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Multitechnique characterisation of gut microbiota dynamics in piglets supplemented with Limosilactobacillus reuteri DSPV002C

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Abstract

The gut microbiota is essential for health and pig growth by aiding digestion, regulating immune responses, and protecting against pathogens. However, weaning disrupts microbial balance, compromising gut integrity and increasing disease susceptibility. Probiotics have been proposed to modulate gut microbiota during the post-weaning period. This study evaluated the effects of *Limosilactobacillus reuteri* DSPV002C supplementation on the gut microbiota of weaned piglets.

Twenty piglets were assigned to a control (CG) or probiotic group (PG), receiving daily *L. reuteri* DSPV002C. Fecal samples were collected on days 0, 7, 14, 21, 28, 35, and 42. Samples were analysed using plate counts and denaturing gradient gel electrophoresis (DGGE). Samples from days 0, 21, and 42 underwent 16S rRNA gene sequencing to assess alpha and beta diversity, relative abundance, linear discriminant analysis, and predicted metabolic pathways.

The supplemented strain was recovered throughout the experimental period in the PG. DGGE profiles revealed temporal clustering, and beta diversity showed a trend toward separation between groups on day 42 ($p < 0.10$). *Firmicutes* and *Bacteroidetes* were the dominant phyla. Some genera showed enrichment trends in the PG. No significant differences were observed in predicted metabolic pathways.

L. reuteri DSPV002C supplementation was associated with temporal shifts in gut microbial composition during the post-weaning period. Functional predictions did not reveal significant pathway differences between groups. The results should be considered exploratory due to the limited number of animals included in the sequencing analyses. Future studies should include larger cohorts and complementary approaches to clarify probiotic-associated microbial changes.

Introduction

The gut microbiota plays a crucial role in the health and development of pigs by facilitating digestion, supporting immune responses, and protecting against pathogens (St-Pierre et al., 2023). Its influence on nutrient absorption and immune system maturation is essential for growth and productivity. However, the weaning process, characterized by abrupt dietary and environmental changes, disrupts microbial balance and gut integrity, often resulting in growth delays and increased susceptibility to diseases such as post-weaning diarrhea syndrome—a condition commonly associated with enterotoxigenic *Escherichia coli* infection (Holman et al., 2021). These challenges highlight the urgent need for strategies to promote gut health, particularly as antimicrobial resistance restricts the use of antibiotics (Tang et al., 2022).

Probiotics have emerged as a promising alternative to antibiotics, restoring microbial balance and improving gut function (Quan et al., 2023; Castillo Zuniga et al., 2024). These live microorganisms, which confer health benefits to the host, have shown efficacy in enhancing nutrient absorption, modulating immune responses, and reducing diarrhea in pigs (Pereira et al., 2022). Studies on probiotic supplementation have shown a reduction in ETEC-associated diarrhea, enhanced growth performance, and improved microbial balance, reinforcing their role as an effective alternative to antibiotics in weaned

pigs (Duddeck et al., 2023). The inclusion of lactic acid bacteria (LAB) strains in pig diets has also been shown to increase the production of short-chain fatty acids (SCFAs), which support gut health, and to maintain intestinal morphology by reducing pathogenic bacterial populations and enhancing nutrient absorption during critical early-life stages (Yoon et al., 2024). Additionally, probiotics reduce gut inflammation, promote villus height in the gastrointestinal tract, and improve barrier intestinal integrity (Canibe et al., 2022; Zhang et al., 2023).

Despite the benefits of probiotics, significant gaps remain in understanding the optimal strains, dosages, and administration timings. Further research is essential to optimize probiotic supplementations and assess their impacts on microbial diversity and host health during critical developmental stages (Song et al., 2024). The variability in probiotic efficacy, influenced by host genetics, dietary composition, and environmental factors, underscores the complexity of optimizing these supplementations. Monger et al. (2024) highlight the need for standardized protocols to evaluate probiotics comprehensively, ensuring consistent outcomes across diverse agricultural settings.

Recent advances in 16S rRNA gene sequencing approaches have significantly enhanced our understanding of microbial contributions to livestock health. These methods have revealed the production of key metabolites that play a critical role in enhancing nutrient metabolism and supporting gut integrity. Additionally, 16S rRNA gene sequencing has provided insights into the mechanisms by which beneficial microbes outcompete pathogens, thereby reducing disease susceptibility and improving overall animal wellness (Wani et al., 2022).

The objective of this study was to characterize temporal changes in fecal microbiota associated with *L. reuteri* DSPV002C supplementation during the post-weaning period using a multitechnique approach that included culture-based methods, denaturing gradient gel electrophoresis (DGGE), and 16S rRNA gene sequencing.

Materials and Methods

Inoculum production and cell entrapment

The strain *Limosilactobacillus reuteri* DSPV002C comb. nov. (GenBank accession number: GQ231436.1), a swine origin strain with probiotic properties (Bertozzi 2012; Fusari 2019) was used in this study. To monitor the strain in feces, a rifampicin-resistant variant of *L. reuteri* DSPV002C was used. Successive cultures of *L. reuteri* were grown in MRS medium with increasing rifampicin concentrations, ranging from 0.1 µg/ml to 100 µg/ml. The rifampicin-resistant strain was then propagated for 24 h at 37°C in MRS broth.

The rifampicin-resistant *L. reuteri* DSPV002C was cultured in a fermenter (Sartorius BIOSTAT® A) with 2% v/v initial inoculum, and incubated 18 h at 37°C. The culture medium consisted of 6% w/v cheese whey permeate (Arla Food Ingredients), 0.8% w/v yeast extract, 0.0003% w/v Manganese (II) sulfate ($MnSO_4$), 1% w/v casein peptone and 2% w/v dextrose. The pH was adjusted to 6 ± 0.2 using 6 N sodium

hydroxide (NaOH). After incubation, the culture was harvested by centrifugation at 5000 × g for 10 min at 17°C, and cells were washed with phosphate buffered saline (PBS) twice.

For cell entrapment, a matrix composed of gelatin (PB LEINER PBGELATINS) and pregelatinized starch (“GLUTAGEL”, Glutal S.A.) was prepared. Gelatin was prepared at 10% w/v and pregelatinized starch at 5%, then both solutions were heated at 70°C for 10 min. Next, solutions were mixed in a 1:1 ratio until complete homogenization obtaining a 7.5% w/v matrix and subsequently combined with the cells in a 9:1 (cells: matrix) ratio. In addition, 10% w/v cheese whey permeate final concentration were added as cryoprotectants. The final mixture was dispensed by extrusion into 10 mm diameter silicone molds for macrocapsules formation. The molds were then placed at − 80°C for 18 h and lyophilized at 0.044 mbar (CHRIST® Alpha1-4 LSC plus. Martin Christ) for 18h.

Experimental design

The trial was conducted on an experimental farm equipped with cage pens. Twenty piglets (*Sus scrofa domesticus*) of both sexes, weaned at 28 ± 1 days of age were randomly assigned to a probiotic group (PG) and a control group (CG), each consisting of 10 animals, with two animals housed per cage. The probiotic strain was administered from day 1 (28 ± 1 days of age) until day 42 of the trial (70 ± 1 days of age). The animals were fed a commercial concentrate diet (starter) and had *ad libitum* access to water throughout the experiment. The diet was based on the TEKNAL animal nutrition program, which included Magnum Starter for piglets from 28 ± 1 d to 35 ± 1 d, then Magnum Initiator 300 until 45 ± 1 d, and Magnum Breed 50 until 70 d. The nutritional composition of the different feeds, expressed on a dry basis is presented in Table 1. In addition, each piglet in the PG received the probiotic macrocapsules, ensuring a daily dose equal to or exceeding 9.80 log CFU/animal throughout the experiment.

Table 1
Nutritional composition of the commercial feeding products used during the experimental period, expressed on a dry matter basis.

Nutritional content			
Diet	Starter	Initiator 300	Breed
Crude protein % (min.)	20	30	31
Ethereal extract % (min.)	5.5	12	-
Crude fiber % (max.)	2.5	2	0.5
Calcium % (max. -min.)	0.5–0.4	2.2–2	9–8.4
Phosphorus % (max. -min.)	0.62–0.58	1.3–1	4.9–4.5
Moisture % (max.)	11	8	3.5
Ash % (max)	4.5	10	40
Metabolizable energy	3500 Kcal.	Not specified	Not specified
"Min." indicates the minimum value and "Max." indicates the maximum value for the given nutrient levels.			

For plate count, fecal samples were collected from six randomly selected piglets in the PG and six piglets in the CG, on days 0, 7, 14, 21, 28, 35 and 42 of the trial. Samples were collected in plastic bags and refrigerated until processing, which occurred within six hours of collection. Animals were individually sampled and considered the biological unit for microbiological analyses. Animals were housed in pairs for management purposes; however, fecal samples were collected and processed individually. For DGGE and 16S rRNA gene sequencing analysis, fecal samples were collected from three randomly selected piglets in the PG and three in the CG, on days 0, 21 and 42 of the trial. Aliquots of 0.2 g were placed in Eppendorf tubes with 1.5 ml of PBS for DGGE analysis and without PBS for 16S rRNA gene sequencing analysis. Due to logistical constraints, 16S rRNA gene sequencing was performed on a subset of representative animals (n = 3 per group and time point), and results should be interpreted as exploratory. All samples were stored at -20°C until processing.

