakes, Lard, Cane molasses, Beet pulp, Sunflower meal, Calcium carbonate, L-Lysine, Sodium bicarbonate, Monocalcium phosphate, Sodium chloride.		**Main ingredients:** Wheat, Barley, Corn Flakes, Oats, Corn, Fish meal, Wheat middlings, Porcine plasma, Soy protein concentrate, Sunflower meal, Soy lecithin, Beet pulp, L-Lysine, L-Valine, Calcium carbonate, Monocalcium phosphate, Sodium Chloride, Choline Chloride.	
Additives: Vitamin A (10,000 IU/kg), Vitamin D3 (100 IU/kg); Fe (as $FeCO_3$; 100 mg/kg), Cu (as sulfate penta-hydrate; 100 mg/kg); Zn (as ZnO; 100 mg/kg); Se (as sodium selenite, 0.2 mg/kg; and selenomethionine, 0.2 mg/kg); Iodine (as potassium iodide; 0.7 mg/kg); Mn (as MnO_2; 50 mg/kg); 6-Phytase (500 PPU/kg); Butyl-hydroxytoluene or BHT (0.3 mg/kg); and *Saccharomyces cerevisiae* NCYC Sc47 (1x10^9 UFC/kg).		**Additives:** Vitamin A (6,000 IU/kg), Vitamin D3 (1,000 IU/kg); Fe (as $FeCO_3$; 31 mg/kg), Cu (as amino acid chelate; 75 mg/kg); Zn (as ZnO; 50 mg/kg); Se (as sodium selenite, 0.08 mg/kg; and selenite CNCM I-3060, 0.008 mg/kg); Iodine (as potassium iodide; 0.4 mg/kg); Mn (as MnO_2; 25 mg/kg); 6-Phytase (0.63 PPU/kg); Endo-1,4-β-xylanase (3,000 EPU/kg); Butylhydroxytoluene or BHT (0.3 mg/kg); and *Saccharomyces cerevisiae* NCYC Sc47 (1x10^9 UFC/kg).	

Table S5.2. List of the 52 genes related to intestinal health included in the custom OpenArray plate and functional group to which they were assigned.

Gene abbreviation	Gene full name	Functional group
OCLN	Occludin	Intestinal barrier
ZO1	Zonula occludens 1	Intestinal barrier
CLDN1	Claudin-1	Intestinal barrier
CLDN4	Claudin-4	Intestinal barrier
CLDN15	Claudin-15	Intestinal barrier
MUC2	Mucin 2	Intestinal barrier
MUC13	Mucin 13	Intestinal barrier
TFF3	Trefoil factor 3	Intestinal barrier
TLR2	Toll-like receptor 2	Pattern recognition receptors (PRRs)
TLR4	Toll-like receptor 4	Pattern recognition receptors (PRRs)
IL1β	Interleukin 1 beta	Immune response
IL6	Interleukin 6	Immune response
IL8	Interleukin 8	Immune response
IL10	Interleukin 10	Immune response
IL17A	Interleukin 17	Immune response
IL22	Interleukin 22	Immune response
IFN-γ	Interferon gamma	Immune response
TNF-α	Tumor necrosis factor alpha	Immune response
TGF-β1	Transforming growth factor beta 1	Immune response
CCL20	Chemokine (C-C motif) ligand 20	Immune response
CXCL2	Chemokine (C-X-C motif) ligand 2	Immune response
IFNGR1	Interferon gamma receptor 1	Immune response
REG3G	Regenerating-islet derived protein 3 gamma	Immune response
PPARGC1α	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	Immune response
FAXDC2	Fatty acid hydrolase domain containing 2	Immune response
GBP1	Guanylate binding protein 1	Immune response
HSP27	Heat shock protein 27	Intestinal homeostasis

HSP70	Heat shock protein 70	Intestinal homeostasis
GPX2	Glutathione peroxidase 2	Digestive enzyme / hormone
SOD2	Superoxide dismutase	Digestive enzyme / hormone
ALPI	Intestinal alkaline phosphatase	Digestive enzyme / hormone
SI	Sucrase-isomaltase	Digestive enzyme / hormone
DAO1	Diamine oxidase	Digestive enzyme / hormone
HNMT	Histamine N-methyltransferase	Digestive enzyme / hormone
ANPEP	Aminopeptidase-N	Digestive enzyme / hormone
IDO1	Indoleamine 2,3-dioxygenase	Digestive enzyme / hormone
GCG	Glucagon	Digestive enzyme / hormone
CCK	Cholecystokinin	Digestive enzyme / hormone
IGF1R	Insulin-like growth factor 1 receptor	Digestive enzyme / hormone
PYY	Peptide YY	Digestive enzyme / hormone
SLC5A1	Solute carrier family 5 (sodium/glucose cotransporter) member 1	Nutrient transport
SLC16A1	Monocarboxylate transporter 1	Nutrient transport
SLC7A8	Solute carrier family 7 (amino acid transporter light chain, L System) member 8	Nutrient transport
SLC15A1	Solute carrier family 15 (oligopeptide transporter) member 1	Nutrient transport
SLC13A1	Solute carrier family 13 (sodium/sulfate symporters) member 1	Nutrient transport
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2	Nutrient transport
SLC30A1	Solute carrier family 30 (zinc transporter) member 1	Nutrient transport
SLC39A4	Solute carrier family 39 (zinc transporter) member 4)	Nutrient transport
CRHR1	Corticotropin releasing hormone receptor 1	Stress indicators
NR3C1-Grα	Glucocorticoid receptor	Stress indicators
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Stress indicators
TBP	TATA-Box binding protein	Housekeeping

Table S5.3. Pearson correlation coefficients between NMR bucket regions and bacterial families. Significant correlations are indicated in bold ($p \leq 0.05$).

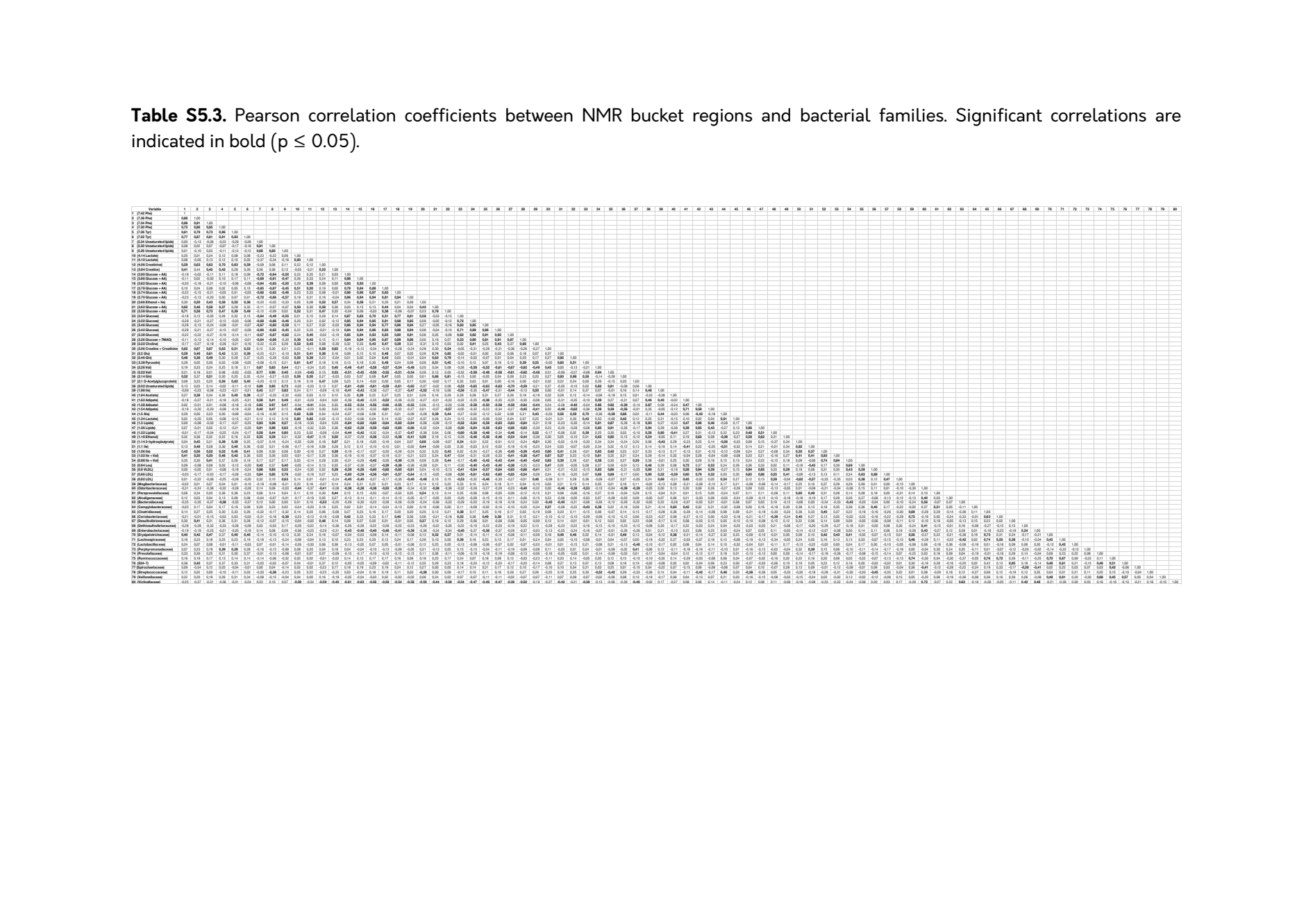


Table S5.4. List of bacterial families that significantly correlated to ^{1}H-NMR buckets ($|r \geq 0.37|$ >, $p \leq 0.05$). The central point of ^{1}H-NMR buckets is indicated (ppm) and the potential metabolite that majority contributed to the signal in this region.

