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Supplementing infant milk formula with a multi-strain synbiotic and osteopontin enhances colonic microbial colonization and modifies jejunal gene expression in lactating piglets†

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A total of ninety-six weaned piglets were assigned to four dietary treatments in a 2 × 2 design. The treatments included: a standard milk formula (CTR); CTR + probiotics (6.4 × 10^8 cfu L^{-1} *Bifidobacterium longum subsp. infantis* CECT 7210 and 1.1 × 10^8 cfu L^{-1} *Lactobacillus rhamnosus* NH001) + prebiotics (galacto-oligosaccharides 4.36 g L^{-1} and human-milk-oligosaccharide 0.54 g L^{-1}) (SYN); CTR + osteopontin (0.43 g L^{-1}) (OPN); and CTR + SYN + OPN (CON). Daily records including feed intake, body weight, and clinical signs, were maintained throughout the 15-day trial. At the end of the study samples from blood, digestive content, and gut tissues were collected to determine serum TNF-α, intestinal fermentative activity (SCFA and ammonia), colonic microbiota (16S rRNA Illumina-MiSeq), histomorphology, and jejunal gene expression (Open-Array). No statistical differences were found in weight gain; however, the animals supplemented with osteopontin exhibited higher feed intake. In terms of clinical signs, synbiotic supplementation led to a shorter duration of diarrhoea episodes. Regarding gut health, the sequenced faecal microbiota revealed better control of potentially dysbiotic bacteria with the CON diet at day 15. In the colon compartment, a significant increase in SCFA concentration, a decrease in ammonia concentration, and a significant decrease in intraepithelial lymphocyte counts were particularly observed in CON animals. The supplemented diets were also associated with modified jejunal gene expression. The synbiotic combination was characterized by the upregulation of genes related to intestinal maturation (ALPI, SI) and nutrient transport (SLC13A1, SLC15A1, SLC5A1, SLC7A8), and the downregulation of genes related to the response to pathogens (GBP1, IDO, TLR4) or the inflammatory response (IDO, IL-1β, TGF-β1). Osteopontin promoted the upregulation of a digestive function gene (GCG). Correlational analysis between the microbiota population and various intestinal environmental factors (SCFA concentration, histology, and gene expression) proposes mechanisms of communication between the gut microbiota and the host. In summary, these results suggest an improvement in the colonic colonization process and a better modulation of the immune response when milk formula is supplemented with the tested synbiotic combined with osteopontin, benefiting from a synergistic effect.

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1. Introduction

The optimal nutrition for newborns is provided by human breast milk, which offers a well-balanced combination of essential nutrients, including proteins, lipids, carbohydrates, minerals, and vitamins. Additionally, breast milk contains trace elements and bioactive components crucial for meeting infants' nutritional requirements and ensuring proper growth and development.[1,2] Among its various health benefits, breast milk plays a pivotal role in shaping the gut microbiota of newborns. The development of intestinal microbiota is a complex process that commences around birth and persists for the first 2–3 years of life. This process can be influenced by several early-life factors, such as gestational age, delivery mode, maternal weight, stress, and notably, diet, which significantly affects the relative proportion of bacteria.[3–5] Due to its profound impact, breast milk is recognized as the "gold standard"

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for infant nutrition, recommended by the World Health Organization (WHO) as the exclusive source of nutrition for the first 6 months of life.[2,6] However, some infants are unable to receive human breast milk and, milk formula becomes the alternative. Unfortunately, most conventional cow's milk-based formulas lack bioactive components crucial for promoting proper gut bacterial colonization.[5] Studies have shown that exclusively breast-fed infants generally exhibit a healthier microbiome compared to exclusively formula-fed infants.[7,8] Nevertheless, recent advancements in milk formulas aim to simulate the functionality of breast milk by incorporating various components, including probiotics, prebiotics and different bioactive components, to enhance early microbiota establishment and maturation in newborns.

Probiotics, defined as live microorganisms that confer health benefits when consumed in adequate amounts,[9] are now commonly added to infant formula. In this study, we selected two probiotic strains, *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, both present in infant gut population and recognized for their beneficial role in infant health.[5,10] However, the lack of long-term effects when supplemented in infant formula suggests that their inclusion as a synbiotic combination (probiotic + prebiotic) could be more effective.[11] Therefore, in this trial, these probiotics were combined with two prebiotics. Prebiotics are defined as substrates selectively utilized by host microorganisms that confer health benefits.[12] In this work, we tested two prebiotics naturally present in human breast milk: galacto-oligosaccharides (GOS) and a human milk oligosaccharide (HMOs). Galacto-oligosaccharides (GOS) are non-digestible short-chain oligosaccharide readily fermented by lactobacilli and bifidobacteria.[13] They are well-documented to improve intestinal barrier, reduce pathogenic bacteria colonization, significantly increase short-chain fatty acid (SCFA) concentration, and modulate gene expression.[14,15] Human milk oligosaccharides (HMOs) represent the third-largest solid component in human milk (following lactose and lipids), yet they are present in bovine milk at 20 times lower concentrations.[16,17] Human milk contains over 200 different HMO structures, with 2′-Fucosyllactose (2′FL), 3-Fucosyllactose (3FL), 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL), lacto-*N*-neotetraose (LNnT), being the most important ones.[18] These human-specific milk oligosaccharides are intricate, non-digestible carbohydrates that reach the infant colon and have been linked to the growth enhancement of *Bacteroides*, *Lactobacillus* and *Bifidobacterium.*[17,19] They play a role in modulating the immune system by inhibiting pro-inflammatory responses, altering intestinal gene expression,[20] and directly influencing intestinal epithelial cells.[21] According to the definition of synbiotic as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host",[12] we could anticipate that the combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001 with GOS and HMOs might exhibit synbiotic properties when added to the milk formula.

Osteopontin is an acidic phosphorylated glycoprotein expressed in various cell types with multiple functionalities. It is involved in processes such as activation and regulation of the immune system, biomineralization, tissue-transformative processes and interactions with bacteria, among other.[22] Recently, osteopontin have been reported to promote intestinal proliferation and maturation, brain myelination, neurodevelopment and immune development.[23] Human milk has the highest concentration of this bioactive protein, being 10 times higher compared to bovine milk.[24] Osteopontin is believed to initiate and regulate developmental, immunological and physiological processes in infants, potentially playing a significant role in early-age development.

Despite extensive literature documenting the key roles of these breast milk components in infants, further research is needed to better understand their mode of action and their effects when combined in infant formulas. We hypothesize that the combination of these components could promote a synergic effect, resulting in earlier maturation of intestinal microbiota and improvement in intestinal development and functionality. Therefore, the aim of this study is to evaluate the impact of enhancing an infant milk formula by adding the described multi-strain synbiotic and/or osteopontin on intestinal health, using a lactating pig model.

2. Material and methods

The experiment was carried out at the Experimental Unit of Universitat Autònoma de Barcelona (UAB) and received prior approval (permit no. CEAAH 4928) from the Animal and Human Experimental Ethics Committee (DMAH 10947). The treatment, management, housing and slaughtering conditions adhered to European Union Guidelines (Directive 2010/63/EU, European Commission, 2010). All efforts were made to minimize animal suffering.

2.1 Animals, housing and experimental design

A total of 96 male piglets (Large White × Landrace) × Pietrain, aged 5 days (±0.14), from a high-sanitary-status commercial farm were included in this study. The animals were transported from a commercial farm to UAB's experimental unit.

The study was organized into four experimental periods of 15 days each, to ensure dedicated care of the animals during the initial days. In each period, 24 new piglets were introduced to UAB's facilities. Animals were chosen from 8 litters, selecting males of intermediate weight. Each period, animals were allocated to two rooms, each with four pens (3 animals per pen). The rooms were equipped with automatic heaters and forced ventilation. Each pen had individual heating lights, a dish with a tank for liquid dispensing (Minitainer, Rotecna, Spain), water nipples, and a plastic toy for environmental enrichment. The experiment was conducted during the autumn season (Sept-Nov).

Upon arrival, animals were distributed among treatments preserving littermates. The trial followed a 2 × 2 factorial

design, encompassing four dietary groups (with/without synbiotic × with/without osteopontin): control formula (CTR), CTR supplemented with probiotics and prebiotics (SYN), CTR supplemented with osteopontin (OPN), and CTR supplemented with the synbiotic and osteopontin (SYN + OPN = CON). Each experimental group had eight replicates across the four experimental periods, with the pen considered as the experimental unit.

2.2 Probiotic strains, prebiotics, bioactive peptide and diets

The tested probiotics were *Bifidobacterium longum* subsp. *infantis* CECT 7210, supplied by Laboratorios Ordesa S.L., and *Lactobacillus rhamnosus* HN001 from Danisco USA Inc. Both strains were supplied in lyophilized form, with concentrations of 5×10^{10} and 3×10^{10} colony forming units (CFU) per gram of product, respectively, in a maltodextrin base. Administered in the milk formula, the final dosage was 6.36×10^{8} and 1.09×10^{8} CFU g^{-1}, respectively. Before the trial, probiotic viability was confirmed through plate counting immediately after suspension in the milk formula and at various storage times on the farm, indicating good stability for both strains up to 24 h.

