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Comparative Analysis of Gut Microbiota in Krškopolje Pigs Reared Under Distinct Organic Farming Systems

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ABSTRACT

Previous studies have revealed that both the production environment (conventional versus pasture-based) and the co-housing of pigs with other farm species can alter gut microbial communities. The present research involved two separate experiments aimed at profiling the 16S fecal microbiota of the native Krškopolje pig breed. Each experiment included three treatment categories: (i) pigs raised on pasture together with small ruminants (group L1, farm 1), (ii) pigs kept indoors without any contact with other livestock (group L2, farm 2), receiving the same organic diet as the remaining groups, and (iii) pigs raised outdoors in shared pastures with cattle (group L3, farm 2). Sampling occurred four times throughout the grower–finisher stage, aligning with seasonal variation. In total, 18 animals were assessed in the first trial and 22 in the second. Alpha-diversity values did not differ notably across groups or age categories. Across all groups, *Firmicutes* and *Bacteroidota* dominated the microbial composition. Clear microbial shifts were associated with age (nonparametric MANOVA, $p < 0.008$) in both trials. Microbial patterns in L1 differed significantly from L2 and L3, while the latter two were more closely aligned. Findings suggest that the specific trial setup and farm location exert a stronger impact on gut microbial profiles than housing type alone.

Keywords: 16S rRNA gene, Gut ecosystem, Krškopolje pig, Organic rearing, Microbial diversity

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Introduction

The intestinal microbial ecosystem of pigs is essential for maintaining optimal health and productive performance. Beneficial impacts include improved immune function, enhanced digestion and nutrient uptake, preservation of intestinal integrity, and lower infection rates [1]. A wide array of factors can shape microbial structure in pigs, such as age, breed, diet, feed composition, medication, physiological status, and the general husbandry system [2–6].

Conventional pig farming typically involves high-density indoor confinement and mechanized care, conditions that may increase stress, disease occurrence, and reliance on antibiotics—thus promoting antimicrobial resistance [7, 8]. In contrast, organic husbandry practices are framed as more sustainable and eco-friendly, focusing on animal welfare through reduced crowding, outdoor access, natural behavior expression, and organic feeding. The expansion of organic livestock production is a key objective of the EU's "Farm to Fork" policy under the Green Deal, which targets converting 25% of EU farmland to organic systems [9]. Such systems promise reduced chemical inputs, limited antimicrobial use, improved welfare, and greater biodiversity.

European organic regulations (Council Regulation 2007/834/EC; Regulation 2018/848) impose tight restrictions on synthetic additives and medical treatments. Notably, free-ranging pigs raised under organic standards display distinct gut microbiomes and resistomes compared with those reared in conventional indoor setups [6]. Interestingly, antimicrobial resistance genes (ARGs) are often found in higher abundance among organically managed pigs [6, 8, 10]. Outdoor housing also enables pigs to engage in natural foraging and extended lactation, supporting better welfare outcomes [11]. Studies have likewise shown that pigs kept on pasture or straw bedding experience higher well-being than those confined to slatted pens [12]. However, outdoor systems pose specific challenges in management and health control, indicating that every housing model presents its own trade-offs regarding welfare, safety, and operational practicality [7].

Mixed free-range systems and study background

Production models that merge organic pig farming with other animal species or crops have the potential to strengthen animal health, enrich biodiversity, and support both ecological and financial sustainability. Despite these advantages, such integrated systems have rarely been explored in detail. A recent investigation by Zhang *et al*. (2022) reported that when pigs share space with goats, microorganisms can be exchanged between the two species — suggesting that social interactions themselves shape the gut microbial environment [13].

The Krškopolje pig, Slovenia's sole indigenous breed, is recognized for its exceptional meat quality and tolerance to demanding or changing conditions. Earlier comparisons between organic and conventional Krškopolje pigs focused on host gene expression [14] and physicochemical as well as sensory traits of raw tissues [15] and fermented meat [16]. Although organic herds performed similarly to conventional ones in productivity, their meat and fat characteristics showed measurable differences [15, 16].

The current work aimed to investigate the gut microbiome of organically managed Krškopolje pigs kept under two contrasting conditions:

i. pigs raised indoors without interaction with other animals, and
ii. pigs grazing outdoors together with additional livestock.

