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Non Ruminant Nutrition

Potential effect of two *Bacillus* probiotic strains on performance and fecal microbiota of breeding sows and their piglets

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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 5×10^8 cfu/kg *B. subtilis* – 541 (BSU); or with 5×10^8 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. Although 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 are evidence of maternal imprinting, with effects lasting beyond weaning.

Lay Summary

The aim of the present study was to determine if the inclusion of probiotic microorganisms in the mother's diet during gestation and the lactation period is capable of modifying the performance of mothers and piglets and the possible effect on the intestinal health of piglets after separation from the mother. For this, 98 females were distributed in three experimental treatments: a control diet, or the same diet in which one of two probiotic strains to be tested (*Bacillus subtilis* or *Bacillus amyloliquefaciens*) were incorporated. The experimental diets were administered during pregnancy and the lactation phase for three consecutive productive cycles. Among the most striking results, it is worth highlighting the impact of probiotic treatments on the reproductive performance of sows. Both supplemented groups showed a higher number of total piglets per sow. Furthermore, sows that received the *Bacillus amyloliquefaciens* diet showed a significant increase in the number of live-born piglets. Probiotic supplementation also showed effects on the fecal microbiota composition of the mothers and their piglets. Changes in the composition of sow milk were also observed. In summary, results demonstrated the potential benefits of supplementing probiotics, and particularly a strain of *Bacillus amyloliquefaciens*, to improve prolificacy, modulate the intestinal microbial composition, and improve the performance of piglets during lactation.

Key words: *Bacillus subtilis, Bacillus amyloliquefaciens*, microbiota, piglet, probiotic, sow

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ANOSIM, analysis of similarities; ASV, amplicon sequence variant; BW, body weight; cDNA, complementary deoxyribonucleic acid; CLDN15, claudin-15; CFU, colony-forming unit; CSS, cumulative sum scaling; DADA2, divisive amplicon denoising algorithm 2; DNA, deoxyribonucleic acid; EFSA, European Food Safety Authority; ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; HSP27, heat shock protein 27; HSPB1, heat shock protein family B member 1; HTS, high-throughput sequencing; IgA, immunoglobulin A; IgG, immunoglobulin G; IGF1R, insulin-like growth factor 1 receptor; IUGR, intrauterine growth restricted; LBW, low birth weight; LPS, lipopolysaccharide; NMDS, non-metric multidimensional scaling; NMR, nuclear magnetic resonance; NRC, National Research Council; PCR, polymerase chain reaction; PERMANOVA,

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permutational analysis of variance; PLS-DA, partial least-squares discriminant analysis; PRRS, porcine reproductive and respiratory syndrome; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; RT-PCR, quantitative reverse transcription polymerase chain reaction; SCFA, short-chain fatty acids; TSP, sodium-3′-trimethylsilylpropionate-2,2,3,3-d4; UPGMA, unweighted pair-wise grouping method with hierarchical arithmetic mean grouping; VIP, variable importance in projection; ZnO, zinc oxide

Introduction

Current 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 et al., 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; Taras et al., 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 et al., 2001; Kenny et al., 2011). Moreover, modulation of the maternal microbiota with probiotics could also have an impact on the intestinal health of their piglets (Baker et al., 2013; Davis et al., 2020; Lan and Kim, 2020). The initial development of the microbiota of piglets is fundamentally dependent on their intimate contact with their sow (Konstantinov et al., 2006; Thompson et al., 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 piglets (Hansen et al., 2012; Everaert et al., 2017; Ferret-Bernard and Le Huërou-Luron, 2019; Jiang et al., 2019).

Bacillus sp. strains have been largely studied as probiotic candidates to be supplemented in pigs. Previous works of Larsen et al. (2014) did an exhaustive screening between 245 *Bacillus* sp. isolates looking for potential probiotics to be used as feed additives in pig feed. Some of these selected strains, particularly *Bacillus amyloliquefaciens,* were shown to improve apparent ileal digestibility of amino acids and gross energy in growing pigs (Blavi et al., 2019). Moreover, strains of *Bacillus subtilis* were also demonstrated to enhanced growth rate and improve gut barrier function of weaned pigs experimentally infected with pathogenic *E. coli* (He et al., 2020), also influencing their mucosal transcriptomic profile (Luise et al., 2019). This inhibitory effect, against pathogens like *E. coli*, could be explained by the production of antimicrobial peptides and an increased mucin production of goblet cells (Bravo-Santano et al., 2020).

Although the potential benefits of supplementing the diets of sows with *Bacillus* sp. probiotics are 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 5×10^8 cfu/kg feed of viable spores of two *Bacillus* probiotic strains (*Bacillus subtilis*—541 and *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.

Materials and Methods

The present study was conducted according to EFSA administrative/technical guidance, and Commission Regulation (EC) No. 429/2008 on detailed rules for the implementation of Regulation (EC) No. 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives. Procedures, documentation, equipment, and records were examined in order to assure that the study was performed following the regulations specified herein and with the protocol and relevant standard operating procedures.

Moreover, 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), and all experimental procedures were approved beforehand by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (permit no. CEEAH 3817).

Animals and housing

The present study was carried out on 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 ± 0.14) and dam body weight (211.8 ± 1.10 kg).

Breeding dams were allocated to individual crates in the service barn where they were inseminated, and pregnancy was confirmed at approximately 30–35 d of gestation. Pregnant sows were then moved to the gestation barn, where they were group-housed (pens of 10 dams/pen) until approximately 110 d gestation when dams were moved to individual farrowing crates in farrowing rooms (5 rooms of 10 pens). Within 24 h 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.

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 Supplementary Tables S1 and S2.

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 days 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 to 5 d post-farrow, and then ad libitum to appetite. Daily feed intake was adjusted according to body condition, assessed via back fat, measured every 3 wk 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 d 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 approximately 7 d of age to weaning at approximately 23 d of age, minimum 21 d.

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. The strains were selected for survival in the gastrointestinal tract, pH and bile resistance, and pathogen inhibition characteristics, primarily *E. coli* and *C. perfringens*. In addition, the safety of the strains was evaluated by genotypic and phenotypic methods for antimicrobial resistance genes and virulence factors. For the BSU treatment, the diet was supplemented with 5×10^8 cfu/kg feed of viable spores of *Bacillus subtilis*—541, and for the BAM treatment, the diet was supplemented with 5×10^8 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 Supplementary Table S3. 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 (minimum guaranteed of 1.25×10^9 CFU/g) was added to approximately 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.

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), was 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 Supplementary Table S4) 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 d 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 15-mL tube was collected and stored at −20 °C. Blood samples were collected from the tail. The tubes containing blood samples were centrifuged (2,500 × g, 15 min) 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 d 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 (approximately 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.

Analytical procedures

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. On this, it should be noted that the farm was PRRS positive and stable and the sows were vaccinated with Aujeszky. 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.

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 ^1H-NMR signal deconvolution routines in MestReNova 8.1. Concentrations of final metabolites were calculated in arbitrary units as the area under the peak.

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). 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).

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) was used. All subsequent steps were performed on the MiSeq Illumina instrument, including cluster generation and paired-end sequencing.

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).

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) as described by González-Solé et al. (2020). For that total RNA was extracted using the Ambion RiboPure Kit (Life Technologies, Carlsbad), according to the manufacturer's protocol. RNA was analyzed using a NanoDrop 1000A spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) to determine if it satisfied the minimum purity and integrity standards for total RNA quality. Ten microliters of total RNA (100 ng/µL) were used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). 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).

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 $P \leq 0.05$, whereas $0.05 > P \leq 0.10$ 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: $Yij = \mu + \alpha i + \beta j + \alpha\beta ij + \varepsilon ijk$, where Yij was the parameter for the observations; μ was the general mean of all observations; αi was the effect of the experimental treatments (CON, BSU, BAM); βj 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. The same model was used for the statistical analysis of piglet performance.