Bacterial family	^{1}H-NMR bucket (ppm)	Potential metabolite	r	*p*-value
Bacteroidaceae	4.06	Creatinine	-0.53	<0.01
Bacteroidaceae	0.98	Isoleucine + Valine	-0.43	0.03
Bacteroidaceae	3.06	Creatine + Creatinine	-0.40	0.04
Bacteroidaceae	2.50	Glutamate	-0.40	0.04
Bacteroidaceae	7.30	Phenylalanine	-0.38	0.05
Campylobacteraceae	1.94	Acetate	0.65	<0.01
Campylobacteraceae	1.62	Adipate	0.42	0.03
Campylobacteraceae	0.90	VLDL[a]	0.40	0.04
Campylobacteraceae	2.26	Valine	0.38	0.05
Campylobacteraceae	1.14	3-hydroxybutyrate	0.38	0.05
Campylobacteraceae	3.06	Creatine + Creatinine	0.37	0.05
Campylobacteraceae	2.38	Pyruvate	-0.43	0.02
Clostridiaceae	1.06	Isoleucine	0.40	0.04
Clostridiaceae	3.54	Glucose	0.38	0.05
Coriobacteriaceae	3.54	Glucose	0.55	<0.01
Coriobacteriaceae	3.90	Glucose + AA	0.42	0.03
Coriobacteriaceae	1.14	3-hydroxybutyrate	0.40	0.04
Coriobacteriaceae	5.26	Unsaturated lipids	-0.39	0.05
Desulfovibrionaceae	4.06	Creatinine	0.48	0.01
Desulfovibrionaceae	7.38	Phenylalanine	0.41	0.03
Desulfovibrionaceae	3.66	Ethanol + Isoleucine	0.37	0.05
Enterobacteriaceae	3.86	Glucose + AA	-0.48	0.01
Enterobacteriaceae	3.54	Glucose	-0.40	0.04
Erysipelotrichaceae	3.58	Glucose + AA	0.57	<0.01
Erysipelotrichaceae	2.50	Glutamate	0.49	0.01
Erysipelotrichaceae	2.14	Glutamine	0.49	0.01
Erysipelotrichaceae	7.34	Phenylalanine	0.47	0.01
Erysipelotrichaceae	1.02	Isoleucine + Valine	0.43	0.03
Erysipelotrichaceae	1.06	Isoleucine	0.42	0.03
Erysipelotrichaceae	7.26	Tyrosine	0.40	0.04
Erysipelotrichaceae	1.94	Acetate	0.38	0.05

Lachnospiraceae	3.54	Glucose	0.39	0.04
Lactobacillaceae	2.10	O-acetyl-glucoprotein	-0.40	0.04
Odoribacteraceae	2.38	Pyruvate	-0.53	<0.01
Odoribacteraceae	3.78	Glucose + AA	-0.50	0.01
Odoribacteraceae	2.50	Glutamate	-0.46	0.02
Odoribacteraceae	4.14	Lactate	-0.44	0.02
Odoribacteraceae	4.06	Creatinine	-0.41	0.04
Odoribacteraceae	3.26	Glucose + TMAO[b]	-0.40	0.04
Odoribacteraceae	2.14	Glutamine	-0.39	0.04
Odoribacteraceae	2.10	O-acetyl-glucoprotein	-0.39	0.05
Paraprevotellaceae	1.14	3-hydroxybutyrate	0.60	<0.01
Paraprevotellaceae	3.66	Ethanol + Isoleucine	0.54	<0.01
Paraprevotellaceae	1.10	Isoleucine	0.49	0.01
Paraprevotellaceae	3.94	Creatine	0.44	0.02
Porphyromonadaceae	2.10	O-acetyl-glucoprotein	0.41	0.03
Porphyromonadaceae	7.26	Tyrosine	0.39	0.04
Porphyromonadaceae	1.10	Isoleucine	0.39	0.04
Porphyromonadaceae	7.30	Phenylalanine	0.39	0.05
Streptococcaceae	1.50	Alanine	0.48	0.01
Streptococcaceae	2.26	Valine	-0.52	0.01
Streptococcaceae	0.90	VLDL[a]	-0.45	0.02
Streptococcaceae	1.58	Adipate	-0.42	0.03
Streptococcaceae	3.66	Ethanol + Isoleucine	-0.38	0.05
S24-7	7.38	Phenylalanine	0.42	0.03
Victivallaceae	3.86	Glucose + AA	-0.63	<0.01
Victivallaceae	4.06	Creatinine	-0.59	<0.01
Victivallaceae	3.54	Glucose	-0.54	<0.01
Victivallaceae	3.06	Creatine + Creatinine	-0.50	0.01
Victivallaceae	3.26	Glucose + TMAO[b]	-0.50	0.01
Victivallaceae	2.50	Glutamate	-0.42	0.03
Victivallaceae	3.94	Creatine	-0.40	0.04
Victivallaceae	2.10	O-acetyl-glucoprotein	-0.40	0.04
Victivallaceae	2.38	Pyruvate	-0.39	0.05

[a]VLDL, very low density lipoprotein; [b]TMAO, trimethylamine-N-oxide.

Figure S5.1. Significant differing caecal microbiota pathways between suckling and weaned piglets (KEGG level 3). All sequence reads were used to predict functions against the KEGG database by means of PICRUSt bioinformatics software package. Figure created with the software STAMP.

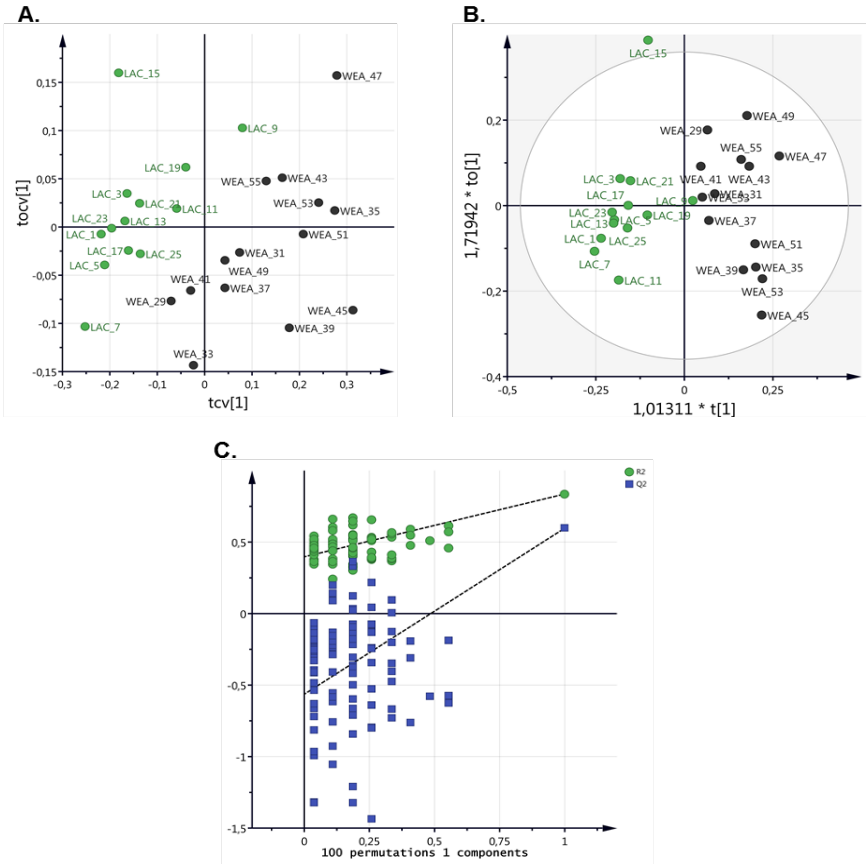


Figure S5.2. Validation of the OPLS-DA model between nursing piglets and after weaning piglets. Cross validation plot (**A**) of the OPLS-DA model. OPLS-DA plot (**B**) derived from ^{1}H-NMR serum spectra of nursing piglets (green) and weaned piglets (black). 100 random permutation test plot (**C**) relative to OPLS-DA model including all samples, where the vertical axis corresponds to R^2 (green circles) and Q^2 (blue squares) values for the model and the horizontal axis corresponds to the correlation coefficient between the original Y and the permuted Y.

The OPLS-DA constructed to discriminate between nursing piglets and after weaning piglets was confirmed by cross validation probe. Comparing both plots, while there was shifting of same spots along the orthogonal axis, the 88.9 % of the spots have the same position respect to the first component, indicating that the OPLS-DA model is devoid of influential observations and it is stable to the inclusion or exclusion of all the different observations. The permutation test plot shows the correlation coefficient between the original y-variable and the permuted y-variable on the x-axis versus the cumulative R^2 and Q^2 on the Y-axis and plots the regression line, the intercept is a measure of the overfitting. The plot of permutation test (100 times) (**Figure S5.1C**) performed for nursing and after weaned piglets shows that the new parameters (R^2=0.40 and Q^2= -0.56) were lower than the original values indicating a lack of over-fitting.

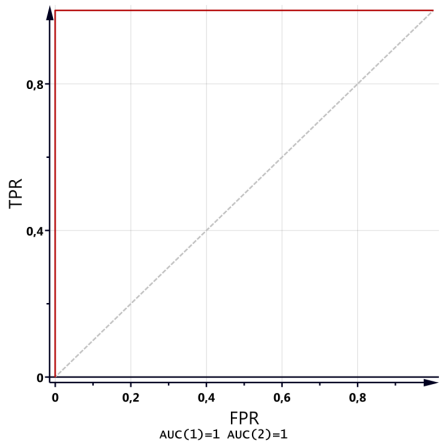


Figure S5.3. Receiver operating characteristic (ROC) plot for the OPLS-DA model between nursing and weaned piglets. The ROC plot displays the TPR for nursing group classification (blue) or for weaned group classification (red) by the constructed model plotted against the corresponding FPR at various threshold settings of the criterion parameter (YPredPS). Both curves have an AUC of the ROC plot of 1.0 indicating high sensitivity and specificity and, in consequence, a high prediction power of the model.