The experimental prebiotics consisted of a mix of galactooligosaccharides (GOS) and human milk oligosacharide (HMO), specifically 2′-Fucosyllactose (2′FL). GOS, in a syrup form, was thoroughly mixed with the milk formula to ensure a homogenous blend with no visual lumps. The final GOS concentration was 4.36 g L^{-1}, simulating the present amount in human milk (5–10 g L^{-1}),[25] as indicated in previous studies.[14,26] HMO, in powder form, was added for a final dosage of 0.436 g L^{-1}. The tested bioactive peptide, osteopontin, provided in powder form, was added for a final dosage of 0.436 g L^{-1}.

The milk formula given to piglets was tailored to their nutritional requirements and manufactured *ad hoc* by Cargill for this study. The formula excluded acidifiers, any other probiotic strains, or derivatives different from those tested, and it reduced immunoglobulin levels. The formula's main ingredients included powdered lactose, whey powder, soybeans' protein concentrate, porcine plasma meal, wheat hydrolysed gluten, sucrose, wheat, monopotassium phosphate, calcium carbonate, fructo-oligosaccharides and magnesium oxide. The chemical composition details are provided in Table 1. Milk was made available continuously, and fresh milk was prepared three times per day (at 7 am, 1 pm and 7 pm). Following milk reconstitution, all additives were incorporated and manually mixed until achieving complete homogeneity. Prior to introducing the probiotic strains, careful verification ensured that the milk temperature remained below 37 °C.

2.3 Experimental procedure

Each experimental period lasted 15 days, during which animals were introduced to the experimental diets upon arrival. Daily observations were conducted, and any clinical signs such as apathy, diarrhoea, dehydration, or anorexia were recorded. No antibiotic treatment was administered.

Table 1 Milk formula composition

Analytical constituents (%)	
Crude protein	20.9
Crudes oils and fats	10.5
Crude fibre	0.10
Ash	9.50
Calcium (Ca)	0.57
Phosphorus (P)	0.79
Sodium (Na)	0.78
Lysine	1.75
Methionine	0.50

Nutritional additives per kg: 25 000 IU vitamin A; 5100 IU vitamin D3; 150 mg vitamin E; 87.0 mg Fe, 130 mg Cu, 50.0 mg Zn, 100 mg Mn, 2.0 mg I, 0.25 mg Se.

Animal performance was monitored, with individual life weight and feed intake recorded daily. Weekly calculations were made for average daily gain (ADG) and average daily feed intake (ADFI). Faecal scores were assessed per pen using the following scale: 1 = solid and dry; 2 = well formed; 3 = very soft or viscous liquid; 4 = watery greyish; 5 = watery yellowish.

For microbiota sequencing, faecal samples were collected on days 3, 9 and 15 through spontaneous defecation or digital stimulation, encompassing all animals (n = 96).

At the end of the trial, two piglets per pen, chosen for higher weight or healthier condition, were euthanized. Euthanasia and subsequent sampling occurred in the morning (between 9:00 and 13:00 h). Remaining animals were euthanized at the end of each experimental period. Deep sedation was induced by intramuscular injection of 20 mg kg^{-1} ketamine (Ketamidor; Wels, Austria) and 2 mg kg^{-1} of xylazine (Xilagesic; Les Franqueses del Vallès, Spain). Blood collection tubes without anticoagulant (Aquisel; Madrid, Spain) and with anticoagulant (Aquisel; Madrid, Spain) were used to collect 10 ml of blood from the cranial cava vein of each animal. After blood sampling, animals were euthanized with an intravenous injection of 200 mg kg^{-1} sodium pentobarbital (Euthasol; Le Vet B.V., Oudewater, The Netherlands). Afterwards, animals were bled, the abdomen was immediately opened, and the gastrointestinal tract was excised and transferred to a tray.

Content from the ileum and proximal colon was collected and homogenized, and their pH measured (Crison 52–32 electrode, Net Interlab; Barcelona, Spain). For ammonia determination, ileal and colonic digesta aliquots were store at −20 °C in 0.2 N H_2SO^4 as a preservative solution (3 ml of digesta + 3 ml of 0.2 N H_2SO^4). Another subsample was kept at −20 °C for SCFA and lactic acid determination.

Jejunal tissue samples were collected for gene expression study. Approximately 0.5 cm^2 tissue from the jejunum was preserved in RNAlater®, initially in the fridge for 24 hours and subsequently at −20 °C.

Additionally, jejunal sections of 2–3 cm length were cut, opened longitudinally, washed thoroughly with sterile PBS, and fixed by immersion in a 4% formaldehyde solution (Panreac; Castellar del Vallès, Spain) for histomorphology.

2.4 Analytical procedures

2.4.1 Short-chain fatty acids, lactic acid and ammonia analysis. Short-chain fatty acids and lactic acid determinations were conducted on ileal and colonic content using gas–liquid chromatography (GLC). The samples underwent an acid–base treatment followed by ether extraction and derivatization with *N*-(tertbutyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1% tertbutyldimethylchlorosilane (TBDMCS) agent, following the method outlined by Richardson *et al.*'s (1989)[27] modified by Jensen *et al.* (1995).[28]

Ammonia concentrations in ileal and colonic content were determined using a gas-sensitive electrode (Hatch Co.; CO, USA) paired with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain). For the determination process, preserved samples were homogenized and centrifuged at 1500× for 10 min. Subsequently, samples were diluted 1 : 4 in distilled water based on ammonia concentration. The ammonia released after the addition of 10 M NaOH was measured in the supernatant as a change in voltage (mV) using a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.).

2.4.2 Blood analysis. Blood samples were centrifuged (2500*g*, 8 min at 4 °C) after 4 hours of refrigeration, and serum samples were stored at −20 °C until analysis. Tumor Necrosis Factor-α (TNF-α) concentration in serum samples was analysed using the Quantikine Porcine TNF-α kit (R&D Systems; Minneapolis, United States) with enzyme-linked immunosorbent assay (ELISA).

2.4.3 Histological analysis. Histological studies were conducted on jejunum, ileal, and colonic samples. Tissues were dehydrated, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin. Morphological measurements were made with a light microscope (BHS, Olympus) following the method outlined by Nofrarías *et al.* 2006.[29] Parameters measured included villus height (VH), crypt depth (CD), the ratio of villus height : crypt depth (VH : CD), intraepithelial lymphocytes (IEL), goblet cells (GC) and mitosis (M). Between 7 and 10 intact villi were measured per animal.

2.4.4 Gene expression analysis. Gene expression was quantified by RT-qPCR to assess the expression of 56 genes in jejunal tissue using an Open Array Real-Time PCR Platform (Applied Biosystems, Waltham, MA, USA), following the method described by González-Solé *et al.* (2020),[30] in the Veterinary Service of Molecular Genetics at the Veterinary Faculty of the Universitat Autònoma de Barcelona (Spain).

A total of 56 genes involved in multiple physiological functions related to intestinal health were analysed and categorized into groups: (1) genes responsible for maintaining the intestinal barrier function, including OCLN, ZO1, CLDN1, CLDN4, CLDN15, MUC2, MUC13, and TFF3; (2) genes crucial for immune responses, including pattern recognition receptors, cytokines, chemokines, and stress proteins, including TLR2, TLR4, IL1B, IL6, IL8, IL10, IL17A, IL22, IFNG, TNF, TGFB1, CCL20, CXCL2, IFNGR1, HSPB1, HSPA4, REG3G, PPARGC1A, FAXDC2, and GBP1; (3) genes encoding enzymes and hormones involved in the digestive process, such as GPX2, SOD2, ALPI, SI, DAO1, HNMT, APN, IDO1, GCG, CCK, IGF1R, and PYY; (4) genes participating in nutrient transport, including SLC5A1, SLC16A1, SLC7A8, SLC15A1, SLC13A1, SLC11A2, MT1A, SLC30A1, and SLC39A4; and (5) genes associated with stress responses, specifically CRHR1, NR3C1, and HSD11B1. Detailed information regarding genes is provided in ESI (Table S1†).

2.4.5 Microbiota sequencing. Microbiota studies were conducted on faecal samples obtained on days 3, 9, and 15. The V3–V4 region of the 16S rRNA gene was amplified and sequenced.