Immune response

The analysis of the immunomodulatory effects (Igs in serum and milk samples) was performed using statistical package R (R Core Team, 2021) The following model was used: $Yi = \mu + \alpha i + \varepsilon i$, where Yi was the variable for the observations; μ was the general mean of all observations; αi was the effect of the experimental treatments (CON, BSU, BAM); and $\varepsilon \sim N$

(0, σ2ε) 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: $Yij = \mu + \alpha i + \beta j + \alpha\beta ij + \varepsilon ijk$, where *Yij* was the parameter for the observations; μ was the general mean of all observations; αi was the effect of the experimental treatments (CON, BSU, BAM); βj was the sampling day (d8 or d21 for sows and d21 or d33 for piglets); αβij was the interaction between the experimental treatments and sampling day; and ε~N (0, σ2ε) 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 et al., 2013a) and the differential abundance analysis was performed following the metagenomeSeq package (v.1.30.0) (Paulson et al., 2013b). 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 Pearson 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) 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, whereas 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, 2021) using the DCrt data matrix. Data were 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, whereas the genes with non-normal distributions were analyzed with a Kruskal–Wallis test. For ANOVA, the following model was used: $Yij = \mu + \alpha i + \varepsilon ij$, where *Yij* was the parameter for the observations; μ was the general mean of all observations; αi was the effect of the experimental treatments (CON, BSU, BAM); βj was weight block effect (medium or small size); αβij was the interaction between the experimental treatments and block of weight; and ε~N (0, σ2ε) 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.

Results

Sow and litter performance

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

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

Piglet performance data were monitored during the first two cycles and are presented in Table 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

Table 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*	CON	BSU	BAM	SEM	*P*
No. of 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
No. of 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
No. of stillborn piglets	1.9	2.1	2.4	0.15	0.418	1.8	2.5	2.1	0.15	0.129
No. of mummified piglets	1.0	1.2	1.2	0.11	0.558	0.9	1.3	1.2	0.11	0.215
No. of 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 = 5×10^8 CFU/kg feed of *Bacillus subtilis*; BAM = 5×10^8 CFU/kg feed of *Bacillus amyloliquefaciens*.
No interaction effect (Productive cycle x treatment) was found significant.

Table 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*	Treatment			SEM	*P*
	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

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 sameroware 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.

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 ($P = 0.024$). In this sense, pig loss includes both the piglets that died during lactation and the culled piglets that had to be removed during the study. No significant interaction between cycles and treatments was found for the performance of piglets.

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 3. Compared to CON, dietary supplementation with BAM significantly decreased the serological titers of IgG specific for Aujeszky at day 21 (ANOVA $P = 0.009$) 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.

Differences in milk metabolites among interventions

The global metabolic profile of a total of 40 milk samples taken 21 d after parturition was 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 1), two clusters could be identified when comparing BSU to CON.

In addition to the PLS-DA, the possible impact of experimental treatments on particular metabolites was evaluated. Supplementary Table S5 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.

Table 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*
	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.

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 (PERMANOVA: $P < 0.001$; ANOSIM: $P = 0.001$) with more dispersed samples on day 8 after parturition (Figure 2).

The alpha diversity indexes of sow fecal samples are presented in Table 4. In general terms, there was a significant increase in the species richness (ANOVA $P = 0.046$) 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 with 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, $P = 0.177$).

The composition of the fecal microbiota of the sows both at the phylum and family level is presented in Supplementary Table S6. Regarding differences in taxonomic groups between sampling days (Figure 3 and Supplementary Table S6), 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 with 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,

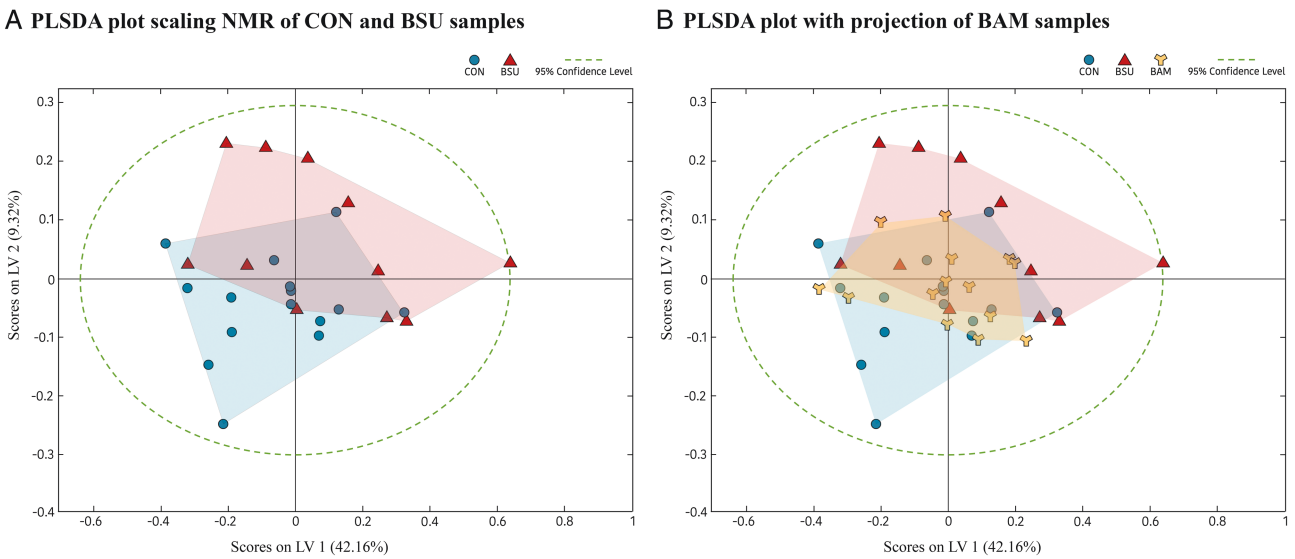

Figure 1. Partial least squares discriminant analysis (PLS-DA) scores plot scaling NMR data from CON and BSU (a); and projection of samples from BAM (b). CON = Control; BSU = *Bacillus subtilis*; BAM = *Bacillus amyloliquefaciens*.

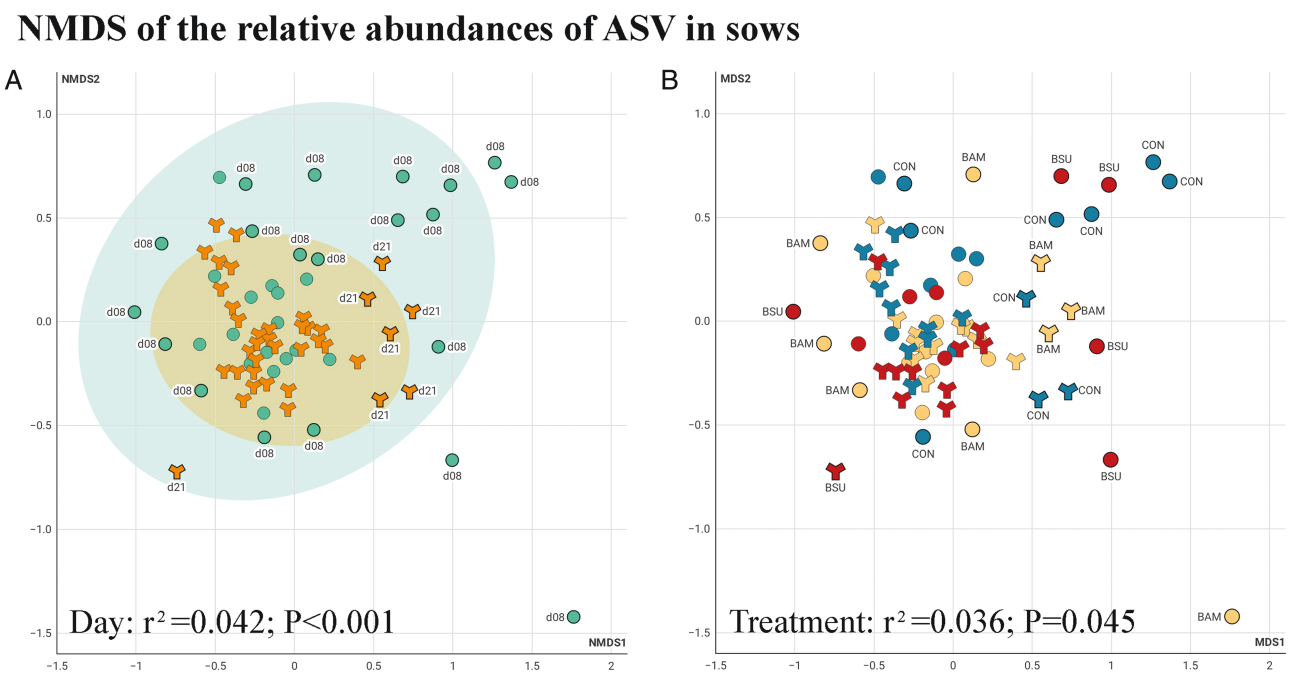

Figure 2. NMDS of the relative abundances of ASV in sow fecal content based on Bray-Curtis distance (stress = 0.157) and grouped by sampling day (d8 after farrowing vs. d21 after farrowing). In order to facilitate the distinction between experimental treatments from (a), the same NMDS figure has been placed in parallel as (b) with the three diets highlighted in color.