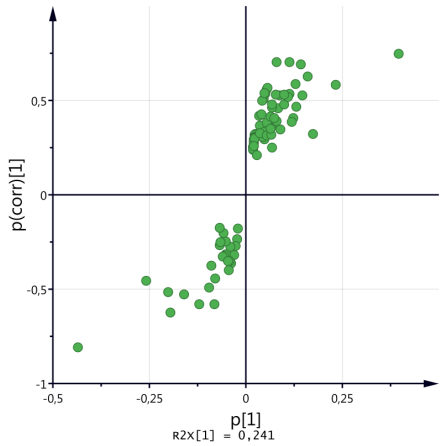


Figure S5.4. S-plot corresponding to OPLS-DA model between nursing and weaned piglets. The covariance value for each variable included is represented on the horizontal axis in the model. The vertical axis represents the correlation values obtained with respect to the dependent variable. The points at both ends of the S-plot curve indicate regions that have a strong discriminant power on the group separation.

Chapter 6

Table S6.1. Sow (standard gestation and lactation feeds) and piglet (mash creep feed) diet formulas.

Ingredients	Gestation (%)	Lactation (%)	Creep feed (%)
Barley	35.00	9.00	13.0
Maize	22.70	27.01	41.3
Wheat	9.00	25.55	12.0
Wheat middlings	15.00	6.00	-
Sweet milk whey	-	-	10.0
HP 300	-	-	15.0
Sunflower meal	5.65	4.50	-
Sugar beet pulp	3.10	2.50	-
Soybean meal 47	2.50	13.50	-
Rapeseed meal	2.50	4.50	-
Fishmeal LT	-	-	5.31
Palm Oil	-	2.0	-
Soybean oil	-	-	0.54
Lard	1.05	1.00	-
Calcium carbonate	1.12	1.08	-
Monocalcium phosphate	-	-	0.87
Dicalcium phosphate	0.99	1.25	-
Vit-Min premix	0.50	0.50	0.40*
Mycofix plus 3.E.**	0.10	0.10	-
Salt	0.40	0.50	0.25
L-Lysine HCL	0.31	0.63	0.52
L-Threonine	0.10	0.18	0.25
Methionine-liquid	-	0.04	-
DL-Methionine 99	-	-	0.27
L-Tryptophan	-	0.02	0.11
L-Valine	-	0.06	0.15

*Premix provides/kg feed: Vitamin A (retinyl acetate) 10,000 IU; Vitamin D_3 (Colecalciferol) 4,800 IU; Vitamin E/acetate de tot-rac-3- tocopheryl) 45 mg; Vitamin K_3 (MNB Menadione nicotinamide bisulphite) 3 mg; Vitamin B1 (Thiamine mononitrate) 3 mg; Vitamin B_2 (Riboflavin) 9 mg; Vitamin B_6 (Pyridoxine Chlorhydrate) 4.5 mg; Vitamin B_{12} (cyanocobalamin) 0.04 mg; Nicotinamide 51 mg; Pantothenic Acid (Calcium D-pantothenate) 16.5 mg; Biotin (D-(+)-biotin) 0.15 mg; Folic Acid 1.8 mg; Choline chloride 350 mg; Iron (Iron sulphate monohydrate) 54 mg; Zinc (Zn, zinc oxide) 66 mg; Manganese (Mn, Manganese oxide) 90 mg; Iodine (I, Calcium Iodine Anhydrus) 1.2 mg; Selenium (Se, Sodium Selenate) 0.18 mg; Copper (Cu, copper Sulphate Penthahydrate) 12 mg; Ethoxyquin 4 mg; D,L-Malic acid 60 mg; Fumaric acid 75 mg; Sepiolite 907 mg; Vermiculite 2,001 mg; Colloidal silica 45 mg.
**Clays, yeast cell wall components, algae.

Table S6.2. Estimated nutrient content of the experimental basal diets (% as fed basis).

Nutrients	Gestation	Lactation	Creep feed
Net energy content, kcal/kg	2261	2455	2480
Dry matter, %	87.7	88.1	89.5
Crude protein, %	13.01	16.69	20.05
Digestible crude protein, %	10.97	14.27	18.05
Neutral detergent fiber, %	18.02	14.56	8.23
Crude fiber, %	5.51	4.51	2.47
Ether extract, %	3.21	5.05	3.47
Ash, %	5.30	5.54	4.82
Starch, %	43.32	40.46	39.98
Total sugars, %	3.21	3.74	8.72
Linoleic acid, %	1.15	1.20	0.943
Lysine, %	0.700	1.110	1.50
SID Lysine, %	0.600	1.000	1.39
Methionine, %	0.240	0.320	0.602
SID Methionine, %	0.220	0.290	0.577
Cystine, %	0.290	0.340	0.117
SID Cystine, %	0.080	0.370	0.156
Methionine + Cystine, %	0.530	0.660	0.895
SID Methionine + Cystine, %	0.450	0.580	0.834
Threonine, %	0.570	0.780	0.990
SID Threonine, %	0.480	0.680	0.904
Tryptophan, %	0.160	0.220	0.333
SID Tryptophan, %	0.140	0.190	0.306
Calcium, %	0.850	0.910	0.482
Total phosphorus, %	0.560	0.570	0.683
STTD phosphorus, %	0.350	0.370	0.410
Magnesium, %	0.180	0.170	0.093
Sodium, %	0.180	0.210	0.240
Chlorine, %	0.340	0.370	0.536
Potassium, %	0.580	0.670	0.827

Table S6.3. Analyzed values of the intended dosage of dam top-dressing and piglet creep feed (Log_{10} CFU/g) and period of administration.

	CON	BSU	BAM
Gestation top-dressing*			
March 2016 – April 2017	<5.00	6.62	6.50
Dam lactation feed**			
July 2016 – May 2017	<5.00	5.78	5.66
Piglet creep feed**			
July – December 2016	<5.00	5.89	5.78
April 2017***	ND	ND	ND

CON=Control; BSU=*Bacillus subtilis* strain EB15; BAM=*Bacillus amyloliquefaciens* strain ZM16.
*: intended dosage (Log_{10}CFU/g): T1<5.0; T2=7.18; T3=7.18.
**: intended dosage (Log_{10}CFU/g): T1<5.0; T2=5.7; T3=5.7
***: No probiotic was supplemented for the 3rd Cycle in the creep-feed
Analyzed data for spore counts are reported as the average of the 3 consecutive cycles

Table S6.4. List of dams that were removed from the study and the reasons for exclusion.

Exclusion date	CON		BSU		BAM	
	Dam ID	**Cause**	**Dam ID**	**Cause**	**Dam ID**	**Cause**
Cycle 1						
Excluded before farrowing	1010	Repeated	930	Dead	943	Repeated
	1276	Repeated	986	Repeated	1017	Unknown cause
	1415	Repeated	1296	Unknown cause	1316	Repeated
	1438	Repeated	1444	Repeated	1443	Repeated
	1487	Repeated and dead later	1458	Repeated	4883	Unknown cause
	4460	Repeated	1459	Repeated	4491	Abortion
			1285	Dead		
Cycle 2						
Excluded before farrowing	1431	Repeated	1318	Dead	1304	Repeated
	1427	Dead	1446	Repeated	1442	Repeated
	1433	Repeated	1451	Repeated	1453	Culled due to claw lesion
	1130	Repeated	4490	Repeated	1439	Repeated
	1484	Repeated	1293	Culled due to claw lesion	1461	Repeated
	1486	Repeated	1467	Repeated	1464	Dead
			1460	Repeated		
Cycle 3						
Excluded before farrowing	1009	Repeated	1485	Repeated	997	Repeated
	1274	Dead	4230	Culled due to abortion	1022	Repeated
	1287	Repeated	4237	Culled due to claw lesion		
	1434	Repeated	4427	Culled due to claw lesion		
	4377	Repeated	4461	Culled due to claw lesion		
			4493	Repeated		

CON=Control; BSU=*Bacillus subtilis* strain EB15; BAM=*Bacillus amyloliquefaciens* strain ZM16.

Table S6.5. List of milk metabolites identified in milk samples with Proton Nuclear Magnetic Resonance (NMR) analysis.

2-Hydroxybutyrate	Glycoprotein
2-Hydroxyisovalerate	Lactose
Betaine	O-Acetylcarnitine
Butyrate	O-Phosphocholine
Choline	sn-Glycero-3-phosphocholine
Creatine	Taurine
Creatine phosphate	UDP-galactose
Creatinine	UDP-N-Acetylglucosamine
Ethanol	Valproate
Galactose	

Table S6.6. Composition of the fecal microbiota of the sows at family level (only families with a relative abundance higher than 0.1% are represented). Relative abundance results are expressed as percentage (%) in decreasing order according to the general mean, and with the standard error of the mean (SEM), followed by the adjusted p-values (adjPvalues) resulting from the comparison between d8 and d21 samplings

Family	**d08**	**d21**	**SEM**	**adjPvalues**
Erysipelotrichaceae	10.31	15.45	1.126	0.0002
Clostridiaceae	9.62	9.72	0.623	0.1307
Prevotellaceae	9.70	9.04	0.461	0.0751
Peptostreptococcaceae	5.78	10.08	0.492	0.0000
Oscillospiraceae	7.20	7.08	0.248	0.2271
Lachnospiraceae	6.99	5.70	0.217	0.4633
Lactobacillaceae	5.85	5.30	0.716	0.7297
Ruminococcaceae	6.47	4.27	0.468	0.8324
Christensenellaceae	4.67	3.49	0.302	0.8346
Bacteroidaceae	5.04	2.90	0.443	0.1794
Spirochaetaceae	2.75	3.29	0.241	0.4217
Rikenellaceae	2.88	2.26	0.114	0.7398
Muribaculaceae	1.37	2.54	0.179	0.0005
Anaerovoracaceae	0.91	0.83	0.050	0.8058
Veillonellaceae	0.63	1.06	0.161	0.2723