For this method, DNA was extracted with the QIAamp Fast DNA Stool Mini Kit (Qiagen; West Sussex, United Kingdom), following manufacturer's protocol. Subsequently, microbiome analysis was performed by sequencing 16S (Illumina MiSeq). For this procedure, a total of 50 ng of DNA was amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). In the first amplification step, primers were designed containing: (1) a universal linker sequence allowing amplicons for incorporation indexes and sequencing primers by Nextera XT Index kit (ILLUMINA); and (2) 16S rRNA gene universal primers.[31] In the second and last assay amplification indexes were included. 16S based libraries were quantified by fluorimetry using Quant–iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher). Libraries were pooled prior to sequencing on the NovaSeq platform (Illumina), 300 cycles paired reads configuration. The size and quantity of the pool were assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 20%). Image analysis, base calling and data quality assessment were performed on the NovaSeq instrument (NovaSeq Control Software (NVCS v1.7)). Raw sequences, forward (R1) and reverse (R2), were imported into QIIME2 plattform.[32] Cutadapt v3.4 [33] plugin was used to filter specific V3–V4 16S region adapters. R1 and R2 reads were processed using 'denoise-paired' command of DADA2 plugin.[34] Low-quality reads were filtered by the function 'filterAndTrim', and were truncated where they started to lose quality (240 for R1, 200 for R2). Error models were generated using 'learnErrors' function, and DADA2 algorithm was applied using 'dada' function. ASVs ('Amplicon Sequence Variants') generated by R1 and R2 reads were merged using 'mergepairs' function. Quimera sequences were removed 'removeChimeraDenovo' function. Taxonomy of resulting ASVs was annotated using blastn v2.2.29+[35] against 16S specific database from the NCBI (v. August 2021). Assigned taxonomies with an identity percentage lower than 97% were reassigned using NBAYES algorithm[36] against SILVA v.138 16S database.

2.5 Statistical analysis

The effects of experimental treatments on various parameters (excluding microbiology) were analysed using the free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA) with the stats package.[37] An

ANOVA was conducted (lm function for a two-way ANOVA) to determine the main effects of synbiotic addition, osteopontin addition, or any possible interaction. The following generalized linear model was employed:

$$Y_{ijkl} = m + \text{Synbiotic}_i + \text{Oseopontin}_j + (\text{Synbiotic} \times \text{Osteopontin})_{ij} + \text{Period}_k + e_{ijkl}$$

where Y represents each observation of the studied variable, m is the global mean, Synbiotic_i is the main effect of adding the synbiotic to the diets, Osteopontin_j is the main effect of adding the osteopontin to the diets, $\text{Synbiotic} \times \text{Osteopontin}_{ij}$ is the possible interaction, and Period_j is the main effect of the period. Finally, e_{ijkl} is the experimental error term.

For the analysis of the evolution of ADFI and faecal scores along time, a mixed-effects model (lme function) was used following:

$$Y_{ijk} = m + \text{Diet}_i + \text{Time}_j + (\text{Diet} \times \text{Time})_{ij} + e_{ijk}$$

where Y is each observation of the studied variable, m is the global mean, Diet_i is the main effect of the experimental diets, Time_j is the main effect of the day, and $\text{Diet} \times \text{Time}_{ij}$ is the possible interaction. Finally, e_{ijk} is the experimental error term.

When the effects of additives were established ($p < 0.05$), the least squares means were separated using the probability function of differences adjusted by Tukey–Kramer.

The analysis of the percentage of casualties was conducted through a frequency analysis using a Fisher test (fisher.test function) with the same statistical package.

The pen was considered the experimental unit for all parameters, and results are expressed as means with their standard errors unless otherwise stated. The α level used to determine statistical significance was $p = 0.05$, with statistical trend levels between $p = 0.05$ – $p = 0.10$ considered.

In the gene expression study, the Open Array data were prepared using ThermoFisher Cloud 1.0 software (Applied Biosystems). The $2 - \Delta\Delta Ct$ method for relative quantification was applied, using the geometric mean of 4 reference genes (ACTB, B2M, GAPDH and TBP) and a reference sample with a representative profile from the CTR group as a calibrator. The maximum allowed cycle relative threshold was set to 26, amplification score <1.240, quantification cycle confidence >0.80, and maximum allowed SD between duplicates was set to <0.38. Three samples showing inadequate amplification were removed. All data underwent a logarithmic transformation to approximate the Gaussian distribution. Statistical analyses were performed using free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA). A two-way ANOVA was conducted following the generalized linear model mentioned before, without considering period effect. Subsequent pairwise *post hoc* comparisons of treatments were executed using Tukey's honest significant difference test.[38] Statistical differences for treatments were considered when p-values were under 0.05 for the ANOVA and Tukey's analysis.

A principal component analysis (PCA) was performed using samples as cases and gene log10-expressions as variables, following the method described by González-Solé *et al.* (2020).[30]

Microbiota sequencing data were treated as follows. The relative taxonomic abundances of the samples were displayed with collapsed histograms plotted by 'ggplot2' library in R (v.4.0). Data were normalized using the rarefaction technique from Phyloseq R package[39] to perform alpha diversity analysis. Shannon, Simpson and Richness indexes were calculated using 'vegan' R package.[40] Boxplots were performed by 'ggplot2' library in R (v.4.0), and Wilcox test was used to find significant differences in alpha diversity between groups.

To illustrate taxonomic dissimilarities based on ASVs, Principal Coordinate Analysis (PCoA) across the samples was carried out using the Bray–Curtis distance matrix. The effects of the factors on taxonomy were evaluated with a permutational multivariate analysis of variance (PERMANOVA) using a Bray–Curtis dissimilarity matrix that was previously calculated considering the relative abundances of functional categories in all samples. The Bray–Curtis dissimilarity matrix and PERMANOVA analysis were performed using 'vegan' R package. PCoA plot was constructed using 'ggplot2'. DESeq2 [41] was used for biomarkers identification between the different diets on each of the days. A taxon was considered differentially abundant if the corrected p-value > 0.1 and if it was present on at least 50% of the samples of one of the compared groups. Heatmaps were constructed using 'ComplexHeatmap' R package (v.2.10.0). MaAslin2 [42] was used to study correlations between microbial abundances and organic compounds, gene expression and histology parameters in day 15 samples. A linear model test was performed for each variable, with the variable as fixed effect. The microbial taxa counts were normalized using DESeq normalization method, and the normalized counts were log-transformed. Only taxa present in more than 10% of the samples were considered.

3. Results

In general terms, the trial proceeded as anticipated. Upon arrival, all animals exhibited good health. However, considering the challenges of rearing early-age piglets without the sows, the mortality rate at the end of the study reached 15.6%, with 5, 1, 3, and 6 casualties for the CTR, SYN, OPN, and CON treatments, respectively. The causes for mortality included: 2 humanitarian euthanasians due to acute meningitis (post-mortem diagnosis), from CON treatment; 1 humanitarian euthanasia due to exudative epidermitis (post-mortem diagnosis), from CON treatment; 10 casualties due to acute diarrhoea and dehydration (6 humanitarian euthanasians, 4 sudden deaths), 3 from CTR, 1 from SYN, 3 from OPN and 3 from CON; and 2 humanitarian euthanasia undiagnosed, from CTR treatment. No significant differences in mortality could be attributed to the experimental treatments (Fisher-test p-value > 0.18).

In total, 8 piglets needed subcutaneous hydration: 3 for CTR (2 unsuccessful); 2 for SYN (1 unsuccessful); 2 for OPN (unsuc-

cessful); 1 for CON (unsuccessful). No significant differences could be attributed to the treatments (Fisher-test p-value = 1).

3.1 Performance parameters

Results for live weight (LW), average daily feed (milk) intake (ADFI) and average daily gain (ADG) are presented in Table 2. Regarding the live weight of the animals, there were no differences due to treatments, but there were differences related to the experimental period at day 0 (1940, 2414, 2666, 2563 g for period 1–4 respectively, p = 0.005). This was related to the age at which animals arrived at the farm. In the first and third periods, animals arrived at 4 days of life, whereas in the second and fourth periods, they arrived at 5 days of life.

Regarding milk intake, the animals adapted well to artificial lactation, and all animals learned how to consume milk from the plates after the first 24 hours. Milk intakes could be considered within normal limits, showing a linear increase over time (Fig. 1). Milk intakes during the first week exhibited variations related to the period, but not with the treatments or the interaction. In the second week, a higher milk intake was observed in animals supplemented with osteopontin as these animals consistently exhibited a higher milk consumption on days 12, 13, and 14 (Fig. 1).

The ADG of the animals also fell within the normal range, although with a high variability between litters (pens). No differences were observed in ADG in relation to the additives or the period.

Ultimately, no significant differences in faecal consistency were noted among treatments over time (mean values of 2.9, 2.6, 2.6, 2.7 for CTR, SYN, OPN, CON respectively, p = 0.739). However, upon examining the percentage of days when animals (pens) exhibited diarrhoea (defined as a score ≥4), a notable effect was observed with the synbiotic supplementation. Animals consuming the synbiotic additives experienced a lower percentage of days presenting diarrhoea (Table 3).

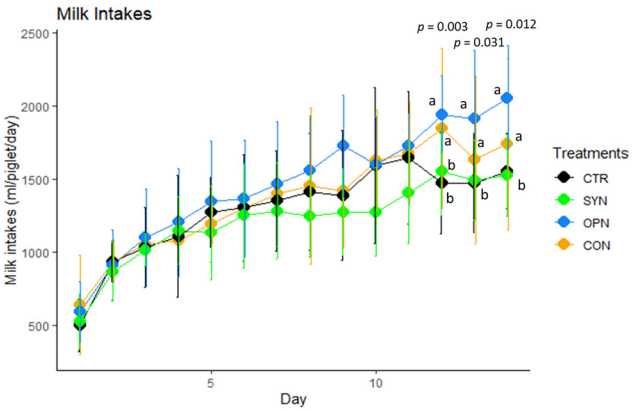

Fig. 1 Effect of experimental treatments on milk consume with standard error bars. Milk intakes (ml) per piglet per day during the 15-day trial. CTR: no additives in the diet; SYN: prebiotic + probiotic additives in the diet; OPN: osteopontin in the diet; CON: synbiotic + osteopontin in the diet. n = 8. ^{a,b} Indicate statistically significant differences between means.