Table 4. Alpha diversity values obtained in each sampling day both on sows and their offspring

Sows	Index	d8			SEM	*P*	d21			SEM	*P*
		CON	BSU	BAM			CON	BSU	BAM		
	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
Piglets	Index	d21			SEM	*P*	d33			SEM	*P*
		CON	BSU	BAM			CON	BSU	BAM		
	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

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

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*.

The impact of the experimental treatments on particular taxonomic groups was analyzed by sampling day since significant effects between days after 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* (metagenomeSeq $P = 0.007$), *Lachnospiraceae* ($P = 0.037$), *Ruminococcaceae* ($P = 0.002$), and *Bacteroidaceae* ($P = 0.001$) than CON (Figure 4a). Regarding particular genera (Figure 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.

Piglet fecal microbiota

The analysis of the piglets' fecal microbiota on days 21 and 33 of life showed that weaning promoted an evident change

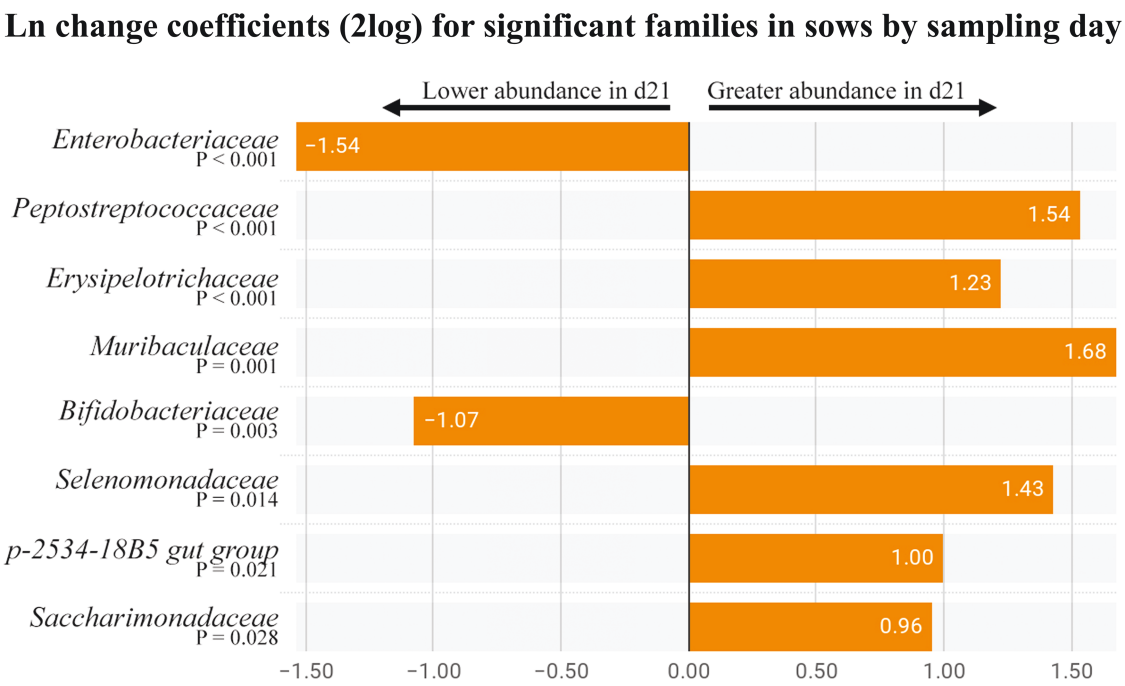

Figure 3. Differentially abundant taxa at family level from sow fecal content [ln change coefficients (2log) and FDR-adjusted $P < 0.05$] between d08 and d21 samplings. Only significant taxa with greater relative abundance than 0.05% are presented; positive and negative values indicate greater and lower abundance, respectively, in d21 animals; taxa are sorted by level of significance (from higher to lower).

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 it shows the NMDS of the relative abundances of ASV based on Bray–Curtis distance in Figure 5. The administration of probiotic supplemented diets to their mothers was not associated with 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: $P = 0.999$).

Concerning alpha diversity (Table 4), weaning promoted a trend for a lower species richness at d33 (1224 vs. 951 for observed species, ANOVA $P = 0.090$; and 1226 vs. 955 for Chao1, $P = 0.092$; for d21 and d33, respectively) and a significant 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 ($P = 0.351$).

The composition of the fecal microbiota of the piglets both at the phylum and family level is presented in Supplementary Table S7. The weaning process promoted significant changes in several taxonomic groups (phylum, family, and genus, Supplementary Tables S7 and S8). As seen in Figure 6, the increase of families such as *Prevotellaceae*, *Spirochaetaceae*, and *Enterobacteriaceae* was observed after weaning, whereas families like *Lactobacillaceae*, *Lachnospiraceae*, *Bacteroidaceae*, and *Clostridiaceae* decreased.

Regarding the impact of supplementing probiotics to the sow on particular microbial taxa of piglets, Supplementary Figure S1 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* (metagenomeSeq $P = 0.043$) 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 7. At the genus level, no significant differences were observed except for minor taxa.

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

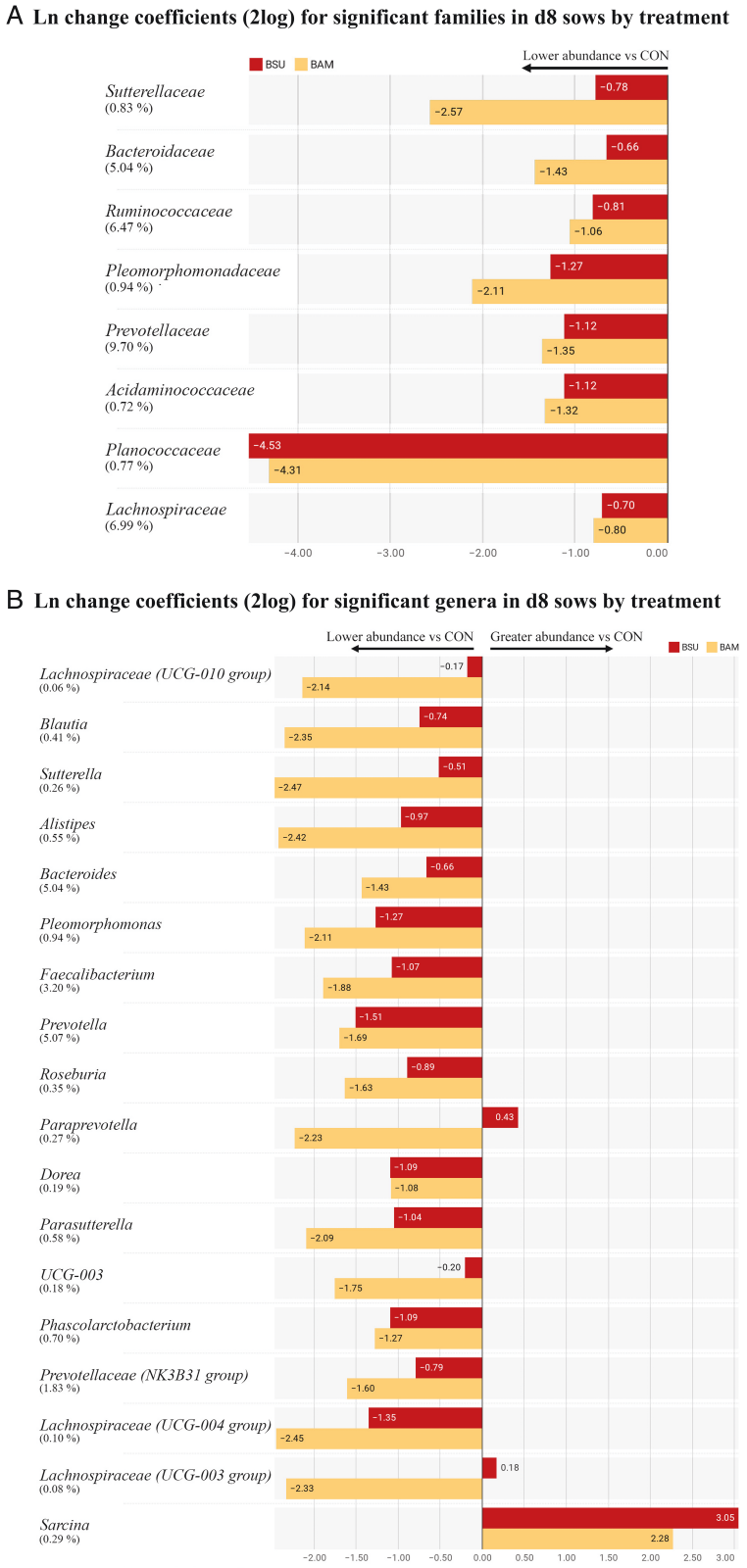

Figure 4. Differentially abundant taxa from fecal content (ln change and FDR-adjusted $P < 0.05$) on day 8 after farrowing between: BSU vs. CON, and BAM vs. CON at family (a) and genus (b) level. Only significant taxa with greater relative abundance than 0.05% are presented; positive and negative values indicate greater and lower abundance, respectively; the average relative abundance of each taxa is expressed in % below the family or genus name; taxa are sorted by level of significance (from higher to lower).