Tannerellaceae	0.86	0.82	0.070	0.3848
Bacteroidales_BS11_gut_group	0.87	0.60	0.174	0.2271
Pleomorphomonadaceae	0.94	0.51	0.095	0.5928
Enterobacteriaceae	1.17	0.27	0.090	0.0000
Acidaminococcaceae	0.72	0.66	0.043	0.3754
Planococcaceae	0.77	0.60	0.235	0.5046
UCG-010	0.66	0.63	0.041	0.4583
Butyricicoccaceae	0.62	0.66	0.046	0.1379
Sutterellaceae	0.83	0.45	0.088	0.2920
Eggerthellaceae	0.60	0.58	0.040	0.7514
p-2534-18B5_gut_group	0.42	0.65	0.041	0.0213
Methanobacteriaceae	0.37	0.44	0.030	0.3749
Pirellulaceae	0.40	0.41	0.047	0.2761
Erysipelatoclostridiaceae	0.37	0.36	0.032	0.5398
F082	0.38	0.26	0.038	0.7104
Desulfovibrionaceae	0.31	0.31	0.023	0.5691
Streptococcaceae	0.38	0.16	0.029	0.0719
Atopobiaceae	0.20	0.26	0.019	0.9026
Barnesiellaceae	0.27	0.16	0.032	0.5667
Geobacteraceae	0.25	0.16	0.029	0.8187
Marinifilaceae	0.28	0.13	0.027	0.0975
Moraxellaceae	0.23	0.16	0.062	0.2271
p-251-o5	0.16	0.21	0.030	0.7474
Monoglobaceae	0.17	0.19	0.015	0.3606
Corynebacteriaceae	0.14	0.21	0.043	0.4583
Saccharimonadaceae	0.14	0.20	0.024	0.0278
Peptococcaceae	0.18	0.15	0.013	0.7514
Fibrobacteraceae	0.16	0.16	0.018	0.7934
Bacillaceae	0.20	0.10	0.032	0.9719
Selenomonadaceae	0.10	0.18	0.020	0.0136
Synergistaceae	0.18	0.06	0.019	0.3076
Pasteurellaceae	0.13	0.08	0.015	0.5413
Akkermansiaceae	0.10	0.08	0.011	0.7331
Coriobacteriaceae	0.08	0.10	0.009	0.6199
Aerococcaceae	0.07	0.10	0.021	0.5275
Bifidobacteriaceae	0.12	0.03	0.014	0.0030
No_match[1]	4.55	4.81	0.166	0.0547
Other (< 0.1%)	7.59	6.26	-	-

[1]No_match: Not assigned taxa.

Table S6.7. Composition of the fecal microbiota of the piglets at family level (only families with a relative abundance higher than 0.05% are represented). Relative abundance results are expressed as percentage (%) in decreasing order according to the general mean (the average of d21 and d33), and with the standard error of the mean (SEM), followed by the adjusted p-values (adjPvalues) resulting from the comparison samplings (during lactation, d21, vs after weaning, d33).

Family	d21	d33	SEM	adjPvalues
Bacteroidaceae	11.76	3.24	1.062	0.0030
Enterobacteriaceae	6.51	8.17	1.465	0.0109
Erysipelotrichaceae	2.95	13.35	1.761	0.2855
Lachnospiraceae	7.65	5.45	0.418	0.0015
Oscillospiraceae	6.24	5.83	0.504	0.4137
Prevotellaceae	2.47	10.62	0.760	0.0000
Lactobacillaceae	7.21	3.32	0.662	0.0009
Ruminococcaceae	6.12	3.98	0.678	0.0506
Muribaculaceae	4.22	3.60	0.581	0.8876
Clostridiaceae	4.44	2.55	0.441	0.0207
Christensenellaceae	3.22	2.78	0.352	0.0972
Rikenellaceae	3.08	2.41	0.310	0.0866
Acidaminococcaceae	3.08	1.99	0.239	0.0022
Comamonadaceae	3.74	0.13	0.595	0.0000
Synergistaceae	2.73	0.78	0.339	0.0000
Spirochaetaceae	1.13	3.09	0.303	0.0013
Tannerellaceae	1.45	2.00	0.266	0.9876
Peptostreptococcaceae	1.95	1.04	0.204	0.0010
Anaerovoracaceae	1.43	0.96	0.110	0.0154
Fusobacteriaceae	0.59	1.94	0.299	0.1986
p-2534-18B5 (gut group)	0.62	1.88	0.241	0.3747
Marinifilaceae	1.18	0.92	0.155	0.0235
Akkermansiaceae	1.68	0.05	0.327	0.0000
Campylobacteraceae	0.61	1.56	0.206	0.1848
Veillonellaceae	0.56	1.37	0.234	0.9876
Desulfovibrionaceae	0.98	0.50	0.082	0.1738
Helicobacteraceae	0.08	1.61	0.344	0.0108
Actinomycetaceae	1.01	0.02	0.173	0.0000
Methanobacteriaceae	0.82	0.17	0.097	0.0000

Eggerthellaceae	0.61	0.42	0.053	0.0504
UCG-010	0.31	0.84	0.075	0.0842
Atopobiaceae	0.75	0.14	0.150	0.0006
Erysipelatoclostridiaceae	0.57	0.40	0.085	0.1448
Streptococcaceae	0.68	0.21	0.086	0.0041
Enterococcaceae	0.75	0.04	0.138	0.0000
Coriobacteriaceae	0.61	0.19	0.073	0.0069
Succinivibrionaceae	0.04	1.01	0.141	0.0000
Peptococcaceae	0.48	0.22	0.180	0.4362
Pirellulaceae	0.33	0.39	0.061	0.7435
Chlamydiaceae	0.00	0.80	0.142	0.0000
Selenomonadaceae	0.15	0.46	0.086	0.0002
Sutterellaceae	0.23	0.16	0.028	0.0943
Bacteroidales BS11 (gut group)	0.17	0.22	0.134	0.0000
Peptostreptococcales-Tissierellales	0.28	0.01	0.070	0.0001
Butyricicoccaceae	0.10	0.25	0.029	0.6163
Rhizobiaceae	0.07	0.30	0.051	0.0056
Monoglobaceae	0.03	0.29	0.037	0.0000
Oscillospirales	0.17	0.07	0.021	0.0002
Porphyromonadaceae	0.15	0.09	0.075	0.5656
Pasteurellaceae	0.07	0.20	0.039	0.9718
Eubacteriaceae	0.20	0.00	0.030	0.0000
p-251-o5	0.00	0.28	0.049	0.0000
Bifidobacteriaceae	0.10	0.09	0.028	0.7599
Bacteroidales (RF16 group)	0.01	0.22	0.029	0.0000
F082	0.01	0.20	0.081	0.0000
Victivallaceae	0.09	0.07	0.019	0.9579
Sphingomonadaceae	0.03	0.13	0.023	0.0014
Oligosphaeraceae	0.03	0.12	0.016	0.0001
Caulobacteraceae	0.02	0.12	0.024	0.0001
Puniceicoccaceae	0.05	0.05	0.028	0.3771
Chitinophagaceae	0.01	0.07	0.011	0.0011
Paludibacteraceae	0.02	0.09	0.015	0.0000

Table S6.8. Composition of the fecal microbiota of the piglets at genus level (only genera with a relative abundance higher than 0.05% are represented). Relative abundance results are expressed as percentage (%) in decreasing order according to the general mean (the average of d21 and d33), and with the standard error of the mean (SEM), followed by the adjusted p-values (adjPvalues) resulting from the comparison between samplings (during lactation, d21, vs after weaning, d33).

Genus	d21	d33	SEM	adjPvalues
Bacteroides	11.76	3.24	1.062	0.0028
Escherichia/Shigella	6.49	8.14	1.460	0.0103
Lactobacillus	7.21	3.31	0.662	0.0008
Turicibacter	0.86	10.88	1.812	0.7972
Clostridium sensu stricto 1	4.16	1.98	0.425	0.0099
UCG-002	3.24	2.45	0.373	0.0549
Christensenellaceae (R-7 group)	2.90	2.43	0.311	0.1473
Phascolarctobacterium	2.99	1.97	0.237	0.0028
Comamonas	3.71	0.12	0.591	0.0000
Rikenellaceae (RC9 gut group)	2.23	2.06	0.281	0.5319
Ruminococcus	2.77	0.62	0.325	0.0000
CAG-873	2.68	0.30	0.502	0.0005
Prevotella	0.81	3.00	0.310	0.0008
Cloacibacillus	2.43	0.33	0.319	0.0001
Alloprevotella	0.31	3.21	0.367	0.0000
Parabacteroides	0.96	1.95	0.258	0.7883
Treponema	0.86	1.96	0.254	0.0021
Subdoligranulum	1.64	0.60	0.357	0.1558
Lachnospiraceae (UCG-004 group)	1.93	0.07	0.227	0.0000
Fusobacterium	0.59	1.93	0.297	0.1977
Akkermansia	1.68	0.05	0.327	0.0000
Campylobacter	0.61	1.56	0.206	0.1825
Lachnoclostridium	1.46	0.26	0.135	0.0000
UCG-005	1.03	0.76	0.141	0.4819
NK4A214_group	0.81	0.99	0.114	0.1731
Romboutsia	1.24	0.09	0.137	0.0000
Butyricimonas	0.99	0.45	0.122	0.0066
Helicobacter	0.08	1.60	0.343	0.0101
Prevotellaceae (NK3B31 group)	0.32	1.21	0.129	0.0019
Prevotellaceae (UCG-003 group)	0.08	1.50	0.143	0.0000
Megasphaera	0.48	0.91	0.208	0.4555
Sphaerochaeta	0.27	1.13	0.116	0.0021
Family XIII (AD3011 group)	0.46	0.77	0.064	0.3389
Alistipes	0.82	0.21	0.084	0.0001
Actinomyces	0.93	0.02	0.160	0.0000
Desulfovibrio	0.67	0.39	0.054	0.1465
Methanobrevibacter	0.82	0.17	0.095	0.0000