3.2 Intestinal fermentation

Concerning the fermentation profile in the ileum and colon, 9 piglets exhibited a substantially altered profile (outliers). Analyzing the history of these animals in the sampling book revealed that they had very little digesta content along gastrointestinal tract (almost empty) and/or presented yellowish ileal content (clear signs of diarrhoea). Therefore, these animals were excluded from the NH_3 and SCFA data subset. These exclusions were as follows: 3 from the CTR group (periods 1 and 2), 2 from SYN (period 3), 2 from OPN (periods 1 and 3)

Table 2 Effect of experimental treatments on growth and milk consumption. Life weight (LW) (g) at the study's onset, day 7, and day 15 (end of study); average daily feed intake (ADFI) (ml day^{-1}) and average daily gain (ADG) (g day^{-1}) during the first experimental week (days 0–7), the second week (days 7–15), and the entire study duration (days 0–15)

	Osteopontin −		Osteopontin +						
Main effects	Synbiotic −	Synbiotic +	Synbiotic −	Synbiotic +		p-Value			
Diets	CTR	SYN	OPN	CON	RSE	Synbiotic	Osteopontin	Interaction	Period
LW (g)									
D0	2376	2436	2390	2383	367.5	0.839	0.885	0.798	0.006
D7	3358	3524	3603	3425	325.5	0.692	0.389	0.313	0.193
D15	4628	4756	5028	4794	536.8	0.225	0.120	0.954	0.549
ADFI (ml)									
D0-d7	1075	1030	1122	1080	108.2	0.172	0.161	0.670	0.004
D8-D15	1504	1397	1788	1626	189.5	0.057	0.002	0.746	0.604
Total	1290	1214	1450	1351	133.0	0.061	0.005	0.962	0.369
ADG (g)									
D0-D7	140.4	155.5	173.3	148.9	46.49	0.691	0.389	0.313	0.193
D8-D15	181.4	175.9	203.6	195.6	89.83	0.883	0.549	0.906	0.194
Total	160.9	165.7	188.4	172.2	38.35	0.680	0.228	0.452	0.545

Osteopontin +/− : presence/absence of osteopontin ingredient in the diet; synbiotic +/− : presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and statistical difference present. RSE, residual standard error.

Table 3 Effect of the experimental treatments on the percentage of days animals were presenting diarrhoea (as a score ≥ 4)

	Osteopontin −		Osteopontin +						
Main effects	Synbiotic −	Synbiotic +	Synbiotic −	Synbiotic +		*p*-Value			
Diets	CTR	SYN	OPN	CON	RSE	Synbiotic	Osteopontin	Interaction	Period
% of days presenting diarrhoea	39.98	25.53	46.63	26.65	18.25	0.039	0.612	0.716	0.062

Osteopontin +/−: presence/absence of osteopontin ingredient in the diet; synbiotic +/−: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and statistical difference present. RSE, residual standard error.

and 2 from CON (period 1). No animals were excluded from the period 4.

The results of fermentation products in ileal and colonic digesta after excluding these individuals are presented in Table 4. Concerning ammonia levels, there was a significant interaction for ileal concentration exhibiting lower values only when all additives were combined in the CON treatment. Significant reductions were observed in colonic ammonia concentration attributed to the synbiotic and the osteopontin supplementation that showed an additive effect in the CON treatment (no significant interaction).

Lactic fermentation predominated in the ileum, with no significant differences noted due to treatments, neither in SCFA. In the colon, however, significant changes were evident concerning diets. The addition of the synbiotic trended to increase total SCFA and acetate and significantly increased propionate, butirate and valerate. Osteopontin supplementation significantly increased total SCFA and acetate concentrations, showing a trend for butyrate. No significant interactions were found showing additive effects for SCFA, acetate and butyrate.

Regarding molar ratio, a significant interaction was found for acetate that only was increased when both additives were added separately.

Lactic acid in the colon was only detected in some animals, with 63.5% below the minimum level of detection (1.69 mM kg^{-1}).

3.3 Intestinal histomorphology and serum TNF-α

Histomorphometry analysis of the jejunum, ileum and colon included parameters such as villus height (VH), crypt depth (CD), ratio between villus height : crypt depth (VH : CD), intraepithelial lymphocytes (IEL) (only in the villus), goblet cells (GC) (only in the villus) and mitosis (only in the crypt) (Table 5).

Table 4 Effect of experimental treatments on ileal and colonic fermentation

	Osteopontin −		Osteopontin +						
Main effects	Synbiotic −	Synbiotic +	Synbiotic −	Synbiotic +		*p*-Value			
Diets	CTR	SYN	OPN	CON	RSE	Synbiotic	Osteopontin	Interaction	Period
Ileum digesta (mM kg^{-1} FM)									
Total SCFA	4.02	4.57	4.66	5.16	2.485	0.746	0.340	0.802	0.039
Acetate	3.91	4.49	4.60	5.08	2.413	0.725	0.308	0.766	0.037
Lactate	20.6	22.3	19.4	20.2	7.449	0.148	0.954	0.495	0.498
mmol NH_3/kg MF	2.99^{xy}	3.64^{x}	3.53^{xy}	2.69^{y}	0.91	0.676	0.453	0.011	<0.001
Colonic digesta (mM kg^{-1} FM)									
Total SCFA	69.0	83.9	89.3	105.4	31.04	0.053	0.026	0.834	0.006
Acetate	44.5	54.4	58.0	66.5	20.19	0.067	0.032	0.958	0.003
Propionate	14.8	19.6	18.6	22.3	6.00	0.005	0.190	0.548	0.558
Butirate	6.90	8.87	8.54	12.17	4.53	0.019	0.059	0.511	0.056
Valerate	1.50	2.40	2.00	2.54	1.00	0.040	0.110	0.903	0.214
Total BCFA	1.50	1.86	2.11	1.88	0.92	0.701	0.292	0.271	0.041
Lactate[a]	13.02	3.70	10.93	6.04	21.33	—	—	—	—
Molar ratio of SCFA (%)									
Acetate	63.71	68.03	66.58	63.13	8.50	0.211	0.685	0.035	0.022
Propionate	21.56	20.35	19.68	21.83	5.51	0.153	0.277	0.715	0.369
Butirate	10.76	10.27	9.29	10.92	2.56	0.350	0.245	0.406	0.007
Valerate	1.88	2.57	2.26	2.36	1.00	0.768	0.329	0.339	0.346
BCFA	2.24	2.05	2.19	1.76	1.00	0.285	0.490	0.574	0.081
mmol NH_3/kg MF	274.8	78.3	147.2	37.25	161.9	0.004	0.033	0.119	0.003

SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids. Osteopontin +/−: presence/absence of osteopontin ingredient in the diet; synbiotic +/−: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and statistical difference present. ^{x,y} Indicate a statistical trend between means. RSE, residual standard error. [a] It is not possible to provide the statistics since in many animals the values were not detectable. Number of detectable animals were 6, 5, 3 and 5 for CTR, SYN, OPN and CON respectively.

Table 5 Effect of experimental treatments on jejunal and colonic histomorphology

	Osteopontin −		Osteopontin +						
Main effects	Synbiotic −	Synbiotic +	Synbiotic −	Synbiotic +		*p*-Value			
Diets	CTR	SYN	OPN	CON	RSE	Synbiotic	Osteopontin	Interaction	Period
Jejunum									
VH (μm)	236	319	319	291	67.59	0.344	0.344	0.098	0.134
CD (μm)	181	177	185	172	20.98	0.254	0.791	0.458	0.111
VH : CD	1.33	2.00	1.80	1.78	0.503	0.364	0.118	0.419	0.228
IEL (cell no)	8.53	14.1	12.9	11.0	5.046	0.294	0.655	0.070	0.339
GC (cell no)	2.20	3.72	2.95	4.34	1.784	0.452	0.920	0.337	0.010
M (cell no)	1.75	1.44	1.42	1.33	0.701	0.338	0.360	0.946	0.604
Colon									
CD (μm)	278	270	269	284	35.06	0.916	0.736	0.497	0.602
IEL (cell no)	2.72	1.98	1.49	1.32	0.866	0.263	0.028	0.688	0.114
GC (cell no)	26.4	24.3	24.8	28.3	5.336	0.697	0.560	0.168	0.760
M (cell no)	0.257	0.157	0.219	0.085	0.123	0.043	0.326	0.794	0.702

Measured parameters: villous height (VH); crypt depth (CD); ratio villous height : crypt depth (VH : CD); intraepeithelial lymphocytes (IEL); goblet cells (GC); mitosis (M). Osteopontin +/−: presence/absence of osteopontin ingredient in the diet; synbiotic +/−: presence/absence of synbiotic ingredients in the diet. $n = 8$. *Indicates $p < 0.05$ and statistical difference present. RSE, residual standard error.