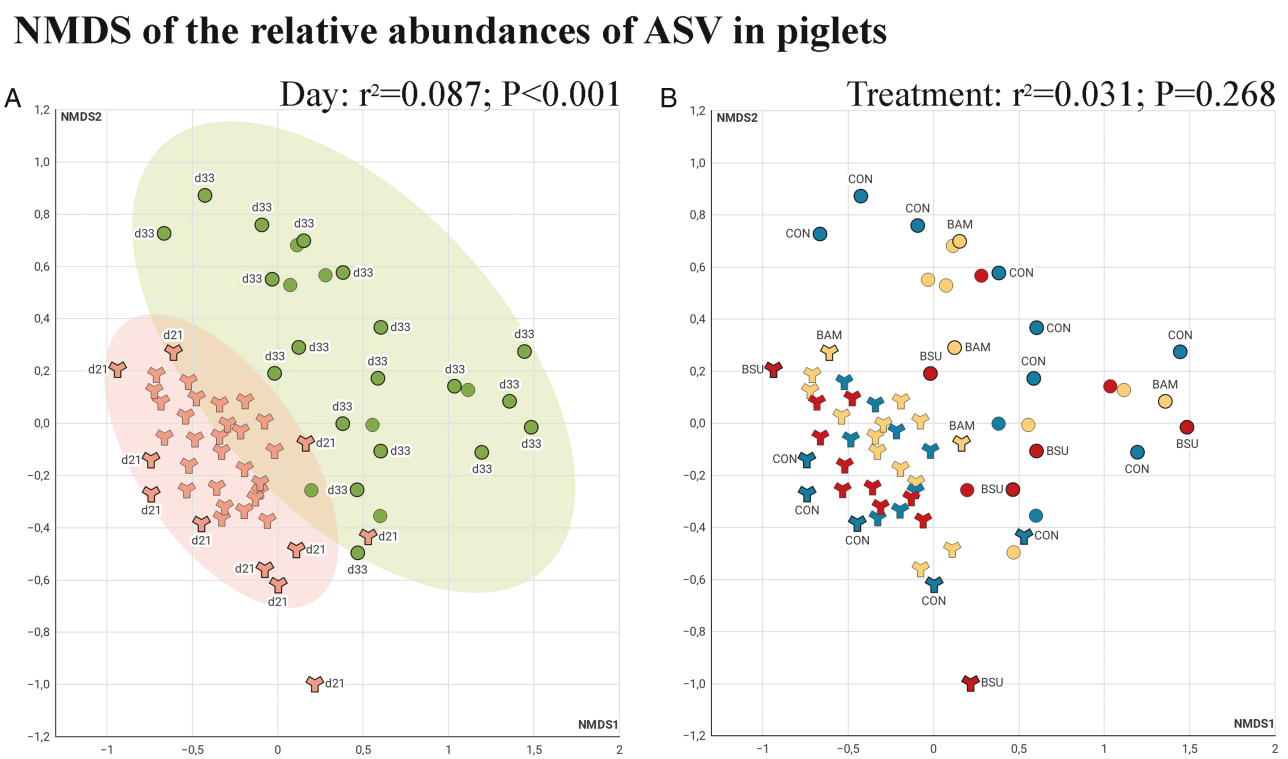

Figure 5. NMDS of the relative abundances of ASV in piglet fecal content based on Bray–Curtis distance (stress = 0.169) during lactation (d21 of life) and after weaning (d33 of life and d12 after weaning). In order to facilitate the distinction between experimental treatments in (a), the same NMDS figure has been placed in parallel as (b) with the three diets highlighted in color.

Interestingly no high correlations were found between the sow microbiota 1 wk 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.

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 days 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.

Intestinal gene expression

Detailed results of jejunal gene expression of medium- and small-sized piglets can be found in Supplementary Table S9 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 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*; ANOVA $P = 0.052$); *HSP27* (*Heat shock protein 27*; ANOVA $P = 0.038$); and *CLDN15* (*Claudin-15*; ANOVA $P = 0.052$) genes compared to medium-sized piglets. No interaction was found between sow's dietary treatment and piglet size.

Discussion

In recent years, dietary supplementation of sows with probiotics has gained considerable attention due to their potential to

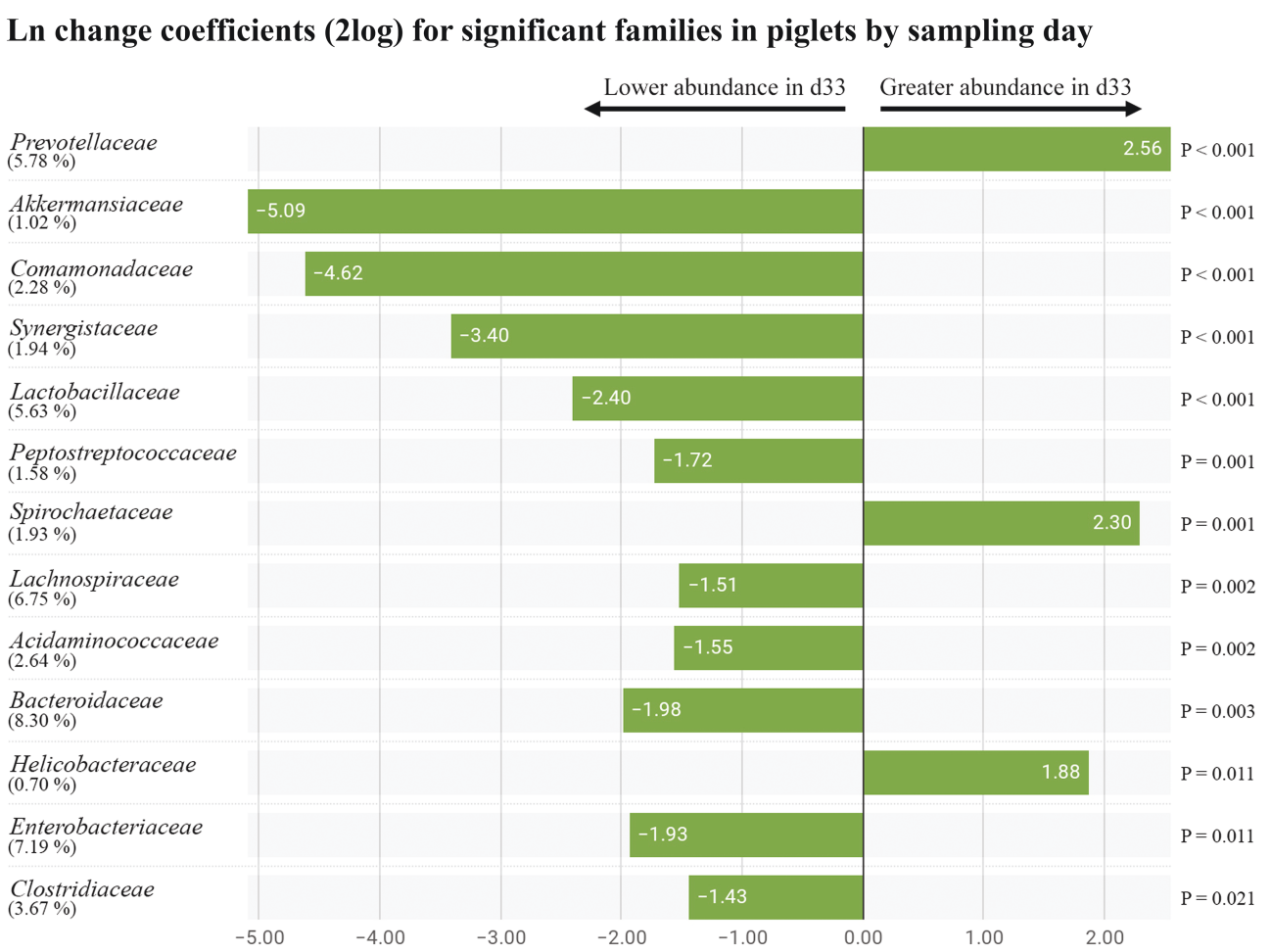

Figure 6. Differentially abundant taxa from fecal content (ln change and FDR-adjusted $P < 0.05$) between d21 and d33 samplings. Only significant taxa with greater relative abundance than 1.5% are presented; positive and negative values indicate greater and lower abundance, respectively, in d33 animals; the mean average relative abundance of each taxa is expressed in % between brackets; taxa are sorted by level of significance (from higher to lower).

improve reproductive performance (Barba-Vidal et al., 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 et al., 2006; Stamati et al., 2006; Larsen et al., 2014; Kritas et al., 2015; Hayakawa et al., 2016; Blavi et al., 2019; Luise et al., 2019; He et al., 2020). Although 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.