Microbiological and molecular analyses

Plate count of microbial populations

Microbial counts were performed using serial decimal dilutions in Ringer's ¼ solution, followed by plating on Petri dishes with specific media and incubation conditions. For the detection of the probiotic strain, lactobacilli were cultured on lactobacillus anaerobic MRS medium with vancomycin and bromocresol green (LAMVAB medium), supplemented with rifampicin (100 µg/ml) (LAMVABrif) and incubated at 37°C under anaerobic conditions for 72 h. LAB were cultured on MRS agar (Oxoid, Basingstoke, United

Kingdom) under anaerobic conditions at 37°C for 72 h. *E. coli* was cultured on Tryptone Bile X-glucuronide agar (TBX agar) (Oxoid, Basingstoke, United Kingdom) and incubated at 44°C for 24 h under aerobic conditions. Yeasts were cultured on Fungi and Yeast medium (HyL) (Britania, Buenos Aires, Argentina) supplemented with 20 g/l dextrose and 5 g/l pluripeptone, incubated at 37°C for 48 h under aerobic conditions. Thermotolerant *Campylobacter* were cultured on modified charcoal cefoperazone deoxycholate agar (mCCDA) and incubated at 42°C under microaerophilic conditions (85% N_2, 10% O_2, and 5% CO_2) for 48 h. Presumptive colonies of thermotolerant *Campylobacter* were confirmed by observing fresh sample under a phase-contrast microscope.

DGGE profiling of fecal microbial communities

Genomic DNA was extracted from fecal samples using Wizard® Genomic Purification DNA Kit (Promega, Madison, USA). DNA concentrations were quantified and adjusted to 25–50 ng/µL using a Take3 device and Gen5 software on a multimodal microplate reader (Sinergy HT, Bio-Tek, Winooski, USA).

The amplification of the 16S gene was performed using the GoTaq DNA Polymerase kit (Promega, Vilnius, Lithuania). Universal primers GC-HDA1 (5'-CGC CCG GGG CGC GCCCCG GGC GGG GCG GGG GCA CGG GGG GAC TCCTAC GGG AGG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTGGCA C-3') were used. The PCR program consisted of an initial denaturation at 94°C for 4 min 30 s, followed by 35 cycles of denaturation at 94°C for 30 S, annealing at 58°C for 30 s, extension at 68°C for 1 min, and a final extension at 68°C for 30 min. Negative controls without DNA were included to verify the absence of contamination. PCR products were verified by electrophoresis on 2% agarose gel containing 0.01% GEL RED (Biotium, Freemont, USA) and visualized under ultraviolet light.

PCR products were separated by DGGE using a D-Code Universal Mutation Detection kit (Bio-Rad Laboratories, Hercules, USA) on 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide 37,5:1) with a 40% to 60% urea formamide gradient. Electrophoresis was conducted at 60°C, 130 V for 4 h (Simpson et al., 2000). A mixture of PCR products from *E. coli* Nissle, *L. reuteri* DSPV002C and *Bifidobacterium animalis* subsp. lactis was used as the DGGE ladder. After electrophoresis, gels were stained with 1% (v/v) SYBR Safe (Invitrogen, Carlsbad, USA) for 15 min and visualized a Safe Imager Blue-Light transilluminator (Thermo Fisher Scientific, Waltham, USA).

Gel images were analysed with BioNumerics software v6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were constructed using the unweighted pair group method with arithmetic mean (UPGMA) based on the Dice similarity coefficient with a 1% position tolerance for band matching. To characterize the microbial ecosystem in pig feces, Shannon diversity index, Pielou homogeneity index and Margalef richness index were calculated.

Representative bands were excised from the polyacrylamide gel and placed in 50 µl of steril distilled water at 4°C for 48 h to allow DNA diffusion. The dissolved DNA was then reamplified using primers

HDA-1 and HDA-2 under the same conditions previously described. The amplified products were purified using the PURO ADN-Clean Up kit (PB-L Productos Bio-Lógicos, Buenos Aires, Argentina). Sequencing was performed by MACROGEN (Seoul, South Korea) and the results were compared with 16S rDNA sequences available in GenBank using the NCBI BLAST software.

16S rRNA gene sequencing análisis

Fecal DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Germany) following the manufacturer's instructions. Subsequently, 16S rRNA genes were analysed using the Ion 16S™ Metagenomics Kit (Cat. A26216, Thermo Fisher, USA), designed for rapid analysis of polybacterial samples with Ion Torrent™ sequencing technology. The kit includes two primer sets: Primer kit V2-4-8 and Primer set V3-6, 7–9, which selectively amplify the corresponding hypervariable regions of the 16S rRNA gene. Combining these two primer sets enables sequence-based identification of a broad range of bacteria within complex samples. All PCR reactions were performed using TaqMan® Fast Universal PCR Master Mix (2X), without AmpErase® UNG (Thermo Fisher, USA). Each PCR run included a positive control (*E. coli* DH10B DNA) and a negative control (water). The following PCR conditions were applied: initial denaturation at 95°C for 10 min, followed by 18–25 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 20 s, and a final extension at 72°C for 7 min. PCR products were quantified with the Qubit™ fluorimeter. The PCR products were pooled and purified via magnetic bead purification using the Agencourt AMPure reagent (Beckman Coulter, USA). The quality of the purified PCR products was assessed with the 2100 Bioanalyzer (Agilent Thecnologies, USA) using a 1000 DNA chip. Sequencing libraries were prepared with the Ion Plus Fragment Library Kit (Cat. 4471252, Thermo Fisher, USA) according to the manufacturer's protocol. The quality of the library was evaluated using the 2100 Bioanalyzer DNA chip. Sequencing was performed on the Ion S5 using Chef's Ion 520 kit plus the Ion 520 On-Chip Sequencing Kit (Cat. A34461, Thermo Fisher, USA). Data analysis was conducted using Ion Reporter, Software 5.2, Workflow Version 1.1.

Sequence quality filtering, denoising, chimera removal and amplicon sequence variant (ASV) inference were performed in R software v4.1.2 using the DADA2 v1.22.0 pipeline. Taxonomic assignment was conducted against the Silva database v138.1 (updated March 10, 2021). Microbiome data processing and visualization were performed using the phyloseq v1.38.0, microbiome v1.16.0, microViz v0.12.1, microbiomeMaker v1.10.0, and vegan v2.6.4 packages.

Alpha diversity indices were calculated using phyloseq and microbiome packages. For beta diversity analyses, count data were transformed using centered log-ratio (CLR) transformation. Principal component analysis (PCA) and principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distance matrices were used to visualize microbial community structure. Data were rarefied prior to diversity analyses to standardize sequencing depth among samples.

Relative abundance analyses were performed at phylum and genus levels. Differentially abundant taxa were identified using linear discriminant analysis effect size (LEfSe). Functional prediction of microbial metabolic pathways was performed using Tax4Fun2 v1.1.5.

Statistical analysis

Microbiological counts and DGGE diversity indices were analysed using repeated measures ANOVA to evaluate temporal changes within groups throughout the experimental period. When significant effects were detected, means were compared using one-way ANOVA tests. Comparisons between groups at individual sampling times were also performed using one-way ANOVA. Data are expressed as mean ± standard deviation.

For 16S rRNA gene sequencing analysis, alpha diversity indices were compared between groups using Kruskal–Wallis tests. Differences in microbial community structure based on weighted and unweighted UniFrac distance matrices were statistically evaluated using PERMANOVA (Adonis function, vegan package). Relative abundance comparisons at phylum and genus levels were performed using Kruskal–Wallis tests.

Differentially abundant taxa and predicted metabolic pathways were identified using linear discriminant analysis effect size (LEfSe). Taxa with LDA scores ≥ 2 and p-values < 0.05 were considered significantly enriched. Values between p = 0.05 and p = 0.10 were interpreted as trends.

Results

Culturable microbial populations

LAB populations remained stable throughout the experimental period in both groups, with no significant differences between the CG and PG ($p > 0.05$). The probiotic strain was not recovered from the CG at any sampling time (detection limit: 2 log CFU/g feces). In contrast, *L. reuteri* DSPV002C was recovered from PG animals from day 7 onward and remained detectable throughout the trial (Table 2).

Table 2
Microbial population counts in the feces of piglets supplemented with *L. reuteri* DSPV002C.