Our main goal was to monitor how the microbial community changed over time and space and to determine how production style influences intestinal composition. As far as available literature suggests, this is the first scientific description of the microbiota associated with organic Krškopolje pigs.

Materials and Methods

This research examined both indoor and outdoor populations of the Krškopolje breed maintained under organic certification. Three environmental settings were evaluated to study spatial differences in gut microbes:

1. Group L1 – pigs living on pasture with small ruminants,
2. Group L2 – pigs confined indoors without other animal contact but fed the same organic feed as the remaining groups, and
3. Group L3 – pigs kept outdoors in shared pastures with cattle.

Group L1 was reared on farm 1, while groups L2 and L3 belonged to farm 2. Because farm 2 supplied growers to farm 1, the genetic background of all pigs originated from the same source. Both farms were situated in the Primorsko-notranjska region and were approximately 13 km apart (straight-line distance). Every group was offered the same commercial organic diet. Prior to the beginning of the experiment, all pigs underwent antiparasitic treatment, were vaccinated against *Erysipelothrix rhusiopathiae*, and examined by a veterinarian at each sampling period to ensure good health.

To track seasonal variation, the same animals were sampled four times across the year (S1–S4), each representing a distinct growth stage and season. The first collection occurred in winter when pigs were roughly 10 weeks old, while the final sampling took place in autumn, at about one year of age. For data reproducibility, two independent experiments were carried out in 2022 and 2023, each involving a new batch of pigs raised under identical conditions.

At the start of each trial, 10 pigs per group were selected and repeatedly sampled throughout the study to limit internal variability. Losses due to natural death or slaughter reduced the final number of individuals. The definitive number of animals and collected fecal samples for each experiment and treatment group are presented in **Table 1**.

Table 1. Overview of pigs included in each group and trial. In total, 160 fecal samples were analyzed for microbial community composition.

Experiment	Pen A1	Pen A2	Pen A3	Total Animals	Total Samples*
1	10	4	4	18	72
2	7	7	8	22	88
Total	17	11	12	40	160

Note: Because each animal was sampled four times, the total sample count equals the number of pigs multiplied by four.

Study design

This research was carried out on organic Krškopolje pigs, the only native Slovenian breed, reared under both indoor and outdoor systems. To examine spatial variation in gut microbial structure, animals were divided across three rearing conditions:

i. pigs grazing with small ruminants (group L1),
ii. pigs kept indoors with no exposure to other livestock and given the same organic feed as all other groups (group L2), and
iii. pigs pastured together with cattle (group L3).

Group L1 animals were maintained on farm 1, while groups L2 and L3 were housed on farm 2. Since farm 2 supplied young pigs to farm 1, all individuals originated from the same breeder. Both farms were positioned within the Primorsko-notranjska region, about 13 km apart (aerial distance). All pigs received identical commercial organic feed throughout the experimental period. Before data collection began, animals were treated for parasites, vaccinated against *Erysipelothrix rhusiopathiae*, and underwent clinical checks at every sampling point to confirm they were in good health.

To capture temporal trends in microbial composition, the same animals were sampled four times per year, each corresponding to a specific season and age group (S1–S4). Sampling started in winter with pigs approximately 10 weeks old (grower stage) and concluded in autumn with animals around one year old (finisher stage). To confirm reproducibility, two independent trials were implemented over two consecutive years (2022 and 2023) at both farms, each with new batches of pigs.

At the beginning of each trial, 10 pigs per group were chosen and repeatedly sampled during the entire study to reduce intra-group variation. Some losses occurred due to natural death or slaughter, resulting in slightly lower final counts. The total number of animals and fecal samples analyzed in each trial and group are listed in **Table 1**.

Sample collection, DNA extraction, and 16S rRNA gene sequencing

A total of 40 pigs were sampled rectally, producing 160 fecal samples in total **(Table 1)**. Samples were collected using sterile gloves, immediately cooled in portable boxes, and transported to the laboratory on the same day. There, they were transferred into 2-ml Eppendorf tubes and stored at –80 °C.

DNA extraction followed a modified version of the QIAamp Fast DNA Stool Mini Kit (Qiagen) protocol for pathogen detection. Frozen samples were thawed and vortexed for 1 min, after which 1 ml of each was moved into tubes containing ≤106 µm glass beads (Sigma-Aldrich, USA). Mechanical lysis was carried out using a MagNA Lyser (Roche Diagnostics, Germany) at 7000 rpm for 70 s. From the resulting mixture, 400 µl of supernatant was transferred to sterile 1.5-ml tubes, incubated at 95 °C for 5 min, and briefly vortexed. Total DNA was then purified according to the kit protocol and kept at –70 °C until sequencing.