In the jejunum sections, a period effect was observed (data not shown). The number of GC showed abnormally high values with the CON treatment in the third period but not in the other periods.

In the ileum, no significant differences were observed (data not shown).

In the colon, the addition of osteopontin significantly affected the number of IEL with lower numbers. An effect of the synbiotic addition was observed regarding mitotic cells with lower mitotic cell counts.

No statistically significant effects were detected in the serum levels of TNF-α (data not shown).

3.4 Gene expression

The genes exhibiting a significant treatment effect are listed in Table 6, while comprehensive results for the entire gene set can be found in ESI (Table S1†).

Within the category of genes associated with enzyme/hormone functions, a significant interaction was observed between additives on intestinal alkaline phosphatase (ALPI) and sucrase-isomaltase (SI) genes, with higher expression in animals only when the synbiotic was supplemented alone (SYN treatment). Significant difference associated to the synbiotic supplementation was noted in the cholecystokinin gene (CCK) gene, with higher expression observed. Among the group of digestive enzymes, an interaction effect was found for indoleamine 2,3-dioxygenase (IDO1) gene, resulting in similar lower expression levels for all three supplemented diets compared to the CTR diet. Regarding proglucagon (GCG), an effect was observed with osteopontin supplementation, resulting in higher expression. In the immune response related genes, results indicated a significant interaction for interleukin 1 beta (IL-1β) with all three experimental treatments showing similar lower values compared to the CTR group. Transforming growth beta factor 1 (TGF-β1) expression also exhibited an interaction effect with reduced levels only when the additives were supplemented separately (SYN or OPN diets). The toll-like receptor 4 (TLR4) gene also showed a significant interaction exhibiting lower expression in the SYN treatment compared to the CTR treatment, with intermediate values observed in the OPN and CON treatments. Lastly, the guanylate binding protein 1 (GBP1) presented lower expression when the synbiotic was supplemented. Regarding the expression of nutrient transport function genes, (SLC13A1, SLC15A1, SLC5A1 and SLC7A8), the synbiotic supplementation promoted a higher expression. In terms of barrier function genes, mucin 13 (MUC13) and mucin 2 (MUC2) displayed an interaction effect. Specifically, when the synbiotic was supplemented alone (SYN diet) resulted in higher values for MUC13, while lower values for MUC2. Ocludin (OCLN) was also more expressed with the synbiotic supplementation.

A principal component analysis (PCA) was conducted to assess the degree of correlation among gene expression values across samples belonging to the four different diets, as illustrated in Fig. 2. The sample identifier numbers are visually represented in the individual factor map (a), with colours corresponding to the assigned diets for each sample. In the variables factor map (b), the diets that exhibit correlations across the gene expression are depicted.

The findings reveal a discernible correlation in the patterns of gene expression within this tissue. In figure (a), samples are visually grouped by treatments in 2D space. Figure (b) clearly shows how the experimental diets with additives differ from the CTR in relation to gene expression.

3.5 Analysis of the microbiota by sequencing the 16S rRNA gene

To analyse the impact of experimental diets on the microbiota of piglets, faecal samples were collected on three different

Table 6 Effect of experimental treatments on jejunal gene expression. Values are expressed relative to a reference sample of the control treatment. The average values for the treatments are presented without the logarithmic transformation employed during statistical analysis

	Osteopontin −		Osteopontin +						
Main effects	Synbiotic −	Synbiotic +	Synbiotic −	Synbiotic +		*p*-Value			
Diets	CTR	SYN	OPN	CON	RSE	Synbiotic	Osteopontin	Interaction	Function
Genes									
ALPI	1.140^{b}	2.204^{a}	1.859^{a}	2.091^{a}	1.588	0.001	0.084	0.027	EH
CCK	2.07	2.252	1.823	2.655	1.581	0.025	0.897	0.257	EH
CXCL2	1.286	0.543	0.586	0.829	2.194	0.066	0.307	0.075	IR
GBP1	1.415	0.781	0.9	0.9	2.289	0.045	0.347	0.217	IR
GCG	0.924	0.891	1.13	1.129	1.468	0.883	0.040	0.900	EH
GPX2	1.826	1.433	1.868	1.433	2.094	0.091	0.846	0.957	EH
IDO1	1.364^{a}	0.284^{b}	0.458^{ab}	0.466^{ab}	5.198	0.024	0.372	0.035	EH
IFNG	3.15	1.002	1.151	1.618	4.136	0.082	0.902	0.145	IR
IL1beta	1.276^{a}	0.406^{b}	0.393^{b}	0.560^{b}	2.389	0.036	0.053	0.027	IR
IL6	2.899	1.103	1.379	1.356	2.304	0.050	0.356	0.103	IR
MUC13	0.815	1.116	0.988	0.871	1.506	0.492	0.605	0.015	BF
MUC2	1.299	1.008	1.094	1.291	1.385	0.447	0.877	0.040	BF
OCLN	1.202	1.571	1.383	1.441	1.302	0.041	0.815	0.212	BF
SI	2.286^{b}	4.729^{a}	2.771^{ab}	3.569^{ab}	1.884	0.004	0.840	0.026	EH
SLC11A2	1.503	1.307	1.409	1.285	1.281	0.096	0.702	0.874	NT
SLC13A1	0.666^{y}	1.086^{x}	0.934^{xy}	1.113^{x}	2.255	0.026	0.072	0.073	NT
SLC15A1	1.632	2.440	1.893	2.171	1.490	0.010	0.731	0.166	NT
SLC39A4	1.24	1.753	1.5	1.529	1.448	0.146	0.566	0.072	NT
SLC5A1	1.694	2.791	1.844	2.349	1.668	0.005	0.909	0.215	NT
SLC7A8	1.077	2.604	2.375	3.175	3.187	0.043	0.296	0.082	NT
TGFbeta1	1.550^{a}	0.904^{b}	0.916^{b}	1.105^{ab}	1.583	0.108	0.163	0.010	IR
TLR2	0.817	0.431	0.635	0.651	2.178	0.073	0.911	0.171	IR
TLR4	2.542^{a}	1.297^{b}	1.577^{ab}	1.620^{ab}	1.755	0.068	0.465	0.017	IR

Osteopontin +/−: presence/absence of osteopontin ingredient in the diet; synbiotic +/−: presence/absence of synbiotic ingredients in the diet. n = 8. Gene abbreviations detailed in ESI (Table S2†). EH – enzyme/hormone; IR – immune response; BF – barrier function; NT – nutrient transport. * Indicates $p < 0.05$ and statistical difference present. ^{a,b} Indicate statistically significant differences between means. ^{x,y} Indicate a statistical trend between means. RSE, residual standard error.

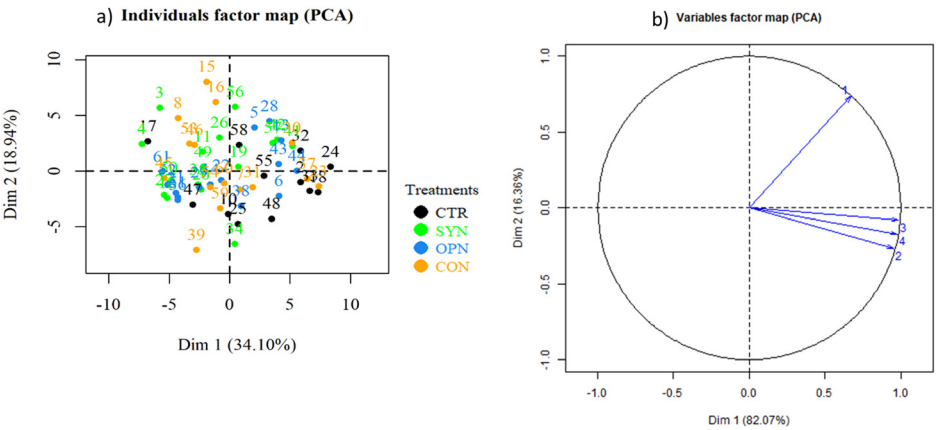

Fig. 2 Principal component analysis (PCA) for jejunal gene expression data: (a) samples' picture from jejunum (individuals factor map); (b) treatment arrow diagram from jejunum (1 – CTR; 2 – SYN; 3 – OPN; 4 – CON). CTR: no additives in the diet; SYN: prebiotic + probiotic additives in the diet; OPN: osteopontin in the diet; CON: synbiotic + osteopontin in the diet. n = 8.

days: 3, 9 and 15. The rarefaction curves for all samples reached the plateau phase, indicating comprehensive identification of bacterial species. The predominant phyla in microbial communities were Firmicutes (46.8 ± 16.65%), Bacteroidetes (36.5 ± 17.86%), and Proteobacteria (9.2 ± 10.76%) (Fig. 3). A total of 69 families were detected, with the most abundant being *Bacteroidaceae* (6.8 ± 6.28%), *Lactobacillaceae* (6.2 ± 9.03%), and *Oscillospiraceae* (4.4 ± 3.67%). A total of 148 genera were identified, with the 30 most abundant ones contributing to 67.6 ± 7.89% of the total abundance. Notably, *Escherichia* (6.2 ± 4.25%), *Bacteroides* (5.8 ± 3.37%), and *Phocaeicola* (5.8 ± 4.54%) exhibited the highest

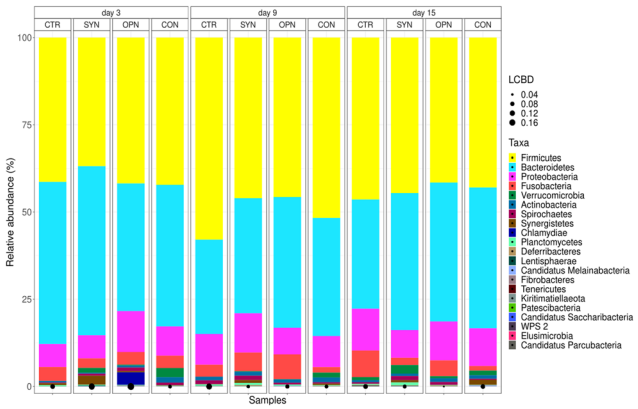

Fig. 3 Effect of the experimental treatments on microbial faecal communities (phylum) at different sampling days (3, 9 and 15). CTR: no additives in the diet; SYN: prebiotic + probiotic additives in the diet; OPN: osteopontin in the diet; CON: synbiotic + osteopontin in the diet. n = 8.