Dorea	0.71	0.27	0.063	0.0061
Streptococcus	0.68	0.21	0.085	0.0015
Terrisporobacter	0.34	0.65	0.143	0.9799
Enterococcus	0.75	0.05	0.138	0.0000
Collinsella	0.61	0.19	0.073	0.0018
Succinivibrio	0.03	0.99	0.140	0.0000
Holdemanella	0.55	0.15	0.073	0.0001
Blautia	0.28	0.53	0.066	0.6997
p-1088-a5 gut group	0.33	0.39	0.061	0.7167
Peptococcus	0.47	0.16	0.180	0.7033
Denitrobacterium	0.35	0.33	0.037	0.2893
Chlamydia	0.00	0.80	0.142	0.0000
Intestinimonas	0.35	0.26	0.049	0.2169
Pyramidobacter	0.24	0.41	0.101	0.0117
Prevotellaceae (UCG-001 group)	0.18	0.41	0.053	0.5075
Prevotellaceae (UCG-004 group)	0.30	0.22	0.056	0.1111
Lachnospiraceae (NK4A136 group)	0.21	0.33	0.045	0.8574
Peptostreptococcus	0.27	0.24	0.074	0.0416
Odoribacter	0.12	0.46	0.077	0.7617
Catenibacterium	0.27	0.23	0.061	0.3013
Faecalibacterium	0.08	0.45	0.056	0.0074
Bilophila	0.30	0.08	0.041	0.0000
Anaerovibrio	0.12	0.32	0.066	0.0000
Colidextribacter	0.13	0.27	0.031	0.0710
Clostridium sensu stricto 6	0.00	0.45	0.059	0.0000
Olsenella	0.23	0.09	0.061	0.0091
Faecalicoccus	0.28	0.00	0.043	0.0000
Veillonella	0.02	0.39	0.060	0.0049
Sutterella	0.21	0.10	0.024	0.0014
Coprococcus	0.09	0.28	0.039	0.0090
Roseburia	0.10	0.23	0.036	0.4803
Clostridium sensu stricto 2	0.26	0.00	0.062	0.0001
Agathobacter	0.00	0.38	0.060	0.0000
UBA1819	0.24	0.02	0.029	0.0000
Frisingicoccus	0.04	0.31	0.089	0.6997
Oscillospira	0.16	0.12	0.036	0.7972
Lachnospiraceae (UCG-010 group)	0.02	0.31	0.031	0.0000
Monoglobus	0.03	0.29	0.037	0.0000
Hydrogenoanaerobacterium	0.17	0.07	0.021	0.0001
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.05	0.24	0.041	0.0000
Porphyromonas	0.15	0.09	0.075	0.5019
Fournierella	0.15	0.10	0.050	0.2676
Marvinbryantia	0.15	0.09	0.023	0.0737
S5-A14a	0.20	0.00	0.035	0.0000
Eubacterium	0.20	0.01	0.030	0.0000

Coriobacteriaceae (UCG-003 group)	0.19	0.00	0.055	0.0000
Lachnospiraceae (UCG-002 group)	0.17	0.01	0.032	0.0000
Sharpea	0.15	0.02	0.049	0.0000
Bifidobacterium	0.10	0.09	0.028	0.7049
Butyricicoccus	0.10	0.07	0.014	0.0630
UCG-004	0.07	0.11	0.025	0.0677
Hungatella	0.14	0.00	0.019	0.0000
Anaerotruncus	0.11	0.03	0.018	0.5249
Eisenbergiella	0.12	0.01	0.016	0.0000
dgA-11 gut group	0.03	0.14	0.018	0.0003
Tuzzerella	0.07	0.08	0.023	0.0443
Atopobium	0.11	0.00	0.020	0.0000
Sphingomonas	0.03	0.13	0.022	0.0002
UCG-003	0.02	0.12	0.016	0.0021
Oscillibacter	0.05	0.08	0.012	0.9799
Caulobacter	0.02	0.12	0.024	0.0000
Paludicola	0.09	0.01	0.011	0.0000
Christensenella	0.09	0.00	0.014	0.0000
UCG-008	0.00	0.14	0.026	0.0000
Solobacterium	0.04	0.08	0.017	0.5432
Eggerthella	0.09	0.00	0.019	0.0000
Candidatus Soleaferrea	0.04	0.07	0.014	0.3120
Oribacterium	0.03	0.07	0.011	0.5835
Erysipelatoclostridium	0.08	0.00	0.015	0.0000
Murdochiella	0.08	0.00	0.017	0.0000
Parvimonas	0.07	0.01	0.023	0.0000
Epulopiscium	0.08	0.00	0.014	0.0000
Sanguibacteroides	0.06	0.02	0.012	0.0001
Victivallis	0.06	0.01	0.015	0.0000
Z20	0.03	0.07	0.010	0.0002
Dielma	0.01	0.09	0.029	0.0000
Mucispirillum	0.00	0.09	0.022	0.0000
Arcanobacterium	0.06	0.00	0.033	0.0492
Parasutterella	0.02	0.06	0.011	0.1427
Sediminibacterium	0.01	0.07	0.011	0.0008
Lachnospiraceae (AC2044 group)	0.01	0.07	0.014	0.0000
Helcococcus	0.06	0.00	0.024	0.0000
Bergeyella	0.00	0.08	0.025	0.0000
Peptoniphilus	0.06	0.00	0.012	0.0000
Lachnospiraceae (ND3007 group)	0.01	0.07	0.016	0.0000
Clostridioides	0.06	0.00	0.014	0.0000
Negativicoccus	0.05	0.00	0.018	0.0000
Mitsuokella	0.01	0.06	0.012	0.0012
Clostridium sensu stricto 13	0.00	0.07	0.016	0.0000
Mesorhizobium	0.01	0.05	0.009	0.0000

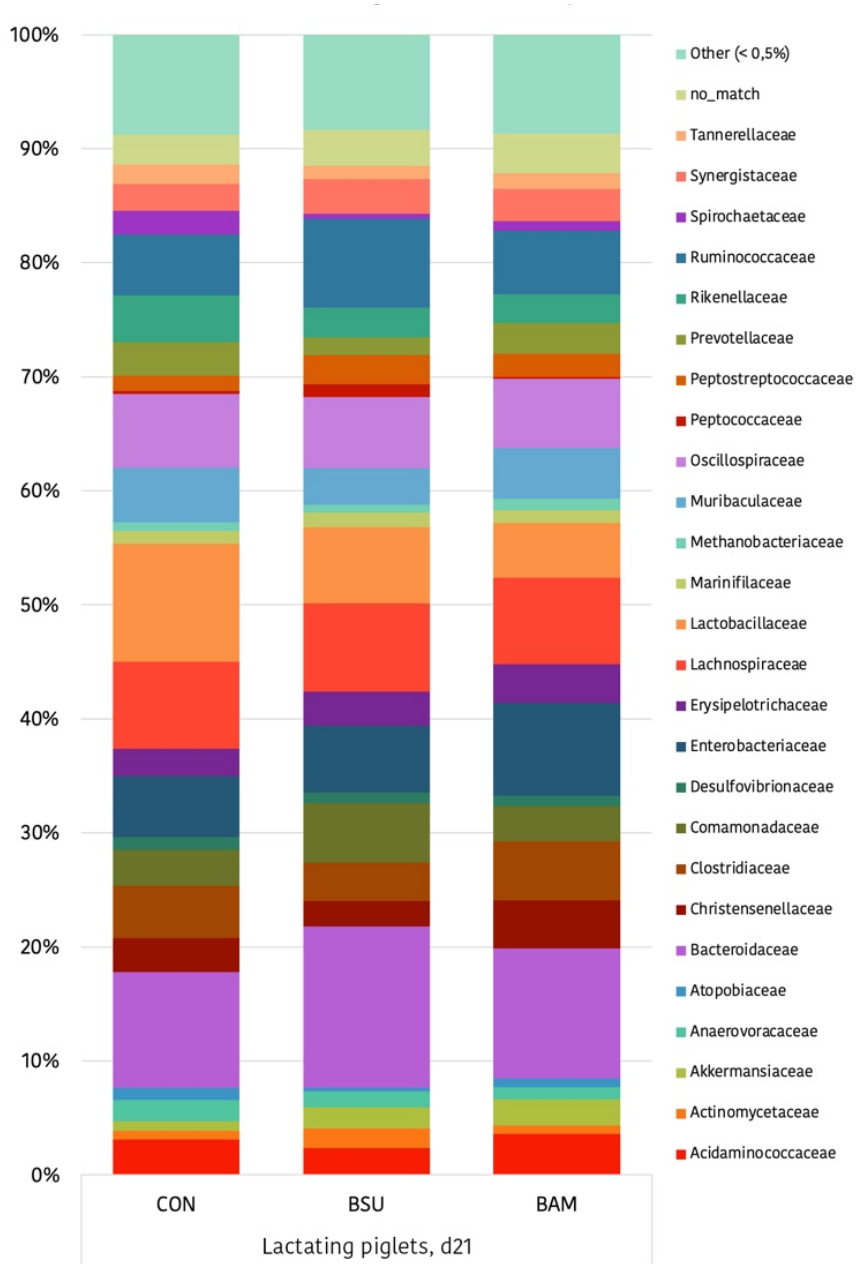
Table S6.9. Statistical analysis obtained for the 51 genes that could be quantitatively determined

Gene	Function	Treatment P-value	Size P-value	Treatment:Size P-value
TFF3	BF	0.918	0.915	0.926
OCLN	BF	0.865	0.971	0.882
ZO1	BF	0.918	0.915	0.776
CLDN1	BF	0.932	0.991	0.882
CLDN4	BF	0.918	0.766	0.982
CLDN15	BF	0.419	0.052	0.926
MUC2	BF	0.532	0.915	0.925
MUC13	BF	0.989	0.915	0.926
SI	EH	0.918	0.788	0.882
DAO1	EH	0.918	0.915	0.882
HNMT	EH	0.843	0.991	0.925
ANPEP	EH	0.843	0.991	0.926
IDO1	EH	0.938	0.991	0.926
GCG	EH	0.406	0.936	0.882
CCK	EH	0.419	0.936	0.737
IGF1R	EH	0.419	0.052	0.882
PYY	EH	0.406	0.740	0.925
GPX2	EH	0.843	0.991	0.882
SOD2.m	EH	0.740	0.936	0.926
ALPI	EH	0.843	0.991	0.772
TLR2	IR	0.999	0.915	0.926
TLR4	IR	0.918	0.915	0.925
IL1B	IR	0.843	0.991	0.979
IL6	IR	0.903	0.971	0.976
IL10	IR	0.800	0.942	0.976
IL17A	IR	0.921	0.942	0.926
IL22	IR	0.800	0.740	0.925
IFNg	IR	0.800	0.942	0.926
TNFa	IR	0.938	0.991	0.926