Microorganisms	**Time (d)**							
	0	**7**	**14**	**21**	**28**	**35**	**42**	***p-value***
Counts (Log CFU/g)								
Lactic Acid Bacteria								
CG	7.65 ± 0.5[a]	8.23 ± 0.6[a]	7.86 ± 0.5[a]	8.11 ± 0.4[a]	8.55 ± 0.4[a]	8.58 ± 0.8[a]	7.62 ± 1.8[a]	0.950
PG	7.56 ± 0.8[a]	8.31 ± 0.6[a]	8.19 ± 0.4[a]	7.68 ± 0.8[a]	8.44 ± 0.5[a]	8.31 ± 0.5[a]	8.20 ± 0.6[a]	
L. reuteri DSPV 002C								
CG	< DL							
PG	< DL	4.25 ± 1.1[a]	3.72 ± 0.7[a]	4.10 ± 0.5[a]	5.96 ± 1.1[a]	5.58 ± 1.1[a]	5.39 ± 0.6[a]	
Campylobacter spp.								
CG	6.53 ± 0.5[a]	5.15 ± 1.3[a]	4.41 ± 0.7[a]	3.10 ± 0.2[a]	3.52 ± 0.4[a]	4.10 ± 0.9[a]	3.44 ± 1.1[a]	0.580
PG	7.18 ± 0.6[a]	4.65 ± 0.8[a]	4.62 ± 0.8[a]	3.62 ± 0.8[a]	3.16 ± 0.9[a]	3.39 ± 1.2[a]	2.99 ± 1.1[a]	
Escherichia coli								
CG	7.48 ± 0.8[a]	6.37 ± 1.1[a]	4.96 ± 1.2[a]	3.02 ± 1.0[a]	5.18 ± 1.0[a]	4.32 ± 1.0[a]	3.82 ± 1.2[a]	0.174
PG	8.4 ± 0.5[a]	7.03 ± 0.9[a]	5.09 ± 1.4[a]	3.04 ± 1.5[a]	4.16 ± 1.0[a]	4.20 ± 1.4[a]	5.54 ± 0.8[a]	
Yeasts								
CG	4.34 ± 0.4[a]	5.42 ± 0.4[a]	3.83 ± 0.5[a]	2.55 ± 0.9[a]	2.98 ± 0.5[a]	2.54 ± 0.7[a]	2.43 ± 0.4[a]	0.422
PG	3.85 ± 1.0[a]	4.62 ± 0.8[a]	3.00 ± 1.3[a]	2.92 ± 0.9[a]	3.01 ± 0.9[a]	2.50 ± 0.8[a]	3.08 ± 0.6[a]	

CG: control group. PG: probiotic group. Data are expressed as Log CFU/g of feces and correspond to the mean ± standard deviation. DL: detection limit > 100 CFU/g. Lowercase letters a and b correspond to significant differences ($P < 0.05$) within each group.

Campylobacter spp., *E. coli*, and yeast populations did not differ significantly between groups or sampling times ($p > 0.05$). However, temporal fluctuations in microbial counts were observed in both groups during the post-weaning period. *Campylobacter* spp. counts ranged from 2.99 ± 1.1 to 7.18 ± 0.6 log CFU/g feces, while *E. coli* populations ranged from 3.02 ± 0.1 to 8.40 ± 0.5 log CFU/g feces. Yeast counts varied between 2.43 ± 0.1 and 5.42 ± 0.3 log CFU/g feces.

DGGE profiling of fecal microbial communities.

Figure 1 shows the DGGE dendrogram profiles obtained from the CG and PG on days 0, 21, and 42. An overall similarity of approximately 40% was observed among all samples. Two profiles from the PG on day 42 (T1 and T5) separated distinctly from the remaining samples, while the rest clustered into a single main group. Within this cluster, three sub-clusters were identified. One sub-cluster grouped all profiles from day 0 together with one CG profile from day 42, showing approximately 55% similarity. The remaining two sub-clusters showed similarities of 60% and 70%, respectively, and included samples from days 21 and 42 from both experimental groups. As shown in Fig. 1, samples tended to cluster according to sampling time rather than treatment group, suggesting progressive restructuring of the fecal microbial community during post-weaning development.

Diversity, richness, and homogeneity indices derived from DGGE profiles are presented in Table 3. No significant differences were observed between the CG and PG for any of the evaluated indices ($p > 0.05$). However, in the PG, Shannon diversity values were higher on day 0 compared with days 21 and 42 ($p < 0.05$). Similarly, richness values were also higher on day 0 in the PG ($p < 0.05$). In contrast, no temporal differences in diversity or richness were observed in the CG. Homogeneity indices remained stable throughout the experimental period in both groups.

Table 3
Indices of diversity, richness and homogeneity of the microbial ecosystem of piglet feces.

Indexes		Time (d)			*p-value*
	Group	0	21	42	
Shannon diversity	CG	1,88 ± 0,38^{a}	1,60 ± 0,37^{a}	1,65 ± 0,15^{a}	0,247
	PG	1,89 ± 0,20^{a}	1,44 ± 0,23^{b}	1,36 ± 0,31^{b}	
Pielou homogeneity	CG	0,91 ± 0,02^{a}	0,96 ± 0,05^{a}	0,94 ± 0,04^{a}	0,162
	PG	0,94 ± 0,03^{a}	0,95 ± 0,02^{a}	0,97 ± 0,03^{a}	
Margalef richness	CG	1,91 ± 0,52^{a}	1,38 ± 0,48^{a}	1,48 ± 0,28^{a}	0,115
	PG	1,64 ± 0,30^{a}	1,13 ± 0,23^{b}	1,27 ± 0,26^{b}	

CG: control group, PG: probiotic group. Values are expressed as mean ± standard deviation at 0, 21 and 42 days (d). Different superscript letters indicate significant differences within the same group ($p < 0.05$).

Representative DGGE bands were excised and sequenced to characterise bacterial groups associated with temporal microbial dynamics (Table 4). On day 0, sequence similarity values ranged from 82.86% to 97.14%. *Firmicutes* was the most represented phylum, followed by *Synergistetes*, *Bacteroidetes*, and *Proteobacteria*. Several sequences were associated with members of the order *Clostridiales*, while others were related to *Cloacibacillus*, *Aminivibrio*, *Barnesiella*, *Blautia*, and *Pyramidobacter*.

Table 4
Identity of partial 16S rRNA sequences of piglets feces on days 0, 21 and 42 of the trial, compared to sequences in the NCBI database

Band	Group	Closest sequence relative		Identity (%)	NCBI accesion number
		Phylum	Specie		
day 0					
1	CG	*Synergistetes*	*Cloacibacillus porcorum*	94.96	MN537495.1
		Synergistetes	*Synergistes* sp. CNM 1720-08	94.96	KU159767.1
2	CG	*Firmicutes*	Uncultured *Garciella* sp. clone 6924	95.65	KP107893.1
3	CG	*Firmicutes*	Uncultured *Clostridium* sp.	91.78	EU809916.1
4	CG	*Synergistetes*	*Aminivibrio pyruvatiphilus* strain	91.84	MG519324.1
5	CG	*Firmicutes*	Uncultured *Ruminococcaceae* bacterium	87.8	KP101892.1
6	CG	-	Uncultured bacterium	82.86	JQ185232.1
7	CG	*Firmicutes*	Uncultured *Clostridiales* bacterium	91.49	KX672592.1
8	PG	*Firmicutes*	Uncultured *Firmicutes* bacterium	93.67	KM650279.1
9	PG	*Bacterioidetes*	Uncultured *Bacteroidetes* bacterium	97.14	KP716742.1
		Bacterioidetes	Uncultured *Sphingobacteriaceae* bacterium	96.57	KP104582.1
10	PG	*Bacterioidetes*	Uncultured *Barnesiella* sp.	97.11	KP104979.1
11	PG	*Firmicutes*	*Blautia* sp.	96.69	MG729425.1
		Firmicutes	Uncultured *Lachnospiraceae*	96.08	KP101979.1
12	PG	*Firmicutes*	Uncultured *Clostridiaceae*	85.71	AB089028.1
13	PG	*Firmicutes*	Uncultured *Clostridium* sp	85.71	MG803277.1
14	PG	*Synergistetes*	Uncultured *Pyramidobacter* sp.	90.82	LT625531.1
		Synergistetes	Uncultured *Synergistaceae*	90.82	LT545834.1
15	PG	*Proteobacteria*	Uncultured *Thiobacillus* sp.	89.16	MF042831.1

CG: control group; PG: probiotic group.

Band	Group	Closest sequence relative		Identity (%)	NCBI accesion number
		Phylum	Specie		
			day 21		
16	CG	*Actinobacteria*	Uncultured *Olsenella* sp.	97.50	KY668857.1
17	CG	*Actinobacteria*	*Leucobacter* sp.	91.84	KT223115.1
18	CG	*Actinobacteria*	*Bifidobacterium* sp	93.71	MN049601.1
19	CG	*Actinobacteria*	*Microbacterium* sp	87.50	KT630604.1
20	CG	*Bacterioidetes*	Uncultured *Barnesiella* sp.	89.14	KP106386.1
		Bacterioidetes	Uncultured *Porphyromonadaceae*	89.14	KP104927.1
21	CG	*Firmicutes*	Uncultured *Lachnospiraceae*	86.16	KP109441.1
22	CG	*Actinobacteria*	*Olsenella umbonata*	96.88	MN537488.1
23	PG	*Firmicutes*	Uncultured *Pseudoramibacter* sp.	88.67	KP107389.1
24	PG	*Firmicutes*	*Lactobacillus parafarraginis*	84.93	KP775924.1
25	PG	*Actinobacteria*	*Olsenella umbonata* strain	96.34	MN537488.1
26	PG	*Actinobacteria*	Uncultured *Olsenella* sp.	96.93	KY668857.1
27	PG	*Firmicutes*	Uncultured *Clostridium* sp.	91.36	LC001061.1
28	PG	*Firmicutes*	*Clostridium paraputrificum*	92.86	MN081661.1
29	PG	*Firmicutes*	Uncultured *Clostridium* sp	91.34	KF507046.1
30	CG	*Firmicutes*	Uncultured *Ruminococcaceae*	91.14	KY663398.1
		Firmicutes	Uncultured *Anaerotruncus* sp	91.14	LT625255.1
			day 42		
31	CG	-	Uncultured prokaryote	94.03	KT979631.1
32	CG	*Firmicutes*	Uncultured *Firmicutes*	90.79	KJ730135.1
33	CG	-	Uncultured bacterium	100.00	AY990827.1
34	CG	*Firmicutes*	Uncultured *Clostridium* sp.	91.84	DQ168164.1
35	CG	*Firmicutes*	Uncultured *Lachnospiraceae* bacterium	90.53	EF700560.1

CG: control group; PG: probiotic group.