Amplification targeted the V3–V4 region of the 16S rRNA gene, with approximately 50,000 reads per sample, using Illumina paired-end sequencing (2 × 250 bp). The primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were applied for PCR. A negative control was included, which yielded no detectable amplicons or sequence reads, confirming the absence of contamination during extraction and amplification. The raw data are publicly accessible in the NCBI SRA under BioProject PRJNA1097363.

Bioinformatic analyses

Raw sequence data were processed using mothur v1.48.0 [17] following the MiSeq SOP (https://mothur.org/wiki/miseq_sop/), with several minor modifications. Reads were aligned against the SILVA SSU 138.1 reference database, and the pre.cluster command was executed with diffs = 4. Sequences were grouped into operational taxonomic units (OTUs) using the cluster.split command at a 0.03 cutoff.

For diversity analysis, datasets were rarefied to 36,648 reads per sample. The following alpha-diversity indices were computed: Shannon, Simpson, Chao1, and the observed OTU count. Statistical analysis and visualization were performed in R v4.2.1, where principal coordinate analysis (PCoA) was generated based on Bray–Curtis dissimilarities using the MicrobiotaProcess package [18]. AMOVA and HOMOVA tests were subsequently performed within mothur to assess molecular variance and homogeneity, respectively.

Results and Discussion

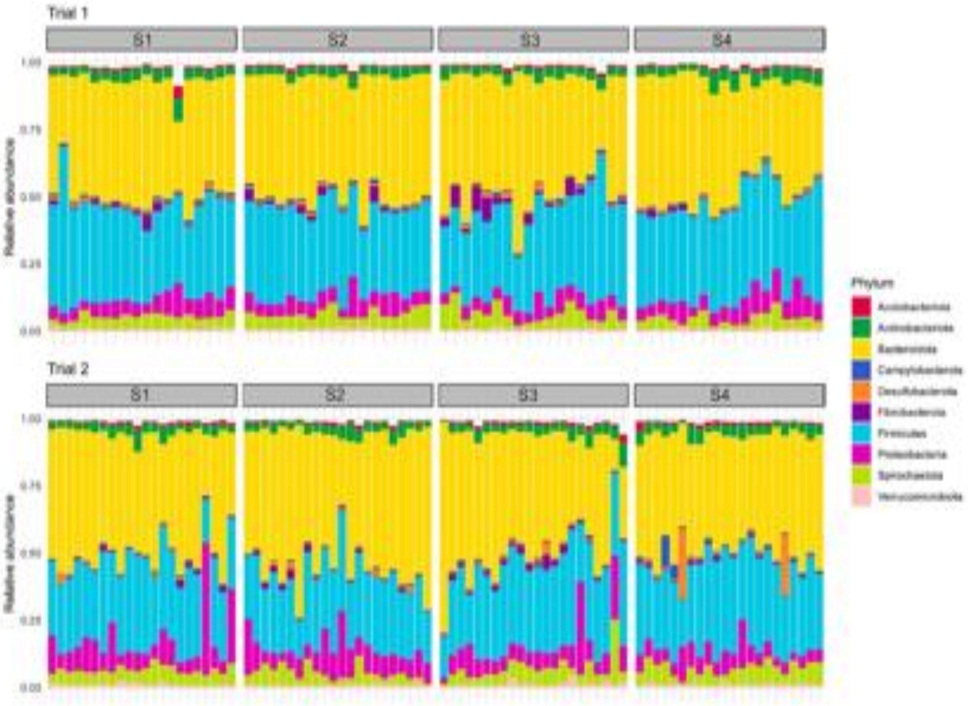
Taxonomic composition

Marked differences were detected in the microbial assemblages between the two independent experimental runs, as supported by HOMOVA (B = 0.4467, $p < 0.001$) and AMOVA (Fs = 6.0840, $p < 0.001$) tests. Owing to this variation, each trial was evaluated and displayed separately in the following analyses.