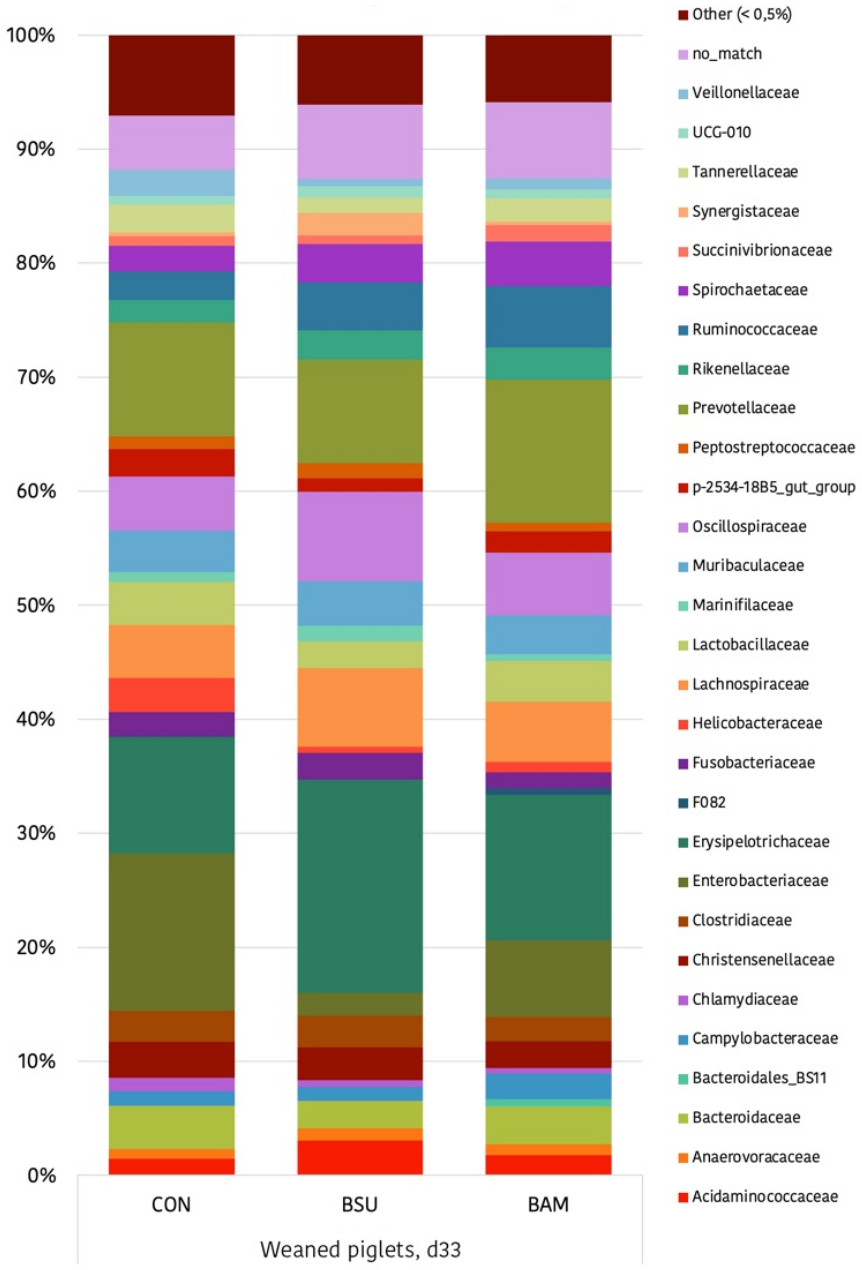
TGFb1	IR	0.918	0.915	0.926
CCL20	IR	0.987	0.942	0.925
CXCL2	IR	0.865	0.991	0.926
IFNGR1	IR	0.918	0.915	0.925
HSPB1/HSP27	IR	0.740	0.038	0.772
HSPA4/HSP70	IR	0.740	0.942	0.926
REG3G	IR	0.918	0.915	0.926
PPARGC1a	IR	0.532	0.915	0.925
FAXDC2	IR	0.987	0.936	0.882
GBP1	IR	0.843	0.971	0.925
IL8	IR	0.843	0.991	0.926
SLC5A1/SGLT1	NT	0.918	0.788	0.882
SLC16A1/MCT1	NT	0.787	0.942	0.956
SLC7A8	NT	0.918	0.352	0.882
SLC15A1/PEPT 1	NT	0.938	0.991	0.925
SLC13A1/NAS1	NT	0.957	0.915	0.882
SLC11A2/DMT1	NT	0.918	0.915	0.882
SLC30A1/ZnT1	NT	0.919	0.936	0.882
SLC39A4/ZIP4	NT	0.918	0.915	0.882
CRHR1	ST	0.830	0.936	0.969
NR3C1-Gra	ST	0.631	0.977	0.926
HSD11B1	ST	0.919	0.936	0.776

BF: Barrier function related genes / EH: Enzymes/Hormones related genes / IR: Immune system related genes / NT: Nutrient Transport related genes / ST: Stress related genes.

Figure S6.1. Barplot of the relative abundances of the families observed in the analysis of the microbiota of lactating piglets (**a**; d21) and weaned piglets (**b**; d33) by massive sequencing of the 16S rRNA gene. Only significant taxa with greater relative abundance than 0.5% are presented. CON=Control; BSU=*Bacillus subtilis* strain EB15; BAM=*Bacillus amyloliquefaciens* strain ZM16



a


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CON
BSU
BAM
Weaned piglets, d33
Other (< 0,5%)
no_match
Veillonellaceae
UCG-010
Tannerellaceae
Synergistaceae
Succinivibrionaceae
Spirochaetaceae
Ruminococcaceae
Rikenellaceae
Prevotellaceae
Peptostreptococcaceae
p-2534-18B5_gut_group
Oscillospiraceae
Muribaculaceae
Marinifilaceae
Lactobacillaceae
Lachnospiraceae
Helicobacteraceae
Fusobacteriaceae
F082
Erysipelotrichaceae
Enterobacteriaceae
Clostridiaceae
Christensenellaceae
Chlamydiaceae
Campylobacteraceae
Bacteroidales_BS11
Bacteroidaceae
Anaerovoracaceae
Acidaminococcaceae

b

Chapter 7

Table S7.1. Phylum relative counts for suckling and weaned piglets (from highest to lowest considering the global average) and for conventional reared piglets (CTR) or with environmental enrichment (ENR). All the detected phyla are included.

	LACT				WEAN			
	CTR	**ENR**	SEM	P-values	**CTR**	**ENR**	SEM	P-values
Firmicutes	38.18	39.45	3.264	0.9626	57.37	43.31	4.182	0.6236
Bacteroidetes	43.69	42.74	3.080	0.9626	30.04	36.35	2.998	0.5997
Proteobacteria	6.32	6.52	0.889	0.9626	5.18	13.00	2.162	0.4410
Spirochaetes	1.96	4.85	1.064	0.9626	3.83	3.31	0.589	0.9941
Fusobacteria	6.80	2.63	1.953	0.7823	0.03	1.96	0.644	0.3571
Planctomycetes	0.34	0.77	0.201	0.9177	0.48	0.19	0.120	0.9228
Cyanobacteria	0.33	0.97	0.297	0.7823	0.32	0.09	0.082	0.5997
Synergistetes	0.47	0.78	0.193	0.9369	0.13	0.26	0.051	0.6236
Verrucomicrobia	0.68	0.29	0.235	0.9634	0.31	0.33	0.083	0.9990
Actinobacteria	0.26	0.11	0.094	0.9626	0.54	0.25	0.113	0.5997
Tenericutes	0.18	0.10	0.036	0.9177	0.67	0.17	0.180	0.4410
Elusimicrobia	0.44	0.14	0.129	0.9177	0.14	0.27	0.103	0.5997
Lentisphaerae	0.16	0.31	0.084	0.9626	0.01	0.07	0.020	0.4410
Chlamydiae	0.00	0.01	0.005	0.6237	0.45	0.01	0.127	0.1537
Deferribacteres	0.04	0.09	0.024	0.6237	0.07	0.23	0.066	0.5413
Fibrobacteres	0.02	0.09	0.038	0.9177	0.19	0.13	0.051	0.9990
TM7	0.01	0.11	0.041	0.4849	0.15	0.02	0.036	0.4390
Euryarchaeota	0.05	0.03	0.008	0.9626	0.07	0.04	0.015	0.5997
WPS-2	0.07	0.01	0.032	0.9177	0.01	0.01	0.003	0.5413

Table S7.2. Genus relative abundance counts for suckling and weaned piglets (from highest to lowest considering the global average) and in conventional reared piglets (CTR) or with environmental enrichment (ENR). Only predominant genera (+1%) are included.