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 et al., 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 et al., 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. 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; Stamati et al., 2006; Taras et al., 2006; Baker et al.,

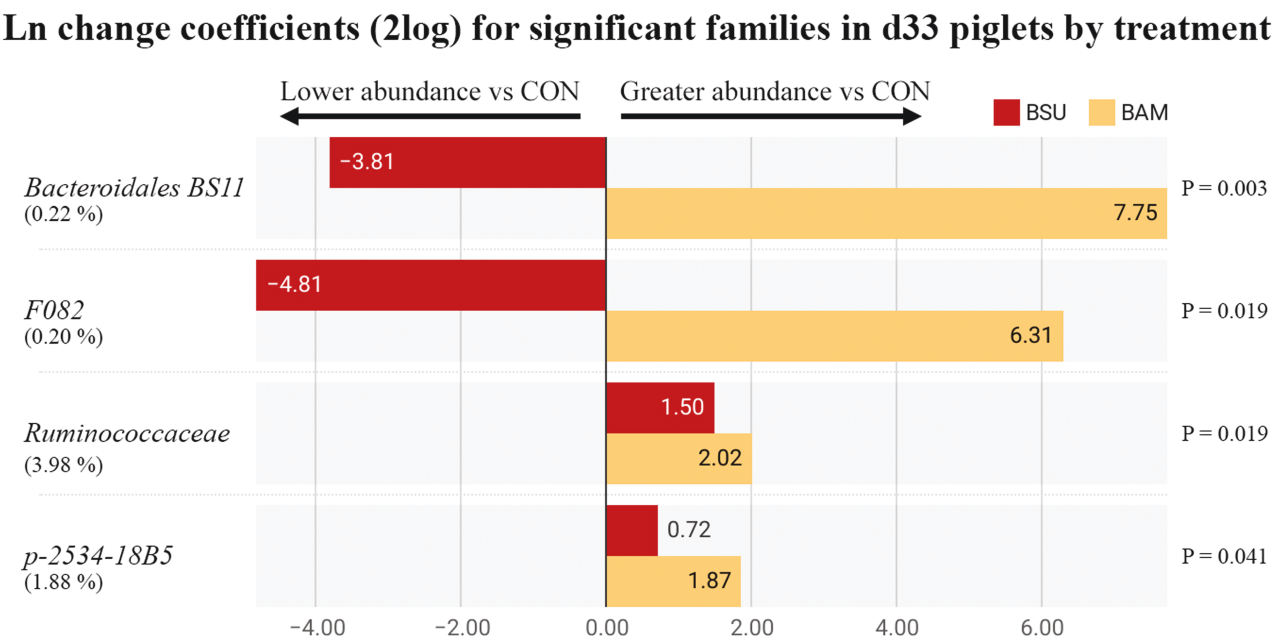

Figure 7. Differentially abundant taxa from fecal content (ln change and FDR-adjusted p < 0.05) of weaned piglets (d33) between: BSU vs. CON, and BAM vs. CON at family level. Only significant taxa with greater relative abundance than 0.05% are presented; positive and negative values indicate greater 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).

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. 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. Although 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.

Impact of probiotics on sow fecal microbiota and maternal milk

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., 2019a), and after farrowing probably needs to establish a new equilibrium. It is 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 was 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 postpartum. 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.

Table 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*
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

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 et al., 2021); however, no significant changes in these groups were observed in our study. It is important to consider here the methodological differences between studies.

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.

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 *B. subtilis* in lactating sows has

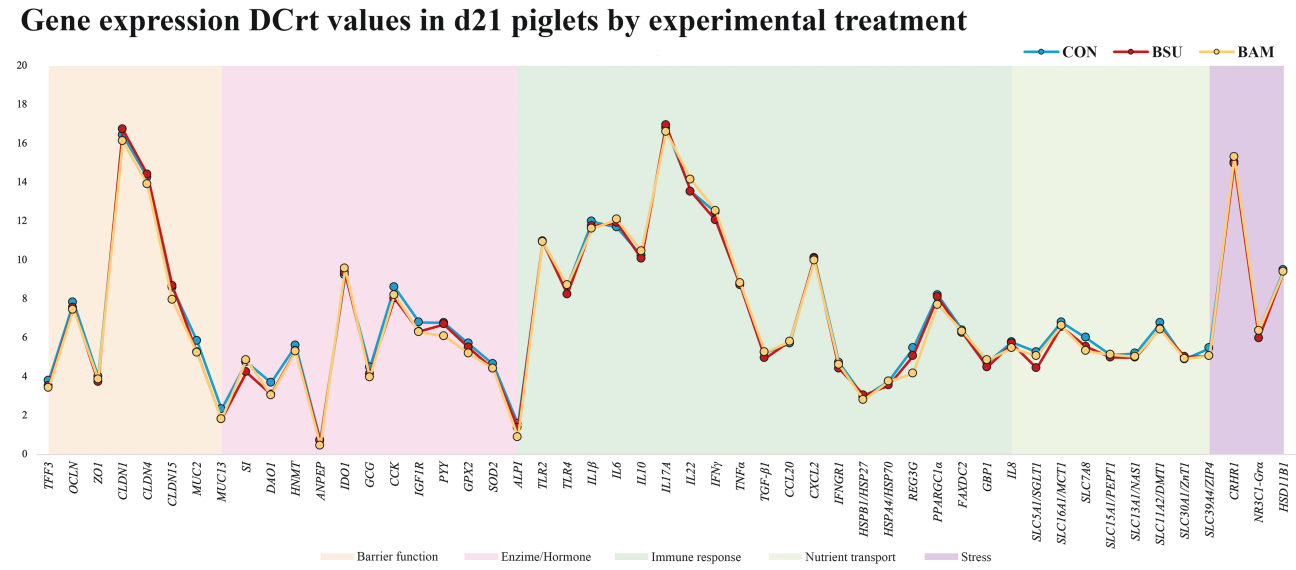

Figure 8. Mean DCrt expression of all the genes analyzed sorted by dietary treatment. Genes have been grouped by function with different background colors. CON = Control; BSU = *Bacillus subtilis*; BAM = *Bacillus amyloliquefaciens*.

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). However, in this work, we were not able to demonstrate any improvement in the immune response based on Aujeszky and PRRS-specific IgG and IgA levels considering that the farm was PRRS positive and stable and the sows were vaccinated with Aujeszky. 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). In the present study, 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.

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 et al., 2016; Picone et al., 2018; Tan et al., 2018).

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; Chen et al., 2018; Jiang et al., 2019; Liu et al., 2019b), 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 et al., 2021). In this context, 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). 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.

Results showed that the diversity and community structure of fecal microbiota were in consonance with the predominant taxa described previously for healthy piglets. *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; Li et al., 2018; Saladrigas-García et al., 2021b). Moreover, and in agreement with previous studies (Saladrigas-García et al., 2021a), 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 although other microbial groups showed a differential impact. It is interesting to note that most of the changes were detected after weaning, suggesting 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. 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. 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; Yang 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* and *Prevotella* genera are associated with the fermentation of complex carbohydrates and are likely important contributors towards the establishment of a more mature microbiota.

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, we were not able to find any significant impact of the probiotic strains on the jejunal expression of the genes selected. This does not discard that probiotic supplementation could have had induced changes in the expression or other genes or tissues.

Despite the lack of the impact of the sows' dietary treatments on jejunal gene expression, differences were found according to the piglet size (medium or small-sized within the same litter). Small-sized piglets showed up-regulated expressions of *IGF1R*, *HSP27*, and *CLDN15* that could suggest a greater genetic effort necessary in smaller piglets to increase their gut maturity and robustness and their intestinal differentiation.

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; Alexopoulos et al., 2004; Taras et al., 2005; Stamati et al., 2006; Baker et al., 2013; Kritas et al., 2015; Hayakawa et al., 2016; Hu et al., 2021), results are not always positive and some others did not find significant changes in the piglet's performance (Böhmer et al., 2006; Menegat et al., 2019; Davis et al., 2020; Menegat 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 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 this adverse impact on body weight was not in BAM piglets. Different studies have described a negative linear correlation between litter size and piglet weight at birth (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. Previous works of Blavi et al. (2019) comparing both strains also showed a clearer impact of *B. amyloliquefaciens* on ileal apparent digestibility in growing pigs that could suggest a higher comparative impact of this strain on the intestinal environment and functionality.

In conclusion, both tested probiotic strains supplemented to reproductive sows were demonstrated a significant impact on prolificacy. Although 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, *B. amyloliquefaciens* (BAM) also increased the survival of piglets at birth and the number of piglets at weaning. The most relevant changes on mothers' intestinal microbiota were observed a few days after delivery (d8 postpartum), suggesting the relevant role of probiotics on the establishment of a new intestinal balance after labor. Microbial shifts were also observed in the piglets, with a clearer impact during the post-weaning than in the lactation period. 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. 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.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

Alexopoulos, C., I. E. Georgoulakis, A. Tzivara, S. K. Kritas, A. Siochu, and S. C. Kyriakis. 2004. Field evaluation of the efficacy of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* spores, on the health status and performance of sows and their litters. *J. Anim. Physiol. Anim. Nutr. (Berl)* 88:381–392. doi:10.1111/j.1439-0396.2004.00492.x.

Alexopoulos, C., A. Karagiannidis, S. K. Kritas, C. Boscos, I. E. Georgoulakis, and S. C. Kyriakis. 2001. Field evaluation of a bioregulator containing live Bacillus cereus spores on health status and performance of sows and their litters. *J. Vet. Med. Ser. A* 48:137–145. doi:10.1046/j.1439-0442.2001.00342.x.

Antwis, R. E., K. L. Edwards, B. Unwin, S. L. Walker, and S. Shultz. 2019. Rare gut microbiota associated with breeding success, hormone metabolites and ovarian cycle phase in the critically endangered eastern black rhino. *Microbiome* 7:27. doi:10.1186/s40168-019-0639-0.

Apic, I., B. Savic, I. Stancic, M. Zivkov-Balas, J. Bojkovski, S. Jovanovic, I. Radovic, D. Zvekic, and Z. Maksimovic. 2014. Litters health status and growth parameters in the sows feeding diets supplemented with probiotic Actisaf Sc 47® within pregnancy or lactation. In: *Proceedings of the International Symposium on Animal Science*. Belgrade-Zemun, Serbia.

Ayala, L., R. Bocourt, M. Castro, M. Martínez, and M. Herrera. 2016. Effect of the probiotic additive *Bacillus subtilis* and their endospores on milk production and immune response of lactating sows. *Cuba. J. Agric. Sci.* 49:71–74. Available from: https://cjascience.com/index.php/CJAS/article/view/550.

Azagra-Boronat, I., A. Tres, M. Massot-Cladera, A. Franch, M. Castell, F. Guardiola, F. J. Pérez-Cano, and M. J. Rodríguez-Lagunas. 2020. *Lactobacillus fermentum* CECT5716 supplementation in rats during pregnancy and lactation impacts maternal and offspring lipid profile, immune system and microbiota. *Cells* 9(3):575–595. doi:10.3390/cells9030575.

Baker, A. A., E. Davis, J. D. Spencer, R. Moser, and T. Rehberger. 2013. The effect of a Bacillus-based direct-fed microbial supplemented to sows on the gastrointestinal microbiota of their neonatal piglets. *J. Anim. Sci.* 91:3390–3399. doi:10.2527/jas.2012-5821.

Barba-Vidal, E., S. M. Martín-Orúe, and L. Castillejos. 2019. Practical aspects of the use of probiotics in pig production: a review. *Livest. Sci.* 223:84–96. doi: 10.1016/j.livsci.2019.02.017. Available from: https://www.sciencedirect.com/science/article/pii/S1871141318302646.

Belzer, C., and W. M. de Vos. 2012. Microbes inside—from diversity to function: the case of Akkermansia. *ISME J.* 6:1449–1458. doi:10.1038/ismej.2012.6.

Betancur, C., Y. Martínez, G. Tellez-Isaias, R. Castillo, and X. Ding. 2021. Effect of oral administration with *Lactobacillus plantarum* CAM6 strain on sows during gestation-lactation and the derived impact on their progeny performance. S. Chen, editor. *Mediators Inflamm.* 2021:6615960. doi:10.1155/2021/6615960.

Bhandari, P., P. Rishi, and V. Prabha. 2016. Positive effect of probiotic *Lactobacillus plantarum* in reversing LPS-induced infertility in a mouse model. *J. Med. Microbiol.* 65:345–350. doi:10.1099/jmm.0.000230.

Blavi, L., J. N. Jørgensen, and H. H. Stein. 2019. Effects of *Bacillus amyloliquefaciens* and *Bacillus subtilis* on ileal digestibility of AA and total tract digestibility of CP and gross energy in diets fed to growing pigs. *J. Anim. Sci.* 97:727–734. doi:10.1093/jas/sky432.

Böhmer, B. M., W. Kramer, and D. A. Roth-Maier. 2006. Dietary probiotic supplementation and resulting effects on performance, health status, and microbial characteristics of primiparous sows. *J. Anim. Physiol. Anim. Nutr. (Berl).* 90:309–315. doi:10.1111/j.1439-0396.2005.00601.x.

Bravo-Santano, N., E. Juncker Boll, L. Catrine Capern, T. M. Cieplak, E. Keleszade, M. Letek, and A. Costabile. 2020. Comparative evaluation of the antimicrobial and mucus induction properties of selected *Bacillus* strains against enterotoxigenic *Escherichia coli*. *Antibiot* 9(12):849–859. doi:10.3390/antibiotics9120849.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13:581–583. doi:10.1038/nmeth.3869.

Chen, L., Y. Xu, X. Chen, C. Fang, L. Zhao, and F. Chen. 2017. The maturing development of gut microbiota in commercial piglets during the weaning transition. *Front. Microbiol.* 8:1–13. doi:10.3389/fmicb.2017.01688. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01688/full.

Chen, X., J. Xu, E. Ren, Y. Su, and W. Zhu. 2018. Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. *Anaerobe* 49:30–40. doi:10.1016/j.anaerobe.2017.12.002. Available from: http://www.sciencedirect.com/science/article/pii/S1075996417302251.

Crespo-Piazuelo, D., G. E. Gardiner, S. Ranjitkar, M. A. Bouwhuis, R. Ham, J. P. Phelan, A. Marsh, and P. G. Lawlor. 2021. Maternal supplementation with Bacillus altitudinis spores improves porcine offspring growth performance and carcass weight. *Br. J. Nutr.* 127(3):403–420. doi:10.1017/S0007114521001203. Available from: https://www.cambridge.org/core/journals/british-journal-of-nutrition/article/abs/maternal-supplementation-with-bacillus-altitudinis-spores-improves-porcine-offspring-growth-performance-and-carcass-weight/0B55BCFAB97EA75CB0C7D354DC8224EC.

Curtasu, M. V., P. K. Theil, and M. S. Hedemann. 2016. Metabolomic profiles of colostrum and milk from lactating sows. *J. Anim. Sci.* 94:272–275. doi:10.2527/jas.2015-9769.

Davis, E., J. Christianson, S. Anderson, T. Rehberger, and J. Sawall. 2020. Administration of a *Bacillus* probiotic to sows improves growth response and health of their progeny after weaning. *J. Anim. Sci.* 98:93.

Everaert, N., S. Van Cruchten, B. Weström, M. Bailey, C. Van Ginneken, T. Thymann, and R. Pieper. 2017. A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. *Anim. Feed Sci. Technol.* 233:89–103. doi: 10.1016/j.anifeedsci.2017.06.011. . https://www.sciencedirect.com/science/article/pii/S037784011631063X.