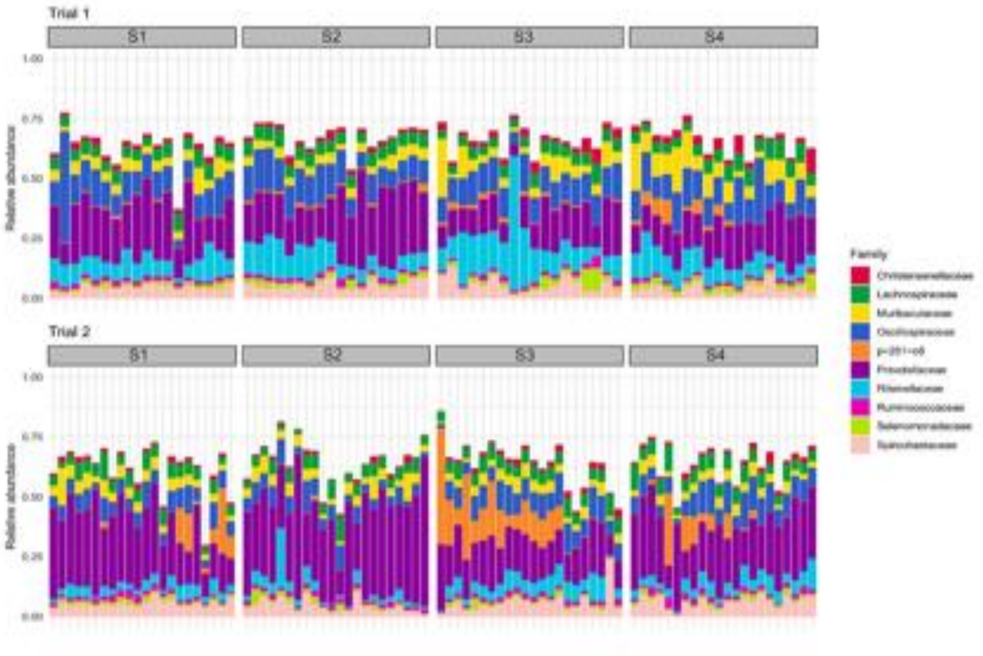
Figure 1 summarizes the community structure across phylum, family, and genus classifications. The dominant bacterial phylum across samples was Bacteroidota (mean relative abundance = 0.45), followed by Firmicutes (0.32) and Proteobacteria (0.08). The Firmicutes:Bacteroidota ratio averaged 0.71. On the phylum scale, patterns remained nearly identical between the two trials and were unaffected by pig age categories **(Figure 1a)**.

Among the ten families with the greatest representation, members belonged mainly to Firmicutes (Oscillospiraceae, Lachnospiraceae, Christensenellaceae, Ruminococcaceae, Selenomonadaceae), Bacteroidota (Prevotellaceae, Rikenellaceae, Muribaculaceae, and p-251-o5), and Spirochaetota (Spirochaetaceae). Prevotellaceae had the highest frequency, while Oscillospiraceae ranked second. Family-level shifts were more noticeable between trials and across age stages. Specifically, p-251-o5 and Prevotella 9 showed greater presence in trial 2, whereas Rikenellaceae and Oscillospiraceae predominated in trial 1 **(Figure 1b)**.

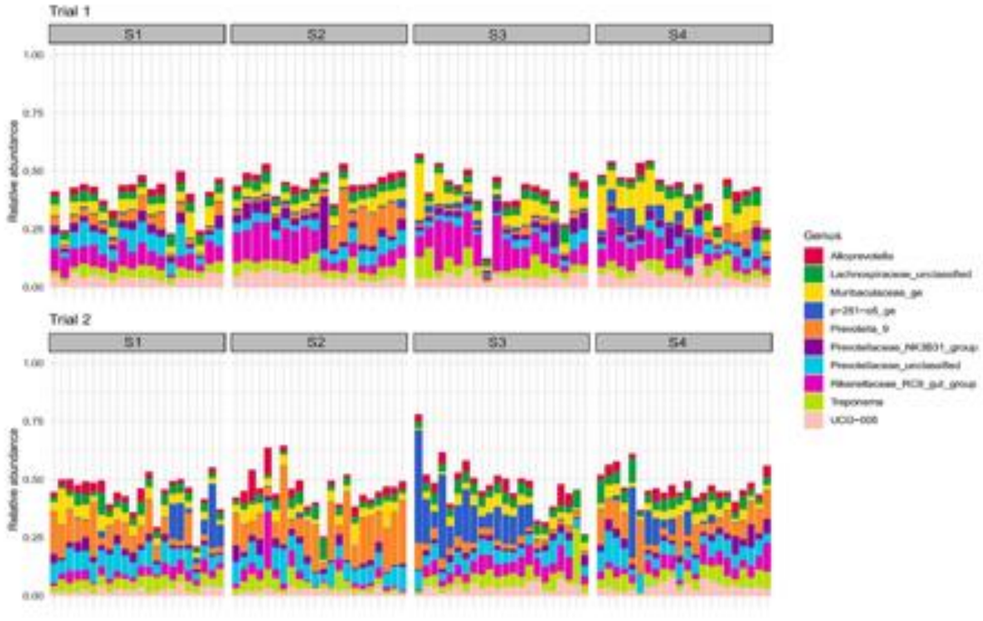
At the genus level, compositional divergence became more evident, both across trials and between age groups **(Figure 1c)**. The genera Rikenellaceae RC9 gut group, Muribaculaceae ge, and Ruminococcaceae UCG-005 consistently ranked as the three most abundant across all samples.