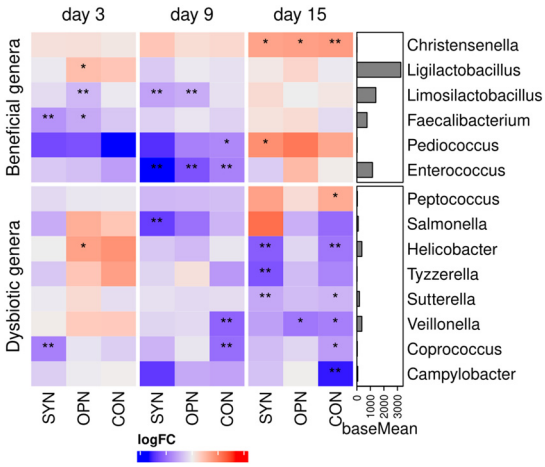

Fig. 4 Heatmap of the impact of experimental treatments on potentially beneficial and dysbiotic genera observed in faecal samples on various sampling days (3, 9 and 15). CTR: no additives in the diet; SYN: prebiotic + probiotic additives in the diet; OPN: osteopontin in the diet; CON: synbiotic + osteopontin in the diet. n = 8. * Indicates $p < 0.1$; ** indicates $p < 0.05$.

relative frequencies, although with a considerable variability among samples.

Regarding alpha diversity of microbiota species, it remained stable over time, and there were no significant effects of the treatment on Richness, Simpson, or Shannon indexes. However, at the beta diversity level, the PERMANOVA test revealed statistical differences related to Treatment (R^2 = 0.023 and $p < 0.001$), although most of the variability was explained by Animal and Day (40.7% and 4.5%, respectively).

A treatment effect was observed in the bacterial populations among the different diets. Significant differences at different taxonomic level are presented in the ESI (Fig. S1–S3†). In general, synbiotic supplementation exhibited a more pronounced effect with longer administration of the additive (day 15) across various populations. These changes were evident at different taxonomic levels, including phylum, family, and genus. For instance, the phylum Synergistetes, family *Synergistaceae*, and genus *Cloacibacillus* showed increased abundance due to synbiotic supplementation on day 15. Conversely, the family *Sutterellaceae* and *Helicobacteraceae*, along with their genera *Sutterella* and *Helicobacter*, respectively, were reduced by synbiotic supplementation on day 15. On the contrary, osteopontin supplementation resulted in a more immediate effect on day 3, which diminished as the study progressed. At the family level, there was an increase in *Lactobacillaceae* on day 3, which disappeared by day 15. At the genus level, on day 3, there was an increase in *Oribacterium*, *Mucispirillum*, *Prevotellaceae*, and *Ligilactobacillus* due to osteopontin supplementation. However, by day 15, the genera *Mediterranea* and *Christensenella* exhibited growth promotion, while populations of *Veillonella* and *Faecalicatena* were reduced.

Fig. 4 illustrates the most notable changes induced by the dietary interventions on specific potentially beneficial or dysbiotic genera according to the literature. As mentioned earlier, these changes gradually consolidated as the study progressed. After 15 days of supplementation, a trend was observed wherein most of the beneficial genera increased, while a notable decrease was registered in most of the potentially dysbiosis genera. However, evolution along the experimental period was not the same for all bacterial genera. For beneficial genera, supplemented diets led to a temporary decrease on day 9, which reversed by day 15, as evidenced by *Enterococcus* and *Limosillactobacillus*. However, for dysbiotic bacteria the changes had the same trend at day 9 and 15 but consolidating at day 15. Concerning potentially dysbiotic genera at day 15, the combination of synbiotic and osteopontin demonstrated superior control over this group. A significant reduction in *Helicobacter*, *Tyzzerella*, and *Sutterella* may be attributed to the synbiotic component, whereas osteopontin appears to impact *Veillonella*. The CON diet benefits from both effects, owing to an additive synergy. However, some outcomes are not solely attributable to the additive effect but suggest an unexpected interaction. For instance, a notable decrease in *Campylobacter* was observed exclusively with the CON diet.

4. Discussion

The benefits of additives supplemented in infant formulas, as used in this trial, are well-documented in the literature. Nevertheless, our aim was to elucidate whether the combination of the synbiotic with osteopontin would result in improvements through a synergistic approach.

While there is inconsistent information about the beneficial effects of combining additives of different nature, notable achievements have been documented with the incorporation of probiotics (specifically *Lactobacillus* and *Bifidobacterium*) in conjunction with prebiotics (such as bovine milk oligosaccharides and GOS) into infant milk formulas. These combinations have successfully created an intestinal

environment in infants that closely resembles that of breastfed infants, as reported by Meli *et al.*, 2014.[43] Regarding osteopontin supplementation in infant milk formulas, limited research has been conducted, especially in combination with a synbiotic. Nevertheless, important bioactive roles have been attributed to osteopontin[44] and when supplemented to infant formula, it has promoted shifts in jejunal gene expression[24] and functional systemic benefits[45] approximating a breast-fed condition. Our hypothesis, therefore, is that each additive would endorse benefits to neonates, and the combination of all would be translated into a better response.

In this trial, suckling piglets were employed as an animal model for newborn infants. Attempting to replicate the characteristics of a neonate in an animal model has proven to be intricate. However, the suckling pig has been accepted as a suitable model for human nutritional interventions,[46–48] presenting a very similar digestive system anatomy and morphology compared to human infants,[49] along with similarities in digestive function and gastrointestinal fermentation profiles.[48] Nevertheless, it is important to consider the distinct predominant bacterial groups that these two species host during the early stages of development. In human infants, the intestinal gut is primarily colonized by bifidobacterial, whereas in newborn piglets, the dominant microbial community comprises lactobacilli.[49] Maternal passive immunity transfer also differs across species. In the case of humans, maternal antibodies are transferred to the fetus through the placenta during gestation, while in piglets, there is no passive immunity transfer. Consequently, the intake of colostrum becomes crucial for them.[50] Despite these unavoidable differences, suckling piglets have already been successfully used as model for humans in nutritional research.[48]

Supplemented formula in this study proved to be safe. Regarding performance, animals supplemented with osteopontin registered statistical higher feed intake during the second week and overall. This effect, however, was more markedly seen in the first experimental period when animals arrived at an earlier age and lighter weights, suggesting that these additives may have a greater impact in more challenging situations. The weights at the end of the study were numerically higher in all treated groups, particularly in the animals receiving osteopontin (p = 0.120), although this difference did not reach statistical significance. The absence of statistical differences in performance could also be attributed to the limitations of the experimental design, which was primarily intended to elucidate possible mechanisms of action rather than assess the impact of diets on performance. Alternative designs, with increased numbers of replicates and extended administration periods, may have resulted in more pronounced differences in growth. Nevertheless, while animal growth remains a reliable indicator of health, other analysed parameters can also offer valuable information in this regard. For instance, the presence or absence of diarrhoea can also be considered a useful indicator. In this case, the addition of the synbiotic demonstrated a significant impact, reducing its prevalence.