	LACT				WEAN			
	CTR	**ENR**	SEM	P-values	**CTR**	**ENR**	SEM	P-values
Prevotella	18.78	13.66	2.243	0.9573	11.93	10.06	2.015	0.9432
[Prevotella]	6.72	6.21	1.080	0.9573	5.28	12.57	1.650	0.4413
Bacteroides	5.90	8.12	1.507	0.9573	2.50	2.66	0.542	0.8931
Phascolarctobacterium	3.33	3.30	0.591	0.9842	4.77	3.23	0.430	0.4413
Campylobacter	1.26	1.10	0.422	0.9573	1.45	8.29	2.092	0.3842
Fusobacterium	6.81	2.63	1.954	0.7993	0.03	1.96	0.645	0.3842
Oscillospira	2.65	2.30	0.333	0.9842	2.57	2.80	0.302	0.9432
Treponema	0.81	2.98	0.863	0.9573	3.16	1.46	0.517	0.6459
p-75-a5	0.72	0.47	0.250	0.9573	3.38	2.47	0.868	0.9432
Lactobacillus	2.72	2.07	0.718	0.9573	0.43	0.82	0.325	0.5380
Megasphaera	2.67	1.86	0.685	0.9573	0.40	0.83	0.316	0.8931
Sphaerochaeta	1.15	1.88	0.466	0.9573	0.67	1.86	0.411	0.5266
Roseburia	0.21	0.17	0.076	0.9573	3.05	1.18	0.703	0.5380
Anaerovibrio	0.94	0.38	0.448	0.9573	1.80	0.94	0.286	0.5266
CF231	1.23	1.09	0.249	0.9573	0.60	0.69	0.164	0.8931
Desulfovibrio	0.73	0.68	0.108	0.9573	1.15	0.77	0.259	0.9432
Butyricimonas	0.59	1.14	0.308	0.9573	0.12	0.61	0.153	0.4746
Ruminococcus	0.33	0.30	0.085	0.9573	1.18	0.65	0.193	0.5226

Table S7.3. PCA and OPLS-DA models parameters for ^{1}H-NMR serum profiles of weaned piglets.

ENR vs CON P-value	n	PCA			OPLS-DA			
		Number of components	$R^2X_{(cum)}$	$Q^2_{(cum)}$	Number of components	$R^2X_{(cum)}$	$R^2Y_{(cum)}$	$Q^2_{(cum)}$
Total spectre	7	1+1	0.64	0.31	1+1	0.62	0.57	0.28
≤ 0.20	7	2	0.85	0.76	1+1	0.83	0.57	0.33
≤ 0.18	7	2	0.88	0.81	1+1	0.87	0.52	0.28
≤0.16	7	2	0.89	0.83	1+1	0.88	0.52	0.29
≤0.14	7	2	0.88	0.81	1+1	0.86	0.61	0.32
≤0.12	7	2	0.90	0.81	1+1	0.86	0.66	0.34
≤0.10	7	2	0.90	0.81	1+1	0.86	0.66	0.34
≤0.08	7	2	0.90	0.81	2	0.89	0.68	0.47
≤0.06	7	3	0.95	0.83	2	0.91	0.68	0.53

Table S7.4. Brief description of the genes analysed.

Gene abbreviation	Gene full name	Functional group
OCLN	Occludin	Intestinal barrier
ZO1	Zonula occludens 1	Intestinal barrier
CLDN1	Claudin-1	Intestinal barrier
CLDN4	Claudin-4	Intestinal barrier
CLDN15	Claudin-15	Intestinal barrier
MUC2	Mucin 2	Intestinal barrier
MUC13	Mucin 13	Intestinal barrier
TFF3	Trefoil factor 3	Intestinal barrier
TLR2	Toll-like receptor 2	Pattern recognition receptors (PRRs)
TLR4	Toll-like receptor 4	Pattern recognition receptors (PRRs)

IL1β	Interleukin 1 beta	Immune response
IL6	Interleukin 6	Immune response
IL8	Interleukin 8	Immune response
IL10	Interleukin 10	Immune response
IL17A	Interleukin 17	Immune response
IL22	Interleukin 22	Immune response
IFN-γ	Interferon gamma	Immune response
TNF-α	Tumor necrosis factor alpha	Immune response
TGF-β1	Transforming growth f actor beta 1	Immune response
CCL20	Chemokine (C-C motif) ligand 20	Immune response
CXCL2	Chemokine (C-X-C motif) ligand 2	Immune response
IFNGR1	Interferon gamma receptor 1	Immune response
REG3G	Regenerating-islet derived protein 3 gamma	Immune response
PPARGC1α	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	Immune response
FAXDC2	Fatty acid hydrolase domain containing 2	Immune response
GBP1	Guanylate binding protein 1	Immune response
HSP27	Heat shock protein 27	Intestinal homeostasis
HSP70	Heat shock protein 70	Intestinal homeostasis
GPX2	Glutathione peroxidase 2	Digestive enzyme / hormone
SOD2	Superoxide dismutase	Digestive enzyme / hormone
ALPI	Intestinal alkaline phosphatase	Digestive enzyme / hormone
SI	Sucrase-isomaltase	Digestive enzyme / hormone
DAO1	Diamine oxidase	Digestive enzyme / hormone
HNMT	Histamine N-methyltransferase	Digestive enzyme / hormone
ANPEP	Aminopeptidase-N	Digestive enzyme / hormone
IDO1	Indoleamine 2,3-dioxygenase	Digestive enzyme / hormone

GCG	Glucagon	Digestive enzyme / hormone
CCK	Cholecystokinin	Digestive enzyme / hormone
IGF1R	Insulin-like growth factor 1 receptor	Digestive enzyme / hormone
PYY	Peptide YY	Digestive enzyme / hormone
SLC5A1	Solute carrier family 5 (sodium/glucose cotransporter) member 1	Nutrient transport
SLC16A1	Monocarboxylate transporter 1	Nutrient transport
SLC7A8	Solute carrier family 7 (amino acid transporter light chain, L System) member 8	Nutrient transport
SLC15A1	Solute carrier family 15 (oligopeptide transporter) member 1	Nutrient transport
SLC13A1	Solute carrier family 13 (sodium/sulfate symporters) member 1	Nutrient transport
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2	Nutrient transport
MT1A	Metallothionein 1A	Nutrient transport
SLC30A1	Solute carrier family 30 (zinc transporter) member 1	Nutrient transport
SLC39A4	Solute carrier family 39 (zinc transporter) member 4)	Nutrient transport
CRHR1	Corticotropin releasing hormone receptor 1	Stress indicators
NR3C1	Glucocorticoid receptor	Stress indicators
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Stress indicators
ACTB	β-actin	Housekeeping
B2M	β2-microglobulin	Housekeeping
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping
TBP	TATA-Box binding protein	Housekeeping

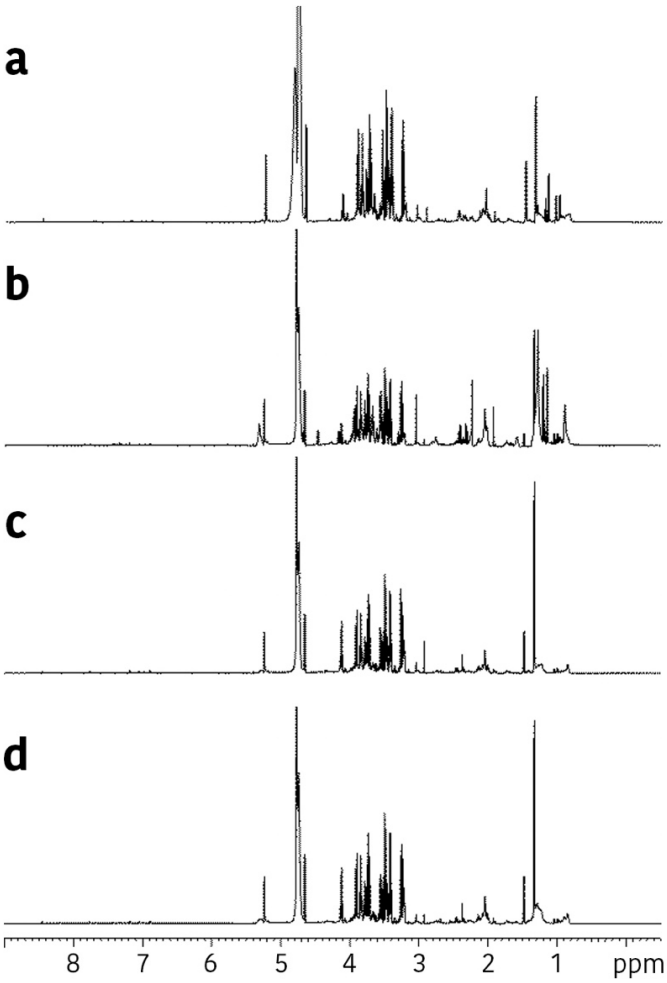


Figure S7.1. Representative ^{1}H-NMR spectra of serum from piglets of different experimental treatment. (**a**) Lactating piglets control group; (**b**) lactating enriched piglets' group; (**c**) weaned piglets control group and (**d**) weaned enriched piglets' group. All spectra were acquired using a 600 MHz spectrometer.