a)



b)



c)

Figure 1. Relative abundance of fecal microbial communities at (a) phylum, (b) family, and (c) genus scales. Only the ten most prevalent taxa are visualized. Residual taxa were merged as “Other,” hence bar totals do not equal 1.0.

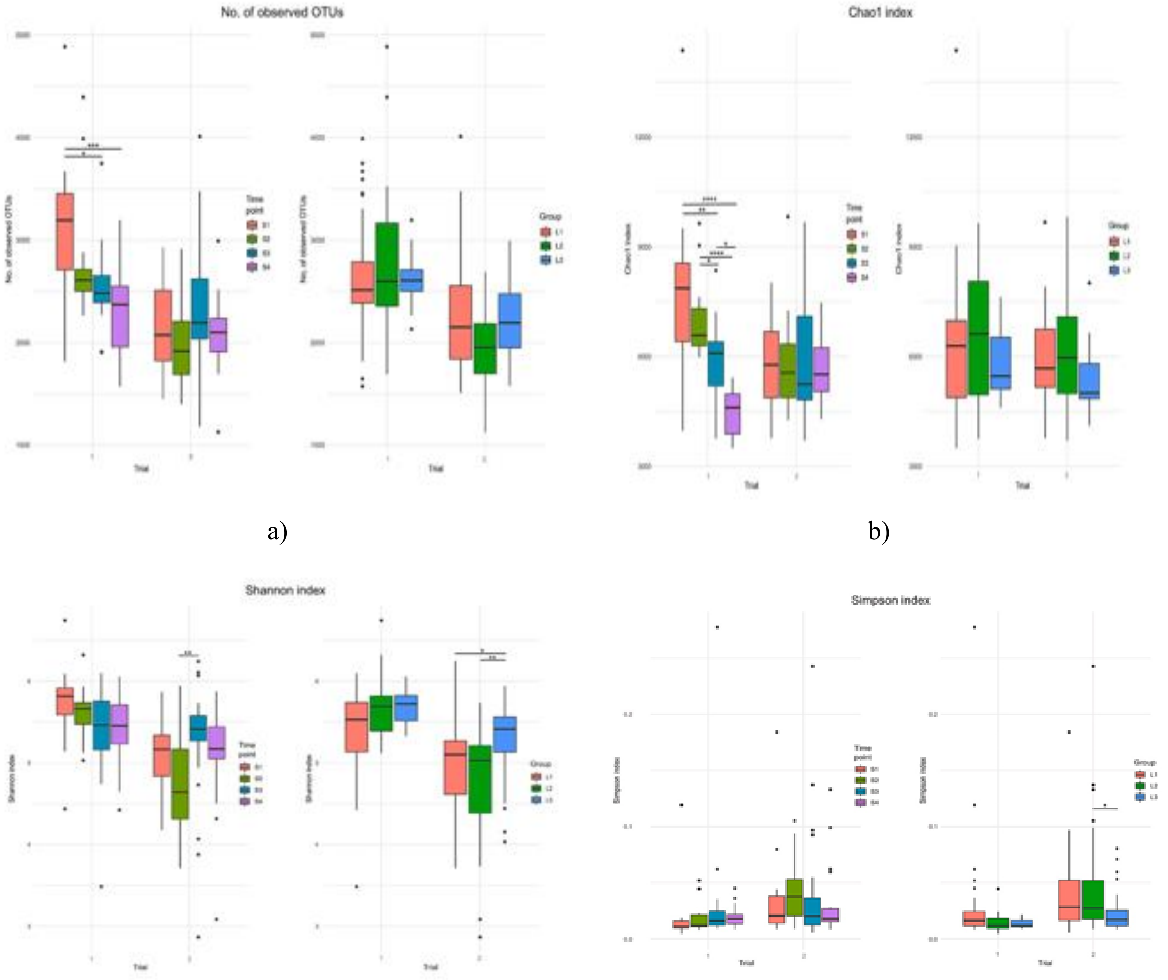
Alpha diversity

Alpha diversity outcomes are presented in **Figure 2**. The mean number of observed OTUs was significantly greater in trial 1 (2660 ± 600) than in trial 2 (2136 ± 473), according to the Wilcoxon test ($p < 0.001$). Rarefaction analysis confirmed adequate sequencing coverage, as nearly all curves approached saturation, implying comprehensive representation of sample diversity.

In trial 1, the observed OTUs dropped with increasing pig age, while in trial 2 the pattern remained stable **(Figure 2a)**. The Chao1 estimator did not differ significantly between trials ($p = 0.0830$), but, similar to OTU richness, declined over time only in trial 1 **(Figure 2b)**.