The presence of beneficial microbiota in the hindgut is generally associated with higher concentrations of SCFA.[5,14,21] In our study, the addition of both synbiotic and osteopontin had an effect increasing colonic SCFA concentration. Notably, the highest values were achieved when the additives were combined in the CON diet due to an additive effect. This result could indicate a greater development of colonic microbiota in these animals, promoted by a synergic effect of these additives. In connection with this finding, higher SCFA denotes a healthier gut environment by enhancing intestinal barrier and promoting an anti-inflammatory condition in the area.[5,51,52] In accordance with that, colonic IEL counts were found significantly lower when all the additives were combined in CON group. Differences in gene expression related to immune response were seen as well, although they were detected in a different intestinal compartment (jejunum). The expression of the pro-inflammatory cytokine IL-1β was found statistically lower in all three supplemented diets compared to CTR. Similar results were seen in other studies where additives of the same nature helped preserve a non-inflammatory tone of the intestinal mucosa.[14,15,53–56] The expression of TGFβ1, IL-1β, GBP1 and TLR4, two inflammation-modulating cytokines[57,58] and two pathogen recognition receptors,[59,60] respectively, was also lower in supplemented diets. In all the cases, a downregulation suggests fewer presence of pathogens and a closer approximation to a homeostatic environment.[61,62]

Continuing with gene expression in the small intestine, the findings suggest that the additives, particularly the synbiotic, influence the expression of various genes. Genes associated with intestinal maturation and protection against pathogens (ALPI and SI)[63] increased with the synbiotic supplementation alone. The synbiotic supplementation also led to increased expression in nutrient transport genes (SCL13A1, SLC15A1, SLC5A1 and SLC7A8). Both results highlight the beneficial effect of the synbiotic combination. In terms of intestinal barrier function, existing literature suggests that both GOS and osteopontin promote higher expression in this gene group.[14,15,53] In our study, synbiotic supplementation led to increased expression of OCLN.[14,15,54] However, contrary to what the literature indicates,[54] the addition of osteopontin did not result in increased expression of OCLN, ZO1, or mucin mRNA. Mucin 2 (MUC2) and mucin 13 (MUC13) showed an upregulation when the synbiotic was supplemented alone (SYN diet). Regarding digestive enzymes and hormones genes, such as proglucagon (GCG), osteopontin exhibited a significant upregulatory effect. This gene encodes for various peptides involved in digestive processes, with the most notable ones being glucagon and glucagon-like peptide-1,[64,65] which play crucial roles in regulating individual intake and satiety. Interestingly, osteopontin supplementation affects milk consumption in animals, suggesting a potential connection with the upregulation of genes related to digestive function.

This study probably represents one of the first to provide detailed information on the early gut microbial colonization in artificially reared suckling piglets. In general, the three dominant phyla present in faecal samples, regardless of the

animals' age, were Firmicutes, Bacteroides and Proteobacteria, consistent with findings from other studies on weaning piglets.[66–70] However, the taxonomic pattern observed in this study during early ages (day 3) was more akin to the pattern reported in previous studies for days 14 and 21 of age.[66] Fusobacterium phyla is typically identified as the third dominant phylum in preweaning piglets, and its abundance tends to decrease around 2–3 weeks of life. In our trial, Fusobacterium, was consistently detected in low abundances from the beginning (day 3), and the three dominant phyla remained relatively stable throughout all the sampling times. This lack of variation could be attributed to the consistent composition of the artificial formula during the trial, unlike sow milk, which undergoes slight changes in composition as lactation progresses. Furthermore, it is important to note the compositional differences between sow's milk and the milk formula used in this trial, particularly regarding its wheat content, providing a certain amount of starch. Major changes in gut microbiota are typically observed when dry feed is introduced to piglets, characterized by complex carbohydrates from dietary fibre and starch.[67,71] A greater diversity of microbiota species is associated with a more mature gut ecosystem and a healthier environment, contributing to resilience and stability during periods of stress.[72,73] However, in our trial, no significant differences in alpha diversity were observed over time (day 7 *vs.* day 20 of age) in any of the treatment groups. These findings contrast with those reported by Saladrigas-García *et al.* (2022),[66] which showed an increase in biodiversity with age. It is possible that the lack of data in the first week of life makes it difficult to discern differences. Added to the previous comment, a milk formula that does not modulate over time may not induce the same changes in gut microbiota. Regarding beta diversity, the PERMANOVA analysis revealed significant differences due to Treatment, Day, Animal, and Period, with Animal being the predominant source of variation. This aligns with previous studies,[66,74] which have noted a high degree of individuality among piglets at 1–2 weeks of age. It is suggested that the gut community in young piglets is highly dynamic and tends to reach a state of greater stability around the age of 4 weeks. Regarding the influence of milk formula supplementation on microbiota composition, significant changes in family and genera abundances induced by the synbiotic were observed by day 15. In contrast, changes induced by osteopontin were predominantly noticed by day 3, diminishing later in some cases. This observation logically aligns with the distinct functions of the additives. The synbiotic, being a biotic compound, aims to gradually alter the microbiota, requiring time for noticeable effects. Conversely, osteopontin, being a peptide with multifaceted functions, appears capable of effecting more rapid changes. The most noteworthy impact induced by the additives was the improved control of potentially dysbiotic genera at day 15, predominantly observed in the CON diet group. This enhanced control could be attributed to an additive effect of the synbiotic and osteopontin. Among these potentially dysbiotic genera, it is worth noting the significant reduction observed in *Campylobacter* genera with the CON diet. Most *Campylobacter* species cannot utilise glucose and therefore rely on free amino and keto acids from the intestinal digesta for growth, making them considered proteolytic bacteria.[75] The reduced levels of colonic ammonia observed in the supplemented formulae, particularly with CON, support a potential shift toward a less proteolytic and more beneficial microbiota due to supplementation. The higher colonic SCFA concentrations found in CON animals reinforces this hypothesis.

Other specific microbial groups were also significantly altered by the experimental treatments; however, drawing conclusions regarding their potential use as diet health-promoting biomarkers is challenging. Identifying which microbiota would best enhance host health remains a complex area of research. Although some progress has been made in recent years, there is still much ground to cover. We now have a better understanding of the gut colonization process in early-life piglets, which involves shifts from initial populations of lactic acid bacteria, such as genera like *Ligilactobacillus* or *Limosilactobacillus*, to other predominant populations as the animals grow.[67] In this regards osteopontin supplementation was associated with increases of *Ligilactobacillus* at day 3. However, despite these insights, the variable environmental conditions influenced by individual hosts and their diets, along with the intricate interactions among microorganisms within the gut ecosystem, complicate the identification of specific microbial biomarkers. This makes it a challenging and likely oversimplified task.

To better understand these complex interactions and how gut microbiota impacts intestinal piglet health, we performed correlation analysis between data from SCFA concentration, histomorphology, and gene expression with sequenced microbiota at day 15. The most relevant correlations ($r > 0.5$; adjusted $p < 0.05$) are shown in Fig. 5–7. Some significant correlations between SCFA bacteria producers and SCFA concentration were detected (Fig. 5). For instance, *Collinsella aerofaciens*, an acetate producer,[76] was positively correlated with acetate concentration; *Butyricicoccus pullicaecorum*, a butyrate producer,[77] positively correlated with butyrate; *Megasphaera elsdenii*, a valerate producer,[78] was positively correlated with valerate; and *Ligilactobacillus salivarius*, a lactate producer,[79] positively correlated with lactate.

In examining the correlation with histomorphology (Fig. 6), it is noteworthy to consider the following genera due to their notable presence in the samples. The genus *Prevotella*, known for harboring species described as butyrate producers that tend to increase with the introduction of a solid cereal-based diet,[80] showed a negative correlation with villus height in the jejunum and ileum. *Bacteroides*, a milk oligosaccharides fermenter genus,[81] exhibited a negative correlation with crypt depths in jejunum. Lastly, *Phocaeicola* was negatively correlated with intraepithelial lymphocytes in the jejunum. These findings could elucidate how bacteria interact with the host, influencing variable absorption surface, villus regenerating area, or immune modulation. Although not detected in large proportion, the recently reviewed *Akkermansia muciniphila*, a promising probiotic well-known to improve the host metab-