Band	Group	Closest sequence relative		Identity (%)	NCBI accesion number
		Phylum	Specie		
36	PG	*Proteobacteria*	Uncultured *Nitrosospira* sp	96.88	JQ904482.1
37	PG	*Synergistetes*	Uncultured *Aminanaerobia* bacterium	91.30	CU925912.1
38	PG	*Synergistetes*	Uncultured *Aminanaerobia* bacterium	88.04	CU920731.1
39	PG	-	Uncultured bacterium	96.77	JF658104.1
40	PG	*Firmicutes*	Uncultured *Firmicutes*	92.78	HM105408.1
41	PG	*Bacterioidetes*	Uncultured *Porphyromonadaceae*	97.16	KP105248.1
		Bacterioidetes	Uncultured *Bacteroidales*	97.16	KM244915.1
42	PG	*Proteobacteria*	Uncultured *delta proteobacterium*	92.98	KC009888.1
43	PG	-	Uncultured bacterium	94.34	MF315152.1
44	PG	*Actinobacteria*	*Arthrobacter* sp.	90.67	FN397645.1

CG: control group; PG: probiotic group.

On day 21, sequence similarity values ranged from 84.93% to 97.50% (Table 4). *Actinobacteria* and *Firmicutes* were the dominant phyla identified, whereas *Bacteroidetes* was represented by a single band. Sequences related to *Olsenella*, *Bifidobacterium*, *Clostridium*, and Lactobacillales-associated genera were detected in both experimental groups.

On day 42, sequence similarity values ranged from 88.04% to 100% (Table 4). *Firmicutes* remained the predominant phylum, followed by *Proteobacteria*, *Synergistetes*, *Bacteroidetes*, and *Actinobacteria*. Bands associated with *Clostridium* sp., *Anaerotruncus* sp., *Nitrosospira* sp., and *Arthrobacter* sp. were identified among the detected sequences.

Bioinformatic analysis

Alpha and Beta Diversity

Figure 2 shows a comparative analysis of alpha diversity, measured by observed amplicon sequence variant (ASV), Shannon and Simpson indices, between CG and PG on days 0, 21, and 42 of the *in vivo* trial. No significant differences were observed in any of the indices between CG and PG at any time point ($p > 0.05$). Supplementary Figure S9 presents the alpha diversity analysis for the same indices (Observed, Shannon, and Simpson) within CG and PG on days 0, 21, and 42. No significant temporal differences were detected for Shannon or Simpson diversity indices in either group. However, the Observed ASV

index showed a tendency to vary over time in both groups ($p < 0.10$). PCA analysis based on CLR-transformed data did not reveal significant differences between CG and PG on days 0 and 21 ($p > 0.05$) (Fig. 3A-B). However, a tendency toward separation between groups was observed on day 42 ($p < 0.10$) (Fig. 3C). Figure 4 presents the beta diversity analysis within each group across the three time points. Temporal differences in microbial community structure were observed within both CG and PG throughout the experimental period ($p < 0.05$), suggesting progressive microbiota shifts during post-weaning development. Weighted and unweighted UniFrac analyses did not reveal significant differences between groups at any sampling time (Supplementary Figure S10). However, temporal differences in microbial community structure were observed within both CG and PG across the experimental period in weighted and unweighted UniFrac analyses (Supplementary Figure S11).*Microbial relative abundances*

Figure 5 illustrates the relative abundance of bacterial phyla in the CG and PG at days 0, 21, and 42, showing the 12 most represented phyla detected in the samples (Fig. 5A-B). In both groups, *Firmicutes*, *Bacteroidota*, and *Proteobacteria* were the dominant phyla throughout the experimental period. A tendency toward differences between groups was observed for the phylum *Synergistota* on day 42 ($p < 0.10$). Statistical comparisons of relative abundances at the phylum level are presented in Supplementary Table S5.

Genus-level relative abundance profiles are presented in Fig. 6, showing the 20 most abundant genera detected at each sampling time, representing more than 60% of the total genus-level relative abundance in the analysed samples. Several genera showed tendencies toward differential relative abundance between groups across the experimental period ($p < 0.10$).

On day 0, *Campylobacter* and *Klebsiella* showed higher relative abundance trends in the PG, whereas *Succinivibrio* and *Megasphaera* tended to be more abundant in the CG. On day 21, *Ligilactobacillus*, *Campylobacter*, and *Butyricimonas* showed higher relative abundance trends in the PG, while *Acidaminococcus* and HT002 tended to be more abundant in the CG. On day 42, *Angelakisella*, [*Eubacterium*] *siraeum* group, and *Peptoclostridium* showed higher relative abundance trends in the PG, whereas *Streptococcus*, *Sphaerochaeta*, and *Mogibacterium* tended to be more abundant in the CG. Statistical comparisons of relative abundances at the genus level are presented in Supplementary Table S6.

Genus level taxa enriched by LefSe

Figure 7 shows the taxa enriched at the genus level in both CG and PG on days 0, 21, and 42. Statistical significance and corresponding LDA scores are detailed in Supplementary Table S7.

On day 0, *Campylobacter* and *Klebsiella* were enriched in the PG, whereas *Succinivibrio* and *Megasphaera* showed the highest enrichment in the CG (Fig. 7A). On day 21, *Ligilactobacillus* and *Campylobacter* were the most enriched genera in the PG, while *Acidaminococcus* and HT002 were enriched in the CG (Fig. 7B). On day 42, [*Eubacterium*] *siraeum* group and *Angelakisella* showed enrichment in the PG, whereas *Streptococcus* and *Sphaerochaeta* were the most enriched genera in the CG (Fig. 7C).

Prediction of Metabolic Pathways

A total of 102 predicted metabolic pathways were identified and compared between CG and PG at days 0, 21, and 42. Most pathways were associated with metabolic functions, including amino acid, carbohydrate, lipid, and energy metabolism. No statistically significant differences in predicted pathway abundances were observed between groups at any sampling time ($p > 0.05$). A heatmap summarizing pathway relative abundances is presented in Fig. 8, while the LEfSe statistical analysis results are shown in Supplementary Table S8.

Discussion

The stability of LAB counts observed throughout the experimental period agrees with previous studies reporting limited changes in cultivable LAB populations following probiotic supplementation. Dang et al. (2024), for example, did not observe differences in LAB counts after probiotic administration, suggesting that culture-dependent methods may underestimate subtle microbial variations. In contrast, Lee et al. (2021) reported increased LAB counts following supplementation with microbial additives, while Pan et al. (2017) demonstrated recovery of LAB populations after enterotoxigenic *E. coli* challenge in probiotic-treated animals. These contrasting findings highlight the variability of probiotic responses depending on the strain used, experimental conditions, and analytical methodology.

L. reuteri DSPV002C was recovered from supplemented animals from day 7 onward and remained detectable throughout the experimental period, indicating gastrointestinal transit and persistence during supplementation. Similar observations were reported by Hu et al. (2022), who detected increased counts of supplemented *Lactiplantibacillus plantarum* in pigs receiving probiotic administration. Although the present study did not demonstrate significant changes in overall LAB counts, the recovery of the supplemented strain suggests its ability to persist within the intestinal environment during the post-weaning period.

No significant differences were detected in *Campylobacter* spp., *E. coli*, or yeast populations between experimental groups. Similar findings were reported by Morales-Partera et al. (2020), who observed limited effects of probiotics on *Campylobacter* counts in pigs. In contrast, Bratz et al. (2015) described inhibitory activity of *Lactobacillus* spp. against *Campylobacter* under *in vitro* conditions, likely associated with organic acid production and pH reduction. Likewise, Hu et al. (2022) reported no differences in *E. coli* populations after probiotic supplementation, whereas Lee et al. (2021) and Yang et al. (2019) described reductions in coliform counts depending on probiotic formulation and experimental conditions. These discrepancies suggest that the effects of probiotics on specific microbial groups may vary according to strain composition, environmental context, and host-related factors.

DGGE sequencing profiles identified several bands associated with members of the *Firmicutes* and *Bacteroidetes* phyla throughout the experimental period, consistent with previous reports describing these phyla as dominant components of the porcine intestinal microbiota (Mach et al., 2015; Brousseau

et al., 2015). *Actinobacteria* were also detected on days 21 and 42, in agreement with observations by Aydin et al. (2015) and Loof *et al*. (2014), although their relative contribution appears to vary among studies and experimental settings.

Several sequences identified by DGGE were associated with members of the *Lachnospiraceae* family and *Clostridiales* order, taxa commonly related to carbohydrate fermentation and short-chain fatty acid production in the porcine gut ecosystem (Biddle et al., 2013; Deusch et al., 2015). These bacterial groups are frequently reported as relevant components of intestinal microbial metabolism during post-weaning development.

Probiotic supplementation did not result in significant differences in DGGE-derived diversity indices between groups. Temporal clustering of DGGE profiles was observed between day 0 and later sampling points, suggesting progressive restructuring of the fecal microbial community during post-weaning development. Similar temporal shifts have been described by Konstantinov et al. (2006), who associated microbial restructuring with the transition from milk-based to solid diets after weaning.