The Shannon diversity index was higher in trial 1 than trial 2 (p < 0.001), though it stayed relatively constant across age categories **(Figure 2c)**. Conversely, the Simpson index was lower in trial 1 (p < 0.001) yet remained steady over time **(Figure 2d)**. Altogether, the data suggest greater richness and evenness in trial 1, but comparable alpha diversity among study groups **(Figure 2)**.

Microbial richness in younger pigs (~10 weeks; S1) was found to be similar—or occasionally higher—than in older fatteners (~1 year; S4), suggesting that gut diversity remained consistent and resilient throughout the growth-to-fattening stages.



a)

b)

c)

d)

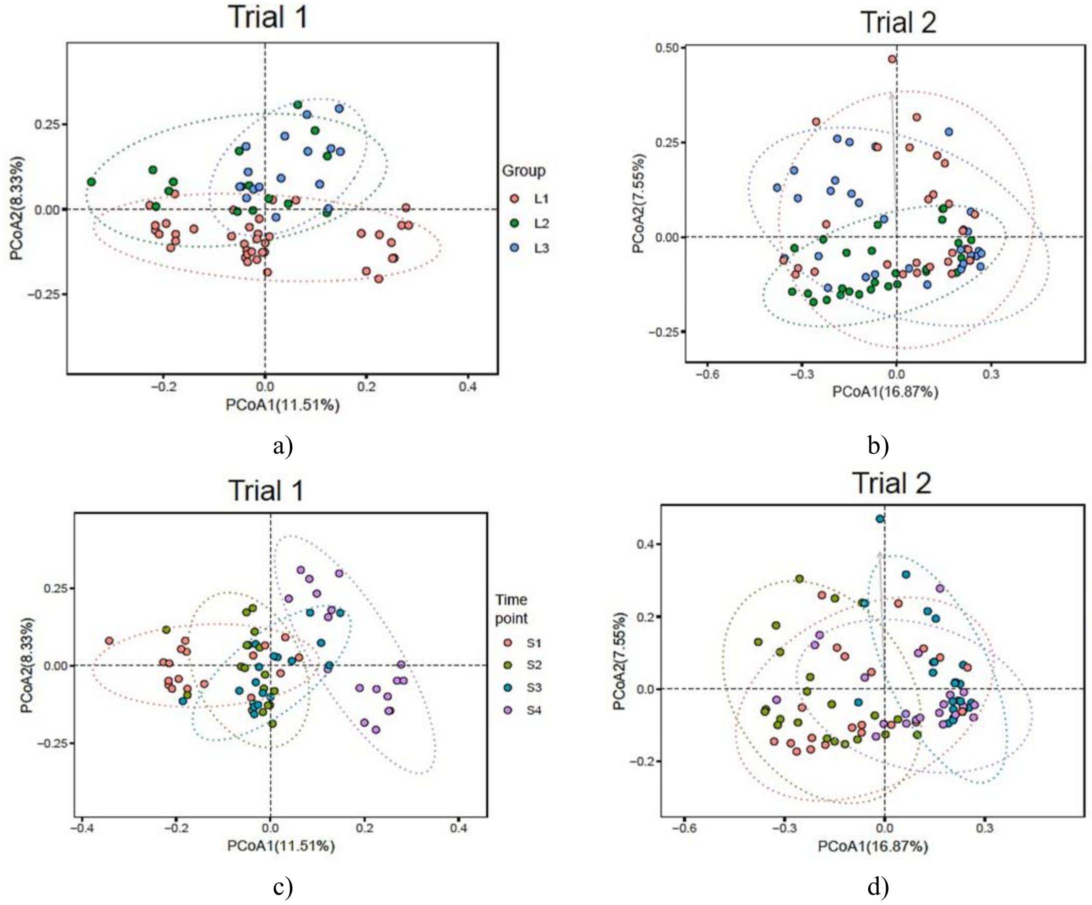
Figure 2. Boxplots illustrating alpha diversity metrics for both trials, displayed by sampling season (left) and study group (right). (a) Observed OTUs; (b) Chao1 index; (c) Shannon index; (d) Simpson index. Significance based on Wilcoxon tests: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001.