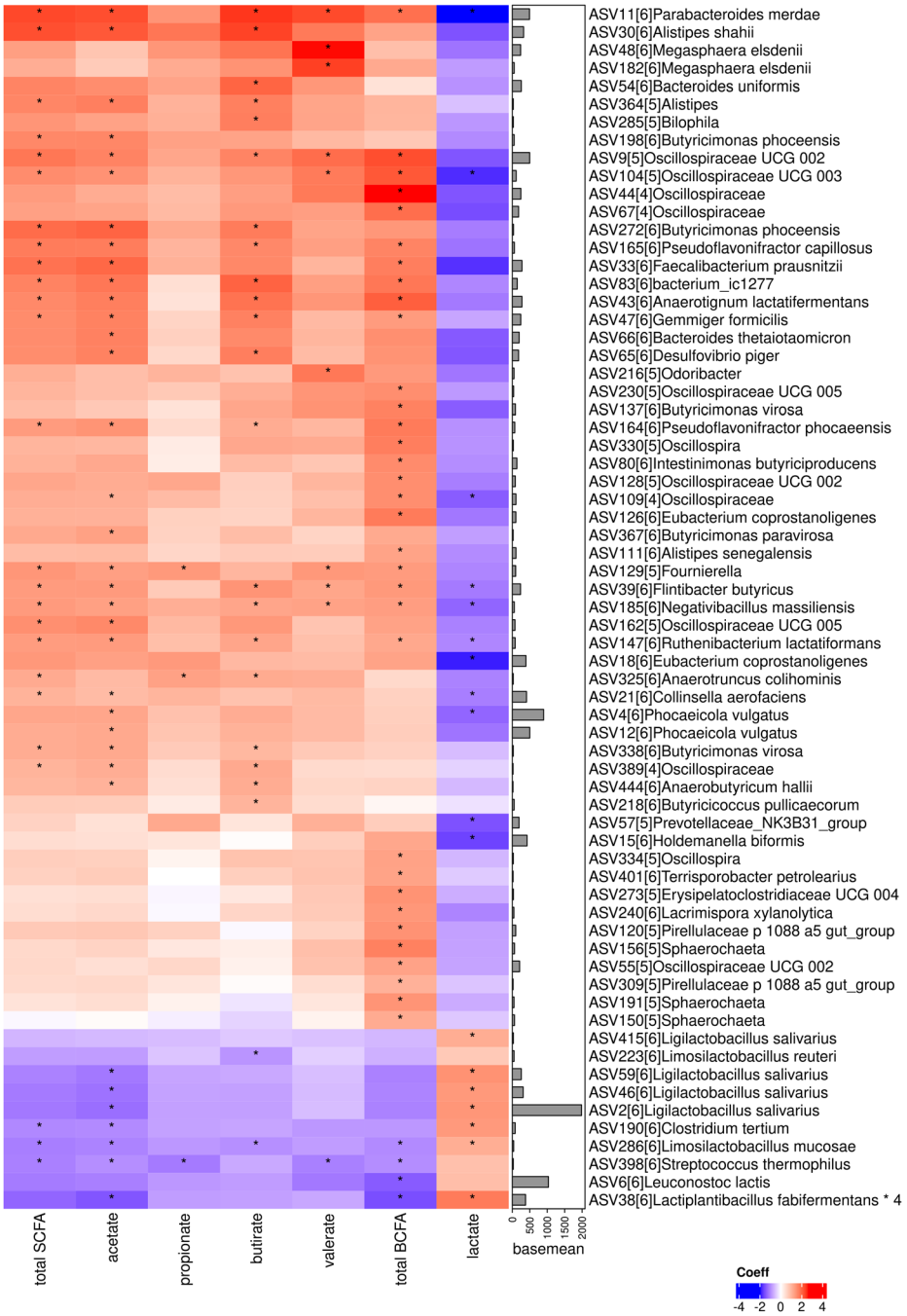

Fig. 5 Heatmap of correlations between colonic SCFA concentration and ASVs detected on faecal samples from day 15. *Indicates $p < 0.05$ and statistical difference present.

olism function and immune responses,[82] was positively correlated with jejunal and ileal villus height. In our trial, this genus was also associated with increases in CCK (digestive hormone involve in satiety)[83] and PPARGC1α (related to obesity and oxidative stress)[84] gene expression. These findings suggest a potential mode of action for this beneficial species. A higher presence of *Akkermansia* could translate into a greater surface area for intestinal absorption and enhanced communication with satiety control mechanisms. It is important to note that these data originate from distinct intestinal compartments. While the microbiota came from feces, gene expression was obtained from jejunum tissue, and histology from the jejunum, ileum and colon. The potential influence that microbiota may exert on intestinal histology and gene expression is unlikely to be only a direct local effect. Instead, it could result from the complex interaction or cross-talk of the microbiota with the host, where, for example, the metabolites produced could instigate alterations in other regions of the intestine.

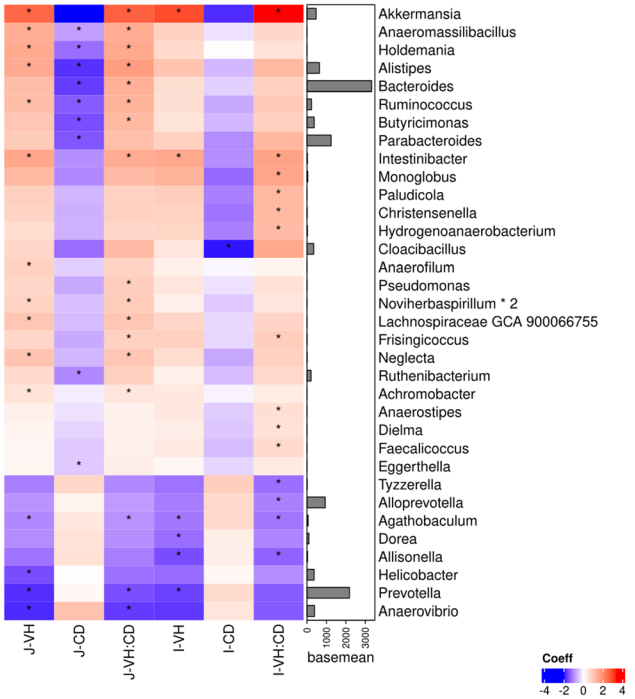

Fig. 6 Heatmap of correlations between histology (jejunal and ileal) and bacterial genera detected on faecal samples from D15. J = jejunal; I = ileal. VH = villus height; CD = crypt depth; VH. CD = ratio villus height : crypt depth. *Indicates $p < 0.05$ and statistical difference present.

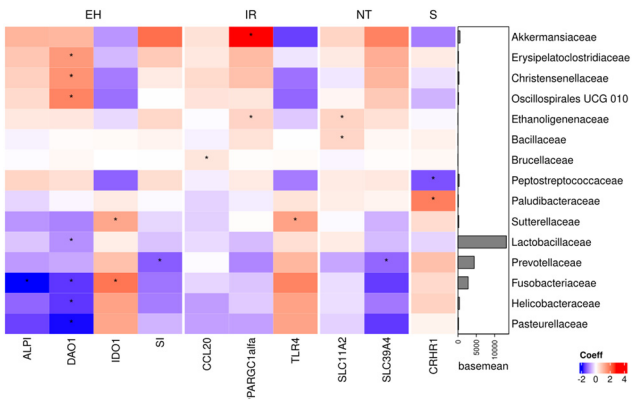

Fig. 7 Heatmap of correlations between jejunal gene expression and bacterial families detected on faecal samples from day 15. EH = hormones/enzymes; IR = immune response; NT = nutrient transport; S = stress. *Indicates $p < 0.05$ and statistical difference present.

Lastly, when gut microbiota was correlated with gene expression (Fig. 7), positive correlations were found between microbiota families known as disbyotic (*Suturella*, *Tyzzerella*)[85] with genes well-known to be linked with a pro-inflammation condition (IDO1, TLR4, TGFβ). These same microbial groups were also found to be significantly reduced by the synbiotic supplementation, suggesting that these reductions could be associated with the lower incidence of diarrhoea registered. Similarly, microbiota families known to be beneficial (*Christensenella*)[86] and that were increased with all the supplemented formulas, were negatively correlated with pro-inflammatory genes (IL6, TLR4, TGFβ). This discovery proposes a way of exerting effects, where the microbiota directly or indirectly seems to modulate different gene expression, developing an inflammatory habitat or not. Complementary information regarding correlation analysis can be found in ESI (Fig. S4–S9†).

5. Conclusions

In conclusion, the findings suggest that supplementing milk formulas with synbiotic and/or osteopontin during the initial days of life may impact the establishment of intestinal microbiota, digestive maturation, and neonate immune response. Synbiotic additives encourage the growth of beneficial microbiota genera and better control of dysbiotic genera in the lower digestive tract after 15 days of administration. Additionally, these additives led to reduced episodes of diarrhoea in these animals. Furthermore, they significantly influenced jejunal gene expression. Osteopontin supplementation resulted in increased milk formula consumption, alongside some promotion of beneficial microbiota genera and a reduction in dysbiotic genera in the lower digestive tract. However, more substantial results were observed with the combination of the synbiotic and osteopontin. Significant increases in SCFA concentration, reduced ammonia levels, and modulation of jejunal gene expression suggest an intriguing synergic effect of the tested products, potentially aiding in controlling the overgrowth of opportunistic pathogen and enhancing the organism's response to challenging situations. Furthermore, the correlations between gut microbiota and various intestinal parameters (SCFA concentration, histology, and gene expression) shed light on the intricate cross-talk between the microbiota and the host. For example, beneficial microbiota genera exhibited negative correlations with pro-inflammatory gene expressions, while dysbiotic genera showed contrary correlations. These findings imply a mechanism of interaction where the microbiota appears to regulate various parameters, including immune response gene expressions, either directly or indirectly.

Author contributions

Conceptualization, S. M. M.-O., J. J., J. A. M.-M. and L. C.; methodology, S. M. M. and L. C.; software, L. F.-S.; validation, S. M. M.-O. and L. C.; formal analysis, L. F.-S., M. S.; investigation, L. F.-S., M. S., S. M. M.-O. and L. C.; resources, L. F.-S., S. M. M.-O., and L. C.; data curation L. F.-S., S. M. M.-O. and L. C.; writing—original draft preparation, L. F.-S., S. M. M.-O. and L. C.; writing—review and editing, L. F.-S., S. M. M.-O., J. A. M.-M. and L. C.; visualization, L. F.-S.; supervision, S. M. M.-O. and L. C.; project administration, S. M. M.-O. and

L. C.; funding acquisition, J. J. and J. A. M.-M. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

J. J. and J. A. M.-M. are employees of Laboratorios Ordesa S. L. company, which provided financial support for this research study, and participated in the study design definition, writing of the manuscript, and contributing to the decision to publish the results.

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