Everard, A., C. Belzer, L. Geurts, J. P. Ouwerkerk, C. Druart, L. B. Bindels, Y. Guiot, M. Derrien, G. G. Muccioli, N. M. Delzenne, et al. 2013. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci.* 110:9066 LP–9069071. doi:10.1073/pnas.1219451110. Available from: http://www.pnas.org/content/110/22/9066.abstract.

Ferret-Bernard, S., and I. Le Huërou-Luron. 2019. Development of the intestinal immune system in young pigs? Role of the microbial environment. In: *The suckling and weaned piglet*. Wageningen, Netherlands: Wageningen Academic Publishers. p. 159–177 SE–6. Available from: https://doi.org/10.3920/978-90-8686-894-0_6

Gómez-Gallego, C., J. M. Morales, D. Monleón, E. du Toit, H. Kumar, K. M. Linderborg, Y. Zhang, B. Yang, E. Isolauri, S. Salminen, et al. 2018. Human breast milk NMR metabolomic profile across specific geographical locations and its association with the milk microbiota. *Nutrients* 10(10):1355–1375. doi:10.3390/nu10101355.

González-Solé, F., L. Criado-Mesas, C. Villodre, W. C. García, M. Farré, E. Borda, F. J. Pérez-Cano, J. M. Folch, D. Solà-Oriol, and J. F. Pérez. 2020. Porcine Digestible Peptides (PDP) in weanling diets regulates the expression of genes involved in gut barrier function, immune response and nutrient transport in nursery pigs. *Anim. an open access J. from MDPI*. 10:2368. doi:10.3390/ani10122368. Available from: https://pubmed.ncbi.nlm.nih.gov/33321976.

Grazul, H., L. L. Kanda, and D. Gondek. 2016. Impact of probiotic supplements on microbiome diversity following antibiotic treatment of mice. *Gut Microbes* 7:101–114. doi:10.1080/19490976.2016.1138197.

Hansen, C. H. F., D. S. Nielsen, M. Kverka, Z. Zakostelska, K. Klimesova, T. Hudcovic, H. Tlaskalova-Hogenova, and A. K. Hansen. 2012. Patterns of early gut colonization shape future immune responses of the host. *PLoS One* 7:e34043–e34043. doi:10.1371/journal.pone.0034043.

Hayakawa, T., T. Masuda, D. Kurosawa, and T. Tsukahara. 2016. Dietary administration of probiotics to sows and/or their neonates improves the reproductive performance, incidence of post-weaning diarrhea and histopathological parameters in the intestine of weaned piglets. *Anim. Sci. J.* 87:1501–1510. doi:10.1111/asj.12565.

He, Y., C. Jinno, K. Kim, Z. Wu, B. Tan, X. Li, R. Whelan, and Y. Liu. 2020. Dietary *Bacillus* spp. enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. *J. Anim. Sci. Biotechnol.* 11:101. doi:10.1186/s40104-020-00498-3.

Holman, D. B., B. W. Brunelle, J. Trachsel, and H. K. Allen. 2017. Meta-analysis to define a core microbiota in the swine gut. *mSystems* 2:1–14. doi:10.1128/msystems.00004-17.

Hu, J., Y. H. Kim, and I. H. Kim. 2021. Effects of two bacillus strains probiotic supplement on reproduction performance, nutrient digestibility, blood profile, fecal score, excreta odor contents and fecal microflora in lactation sows, and growth performance in sucking piglets. *Livest. Sci.* 244:104293. doi: 10.1016/j.livsci.2020.104293 . https://www.sciencedirect.com/science/article/pii/S1871141319315306.

Hu, J., Y. Nie, J. Chen, Y. Zhang, Z. Wang, Q. Fan, and X. Yan. 2016. Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* 7:1–15. doi:10.3389/fmicb.2016.01727.

Jadamus, A., W. Vahjen, and O. Simon. 2001. Growth behaviour of a spore forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets . *Arch. für Tierernaehrung*. 54:1–17. doi:10.1080/17450390109381962.

Jeong, J., J. Kim, S. Lee, and I. Kim. 2015. Evaluation of *Bacillus subtilis* and *Lactobacillus acidophilus* probiotic supplementation on reproductive performance and noxious gas emission in sows. *Ann. Anim. Sci.* 15:699–710. doi: 10.1515/aoas-2015-0018. Available from: https://content.sciendo.com/view/journals/aoas/15/3/article-p699.xml.

Jiang, L., C. Feng, S. Tao, N. Li, B. Zuo, D. Han, and J. Wang. 2019. Maternal imprinting of the neonatal microbiota colonization in intrauterine growth restricted piglets: a review. *J. Anim. Sci. Biotechnol.* 10:88. doi:10.1186/s40104-019-0397-7.

Jost, T., C. Lacroix, C. P. Braegger, F. Rochat, and C. Chassard. 2014. Vertical mother–neonate transfer of maternal gut bacteria via breastfeeding. *Environ. Microbiol.* 16:2891–2904. doi: 10.1111/1462-2920.12238.

Kelly, D., and S. Conway. 2005. Bacterial modulation of mucosal innate immunity. *Mol. Immunol.* 42:895–901. doi:10.1016/j.molimm.2004.12.003.

Kenny, M., H. Smidt, E. Mengheri, and B. Miller. 2011. Probiotics—do they have a role in the pig industry? *Animal* 5:462–470. doi:10.1017/S175173111000193X. Available from: https://www.sciencedirect.com/science/article/pii/S175173111000193X.

Konstantinov, S. R., A. A. Awati, B. A. Williams, B. G. Miller, P. Jones, C. R. Stokes, A. D. L. Akkermans, H. Smidt, and W. M. De Vos. 2006. Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8:1191–1199. doi:10.1111/j.1462-2920.2006.01009.x.

Kritas, S. K., T. Marubashi, G. Filioussis, E. Petridou, G. Christodoulopoulos, A. R. Burriel, A. Tzivara, A. Theodoridis, and M. Pískoriková. 2015. Reproductive performance of sows was improved by administration of a sporing bacillary probiotic (*Bacillus subtilis* C-3102). *J. Anim. Sci.* 93:405–413. doi:10.2527/jas.2014-7651.

Lahti, L., S. Shetty, T. Blake, and J. Salojarvi. 2017. *Microbiome R package*. London (UK): Tools Microbiome Anal. R.

Lan, R., and I. Kim. 2020. Enterococcus faecium supplementation in sows during gestation and lactation improves the performance of sucking piglets. *Vet. Med. Sci.* 6:92–99. doi:10.1002/vms3.215.

Larsen, N., L. Thorsen, E. N. Kpikpi, B. Stuer-Lauridsen, M. D. Cantor, B. Nielsen, E. Brockmann, P. M. F. Derkx, and L. Jespersen. 2014. Characterization of *Bacillus* spp. strains for use as probiotic additives in pig feed. *Appl. Microbiol. Biotechnol.* 98:1105–1118. doi:10.1007/s00253-013-5343-6.

Li, Y., Y. Guo, Z. Wen, X. Jiang, X. Ma, and X. Han. 2018. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. *Sci. Rep.* 8:1–12. doi:10.1038/s41598-018-33649-8.

Liu, H., C. Hou, N. Li, X. Zhang, G. Zhang, F. Yang, X. Zeng, Z. Liu, and S. Qiao. 2019a. Microbial and metabolic alterations in gut microbiota of sows during pregnancy and lactation. *FASEB J.* 33:4490–4501. doi:10.1096/fj.201801221RR.

Liu, H., X. Zeng, G. Zhang, C. Hou, N. Li, H. Yu, L. Shang, X. Zhang, P. Trevisi, F. Yang, et al. 2019b. Maternal milk and fecal microbes guide the spatiotemporal development of mucosa-associated microbiota and barrier function in the porcine neonatal gut. *BMC Biol.* 17:106. doi:10.1186/s12915-019-0729-2.

Luise, D., M. Bertocchi, V. Motta, C. Salvarani, P. Bosi, A. Luppi, F. Fanelli, M. Mazzoni, I. Archetti, G. Maiorano, et al. 2019. *Bacillus* sp. probiotic supplementation diminish the Escherichia coli F4ac infection in susceptible weaned pigs by influencing the intestinal immune response, intestinal microbiota, and blood metabolomics. *J. Anim. Sci. Biotechnol.* 10:74. doi:10.1186/s40104-019-0380-3.

Mach, N., M. Berri, J. Estellé, F. Levenez, G. Lemonnier, C. Denis, J. J. Leplat, C. Chevaleyre, Y. Billon, J. Doré, et al. 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environ. Microbiol. Rep.* 7:554–569. doi:10.1111/1758-2229.12285.