Beta diversity

The principal coordinate analysis (PCoA) results for both experimental trials are illustrated in **Figure 3**. In trial 1, the three experimental groups exhibited distinct separation across the PCoA axes, while in trial 2, the distance between groups L1 and L2 appeared less pronounced **(Figure 3)**. The sample distribution in trial 1 followed a clear pattern based on age category, whereas such clustering was less distinct in trial 2. These visual patterns were supported by the pairwise ADONIS test, which indicated that in trial 1, group L1 differed significantly from groups L2 and L3 (adjusted p = 0.003), while the comparison between L2 and L3 revealed no significant divergence (adjusted p = 0.705).

In trial 2, L1 remained statistically distinct from L2 (adjusted p = 0.006), and the difference between L1 and L3 was marginally significant (adjusted p = 0.057).

Across both trials, the microbial communities of L2 and L3 showed greater similarity in structure when compared with L1. Moreover, when evaluating pigs across age categories, noticeable temporal shifts were recorded in the microbiota structure, as all pairwise age comparisons displayed significant variation (*adjusted* $p \leq 0.03$).



a) b)

c) d)

Figure 3. Principal coordinate analysis (PCoA) plots derived from Bray–Curtis dissimilarity for both trials. Samples are colored by study group (upper plots) and sampling time point (lower plots). The explained variance (%) for each axis is shown beside the axis label.

Understanding how different housing environments influence the pig gut microbiome is essential for enhancing animal health, reducing antibiotic dependency, boosting efficiency, and supporting sustainable pig production. This work represents the first exploration of the fecal microbiota of Krškopolje pigs reared under varying housing systems, highlighting that the farm of origin plays a major role in shaping gut microbial composition.

At the phylum level, the intestinal microbiota was dominated by Bacteroidota and Firmicutes, aligning with previous findings in both commercial and local pig breeds [4, 19–24]. Notably, Bacteroidota emerged as the most prevalent phylum in this research and in the Duroc × Iberian crossbred study by Sebastià *et al*. (2024), while earlier reports typically identified Firmicutes as the dominant group [4, 19–25].

At the family rank, Prevotellaceae showed the highest abundance, followed by Oscillospiraceae and Rikenellaceae. The prominence of Prevotellaceae agrees with earlier reports in commercial and indigenous pig breeds [19, 20, 23, 24, 26]. Conversely, Clostridiaceae, Lactobacillaceae, and Streptococcaceae—all belonging to Firmicutes and often described as common in commercial breeds [19, 20, 26]—were absent from the ten most dominant families in this study. These families are typically associated with protein fermentation and the formation of branched-chain short-chain fatty acids (BCFAs) [27].

At the genus level, the Rikenellaceae RC9 gut group was the most prevalent, followed by Prevotella 9 and an unclassified Prevotellaceae genus, all of which belong to the order Bacteroidales. The RC9 gut group has been characterized as part of the core porcine microbiome and the dominant genus in the Duroc × Iberian hybrid [23], yet it has not been identified as predominant in other breeds [4, 22, 24, 28]. Meanwhile, Clostridium and Ruminococcus—key Clostridiales genera and frequently cited as core taxa in pig gut microbiota [4, 28]—were not included among the ten leading genera in this study or in Sebastià *et al*. (2024) [23]. Similarly, Lactobacillus and Streptococcus, both Lactobacillales members often observed in adult pigs [4, 26, 29, 30], were not abundant in either dataset.

Nevertheless, such taxonomic discrepancies are unlikely to translate into major functional variation, given the redundancy within the gut microbiome. For instance, species within Lachnospiraceae, Prevotellaceae, Rikenellaceae, and Ruminococcaceae can ferment complex host-indigestible carbohydrates, generating short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which are critical for metabolic regulation and intestinal barrier integrity [27, 31, 32].

The microbial ecosystem in Krškopolje pigs shows a closer resemblance to that of the Duroc × Iberian crossbreed than to other porcine lines across multiple taxonomic hierarchies. Like the Iberian and other traditional breeds, Krškopolje pigs exhibit a pronounced capacity for intramuscular and subcutaneous fat accumulation compared to modern commercial types [23, 33]. Recent investigations into Duroc × Iberian hybrids revealed that *Prevotella* and *Rikenellaceae RC9 gut group* may serve as microbial signatures linked to fatty acid profiles in both muscle and backfat tissues [23]. These taxa were likewise found in high abundance within the Krškopolje microbiome. Further investigation is needed to verify whether similar functional associations exist in this breed.

This research encompassed animals from early growth (sampling point S1) through the late fattening phase (sampling point S4). Across both trials, the overall α-diversity — measuring microbial richness and evenness — showed no significant differences among age categories, apart from the number of observed OTUs, which was higher in growers than in fatteners in trial 1 but unchanged in trial 2. Consequently, microbial diversity and richness remained generally constant between these two physiological stages. Earlier publications have reported inconsistent trends, with gut diversity either rising [4] or declining [29] as pigs mature.

Multivariate ADONIS testing revealed distinct microbial assemblages between the two age groups, supporting earlier research that reported compositional separation between growing and finishing pigs [4, 20, 29]. However, bacterial genera such as *Clostridium*, *Streptococcus*, and *Lactobacillus*—commonly identified as age-associated in previous literature [4, 29]—were not among the dominant taxa in the current dataset. Prior analyses have also emphasized that breed identity can substantially shape gut microbial profiles [20, 34].

In this investigation, no marked differences in community composition were found between the indoor-kept pigs (group L2) and the outdoor group (L3) from farm 2, although both differed notably from the outdoor pigs (L1) originating from farm 1. These findings suggest that the specific farm environment and husbandry personnel might exert a stronger influence on the microbiome than housing type. This observation agrees with previous conclusions that inter-farm variability exceeds within-farm differences [35, 36]. Since the L2 pigs were kept in spacious, straw-bedded barns exceeding national space requirements, their welfare likely did not differ substantially from that of the outdoor animals (L3) on the same farm. Earlier evidence has shown that pigs maintained on deep-litter systems display greater exploratory behavior than those kept conventionally [37]. Moreover, because L2 pigs were isolated from other livestock species, it can be inferred that mixed-species rearing exerts minimal influence on intestinal microbial structure. All animals received the same organic feed and were offspring of the same sows, minimizing dietary and genetic variability.

Notable differences in microbial profiles also appeared between the two experimental trials, despite identical pig suppliers and farm settings. This pattern highlights a strong "trial effect," a phenomenon previously documented yet often neglected in microbiome research [38]. Hence, repeated trials are essential for confirming consistent microbial trends—or such consistency may not emerge due to the inherently dynamic nature of the microbiome. Furthermore, both farm identity and management staff had measurable effects on microbial structure. Collectively, factors such as experimental trial, animal age, and farm of origin should be carefully controlled in future microbiome investigations.

Conclusion

This study offers the first comprehensive overview of the gut microbial landscape of organically farmed Krškopolje pigs. Significant shifts in community composition were observed between growers and fatteners, as well as across trials. However, α-diversity metrics—reflecting richness and evenness—remained generally stable throughout both developmental phases. Combined with the pigs' overall good health, these findings imply that the Krškopolje gut microbiota is resilient under organic rearing conditions. Additionally, in both experimental rounds, the microbiota of indoor and pasture pigs from farm 2 were more alike than either was to the outdoor group from farm 1, underscoring the pronounced role of farm-specific factors. Future studies examining host–microbiome interactions should account for these environmental and experimental influences.

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Ethics Statement: None

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