Regarding 16S rRNA gene sequencing diversity analyses, no significant differences in alpha diversity indices were detected between groups throughout the study. Similar findings were reported by Vieira et al. (2021) and Li et al. (2024), whereas Duddeck et al. (2023) and Sun et al. (2023) observed diversity changes after probiotic supplementation. These contrasting observations likely reflect differences in probiotic strains, dietary conditions, animal age, and experimental design.

PCA analyses suggested modest differentiation in microbial community structure between groups on day 42, while temporal differences were observed within both groups throughout the post-weaning period. Similar temporal microbial shifts have been described in previous probiotic studies (Yang et al., 2022; Du et al., 2022). However, weighted and unweighted UniFrac analyses did not reveal significant differences between groups, indicating that microbial changes associated with supplementation were relatively subtle under the conditions evaluated.

Relative abundance analyses confirmed *Firmicutes* and *Bacteroidota* as the dominant phyla across all sampling times, consistent with previous studies in weaned piglets (Sun et al., 2023; Li et al., 2024). A tendency toward differences in *Synergistota* abundance was observed on day 42, suggesting temporal variation in less abundant microbial groups during the post-weaning period.

At the genus level, several taxa associated with microbial fermentation processes showed tendencies toward differential abundance between groups. On day 21, *Ligilactobacillus* and *Lachnospiraceae* UCG-002 showed higher relative abundance trends in the probiotic group, whereas on day 42, taxa such as [*Eubacterium*] *siraeum* group and *Peptoclostridium* tended to be more abundant in supplemented animals. Similar genera have previously been associated with intestinal microbial metabolism and fermentation processes in pigs (Apiwatsiri et al., 2022; Muwonge et al., 2021). Conversely, genera such as *Streptococcus* and *Sphaerochaeta* tended to show higher relative abundance in the control group at later sampling times.

LEfSe analyses complemented the relative abundance results by identifying taxa differentially enriched between groups across the experimental period. *Ligilactobacillus* and *Campylobacter* showed enrichment in the probiotic group on day 21, whereas [*Eubacterium*] siraeum group and *Angelakisella* were enriched on day 42. In contrast, *Streptococcus* and *Sphaerochaeta* predominated in the control group at the final sampling point. Similar microbial shifts following probiotic supplementation have been described by Duddeck et al. (2023) and Sun et al. (2023), although the biological implications of these differences remain uncertain.

No significant differences were detected in predicted metabolic pathways between groups. Similar findings have been reported in studies where modest taxonomic changes were not accompanied by detectable functional divergence. Although previous studies have described modulation of metabolic pathways following probiotic supplementation (Liang et al., 2021; Lee et al., 2022) such effects were not observed under the conditions evaluated in the present study.

The integration of cultivable methods, DGGE profiling, and 16S rRNA gene sequencing analysis provided complementary perspectives on temporal microbial dynamics during the post-weaning period. Culture-dependent techniques allowed recovery of the supplemented strain, whereas DGGE and 16S rRNA gene approaches identified broader temporal shifts in microbial community structure. Together, these observations suggest that post-weaning microbial development may be associated with modest microbiota changes during probiotic supplementation.

This study presents some limitations that should be considered when interpreting the results. 16S rRNA gene sequencing analysis were performed using a limited number of animals per group, which reduces statistical power and limits the detection of subtle microbial differences. Consequently, the findings should be interpreted as exploratory and hypothesis-generating.

The multitechnique approach employed in this study contributed to a broader characterisation of temporal microbiota dynamics by integrating culture-dependent and culture-independent methodologies. Future studies including larger cohorts and complementary approaches such as metabolomics, short-chain fatty acid quantification, and productive or physiological parameters would help clarify the biological relevance of the microbial shifts observed during probiotic supplementation.

Conclusions

The present study suggests that supplementation with *Limosilactobacillus reuteri* DSPV002C may be associated with temporal modulation of the fecal microbiota during the post-weaning period. The multitechnique approach employed allowed a broader characterisation of microbial dynamics by integrating culture-dependent and culture-independent methodologies. Although only modest microbial differences were detected between experimental groups, the findings support the relevance of exploring probiotic-associated microbiota changes during early-life dietary transitions. However, the absence of clear functional differences and the limited number of animals included in 16S rRNA gene sequencing analysis indicate that the results should be interpreted cautiously and considered exploratory. Future

studies including larger cohorts and complementary physiological, metabolomic, or transcriptomic approaches are necessary to better understand the biological significance of probiotic-associated microbial modulation in piglets.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Conceptualization: JAZ and CLS; Methodology: JAZ, CLS, MJR, FAL; Software: NDP, JAZ; Validation: LSF, LPS; Formal analysis: JAZ, CLS; Resources: LMC, CLS, LP; Writing – Original Draft Preparation: JAZ, CLS; Writing – Review and Editing: MVZ, LSF, LPS; Visualization: NDP, JAZ; Supervision: LPS; Project Administration: LPS; Funding Acquisition: LSF, LPS. All authors have read and agreed to the published version of the manuscript.

Data Availability

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

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Ethics Approval

All animal procedures were approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE) of INTA CRBAN (Instituto Nacional de Tecnología Agropecuaria, Centro Regional Buenos Aires Norte) under protocol 2019/61.

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Figures

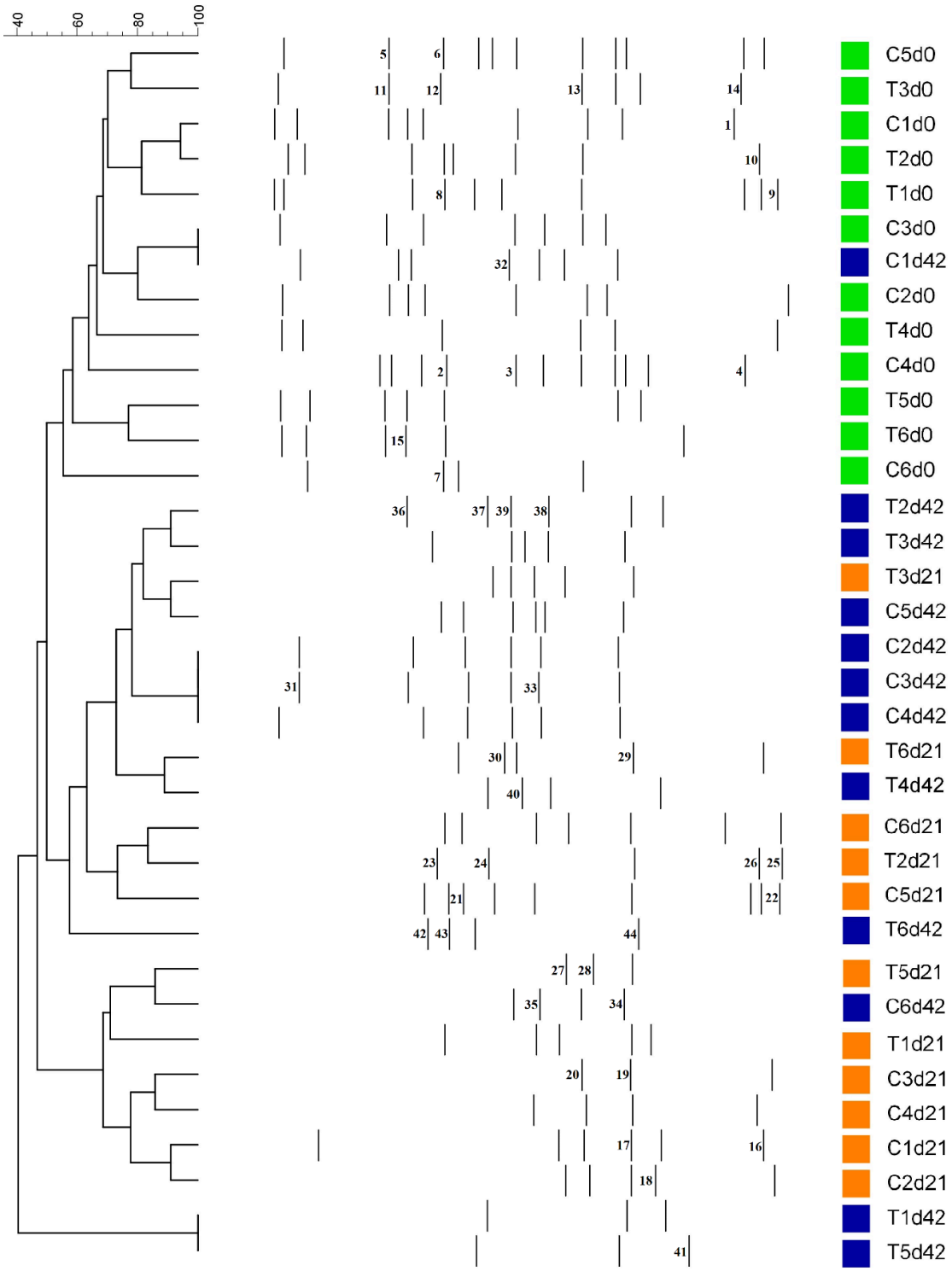


Figure 1

Cluster analysis of denaturing gradient gel electrophoresis profiles of fecal microbiota of piglets fed *Limosilactobacillus reuteri* DSPV002C strain. The dendrogram was band-based and was constructed using unweighted pair group method with arithmetic means in BioNumerics software. Band profiles obtained from piglet fecal samples with bands selected for sequencing (numbers). Control group: C1-6; Probiotic group: T1-6. Day 0 green (d0); day 21 orange (d21); day 42 blue (d42).