McMurdie, P. J., and S. Holmes. 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8. doi:10.1371/journal.pone.0061217.

Medina, M., E. Izquierdo, S. Ennahar, and Y. Sanz. 2007. Differential immunomodulatory properties of Bifidobacterium logum strains: relevance to probiotic selection and clinical applications. *Clin. Exp. Immunol.* 150:531–538. doi:10.1111/j.1365-2249.2007.03522.x.

Menegat, M. B., J. M. DeRouchey, J. C. Woodworth, S. S. Dritz, M. D. Tokach, and R. D. Goodband. 2019. Effects of *Bacillus subtilis* C-3102 on sow and progeny performance, fecal consistency, and fecal microbes during gestation, lactation, and nursery periods. *J. Anim. Sci.* 97:3920–3937. doi:10.1093/jas/skz236.

Menegat, M. B., J. M. DeRouchey, J. C. Woodworth, M. D. Tokach, R. D. Goodband, and S. S. Dritz. 2020. Effects of oral administration of Bacillus subtilis C-3102 to nursing piglets on preweaning growth performance, fecal consistency, and fecal microbes. *J. Swine Heal. Prod.* 28:12–20.

Mu, C., G. Bian, Y. Su, and W. Zhu. 2019. Differential effects of breed and nursing on early-life colonic microbiota and immune status as revealed in a cross-fostering piglet model. *Appl. Environ. Microbiol.* 85(9):2510–2518. doi:10.1128/AEM.02510-18.

Nasiri, A. H., A. Towhidi, M. Shakeri, M. Zhandi, M. Dehghan-Banadaky, and M. G. Colazo. 2018. Effects of live yeast dietary supplementation on hormonal profile, ovarian follicular dynamics, and reproductive performance in dairy cows exposed to high ambient temperature. *Theriogenology* 122:41–46. doi:10.1016/j.theriogenology.2018.08.013. Available from: https://www.sciencedirect.com/science/article/pii/S0093691X18306393.

Nowland, T. L., R. N. Kirkwood, V. A. Torok, K. J. Plush, and M. D. Barton. 2021. Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets. *Life (Basel, Switzerland)* 11(4):312–326. doi:10.3390/life11040312.

Nowland, T., K. Plush, M. Barton, and R. Kirkwood. 2019. Development and function of the intestinal microbiome and potential implications for pig production. *Animals* 9:76. doi:10.3390/ani9030076. Available from: https://www.mdpi.com/2076-2615/9/3/76.

NRC. 2012. *Nutrient requirements of swine. Eleventh R.* National Academies Press, Washington, DC.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, et al. 2013. Package "vegan." *Community Ecol.* Package version. 2:1–295.

Paulson, J. N., O. C. Stine, H. C. Bravo, and M. Pop. 2013a. Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* 10:1200.

Paulson, J. N., H. Talukder, M. Pop, and H. C. Bravo. 2013b. metagenomeSeq: statistical analysis for sparse high-throughput sequencing. *Bioconductor Packag.* 1:1–191. Available from: http://bioconductor.jp/packages/2.14/bioc/vignettes/metagenomeSeq/inst/doc/metagenomeSeq.pdf.

Picone, G., M. Zappaterra, D. Luise, A. Trimigno, F. Capozzi, V. Motta, R. Davoli, L. Nanni Costa, P. Bosi, and P. Trevisi. 2018. Metabolomics characterization of colostrum in three sow breeds and its influences on piglets' survival and litter growth rates. *J. Anim. Sci. Biotechnol.* 9:23. doi:10.1186/s40104-018-0237-1.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41:D590–D596. doi:10.1093/nar/gks1219.

R Core Team. 2021. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Reyes-Camacho, D., E. Vinyeta, J. F. Pérez, T. Aumiller, L. Criado, L. M. Palade, I. Taranu, J. M. Folch, M. A. Calvo, J. D. Van der Klis, et al. 2020. Phytogenic actives supplemented in hyperprolific sows: effects on maternal transfer of phytogenic compounds, colostrum and milk features, performance and antioxidant status of sows and their offspring, and piglet intestinal gene expression. *J. Anim. Sci.* 98. doi:10.1093/jas/skz390.

Saladrigas-García, M., M. D'Angelo, H. -L. Ko, P. Nolis, Y. Ramayo-Caldas, J. M. Folch, P. Llonch, D. Solà-Oriol, J. F. Pérez, and S. M. Martín-Orúe. 2021a. Understanding host-microbiota interactions in the commercial piglet around weaning. *Sci. Rep.* 11:23488. doi:10.1038/s41598-021-02754-6.

Saladrigas-García, M., M. D'Angelo, H. -L. Ko, S. Traserra, P. Nolis, Y. Ramayo-Caldas, J. M. Folch, P. Vergara, P. Llonch, J. F. Pérez, et al. 2021b. Early socialization and environmental enrichment of lactating piglets affects the caecal microbiota and metabolomic response after weaning. *Sci. Rep.* 11:6113. doi:10.1038/s41598-021-85460-7.

Scharek-Tedin, L., S. Kreuzer-Redmer, S. O. Twardziok, B. Siepert, R. Klopfleisch, K. Tedin, J. Zentek, and R. Pieper. 2015. Probiotic treatment decreases the number of CD14-expressing cells in porcine milk which correlates with several intestinal immune parameters in the piglets. *Front. Immunol.* 6:108. Available from: https://www.frontiersin.org/article/10.3389/fimmu.2015.00108.

Sommer, F., J. M. Anderson, R. Bharti, J. Raes, and P. Rosenstiel. 2017. The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* 15:630–638. doi:10.1038/nrmicro.2017.58.

Stamati, S., C. Alexopoulos, A. Siochu, K. Saoulidis, and S. C. Kyriakis. 2006. Probiosis in sows by administration of *Bacillus toyoi* spores during late pregnancy and lactation: effect on their health status/performance and on litter characteristics. *Int. J. Probiotics Prebiotics*. 1:33.

Starke, I. C., R. Pieper, K. Neumann, J. Zentek, and W. Vahjen. 2013. Individual responses of mother sows to a probiotic *Enterococcus faecium* strain lead to different microbiota composition in their offspring. *Benef. Microbes* 4:345–356. doi:10.3920/BM2013.0021. Available from: https://www.wageningenacademic.com/doi/10.3920/BM2013.0021.

Tan, C., Z. Zhai, X. Ni, H. Wang, Y. Ji, T. Tang, W. Ren, H. Long, B. Deng, J. Deng, et al. 2018. Metabolomic profiles reveal potential factors that correlate with lactation performance in sow milk. *Sci. Rep.* 8:10712. doi:10.1038/s41598-018-28793-0.

Taras, D., W. Vahjen, M. Macha, and O. Simon. 2005. Response of performance characteristics and fecal consistency to long-lasting dietary supplementation with the probiotic strain *Bacillus cereus* var. toyoi to sows and piglets. *Arch. Anim. Nutr.* 59:405–417. doi:10.1080/17450390500353168.

Taras, D., W. Vahjen, M. Macha, and O. Simon. 2006. Performance, diarrhea incidence, and occurrence of *Escherichia coli* virulence genes during long-term administration of a probiotic *Enterococcus faecium* strain to sows and piglets. *J. Anim. Sci.* 84:608–617. doi:10.2527/2006.843608x.

Thompson, C. L., B. Wang, and A. J. Holmes. 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J.* 2:739–748. doi:10.1038/ismej.2008.29.

Whittaker, R. H. 1960. Vegetation of the Siskiyou mountains, Oregon and California. *Ecol. Monogr.* 30:279–338. doi:10.2307/1943563. Available from: https://esajournals.onlinelibrary.wiley.com/doi/abs/10.2307/1943563.

Yang, H., Y. Xiang, K. Robinson, J. Wang, G. Zhang, J. Zhao, and Y. Xiao. 2018. Gut microbiota is a major contributor to adiposity in pigs. *Front. Microbiol.* 9:3045. doi:10.3389/fmicb.2018.03045. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.03045.

Zhang, Q., J. Li, M. Cao, Y. Li, Y. Zhuo, Z. Fang, L. Che, S. Xu, B. Feng, Y. Lin, et al. 2020. Dietary supplementation of *Bacillus subtilis* PB6 improves sow reproductive performance and reduces piglet birth intervals. *Anim. Nutr.* 6:278–287. doi: 10.1016/j.aninu.2020.04.002. Available from: https://www.sciencedirect.com/science/article/pii/S2405654520300615.