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## Impact of a microencapsulated probiotic consortium on lactose digestion and intestinal health in piglets

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### ABSTRACT

In weaned piglets, the decline of intestinal lactase activity predisposes animals fed lactose-rich diets to maldigestion and diarrhea. Microbial pathways can compensate via the Leloir or tagatose-6-phosphate routes, depending on transport systems and microbiota composition. This study assessed the effect of microencapsulated probiotic consortia on lactose utilization, gut microbiota, and performance in 108 piglets assigned to four diets: two probiotic blends (D1, D2), an antibiotic group (D3), and a copper control (D4). 16S rRNA sequencing and metagenomics showed that probiotics, especially D2, stabilized the microbiota by enriching *Firmicutes*, increased *Lactobacillus* and *Prevotella*, and suppressing *Escherichia coli*. Functional profiling revealed distinct metabolic strategies: D4 relied on broad extracellular hydrolysis, whereas D1 and D2 utilized active Lactose-Phosphotransferase transport linked to intracellular catabolism. D2 exhibited the highest glycolytic engagement, indicating optimized energy harvest. Dietary probiotics enriched lactose-metabolizing pathways and stabilized the gut microbiota, alongside indicative trends toward better stool consistency and feed utilization.

### KEYWORDS


Microencapsulated probiotics; Lactose digestion; Weaned piglets; Gut microbiota; Leloir and tagatose-6P pathways

## Introduction

In commercial swine production, weaning is a critical stress phase marked by dietary transitions, loss of passive immunity, and social disruption, often resulting in reduced feed intake and impaired growth (Gaggia et al., 2010; Gresse et al., 2017). A primary physiological challenge during this period is the development of lactose intolerance (Modesto et al., 2009; Dowarah et al., 2017). While piglets are born with high intestinal lactase activity to digest maternal milk, this expression declines sharply around weaning (3–4 weeks), limiting the capacity to hydrolyze lactose from solid feeds (Pluske et al., 2003; J. Zhao et al., 2021a). Although moderate lactose fermentation can exert prebiotic benefits, excessive undigested lactose reaching the colon triggers osmotic diarrhea, dehydration, and weight loss (Szilagyi, 2004; J. Zhao et al., 2021a).

This challenge is exacerbated by the use of milk replacers in hyper-prolific litters. These diets, though necessary to reduce pre-weaning mortality, are often lactose-rich and may worsen nutritional diarrhea during periods of declining lactase activity (Kobek-Kjeldager et al., 2021; Quiniou, 2021). Historically, antibiotic growth promoters and pharmacological levels of zinc or copper were used to manage these gastrointestinal disturbances (López-Gálvez et al., 2021). However, rising antimicrobial resistance and regulatory restrictions have intensified the search for effective non-antibiotic alternatives to support gut health and lactose utilization during the post-weaning transition (Lekagul et al., 2019).

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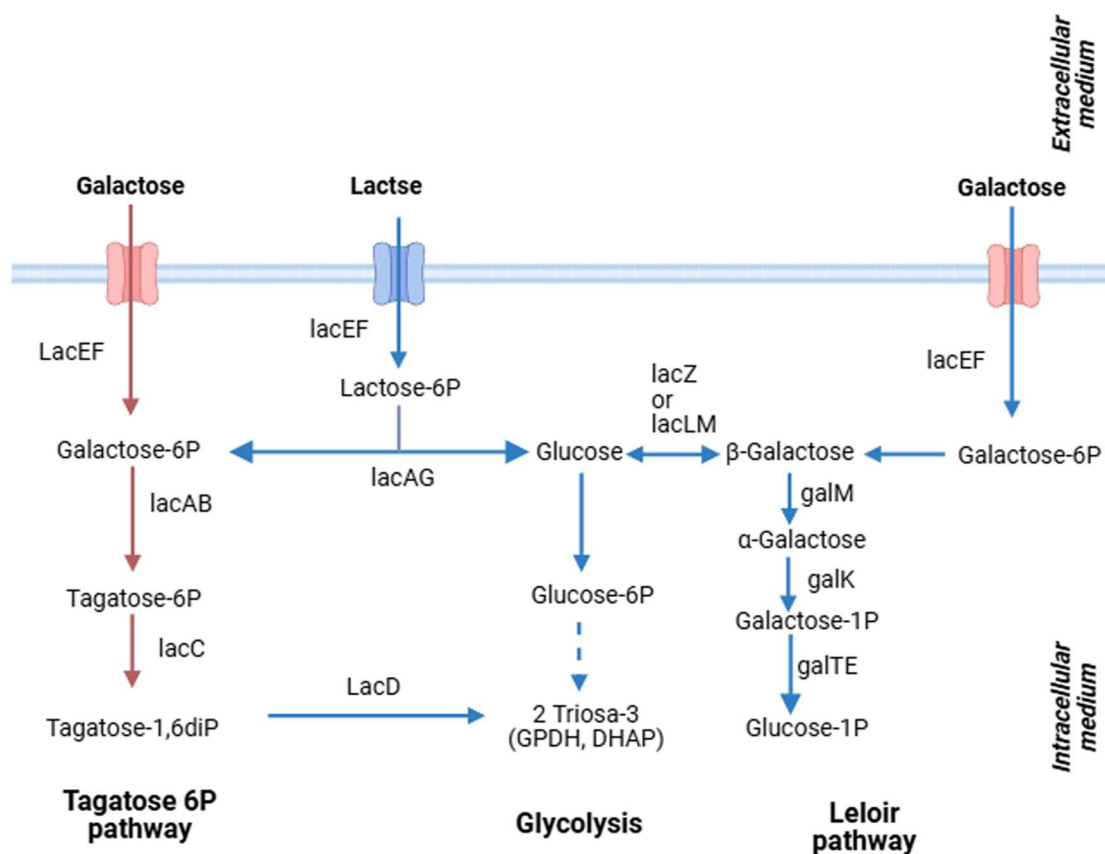
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To be effective, such alternatives must bolster the microbial pathways responsible for lactose degradation, primarily the Leloir pathway and the lactose and galactose degradation I pathway (Tagatose 6-P pathway) (Iskandar et al., 2019). These routes depend on a coordinated set of enzymes— $\beta$ -galactosidase, 6-phospho- $\beta$ -galactosidase, and various isomerases – which mediate the hydrolysis of lactose and its further transformation into glycolytic intermediates (Figure 1).

In this context, probiotics have emerged as a promising strategy to enhance digestion and gastrointestinal health in piglets (Markowiak and Ślizewska, 2018; Galli et al., 2024). Multiple studies in swine have shown that probiotic supplementation can improve intestinal microbial balance and strengthen gut barrier function, leading to reduced diarrhea incidence and improved growth (Dowarah et al., 2018; Zhao et al., 2021a; Jiang et al., 2024; K. Zhao et al., 2023). However, their efficacy is often inconsistent due to poor stability during industrial feed processing and gastric transit. High temperatures, mechanical shear, and mineral premixes can drastically reduce microbial viability, limiting the effective dose that reaches the intestine (Tripathi and Giri, 2014; Stratford et al., 2019). This instability is especially problematic in the context of lactose digestion, as the potential benefits of probiotics depend not only on strain selection but also on their survival through processing, gastric passage, and early intestinal transit.