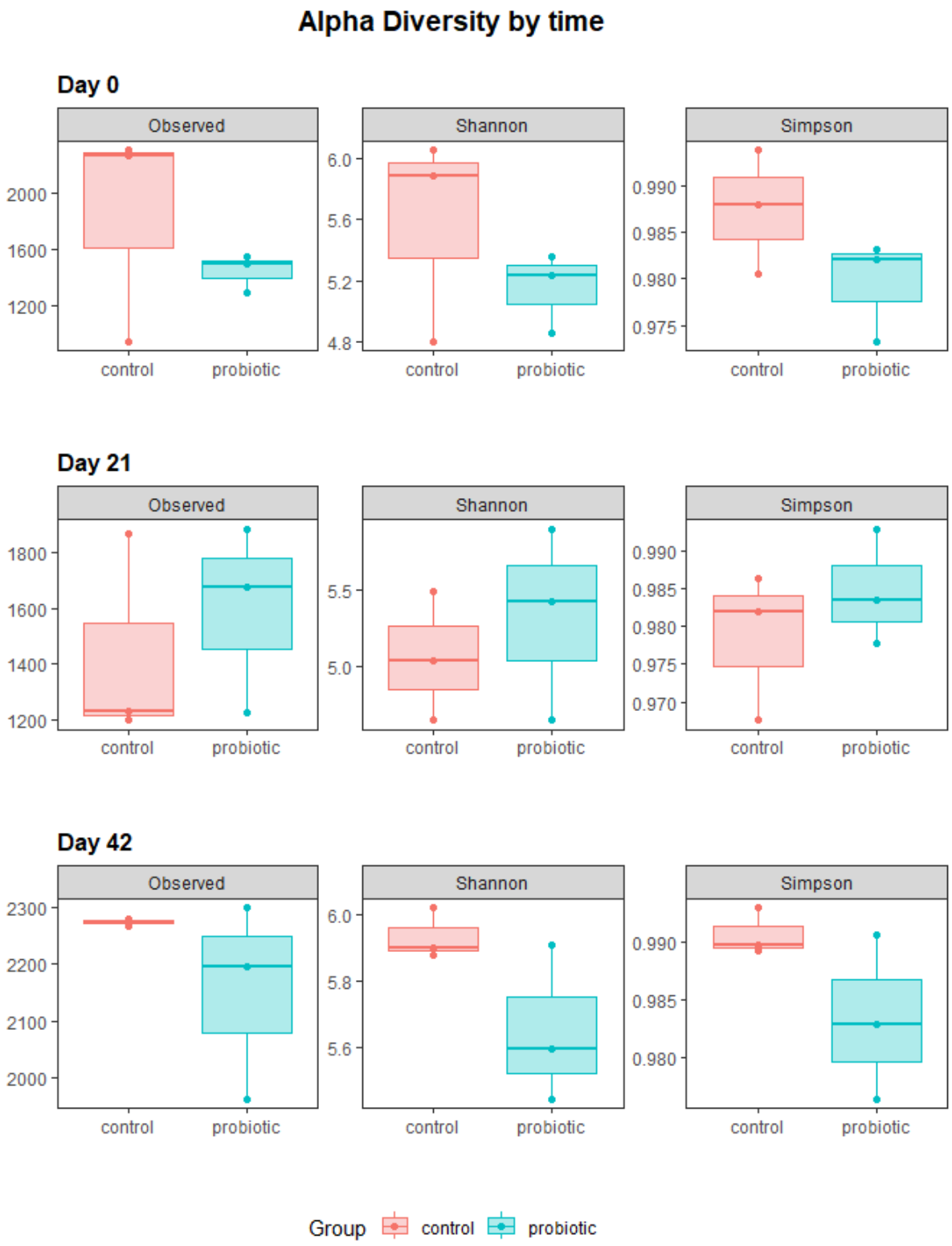


Figure 2

Alpha diversity analysis between the control group (control) and the probiotic group (probiotic). Box plots showing the observed ASV index (Observed), the Shannon index (Shannon), and the Simpson index (Simpson) for each group. Control group (pink) and probiotic group (sky blue) for days 0 (Day 0), 21 (Day 21), and 42 (Day 42).

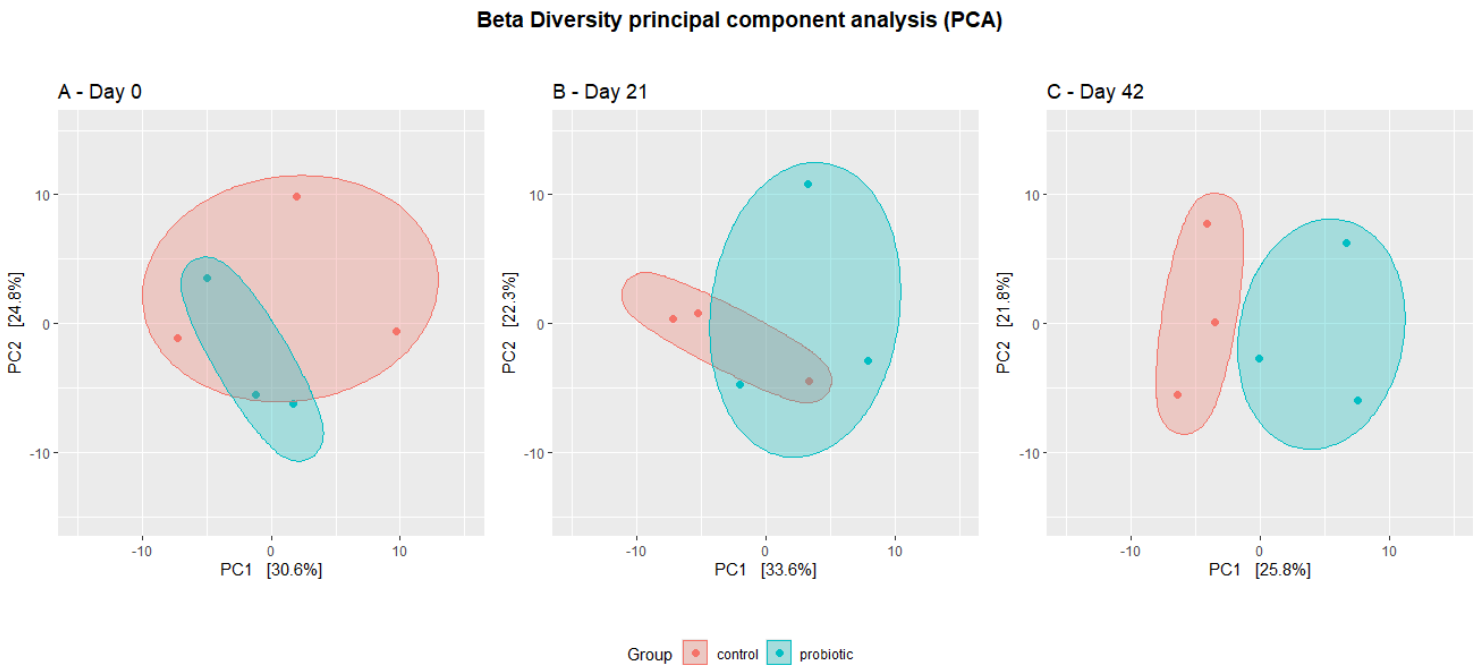


Figure 3

Beta diversity analysis. Comparison of microbiota profiles between control group (pink) and probiotic group (sky blue) using principal component analysis. The two principal components (PC1 and PC2) are presented for day 0 (A), day 21 (B), and day 42 (C).