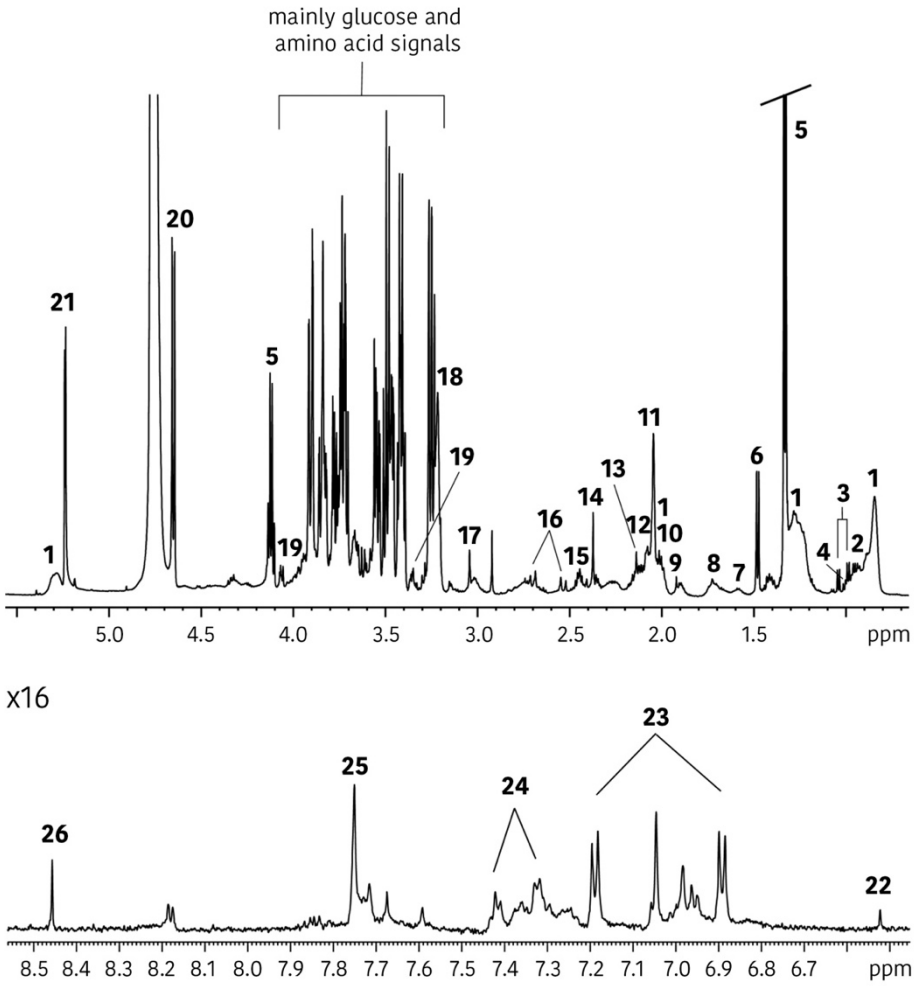


Figure S7.2. Representative ^{1}H CPMG spectrum (600MHz) of the serum from a nursing piglet. Assignments: **1**, LDL/VLDL; **2**, leucine; **3**, valine; **4**, isoleucine; **5**, lactate; **6**, alanine; **7**, adipate; **8**, arginine; **9**, acetate; **10**, proline; **11**, N-acetyl glycoproteins; **12**, O-acetyl glycoproteins; **13**, glutamine/glutamate; **14**, pyruvate; **15**, glutamate; **16**, citrate; **17**, creatine; **18**, choline; **19**, myo-inositol; **20**, β-glucose (anomeric proton); **21**, α-glucose (anomeric proton); **22**, fumarate; **23**, tyrosine; **24**, phenylalanine; **25**, methyl histidine; **26**, formic acid.

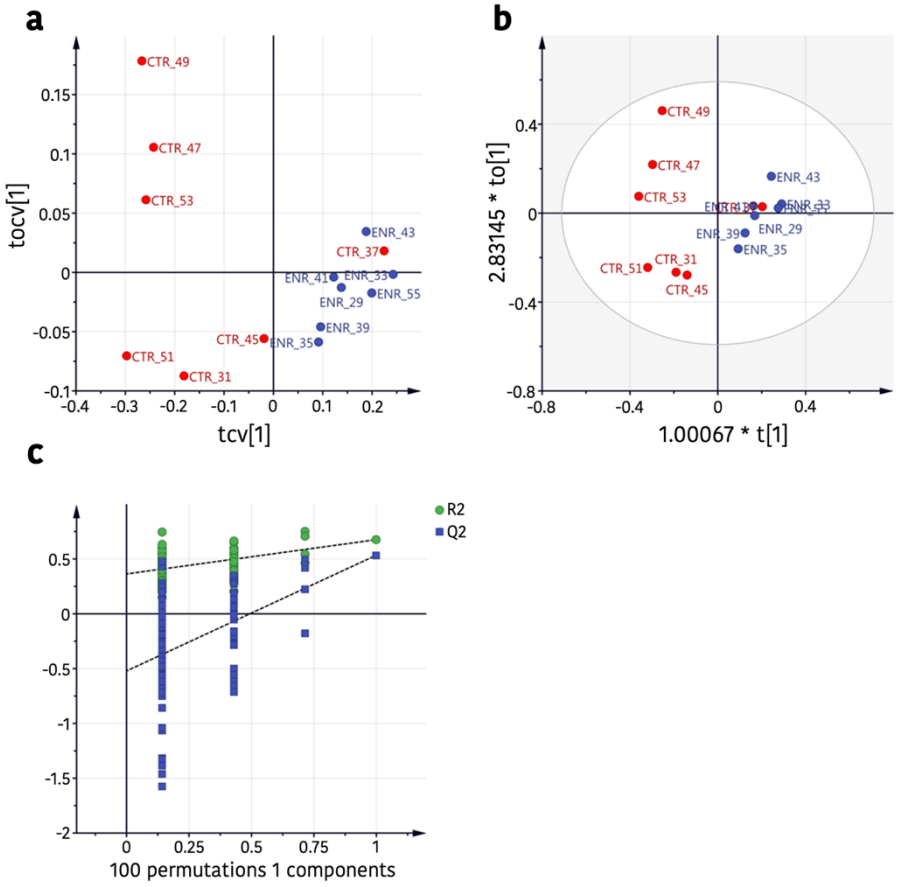


Figure S7.3. Validation of the OPLS-DA model between enriched and control weaned piglets. OPLS-DA (P≤0.06) plot (**a**) derived from ^{1}H-NMR serum spectra of weaned enriched piglets (blue) and weaned control group (red). Cross validation plot (**b**) of the OPLS-DA (P≤0.06) model. 100 random permutation test plot (**c**) relative to OPLS-DA (P≤0.06) model for all samples including enriched and control piglets, where the vertical axis corresponds to R^2 (green circles) and Q^2 (blue squares) values for the model and the horizontal axis corresponds to the correlation coefficient between the original Y and the permuted Y.

The OPLS-DA (P≤0.06) model constructed to discriminate between enriched and control piglets after weaning was confirmed by cross-validation, the score plot of the regular scores (**Figure S7.3a**) compared with the score plot of the CDs course (**Figure S7.3b**) were are almost the same with very little shifting of the spots which is a strong indication that the OPLS-DA (P≤0.06) model is devoid of influential observations and it is very stable to the inclusion or exclusion of all the different observations. Furthermore, the plot of permutation test (100 times) (**Figure S7.3c**) performed for all samples including enriched piglets and control piglets shows that the new parameters (R^2=0.36 and Q^2= -0.52) were lower than the original values indicating a lack of over-fitting.

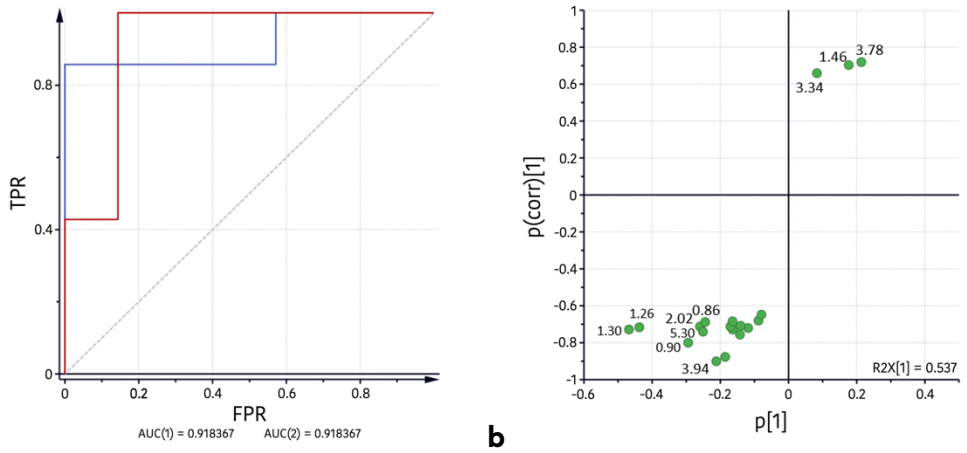


Figure S7.4a. Receiver operating characteristic (ROC) plot for the OPLS-DA (P≤0.06) model after weaning. Weaned enriched piglets (blue) and weaned control group (red).

The operating characteristic (ROC) plot (**Figue S7.4a**) for the OPLS-DA (P≤0.06) model displays the true positive classification rate (TPR) for enriched group classification (blue) or for control group classification (red) by the constructed model plotted against the corresponding false positive classification rate (FPR) at various threshold settings of the criterion parameter (YPredPS). Thus, for reed curve, TPR (or Sensitivity) represents the probability that a test result will be positive when the enrichment practice is present, and TNR (or Specificity) corresponds to the probability that a test result will be negative when the enrichment practice is not present. Every point on the ROC curves represents a pair sensitivity/specificity values corresponding to a particular decision threshold. For both curves the area under the curve (AUC) of the ROC plot has a value of 0.92 indicating high sensitivity and specificity and thus, a high prediction power of the model

Figure S7.4b. S-plot corresponding to OPLS-DA (P≤0.06) model between enriched and control piglets at after weaning period. The covariance value for each variable included is represented on the horizontal axis in the model. The vertical axis represents the correlation values obtained with respect to the dependent variable. The points at the ends of the S-plot curve indicate higher contributions to the classification.

The process of microbial colonization of the gut after birth plays an important role in the development of the neonatal immune system of mammals with implications during their whole life. The intestinal microbiota protects against colonization by pathogens by bacterial competition and interaction. Moreover, the disruption of the healthy microbial community during the neonatal period may lead to the overgrowth of indigenous pathobionts and the induction of pro-inflammatory status. It has been shown that stress, diet, management practices, and antimicrobial compounds during the early-life period may induce a long-lasting impact on the establishment of gut microbiota, disease susceptibility, and growth performances of offspring pigs. This is especially relevant in swine production with each farm microbial environment being different and possibly impacting animal health status and the productive outcome.

The present doctoral thesis aims to focus on those early events that occur in the first days of life of the piglets that could determine significant changes in the performance of the animals in the following stages of life and to explore specific applications in the commercial practice addressed to improve the health and productivity of pigs and to reduce the use of antibiotics.