Microencapsulation addresses these limitations by physically shielding probiotic cells from environmental and physiological stressors (Stratford et al., 2019; Ordway et al., 2020; Barajas-Álvarez et al., 2023). Encapsulation matrices systems have been shown to improve probiotic survival during feed manufacturing and simulated gastrointestinal digestion, while also modulating release kinetics in the intestine (Baek et al., 2023; Barajas-Álvarez et al., 2023; Arroyave Manco et al., 2024). However, despite growing interest in encapsulated



**Figure 1.** Schematic representation of the lactose and galactose metabolic pathways (This schematic was adapted from Iskandar et al., 2019 and is not an experimental result). Enzymes encoded by the genes: lacAB, galactose-6-phosphate isomerase; lacC, tagatose-6-phosphate kinase; lacD, tagatose-1,6-diphosphate aldolase; lacEF, EII complex of the phosphotransferase system; lacG, phospho- $\beta$ -galactosidase; lacZ,  $\beta$ -galactosidase; lacLM,  $\beta$ -galactosidase; galP, galactose permease; galM, galactose epimerase; galK, galactose kinase; galE, UDP-glucose-4-epimerase; pgm, phosphoglucomutase; glk, glucokinase. GPDH: glyceraldehyde 3 phosphate, DHAP: dihydroxyacetone phosphate.

probiotics, relatively few studies have evaluated their functional impact on lactose-related metabolic pathways or their ability to support intestinal adaptation during the critical post-weaning transition (Park et al., 2024). Furthermore, multi-strain formulations may offer superior functional resilience through synergistic metabolic interactions compared to single-strain products (Timmerman et al., 2004). Despite this potential, direct evidence of such synergy in optimizing lactose digestion remains scarce.

The objective of this study was to evaluate the impact of two different probiotic supplementation in comparison to a conventional antibiotic, and copper-based control on intestinal microbiota composition, lactose-related metabolic pathways, digestive enzyme production, and growth performance in piglets fed lactose-rich solid diets.

## Materials and methods

### Animal ethics

The feeding trial was carried out in strict compliance with the national legislation that regulates the use, care, and protection of animals in research activities stipulated in the Institutional Committee for the Care, and Use of Animals (CICUA established by the CES University Medellín, Antioquia Colombia. This is under act number 043 of the project: 'Porcicesbialtecudea: un modelo de optimizacion para la alimentacion de precision en cerdos' Code: Ae-025. Medellín, November 23, 2020.

### Experimental design and animals

A total of 108 piglets (Landrace × Large White × Duroc) were used. They were weaned at 21 days of age and housed on the experimental farm. The piglets were randomly assigned to one of four dietary treatments. Each pen contained nine pigs, and each treatment was replicated three times (nine piglets × four diets × three pen replicates). The experiment lasted from day 21 to 35 of age. Pigs were weighed individually at the end of each period to calculate body weight gain (BWG), feed intake (FI), final body weight (FBW), feed conversion ratio (FCR), and average daily feed intake (ADFI).

### Feeding program and diets

Piglets had ad libitum access to feed and water and did not receive creep feed prior to weaning. Experimental diets were custom-formulated and produced in a commercial feed mill by Compañía Industrial de Productos Agropecuarios (CIPA, Medellín, Colombia) to meet the nutritional requirements of weaned piglets (PIC® Nutrition and Feeding Guidelines, 2021). Although not commercial products with guaranteed analyses, nutritional equivalence among treatments was ensured through standardized formulations and identical ingredient inclusion rates (Table 1). All diets were iso-nitrogenous and iso-energetic and based on a

**Table 1.** Ingredients and chemical composition of dietary treatments characteristics (% dry weight as fed basis).

| Items                                   | D1 (%) | D2 (%) | D3 (%) | D4 (%) |
|---|--------|--------|--------|--------|
| Corn                                    | 46,46  | 46,46  | 46,46  | 46,46  |
| Soybean meal                            | 15     | 15     | 15     | 15     |
| Milk powder (54% protein)               | 15     | 15     | 14,77  | 15     |
| Lactose (80%)                           | 7      | 7      | 7      | 7      |
| Fish meal                               | 8      | 8      | 8      | 8      |
| Vitamin premix                          | 3,25   | 3,25   | 3,25   | 3,25   |
| Spray-dried animal plasma (78% protein) | 3      | 3      | 3      | 3      |
| Calcium carbonate                       | 1,4    | 1,4    | 1,4    | 1,4    |
| Salt                                    | 0,38   | 0,38   | 0,38   | 0,38   |
| Essential aminoacids                    | 0,45   | 0,45   | 0,45   | 0,45   |
| Copper sulfate (25%)                    | 0      | 0      | 0,04   | 0,05   |
| Antibiotics <sup>1</sup>                | 0      | 0      | 0,19   | 0      |
| Fortcell Feed® Cria <sup>2</sup>        | 0,05   | 0      | 0      | 0      |
| Fortcell Feed® Lactantes <sup>3</sup>   | 0      | 0,05   | 0      | 0      |

<sup>1</sup>Antibiotics: doxycycline 80 mg/kg + zinc bacitracin 50 mg/kg;

<sup>2</sup>Fortcell Feed® Cria: *Saccharomyces cerevisiae* ( $8.5 \times 10^8$  CFU/g), *Lactobacillus spp* ( $2.72 \times 10^8$  CFU/g), *Bacillus subtilis* ( $2 \times 10^8$  CFU/g), and *Enterococcus faecium* ( $7.5 \times 10^6$  UFC/g);

<sup>3</sup>Fortcell Feed® Lactantes: *Saccharomyces cerevisiae* ( $1.5 \times 10^9$  UFC/g), *Bacillus subtilis* ( $1 \times 10^9$  CFU/g), and *Enterococcus faecium* ( $1.88 \times 10^7$  UFC/g)

common lactose-containing starter formulation; treatments differed only in the additive included. The dietary treatments included: diet 1 (D1), supplemented with a probiotic blend (Fortcell Feed® Cría, Bialtec); diet 2 (D2), supplemented with a probiotic blend (Fortcell Feed® Lactantes, Bialtec); diet 3 (D3), supplemented with conventional antibiotic growth promoters at sub-therapeutic doses in combination with copper sulfate provided at nutritional levels; and diet 4 (D4), supplemented with nutritional-level copper sulfate (12 mg Cu/kg). The microorganisms included in D1 and D2 were microencapsulated using an emulsion-based formulation stabilized by fatty acids and processed using a pan granulator, as previously described (Arroyave Manco et al., 2024) The probiotic products were incorporated during the pelleting process at a rate of 500 g/ton.

### ***Rectal faeces collection***

For microbiota analysis, in total, 28 samples were analyzed, comprising 24 individual samples from post-weaning piglets (12 per sampling time point). Two individual samples from suckling piglets at 10 days of age were included to serve as an exploratory baseline of the pre-weaning microbiota, allowing for a qualitative assessment of the microbial restructuring that occurs following the transition to solid diets, along with two environmental negative controls to monitor potential external contamination. Faecal samples were collected on days 25 and 34 age, corresponding to the post-weaning period and three different piglets per dietary treatment were randomly selected, resulting in twelve individual fecal samples per time point (3 piglets × 4 treatments). Samples were analyzed at the individual level without pooling to capture inter-individual microbiota variability. Analyses used individual animals as biological replicates and were interpreted as exploratory due to limited replication and statistical power. Samples were collected using sterile 20–30 mm cotton swabs (Zymo Research, Irvine, CA, USA), which were gently inserted into the rectum and rotated against the intestinal wall to ensure adequate sample collection. Immediately after collection, total DNA was extracted using the QIAGEN DNeasy PowerSoil Pro Kit according to the manufacturer's instructions, which include bead-beating-based mechanical lysis and chemical disruption to ensure efficient recovery of microbial DNA from faecal material. DNA concentration was quantified using a Qubit 4 Fluorometer and the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific).

### ***Taxonomy analysis***

The samples from the piglets were processed for 16S rRNA taxonomy analysis using the Illumina NovaSeq 6000 (BaseClear B.V., Netherlands). Paired-end sequence reads were collapsed into so-called pseudoreads using sequence overlap with USEARCH version 9.2 (SEC, 2010). Classification of these pseudoreads is performed based on the results of alignment with SNAP version 1.0.23 (Zaharia et al., 2011) against the RDP database (Cole et al., 2014)

### ***Shotgun metagenomic sequencing***

The DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes with total DNA input of 1 ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Unique dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. The DNA libraries were purified using AMPure magnetic Beads (Beckman Coulter) and eluted in QIAGEN EB buffer. The DNA libraries were quantified using Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit. Libraries were then sequenced on an Illumina NovaSeq platform (2 × 150 bp). Shotgun metagenomic sequencing yielded a median of  $1.34 \times 10^7$  paired-end reads per sample (range:  $7.3 \times 10^4$  to  $6.16 \times 10^7$ ; mean:  $1.46 \times 10^7$ ).

### ***Bioinformatics for functional analysis***

Adapter trimming, QC, and preprocessing of reads were performed using BBduk (Bushnell, 2021). Quality filtering included removal of adapter sequences, trimming based on a minimum Phred quality score of Q20 and discarding reads shorter than 50 bp after trimming to ensure high-quality input data. Reads

were mapped against the UniRef90 protein database, clustering sequences with  $\geq 90\%$  identity (UniProt, 2016). Taxonomic and functional assignments were performed using curated reference databases with internal confidence thresholds to minimize false-positive identifications. Metagenomic reads were mapped to gene sequences, and gene family abundances were estimated as described by Franzosa et al., 2018. Gene families were annotated to MetaCyc reactions and further categorized into enzyme classes (Enzyme Commission, Pfam, CAZy, and GO Terms) to assess gene function in the community (Caspi et al., 2016). Abundance values were normalized using Total Sum Scaling (TSS) to produce copies per million (CPM) units.

### Statistical analysis

Growth performance variables included body weight gain (BWG), feed intake (FI), final body weight (FBW), and feed conversion ratio (FCR). Feed intake and FCR were determined on a pen basis, with the pen considered the experimental unit for growth performance. For microbiota outcomes, individual piglets ( $n = 3$  per treatment) were used as the experimental unit to explore inter-individual variation. Growth performance data were analyzed using R programming language through one-way ANOVA and Tukey's post-hoc tests.

For all microbiota and functional outcomes, individual piglets ( $n = 3$  per treatment) were used as the experimental unit. Metagenomic data were analyzed using a specialized Microbiota Analysis Pipeline in MATLAB (The MathWorks Inc.). Taxonomic profiles were processed at both phylum and genus levels. Beta diversity was assessed using Bray–Curtis dissimilarity matrices, with Global and Pairwise PERMANOVA (9,999 permutations) employed to determine structural differences between dietary groups. Furthermore, differential abundance of taxa was evaluated using  $\log_2$  Fold Change ( $\log_2$ FC) analysis, with significance validated through bootstrap 95% confidence intervals ( $n = 2,000$ ).

For the functional potential (metabolic pathways and enzymatic profiles), quantitative statistical comparisons were performed using one-way ANOVA followed by Tukey–Kramer post-hoc tests for all pairwise diet comparisons. The magnitude of these functional shifts was expressed as  $\log_2$ FC with 95% confidence intervals (CI) derived from the studentized range distribution. To control the false discovery rate (FDR) inherent in high-dimensional functional data, all p-values were adjusted using the Benjamini-Hochberg procedure. This comprehensive dual-software approach ensured that both performance and high-dimensional sequencing data were analyzed with the most robust tools available. No a priori power calculation was performed for microbiota outcomes due to the exploratory nature of the sequencing analysis and the high inter-individual variability typically observed in gut microbial data.

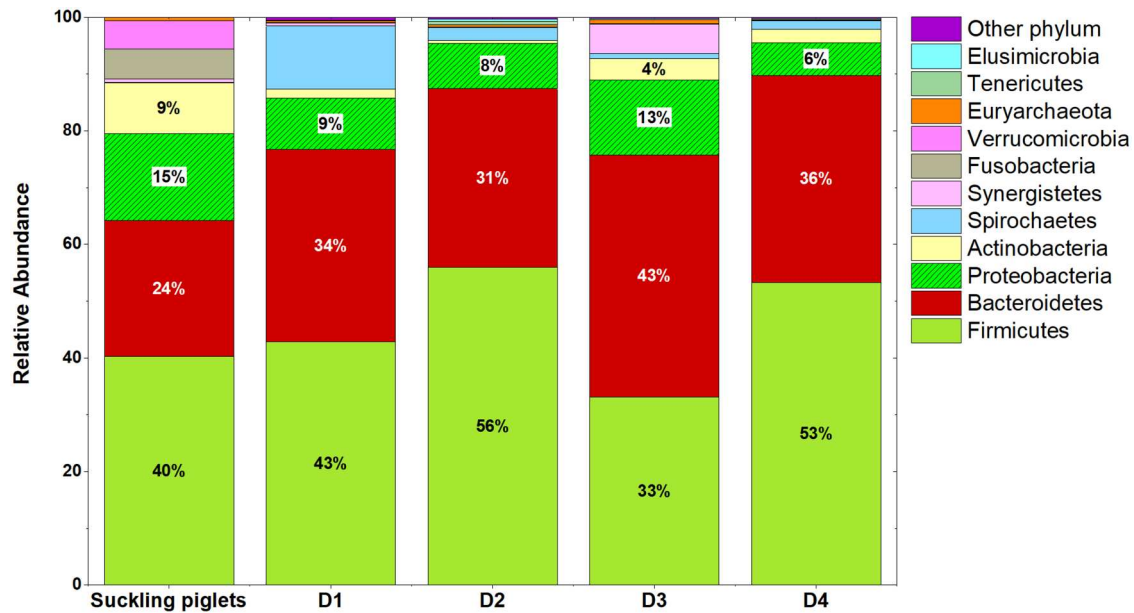
## Results

In general, piglets from all treatment groups actively consumed solid diets throughout the study period. No feed refusals or evident adverse effects attributable to dietary additives were observed, indicating good palatability and safety of the tested formulations.

### Intestinal microbiota profiling

Collectively, 27 bacterial phyla, 217 genera and 220 species (OTU) were identified in the faecal samples of piglets. At the phylum level, the PERMANOVA revealed that the global microbial architecture remained statistically stable across dietary treatments (Pseudo-F = 1.46,  $p = 0.183$ , Supplementary material Figure A1), where *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the three predominant phyla across all groups (Figure 2). In suckling piglets, *Firmicutes* accounted for 40% of the microbiota, followed by *Bacteroidetes* (24%) and *Proteobacteria* (15%).

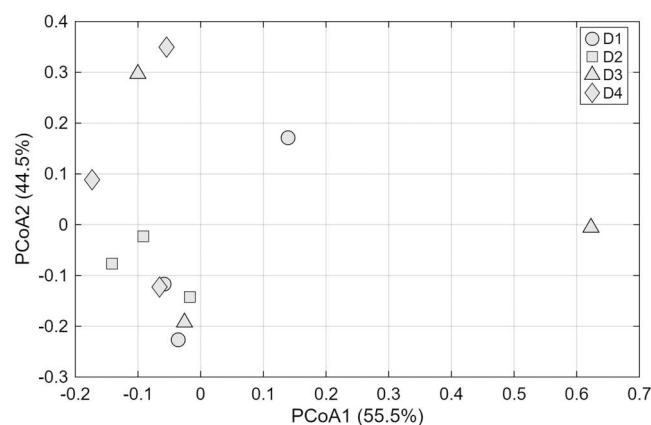
Following weaning, specific taxonomic modulations were identified through differential abundance analysis. The D1-supplemented group exhibited a notable numerical increase in *Spirochaetes* compared to the other treatments ( $\log_2$ FC = 2.81,  $p = 0.10$  nominal). In contrast, the D3-supplemented group showed a microbiota profile dominated by *Bacteroidetes* (43%), with a reduction in *Fibrobacteres* ( $\log_2$ FC = -4.33,  $p = 0.10$  nominal) compared to the D4 group (Supplementary material Table A1). The D4-supplemented group (copper sulfate) supported a composition similar to D2, characterized by elevated *Firmicutes* (53%) and the lowest proportion of *Proteobacteria* (7%).



**Figure 2.** Relative abundance of major bacterial phyla in fecal samples across dietary treatments. Stacked bars represent the mean relative abundance (%) of dominant phyla, with low-abundance taxa grouped as ‘Other phylum.’ This figure is presented for descriptive purposes. Statistical evaluation of overall community structure at the phylum level was performed using Bray–Curtis–based PCoA and PERMANOVA and is shown in Supplementary Figure 1.

Interestingly, within the genus *Escherichia* (Phylum *Proteobacteria*), distinct treatment-associated shifts were observed. Suckling piglets presented high *Escherichia* spp., counts (6746 reads), whereas post-weaning groups D1, D2, and D4 showed nearly complete suppression. However, the D3 group maintained a considerable presence (2659 reads), suggesting a potential resistance or niche persistence despite antibiotic supplementation ( $\log_2FC = 1.47$ ,  $p = 0.10$  nominal in comparison with D4)

Global community structure at the genus level was analyzed using Bray–Curtis dissimilarity matrices. The PERMANOVA revealed that the global microbial architecture remained statistically stable across dietary treatments (Pseudo-F = 1.19,  $p = 0.207$ ), indicating that the microencapsulated probiotic consortium does not disrupt the core microbial equilibrium. The PCoA explained 99.65% of the total variance (PCoA1: 55.5%; PCoA2: 44.15%), showing a high degree of representativeness of the model (Figure 3). Special attention was given to the abundance of key bacterial genera associated with dairy digestion: *Lactobacillus* spp.,



**Figure 3.** Structural stability of the fecal microbiota across dietary treatments. Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarity at the genus level. Each point represents an individual animal sample. The first two axes explain 99.65% of the total variance. Global PERMANOVA analysis (Pseudo-F = 1.19,  $p = 0.207$ ) indicates no significant differences in overall microbial community structure among dietary treatments. Pairwise PERMANOVA comparisons similarly showed no significant differences ( $p > 0.05$ ).

(lactase producers and lactose fermenters), as well as other relevant genera (*Eubacterium*, *Prevotella*) involved in carbohydrate and protein fermentation.

The relative abundance of bacterial genera in piglets during the first two weeks post-weaning was significantly influenced by dietary treatments. Suckling piglets showed a highly diverse and unstructured microbiota, with a large proportion of taxa grouped under 'Other Genus' and dominance of *Eubacterium* spp., (15%) and *Bacteroides* spp., (15%) were the most abundant genera, followed by *Ruminococcus* spp., and *Catenibacterium* spp., *Prevotella* spp., and *Lactobacillus* spp., were nearly absent (1%) in this group (Figure 4).

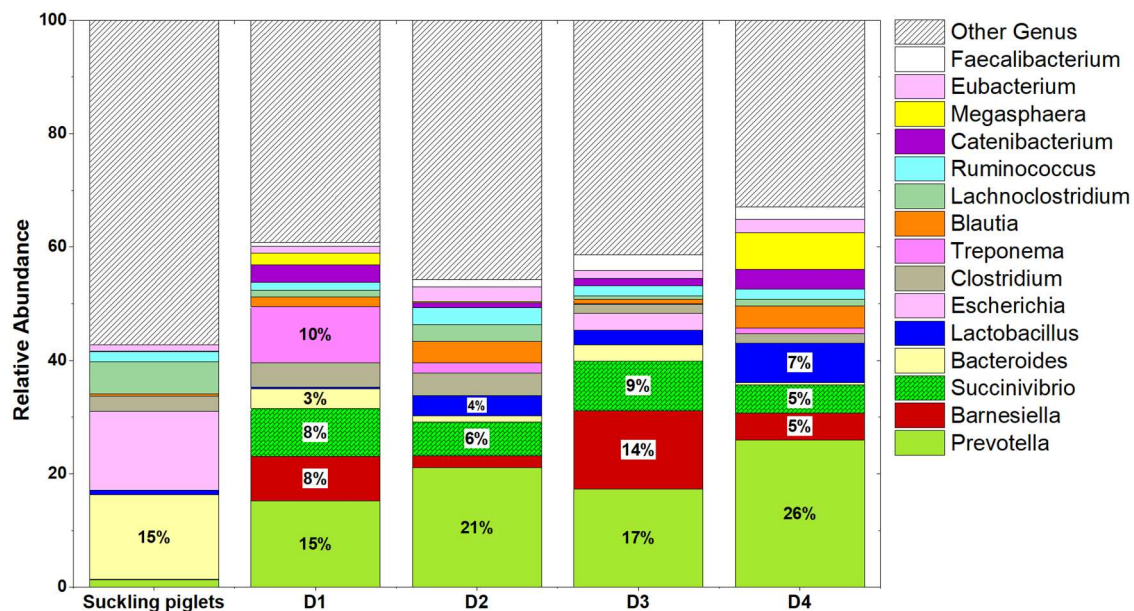
Following weaning, all dietary supplementations promoted a rapid expansion of *Prevotella* spp.,. In the D1 group, *Prevotella* spp., increased to 15%, supported by moderate levels of *Succinivibrio* spp., (8%) and *Barnesiella* spp., (8%), while *Lactobacillus* spp., remained low. Dietary group D2 showed a higher *Prevotella* spp., expansion (21%) and a moderate rise in *Lactobacillus* spp., (4%), alongside a strong numerical enrichment in *Acholeplasma* spp., ( $\log_2FC = 12.07$ ,  $p = 0.40$  nominal) (Figure 5, Supplementary material Table A2).

In contrast, in the D3-supplemented group *Prevotella* spp., reached 17%, but the most distinct feature was a marked increase in *Barnesiella* spp., (14%). Notably, D4-supplemented piglets exhibited the most robust expansion of beneficial taxa, with the highest *Prevotella* spp., abundance (26%) and elevated *Lactobacillus* spp., levels (5%) the D2 – group achieved a comparable and targeted modulation. The proportion of *Eubacterium* spp., decreased dramatically across all post-weaning groups compared to suckling piglets, highlighting the profound shift in microbial composition during the transition to solid feed.

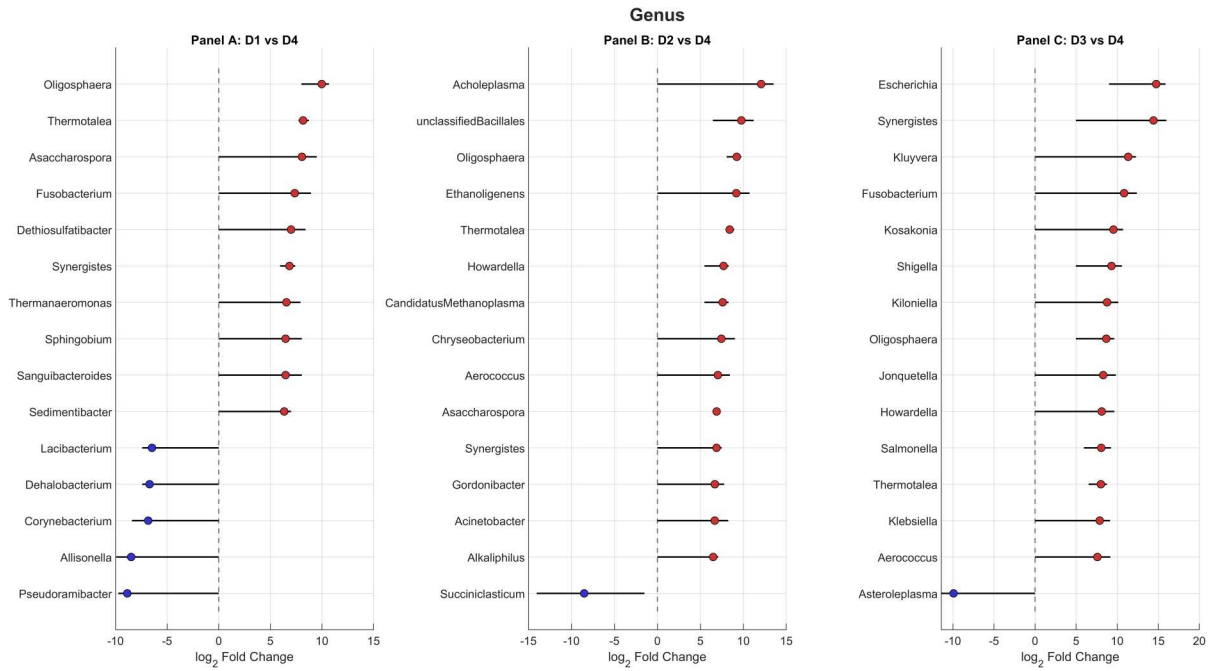
To evaluate the dissimilarity between microbiota profiles of treated and suckling piglets, Euclidean distances were calculated using genus-level abundance data across supplemented groups (Supplementary material Figure A1). This metric reflects the overall compositional divergence in microbial communities, with lower values indicating greater similarity. The lowest dissimilarity (9,779) was recorded between D1 and D2. Conversely, the D3 group showed the highest dissimilarity to suckling piglets (24,770). D4-supplemented group exhibited intermediate distances (D4–Suckling: 22,270).

## Digestive enzyme production

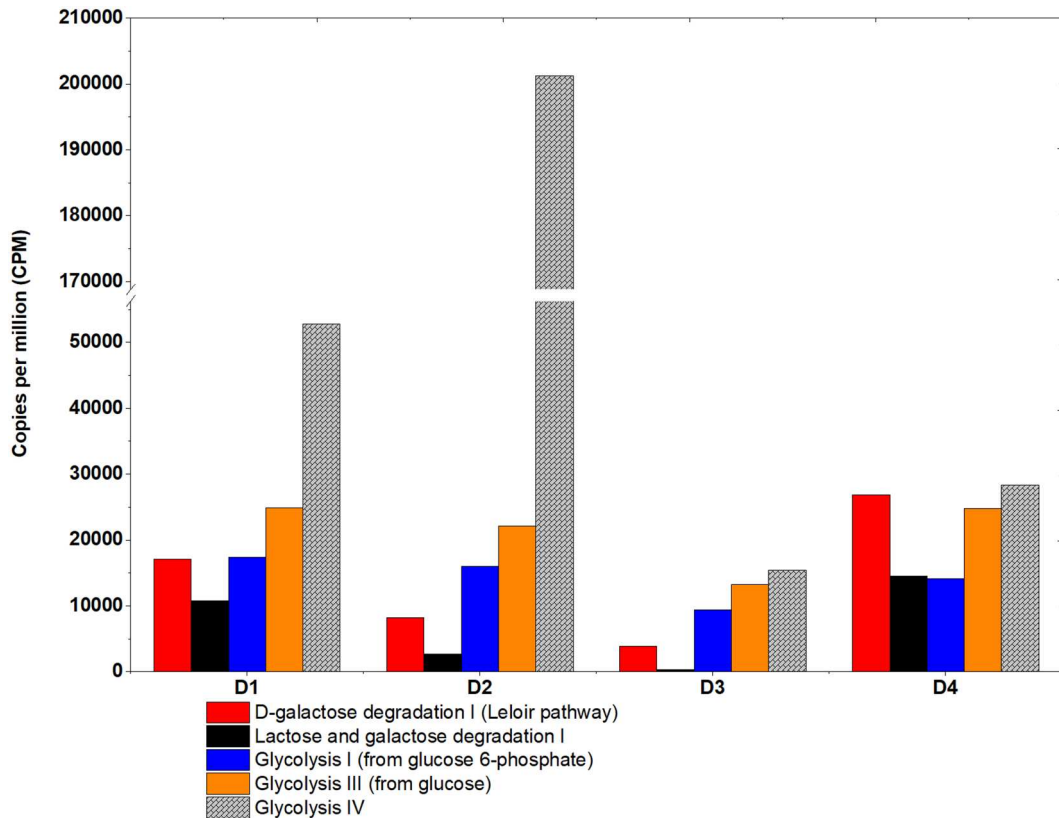
To evaluate the functional capacity of the microbiota under different dietary regimens, we characterized the abundance of key enzymes and the expression of metabolic pathways (Figure 6 and 8). This analysis allowed



**Figure 4.** Relative abundance of dominant bacterial genera in fecal samples across dietary treatments. Stacked bars represent the mean relative abundance (%) of the most abundant genera, with remaining taxa grouped as 'Other genus.' This figure is presented for descriptive purposes to illustrate compositional patterns; statistical differences in microbial community structure were assessed using Bray–Curtis–based PCoA and PERMANOVA (Figure 3), and taxon-specific differences were evaluated using  $\log_2$  fold change analyses.



**Figure 5.** Log<sub>2</sub> fold change–based differential abundance of bacterial genera relative to diet D4. Points represent log<sub>2</sub> fold change relative to D4, and horizontal lines indicate 95% bootstrap confidence intervals. Genera with confidence intervals not crossing zero were considered significantly different after FDR correction.



**Figure 6.** Normalized Functional Pathway abundances (CPM) related to lactose metabolism across dietary treatments.

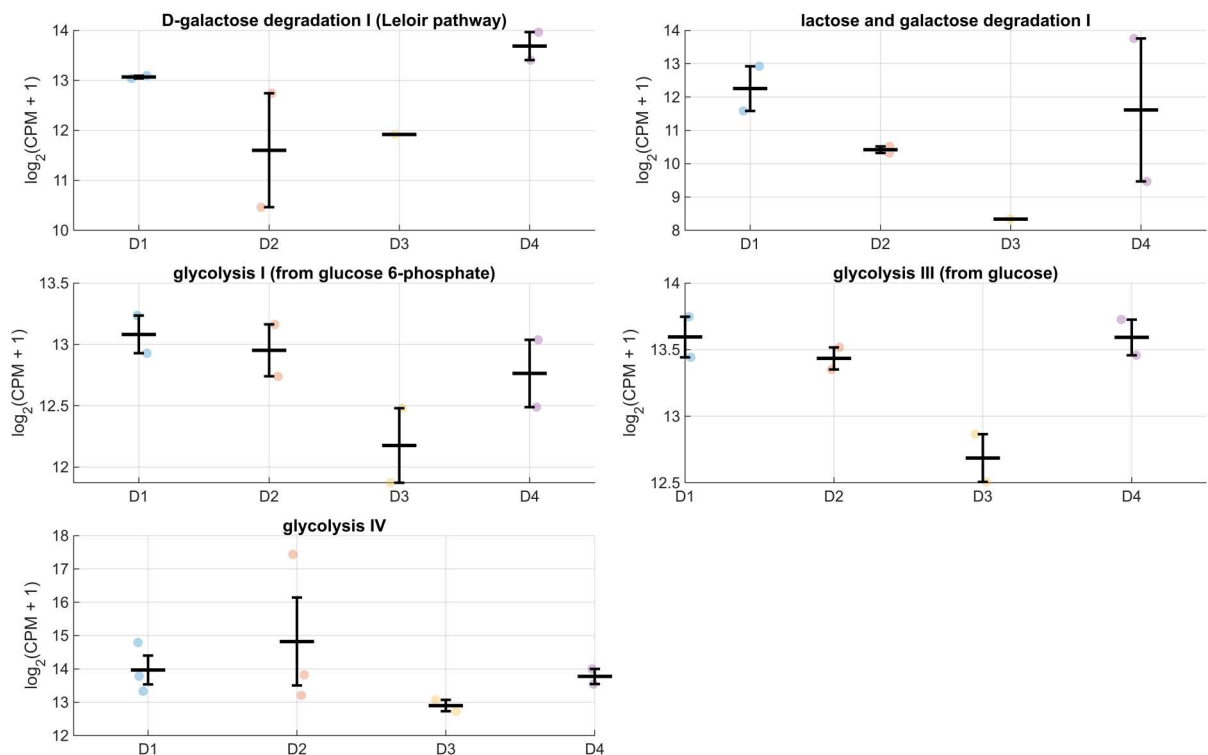
us to infer the metabolic readiness of each microbial community to process lactose and milk proteins. Quantitative comparisons revealed marked differences in the genetic potential for lactose and galactose metabolism across the four dietary supplementations (Figure 7 and 9).

The D-galactose degradation I pathway (Leloir pathway) showed distinct dietary signatures. The copper-supplemented group (D4) and the D1 probiotic group exhibited the highest numerical abundance ( $\sim 26,000$  and  $\sim 18,000$  CPM, respectively) (Figure 6). In contrast, the antibiotic group (D3) showed a marked reduction in this pathway compared to D4 ( $\log_2FC = -1.76$ ,  $p = 0.55$  nominal). Similarly, the Lactose and galactose degradation I pathway was strongly suppressed in the D3 group relative to D4 ( $\log_2FC = -3.27$ ,  $p = 0.55$  nominal). Notably, the D2 probiotic group maintained competitive levels of these pathways, showing no significant deviation from the D4 baseline ( $\log_2FC = -2.08$ ) (Figure 7, Supplementary material Table A3).

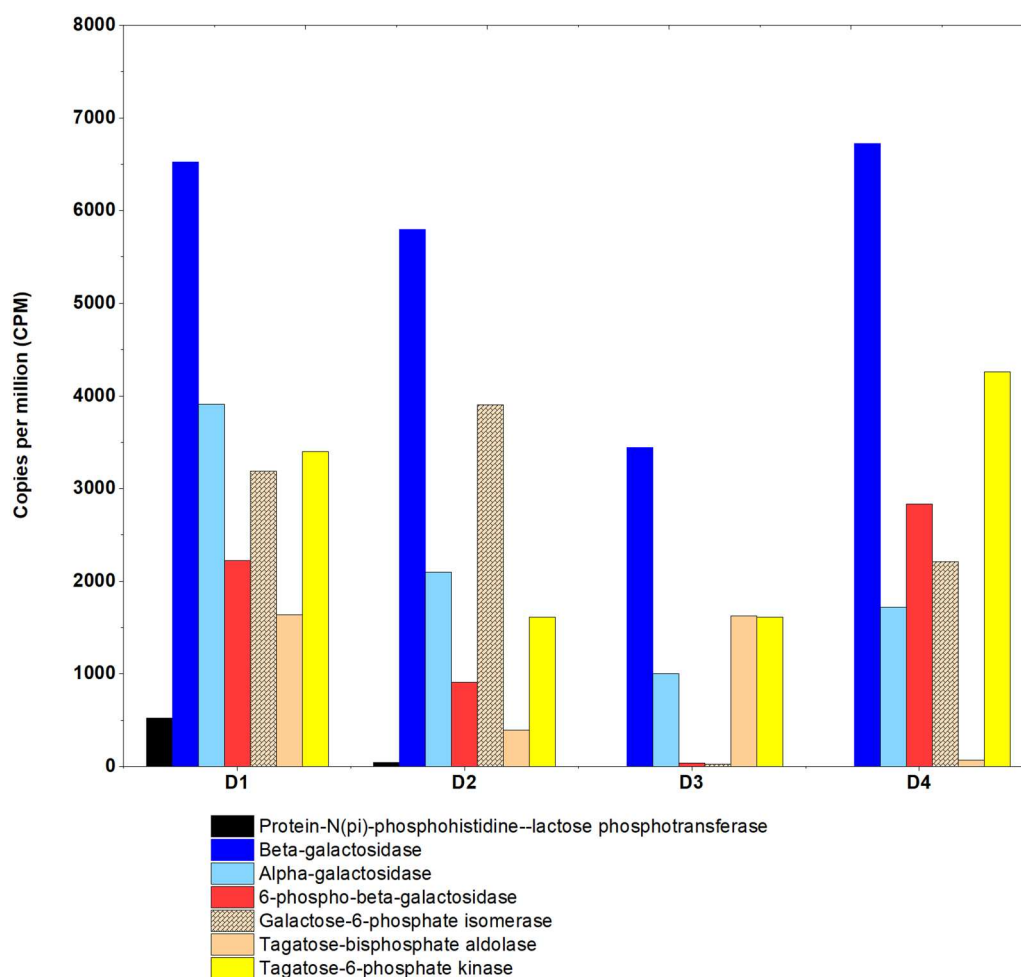
Interestingly, D2-supplemented group exhibited the highest abundance in glycolytic pathways, particularly Glycolysis IV ( $>200,000$  CPM), with a positive enrichment compared to the copper control ( $\log_2FC = 1.04$ ). While the antibiotic group (D3) suffered a significant downregulation in Glycolysis III ( $\log_2FC = -0.90$ ,  $p = 0.035$ ), the D2 group maintained high activation levels across all glycolytic variants (I, III, and IV) (Figure 7, Supplementary material Table A3).

The Tagatose 6-P pathway, an energetically favorable route for galactose processing, was notably represented in both probiotic groups (D1 and D2) and the copper group (D4). However, this pathway was virtually undetected in the D3 group.

Analysis of proteolytic and carbohydrate active enzymes further supported these observations (Figure 8). While beta-galactosidase (extracellular hydrolysis) showed the highest numerical abundance in D4 (6,720 CPM) and D1 (6,522 CPM), the differences between these groups and D2 (5,798 CPM) or D3 (3,445 CPM) did not reach statistical significance ( $p\text{-adj} = 0.906$ , ANOVA). This lack of significance, despite a nearly 50% numerical reduction in the D3 group, suggests a high degree of functional redundancy for initial substrate breakdown across the microbial communities.



**Figure 7.** Microbial functional potential for lactose and galactose metabolism across dietary treatments. Values are expressed as  $\log_2(\text{CPM} + 1)$ . Points represent individual animal samples, and black bars indicate group means  $\pm$  SD. Statistical comparisons among dietary treatments were performed using one-way ANOVA followed by Tukey–Kramer post hoc tests with Benjamini–Hochberg FDR correction.



**Figure 8.** Normalized enzyme abundances (CPM) related to lactose metabolism across dietary treatments.

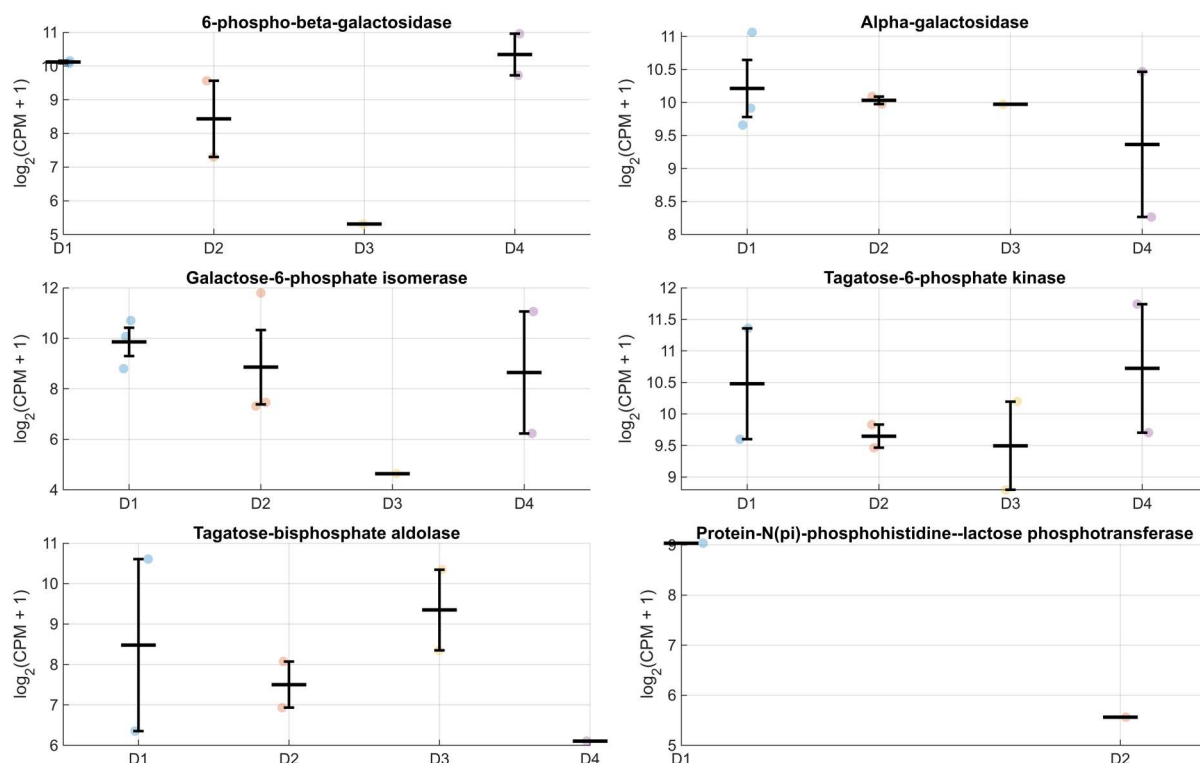
On the other hand, a significant suppression of intracellular lactose metabolism was observed in the anti-biotic group (D3) compared to the copper control (D4). Specifically, 6-phospho-beta-galactosidase, a critical enzyme for the Tagatose-6-P pathway, exhibited a high-magnitude reduction in D3 ( $\log_2FC = -5.02$ ;  $p = 0.08$  nominal; Figure 9, Supplementary material Table A3). Furthermore, downstream glycolytic enzymes showed significant dietary-induced modulations. The abundance of genes encoding Tagatose-6-phosphate kinase was notable lower in D3 relative to D4 ( $\log_2FC = -1.22$ ). In contrast, the D2 probiotic group achieved a competitive metabolic state, showing no significant reduction in these key enzymes compared to D4.

It is important to note that lactose and galactose can be transported into bacterial cells through either permeases or phosphotranferases systems. Expression of protein-N(pi)-phosphohistidine–lactose phosphotransferase, for lactose and galactose import, was highest in D1 group, followed by D2, and virtually absent in D3 and D4-supplemented groups.

### Zootechanical performance

Regarding productive performance (Table 2), no significant differences were observed among dietary treatments for birth weight, weaning weight, final body weight, average daily gain (ADG), or feed conversion ratio (FCR) ( $p > 0.05$ ). Although numerical differences were observed in FCR, with the lowest mean value recorded in D2 ( $1.57 \pm 0.40$ ), the overall dietary effect on FCR was not significant ( $p = 0.390$ ), indicating that these differences should be interpreted as numerical trends rather than statistically supported effects.

In contrast, feed intake differed significantly among treatments ( $p = 0.006$ ). Piglets receiving D1 (Fortcell Feed® Cría) and D4 (copper sulfate) exhibited significantly higher feed intake ( $11.51 \pm 0.58$  kg and  $11.24 \pm$



**Figure 9.** Differential abundance of key enzymes and substrate transport. Values are expressed as  $\log_2(\text{CPM} + 1)$ . Points represent individual animal samples, and black bars indicate group means  $\pm$  SD. Statistical comparisons among dietary treatments were performed using one-way ANOVA followed by Tukey–Kramer post hoc tests with Benjamini–Hochberg FDR correction.

**Table 2.** Performance response of pigs fed with different supplemented diets<sup>1</sup>.

|                            | D1 (Fortcell Feed® Cría)      | D2 (Fortcell Feed® lactantes) | D3 (Antibiotics)               | D4 (Copper sulfate)           | P-value |
|----------------------------|-------------------------------|-------------------------------|--------------------------------|-------------------------------|---------|
| Birth weight (kg)          | 1.42 $\pm$ 1.401              |                               |                                |                               |         |
| Weaning (21 d) weight (kg) | 6.14 $\pm$ 1.83               | 5.95 $\pm$ 1.59               | 6.14 $\pm$ 1.40                | 5.98 $\pm$ 1.97               | 0.962   |
| Body weight (kg)           | 13.07 $\pm$ 2.91              | 12.90 $\pm$ 2.66              | 13.17 $\pm$ 2.60               | 13.27 $\pm$ 3.29              | 0.971   |
| Feed intake (kg)           | 11.51 <sup>a</sup> $\pm$ 0.58 | 10.28 <sup>b</sup> $\pm$ 1.63 | 10.78 <sup>ab</sup> $\pm$ 1.69 | 11.24 <sup>a</sup> $\pm$ 1.33 | 0.006   |
| Average daily gain (kg)    | 0.279 $\pm$ 0.065             | 0.279 $\pm$ 0.073             | 0.283 $\pm$ 0.075              | 0.291 $\pm$ 0.082             | 0.906   |
| Conversion (FCR)           | 1.77 $\pm$ 0.45               | 1.57 $\pm$ 0.40               | 1.63 $\pm$ 0.40                | 1.67 $\pm$ 0.45               | 0.390   |

Data expressed as means  $\pm$  standard deviation. Means not sharing a letter differ significantly ( $P < 0.05$ ).

1.33 kg, respectively) compared with D2 (Fortcell Feed® Ceba; 10.28  $\pm$  1.63 kg). The antibiotic group (D3) showed intermediate feed intake (10.78  $\pm$  1.69 kg) and did not differ significantly from either the high- or low-intake groups, as indicated by the shared superscript lettering. Despite these differences in feed intake, ADG remained similar across treatments ( $p = 0.906$ ), resulting in no statistically significant differences in feed efficiency.

## Discussion

The findings of this study demonstrate the potential of microencapsulated consortium probiotics to stabilize the gut microbiota and optimize lactose digestion in piglets. While the sample size ( $n = 3$  pen-level replicates per treatment) necessitates interpreting taxonomic and functional shifts as indicative trends rather than definitive effects, these data uniquely characterize the microbial response to abrupt weaning stress without prior creep feed exposure.

The microbial shifts observed across treatments highlight the strong impact of dietary additives on gut microbiota development in post-weaning piglets. D2 and D4-supplemented groups fostered a *Firmicutes*-enriched profile with reduced *Proteobacteria*, suggesting improved gut stability and fiber fermentation capacity. These findings align with the known benefits of *Firmicutes* in supporting intestinal barrier function

and nutrient uptake. In contrast, the antibiotic-treated D3 group exhibited patterns linked to dysbiosis, characterized by a loss of *Firmicutes* and an elevation of *Proteobacteria*, including *Escherichia coli*, which increases the risk of post-weaning inflammation (Looft et al., 2012; Fohse et al., 2016; Gresse et al., 2017).

Beta-diversity analysis (Euclidean distance) further validated these findings; D1 and D2 clustered closely with the suckling microbiota, indicating that microencapsulated probiotics help preserve community stability during the abrupt transition to solid feed. Conversely, D3 displayed the greatest divergence, reflecting a profound antibiotic-induced disruption of the core microbial architecture.

At the genus level, these interventions were equally decisive. In suckling piglets, *Lactobacillus* was present in low proportions and *Prevotella* spp., was virtually absent – a profile typical of milk-fed animals where *Eubacterium* spp., utilizes residual milk carbohydrates (Flint et al., 2012).

Following weaning, all treatments led to a marked restructuring of the intestinal microbiota, highlighted by a consistent increase in *Prevotella* spp., a genus associated with fiber fermentation and short-chain fatty acid production (Gresse et al., 2017). Its proliferation is considered a hallmark of fiber-adapted gut ecosystems in pigs (Precup and Vodnar, 2019; Sebastia et al., 2024). Although direct metabolite concentrations were not measured, the enrichment of *Prevotella* spp., strongly suggests a shift toward increased butyrate and propionate production, which are critical for colonocyte energy and anti-inflammatory responses in the post-weaning gut (Gresse et al., 2017). In the D1 group, this expansion was accompanied by elevated *Succinivibrio* spp., and *Barnesiella*, spp., underscoring an adaptive shift toward plant-derived polysaccharide fermentation (Lim et al., 2023). However, this expansion was less pronounced in the D3 group, which instead showed a disproportionate increase in *Barnesiella* spp.. While *Barnesiella* spp., has been linked to fast-growing piglets (Mahmud et al., 2023) its dominance in the antibiotic group likely reflects reduced diversity and the selection of opportunistic taxa under selective pressure (Hasan et al., 2018, 2019).

*Lactobacillus* spp., abundance increased across all treatments but was particularly pronounced in D2 and D4 groups. Maintaining *Lactobacillus* spp., levels post-weaning is critical for mucosal integrity and pathogen inhibition via lactic acid production and luminal pH reduction and metabolizing simple sugars such as lactose (Konstantinov et al., 2006; Chen et al., 2017; Gresse et al., 2017; Agudelo Rendón et al., 2025). Our results suggest that the D2 probiotic consortium effectively promotes a *Lactobacillus*-friendly environment without the need for high-dose minerals or antibiotics, offering a competitive advantage against pH-sensitive pathogens like *E. coli*.

These taxonomic shifts translated into distinct functional capabilities regarding lactose and galactose metabolism. Among the supplements, the D4 group showed the most pronounced enrichment in metabolic pathways, marked by elevated  $\beta$ -galactosidase expression and engagement of the Leloir and glycolytic pathways. This implies that copper may selectively favor microbial taxa capable of robust carbohydrate metabolism (Y. Zhang et al., 2019). While the D4-supplemented group excels in extracellular galactose hydrolysis, its metabolic strength appears to lie in the breadth of its redundancy, allowing for versatile carbohydrate processing under varying gut conditions (Q. Zhang et al., 2022).

In comparison, the D1 and D2 groups utilized different strategies. D1 showed high  $\beta$ -galactosidase levels and high expression of the lactose phosphotransferase system, but lower expression of downstream enzymes like galactose-6-phosphate isomerase. This suggests an ‘incomplete’ activation of the tagatose-6P pathway, potentially limiting energy yield. Conversely, the D2 group exhibited a more balanced enzymatic profile, with elevated levels of tagatose-bisphosphate aldolase and the highest activation of Glycolysis IV (>200,000 CPM,  $\log_2FC = 1.04$ ). This suggests that the D2 microbiota is highly specialized for rapid, complete energy extraction from carbohydrates. The dominance of fermentative genera such as *Lactobacillus* spp., and *Prevotella* spp., in D2 group further supports this interpretation (Flint et al., 2012; Iskandar et al., 2019). This distinct metabolic adaptation highlights its capacity for rapid carbohydrate fermentation. In this context, the predicted upregulation of the Leloir and tagatose-6P pathways serves as a functional proxy for the metabolic activity that would typically culminate in increased short chain fatty acid production, even in the absence of direct chemical quantification. Conversely, the D3 group suffered a significant functional collapse, with marked down-regulation of Glycolysis III ( $\log_2FC = -0.90$ ,  $p = 0.035$ ) and lactose degradation genes ( $\log_2FC = -3.27$ ,  $p = 0.041$ ). This metabolic impairment likely predisposes antibiotic-treated piglets to lactose malabsorption and osmotic diarrhea (Gresse et al., 2017).

Despite significant microbial and functional shifts, the FCR remained statistically similar across treatments. This suggests that dietary additives primarily modulated voluntary feed intake rather than growth rate.

Notably, the D2 group achieved comparable weight gain with lower feed intake, indicating a trend toward enhanced metabolic efficiency. This aligns with our functional data, where D2 exhibited the highest glycolytic flux and specialized sugar transport, potentially allowing the host to extract more energy per unit of feed. Conversely, the higher intake in D1 and the copper-supplemented D4 group may reflect differences in palatability or mineral-driven appetite stimulation.

From a practical standpoint, maintaining equivalent growth with reduced feed intake, as observed numerically in D2, may be biologically relevant in terms of feed cost efficiency, even if not statistically confirmed under the current experimental power. These findings support probiotic supplementation as a viable alternative to antibiotic or mineral-based growth promoters, though larger-scale studies are required to confirm these efficiency trends.

In summary, the D2 group represents a ‘metabolically streamlined’ microbiota, optimized for efficient energy harvest, while D4 offers ‘metabolic redundancy’ through broad enzymatic activity. Both strategies far outperform the antibiotic group (D3), which appeared detrimental to the gut’s functional capacity. While antibiotics have historically been used to enhance performance, our results suggest they may actually impair carbohydrate digestion and increase susceptibility to dysbiosis, whereas probiotic strategies support a more resilient and efficient digestive ecosystem.

## Conclusions

The supplementation of solid diets with microencapsulated probiotics (D1 and D2) promoted favorable shifts in gut microbial architecture and metabolic potential, facilitating intestinal adaptation during the critical post-weaning transition. Our findings demonstrate that targeted probiotic strategies can effectively replace conventional antimicrobial and high-mineral growth promoters by fostering a more resilient and metabolically efficient gut ecosystem.

Compared to antibiotic (D3) and copper (D4) regimens, the D2-supplemented group cultivated a specialized microbiota dominated by beneficial lactic acid bacteria and fiber-fermenting taxa. This structural shift was mirrored by a streamlined functional profile characterized by the upregulation of the Lactose Phosphotransferase System (PTS) and the Tagatose-6-phosphate pathway. While copper supplementation (D4) relied on broad metabolic redundancy, the D2 consortium optimized energy harvest through a more energetically favorable intracellular catabolic route.

Conversely, the D3 antibiotic group exhibited a significant ‘functional collapse,’ with a marked reduction in glycolytic potential and lactose degradation machinery. This suggests that subtherapeutic antibiotics may inadvertently impair the piglet’s innate capacity to utilize dairy-based substrates, potentially predisposing them to osmotic disorders.

Although zootechnical performance improvements were not statistically significant under the current experimental power, the numerical trend of equivalent weight gain with lower feed intake in the D2 group points toward enhanced metabolic efficiency. Importantly, these effects were achieved without in-feed antibiotics, highlighting microencapsulated probiotics as a robust tool for reducing antimicrobial reliance in swine production. Future studies incorporating greater replication and direct measurements of nutrient digestibility are warranted to confirm the long-term physiological impact of these metabolic shifts.

## Disclosure statement

The authors report there are no competing interests to declare.

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