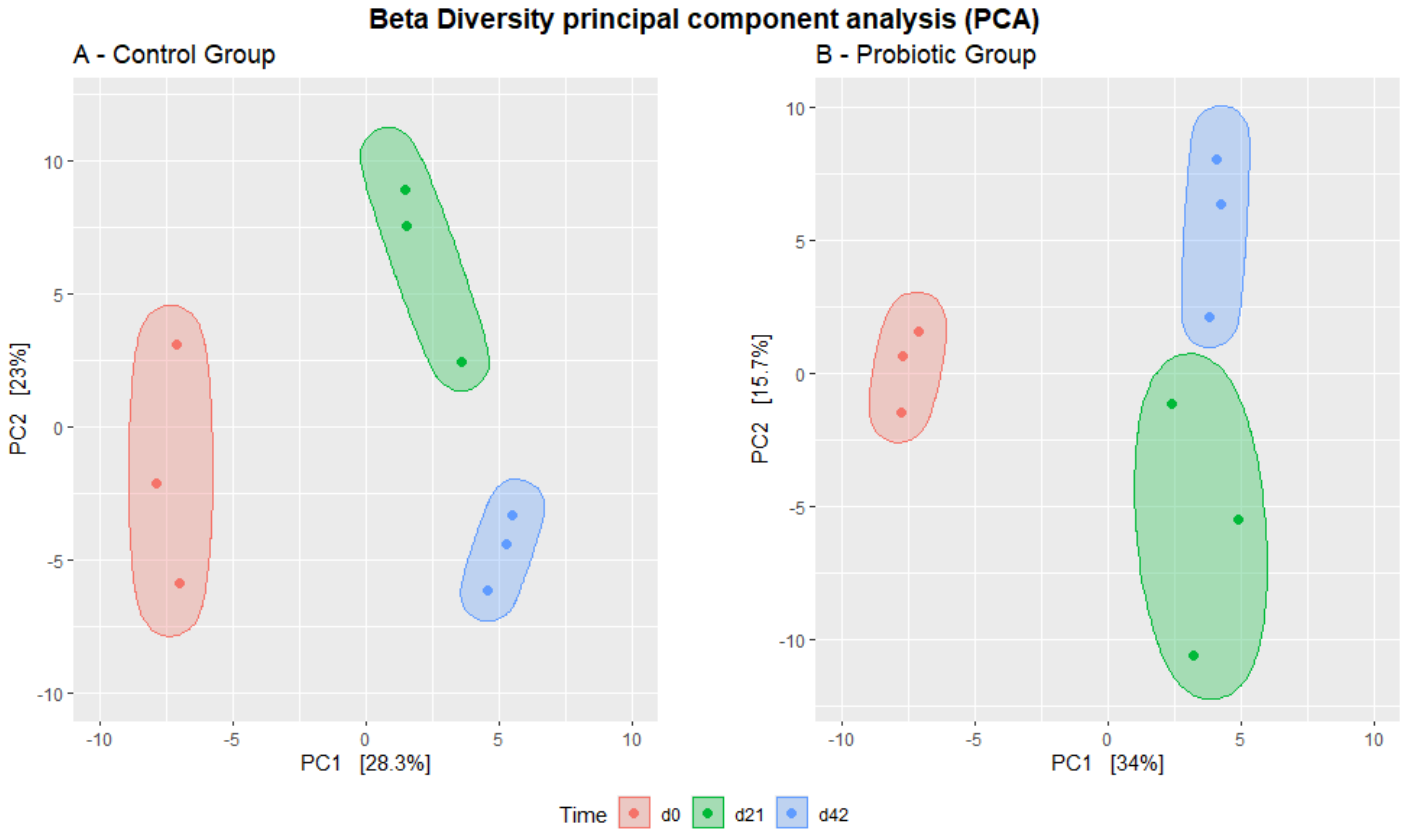


Figure 4

Beta diversity analysis. Comparison of microbiota profiles per treatment day, day 0 (pink), day 21 (green), and day 42 (sky blue) using principal component analysis. The two principal components (PC1 and PC2) are presented for control group (A) and probiotic group (B).

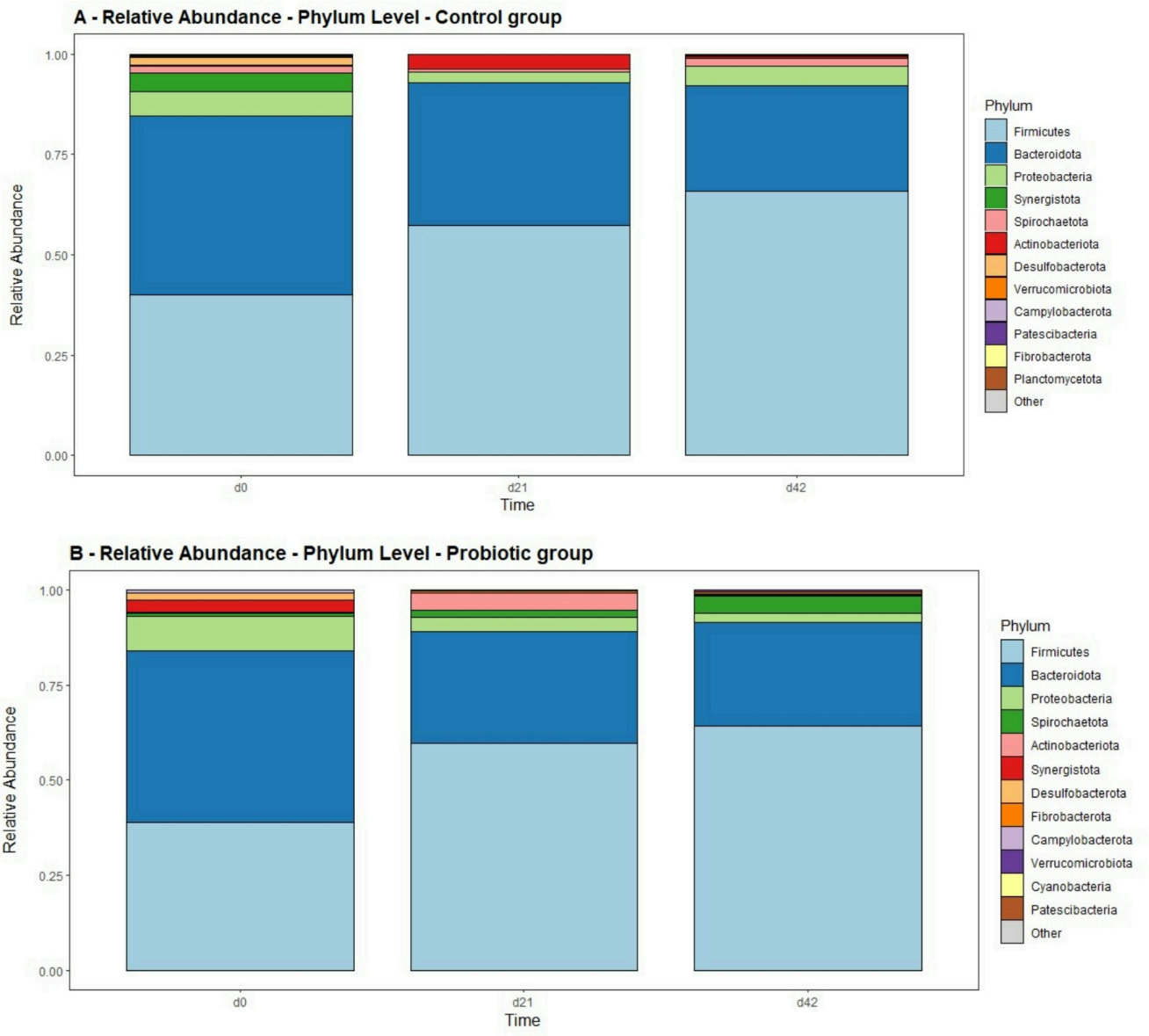


Figure 5

Effect of probiotic supplementation over time. Microbiota composition at phylum level in control group (A) and probiotic group (B) at days 0, 21 and 42. The 12 main phyla found in the samples are presented. The rest are presented as “others”.

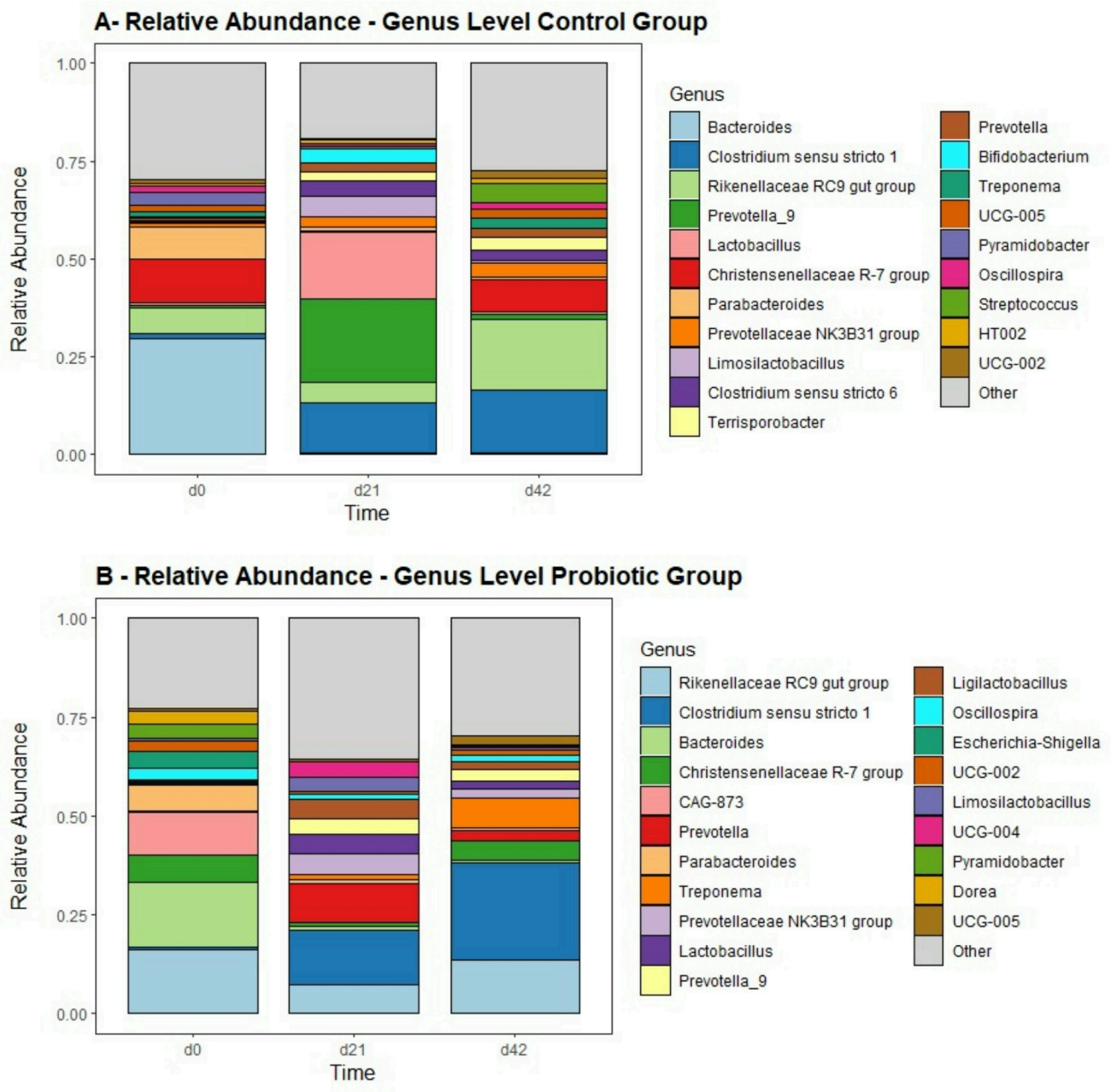


Figure 6

Effect of probiotic supplementation over time. Composition of the microbiota at the genus level in the control group (A) and probiotic group (B) at days 0, 21 and 42. The top 20 genera found in the samples are presented. The rest are presented as “others”.

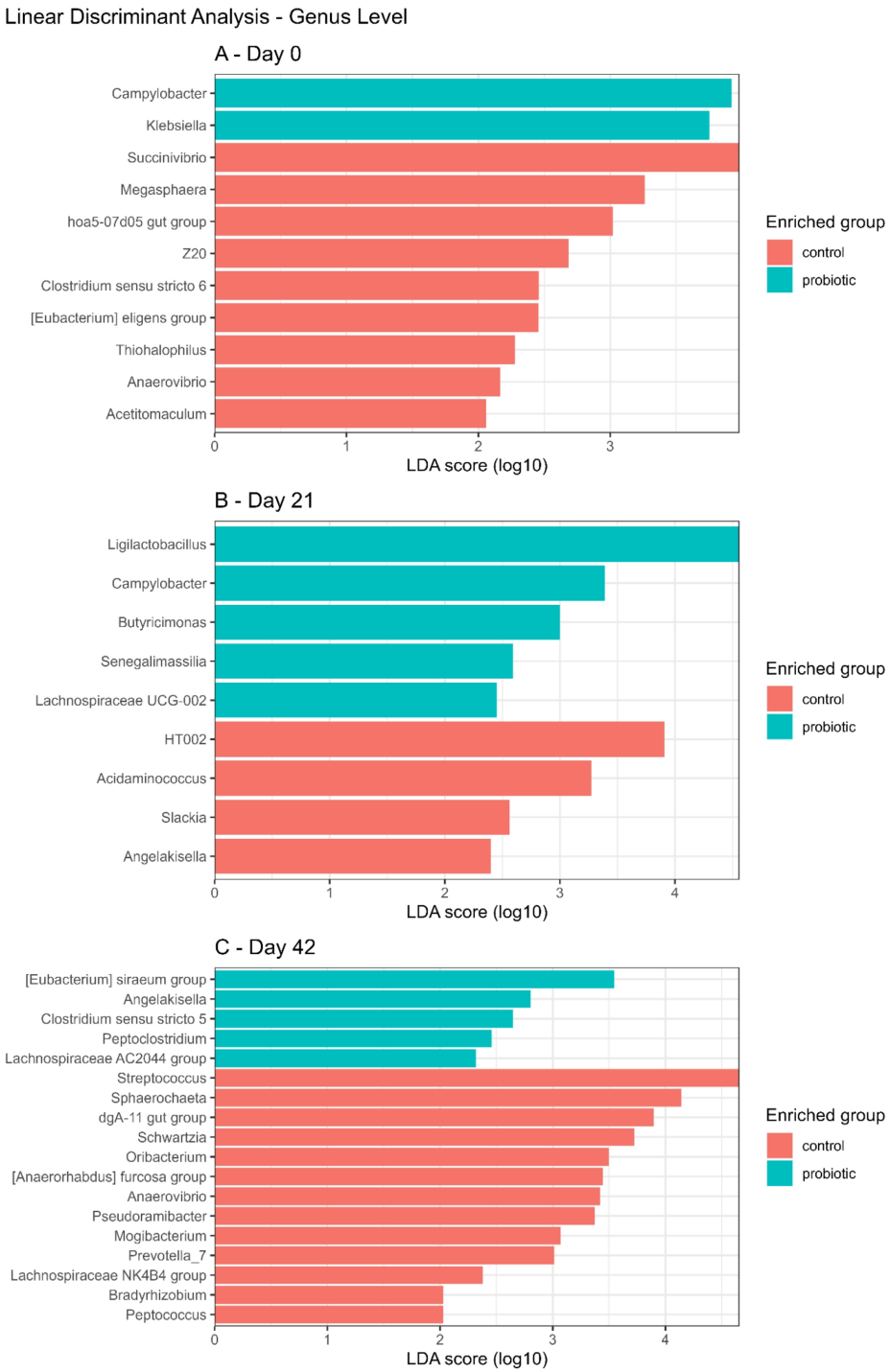


Figure 7

Linear discriminant analysis (LDA) with effect size measurements (LEfSe) at the genus level over time. The bar charts represent the taxa that are significantly different between the control group (pink) and the probiotic group (blue) on days 0 (A), 21 (B) and 42 (C).

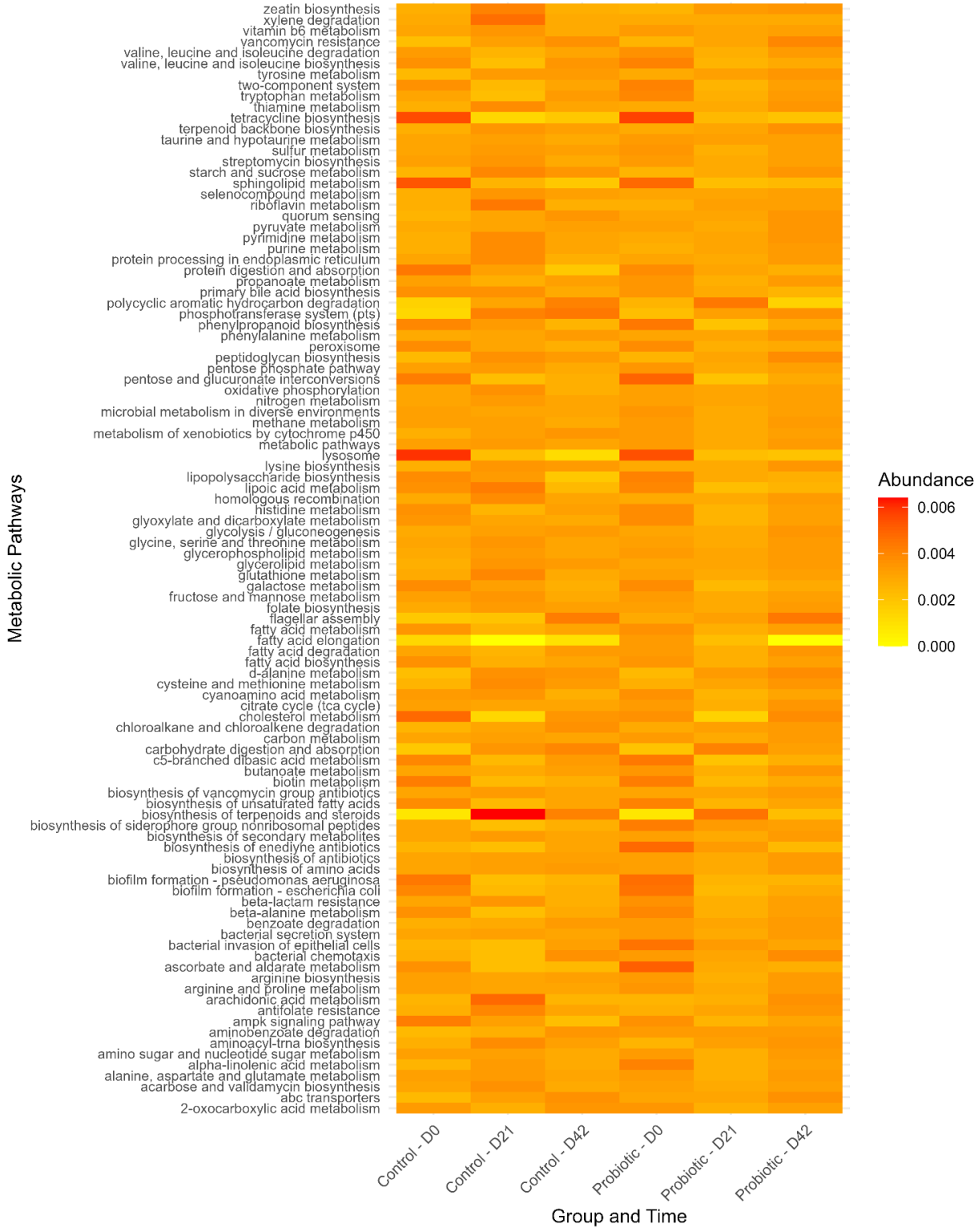


Figure 8

Heatmap of the relative abundances of predicted metabolic pathways in the gut microbiome. Each row represents a metabolic pathway, and each column represents a sample group at a specific time point: Control (D0, D21, D42) and Probiotic (D0, D21, D42). The color gradient (yellow to red) indicates the relative abundance of each pathway, with yellow representing lower abundance and red representing higher abundance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx