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Dynamic picture of the pig gut's microbiota under normal and pathological conditions

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Abstract

Background Recent advancements in sequencing technologies and associated bioinformatic tools have eased the analyses of the factors influencing variability in host-associated microbial communities in the gastrointestinal tract (GIT). Although extensive research has focused on fecal microbiota, the small intestine represents a critical, yet less explored, site for understanding the interplay between microbiota, diet, and host health. Study 1 employed CapSa, a non-invasive sampling capsule, to collect microbiota at five distinct administration time points. The aim was to use amplicon sequencing to investigate changes in the small intestine microbiome composition throughout the grower-finisher pig lifespan. Study 2 examined the long-term impact of enterotoxigenic *Escherichia coli* (ETEC) F4 infections, which cause post-weaning diarrhea (PWD), on small intestine microbiota dynamics. The study provides insights into the long-term responses of microbiota after a short pathological challenge.

Results In both studies, microbiota analysis of the small intestinal content revealed that Firmicutes predominated across all samples, and at weaning, Lactobacillaceae and Lactobacillus were the most abundant. In Study 1, following CapSa administration, *Clostridium sensu stricto* 1 and *Terrisporobacter* increased with age/body weight and at slaughter, Streptococcaceae dominated. Significant differences in microbial composition were observed based on sample type and diet, indicating dynamic shifts throughout the pigs' lives under normal conditions. In Study 2, the abundance of Lactobacillaceae was consistently lower in ETEC-infected pigs. At slaughter, only minimal differences in microbial composition emerged based on the early post-weaning infection status in specific small intestine segments, indicating dynamic infection-induced shifts in the gut microbiota composition. The CapSa sampling method was successful, with a retrieval rate higher than 70% in both studies.

Conclusions This study monitored porcine intestinal microbiota dynamics using an ingestible capsule. In healthy pigs, microbial composition changes occurred from post-weaning to slaughter. In contrast, ETEC infection only minimally altered communities, though small differences at slaughter suggest lasting impacts.

Keywords Microbiota, ETEC infection, Capsule sampling, Small intestine

Background

The largest density of bacteria is found in the gastrointestinal tract (GIT), where they produce essential nutrients such as thiamine, folate, and biotin [1] and participate in feed digestion [2]. Furthermore, GIT bacteria create an ecological barrier to pathogenic bacteria by stimulating the host to produce IgA and antimicrobial proteins [3]. Intestinal bacteria also influence central physiological functions, such as lymphatic tissue development,

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mucosal tolerance induction, angiogenesis, and fat storage [4].

However, understanding fluctuations in microbiota composition over time and the diversity among individuals poses constraints on microbiome experimentation [5]. Advances in sequencing and computational tools have opened up a new era of research, promoting our understanding of the parameters that influence the variation of host-associated microbial communities [4]. The temporal stability of an individual's gut microbiome remains a critical question [6]. Shotgun sequencing has revealed that over a five-year period, an individual conserves on average 60% of ~200 phylotypes (highest taxonomic level) [7]. At lower taxonomic levels, each individual's microbiome may be as unique as a fingerprint [8], although its stability remains unclear and poses methodological challenges [9]. In pigs, fecal microbiome research indicates relative stability at approximately three months of age, suggesting that gut microbiome development and maturation are primarily age-driven [10]. By contrast, the development of small intestine (SI) microbiota remains less understood due to the lack of longitudinal sampling methods [5]. There is a demand for innovative, minimally invasive techniques to study pigs' gut microbiota while ensuring animal welfare.

The swine industry and academia have increasingly focused on the interaction between intestinal health and microbiota concerning pig growth [11]. Understanding the roles of intestinal microbiota and their interactions with the host is essential in feed formulation [12]. Manipulating the intestinal microbiota can have both immediate and long-term effects on pig intestinal health [13, 14]. Although most research in this area emphasizes fecal microbiota [5, 15], it is important to note that the SI plays a crucial role in understanding the interactions between microbiota, diet, and the host, offering insights that fecal samples alone cannot provide. The SI is the primary site for digestion and nutrient absorption, harboring the majority of immune system cells [12, 16]. Studies show that microbiota from feces contain fewer microbial functions than those from the SI, indicating significant differences between the microbial profiles [15]. The pig's gut microbiota undergoes remarkable changes throughout its life, influencing the nutritional and health status of the host [17].

There is also growing interest in understanding microbiota evolution under pathological conditions, specifically following post-weaning diarrhea (PWD). PWD is of significant economic concern in global pig production, affecting pigs during the first two weeks after weaning and characterized by sudden death or diarrhea, dehydration, and growth retardation if the piglet survives [18]. PWD is commonly associated with enterotoxigenic

Escherichia coli (ETEC), with F4 and F18 being the predominant adhesins [18–21]. Recent studies indicate that PWD caused by ETEC has a lasting impact on piglet fecal microbiota [22]. Agroscope has developed and validated a PWD model using ETEC with F4 attachment factors, inducing diarrhea without causing fatalities [23].

Our first experiment (Study 1) aimed to capture a comprehensive view of the SI microbiome across various life stages, from early post-weaning to slaughter (140 ± 5 days of age) under normal conditions. We used a novel sampling capsule called CapSa, enabling the non-invasive collection of intestinal microbiota samples, and which has been validated in vitro [25] and in vivo [24]. The device opens, collects the sample and closes depending on the physico-chemical properties of its environment. It moves along the digestive tract purely passively, and the speed at which it passes depends entirely on intestinal peristalsis. This study uses amplicon sequencing to track temporal changes in species richness (alpha diversity) and community structure (beta diversity) within the same pig's small intestinal microbiota. The second study (Study 2) aimed to examine the evolution of the gut microbiota following an ETEC infection, using CapSa to sample the microbiota from the same pigs from two weeks post-infection (52 ± 3 days of age) until slaughter (140 ± 5 days of age). To the best of our knowledge, these results are the first to describe the shift in the SI microbiome following an ETEC F4 infection through repeated sampling from the same pigs.

Methods

Experimental design

A total of 48 Swiss Large White piglets were selected before weaning from 6 multiparous sows (parities 2 to 4) and allocated to the 2 studies (Studies 1 and 2 with 24 piglets each). The sows from each study were inseminated with the same boar, and no antibiotic treatments were necessary during gestation or lactation.

In Study 1, 24 piglets from four litters (12 castrated males and 12 females) were included. Eight piglets (two from each litter, including one castrated male and one female) were randomly chosen and euthanized before weaning to collect digestive tract content, establishing a microbiome baseline. The SI was separated into three segments for analysis. The remaining 16 piglets received the CapSa at five different time points: T1: 52 ± 3 , T2: 70 ± 3 , T3: 83 ± 3 , T4: 110 ± 3 , and T5: 126 ± 3 days of age (mean $\pm$ standard deviation), following the standard operating procedure for CapSa administration [26].

In Study 2, we included 24 piglets from 2 sows known to be of the heterozygote susceptible (SR) genotype, harboring a genetic marker for F4ab/ac receptor. These sows were also inseminated with the same boar (homozygote

susceptible (SS) to ETEC F4). Piglets were genotyped at 14 ± 2 days of age using a 3 mm piece of ear tissue, analyzed by PCR to determine their genotype (SS, SR, RR), as previously described [27]. From each litter, five castrated males (SR) and five females (SR) were included. Four piglets (two per litter, one male and one female) were randomly selected and euthanized before weaning to sample digestive tract content from three segments of the SI. The remaining 16 piglets were divided into two groups: 12 were infected with the Agroscope's ETEC F4 strain ("infected" [INF] group), and 4 received a placebo ("non-infected" [N-INF] group). All pigs in study 2 received CapSa at the same five time points (T1–T5) as in Study 1.

Throughout both studies, all pigs were weighed every seven days from birth until slaughter. All pigs from both studies were slaughtered at 140 ± 5 days of age.

ETEC infection

Piglets from Study 2 ($n = 12$, 6 per litter) were infected at weaning (28 days of age) by oral administration of a solution containing 10^{10} colony-forming units ETEC F4. The infective solution was administered through gelatin capsules (approx. 2 capsules per piglet) and deposited behind the tongue, using a capsule applicator. Gelatin capsules were 000 size. The health status of the piglets was regularly observed within 4 h after infection. The infective strain (35H4) was originally isolated from diarrheic piglets at Agroscope Posieux [23].

One week after infection, the piglets were allocated by body weight (BW) and litter to the INF and N-INF groups. They were allocated to a box (three piglets per box) and fed a starter diet (Supplementary Table 1, Additional File 1). Straw was provided as enrichment. In addition to daily monitoring of the pigs, fecal scores and ETEC counts were measured on day 0 (before infection) and on days 1, 2, 3, 6, and 7 post-infection.

Rearing conditions and feeding

One week before the first CapSa administration (45 ± 3 days of age), all animals were placed in boxes (total surface area of 4.47m^2) with four pigs by boxes respecting the litter origin.

For Study 1, the pigs were allocated to four pens, with four pigs from the same litter assigned to each pen. In Study 2, each litter of pigs was split into two pens two weeks post-infection. One pen housed four INF pigs (referred to as the "infected" pen), and the other pen housed a mix of two N-INF pigs and two INF pigs (referred to as the "mixed" pen).

During the whole experiment, pigs were fed standard starter, grower, and finisher diets formulated to meet their nutritional requirements [28]. The starter, grower,

and finisher diets were offered from days 25 ± 3 to days 72 ± 3 , from days 73 ± 3 to days 112 ± 3 , and from days 113 ± 3 to 140 ± 5 days of age, respectively (see Additional File 1, Supplementary Tables 1, 2, and 3). Each diet period included two capsule administrations: one at the beginning and one at the end, except for starter and the finisher diet periods, which had only a single capsule administration. Feed was provided ad libitum, except for the two days prior to the CapSa administration, when they were fed a liquid diet as specified in the capsule administration protocol [24]. Pigs had ad libitum access to water via nipple drinkers.

CapSa administration and retrieval protocol and CapSa sample collection procedure

The detailed protocol for CapSa administration, retrieval, and intestinal sample collection from CapSa has been reported in detail by Garcia-Vinado et al. [26]. Briefly, three days before CapSa administration, pigs were moved to dedicated pens. They were adapted to a liquid diet two days prior, and straw was removed from their pens. The day before administration, pigs received half their usual liquid feed and were fasted for 12 h before administration. Forty minutes prior to CapSa administration, pigs received 0.18 ± 0.02 mg/kg BW prucalopride (Resolor®, Takeda Pharma AG) by sondage. Pigs were then placed in slings and given two individually identified CapSas, followed by 10 mL of orange juice. Four hours post-administration, they had ad libitum access to feed. Three days later, the pigs returned to conventional pens. CapSa recovery from feces was monitored six times daily for 2 days. The volume of CapSa content was measured, and its pH was assessed using Litmus paper. The samples were then stored at -80°C until analysis.

Fecal score monitoring and ETEC count

The fecal score was assessed individually the day before infection and on days, 0, 1, 2, 3, 6 and 7 after infection, as well as one day after every capsule administration and at slaughter. This was determined using a 4-level scoring scale: 1 = normal (firm but not hard), 2 = soft (does not hold form, piles but spreads slightly), 3 = runny (spreads readily), and 4 = watery (liquid consistency, splatters).

The ETEC F4 infective strain used in this study was resistant to rifampicine (rif) and harbored the F4 fimbriae (K88ac+), the heat-labile toxin (LT+) and the heat-stable toxin (STb+). The amount of F4 ETEC excreted in the feces was measured by counting the number of colony-forming units per culture on an E. coli-selective medium (Eosin Methylene Blue Agar plates) containing rifampin (50 µg/mL).

Post-mortem sampling at weaning and at slaughter

For euthanasia, all pigs were stunned with CO₂ (140 ± 5 days old pigs) or with an electrical shock (28-day old piglets) and bled. The GIT was extracted. For those pigs euthanized at weaning, samples from three segments of the SI equally divided were collected in sterile 2 mL Eppendorf tubes (Eppendorf SE, Hamburg, Germany) and stored at −80°C until analysis. At slaughter, samples from six segments of the SI were collected in sterile 2 mL Eppendorf tubes (Eppendorf SE, Hamburg, Germany) and then stored at −80°C until analysis. Any CapSa that was not retrieved in the feces was searched for in the intestines (stomach, SI, or large intestine) on the day of slaughter.

DNA extraction and bioinformatics analyses

Microbiota analysis was performed on all post-mortem samples from weaning and slaughter. Additionally, one capsule expelled per pig per administration time was chosen for analysis. The selected CapSa samples had to meet the following criteria: a pH greater than 5.5 and recovery within 48 h of administration.

Bacterial DNA was extracted using the HostZERO™ Microbial DNA Kit (Zymo Research, California, USA) following the manufacturer's instructions. The DNA concentration (ng/μL) and purity (absorbance ratio 260/280 and 260/230, respectively) were verified spectrophotometrically on NanoDrop™ (Fisher Scientific, 13 Schwerte, Germany). The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified by PCR using Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy) and the universal primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAGCCTACG GGNBGCASCAG-3' and Pro805R: 5'-GTCTCGTGGGCTCGGA GATGTGTAT AAGAGACAGGACTACNVGGGTATCTAATCC-3' [29]. The PCR reaction conditions for amplification of DNA were as follows: initial denaturation at 94 °C for 1', followed by 25 cycles of denaturation at 94 °C for 30", annealing at 55 °C for 30" and extension 65 °C for 45", ending with 1 cycle at 68 °C for 7' [29]. Amplicons were then sequenced by Illumina MiSeq 300 × 2 bp with the MiSeq® V3-V4 reagent kit on the MiSeq-Illumina® platform (Illumina, USA). Microbiota analysis was performed using the DADA2 pipeline [30] according to the Silva database taxonomy, version 138.1 [31]. For the DADA2 pipeline, primers were removed from the raw sequences, and based on the average quality score, forward and reverse reads were trimmed at positions 280 and 260. All other DADA2 parameters were left in their default settings.

Statistical analysis

The statistical analysis of alpha and beta diversity and taxonomics was carried out with R (version 4.3.2), using "phyloseq" [32] v1.38, "vegan" v2.6 [33] and "microbiomeutilities" v1.0. For beta diversity at ASV level, a dissimilarity matrix using Euclidean distances of centered log ratio (clr) transformed data was constructed, and results were plotted using a Principal Coordinates Analysis (PCoA) plot. Differences were tested using a PERMANOVA model (Adonis) with 9,999 permutations, including sample type, group, and diet as factors. Pairwise contrasts among sample types were carried out using the pairwise Adonis function included in the "PairwiseAdonis" package [34]. For alpha diversity, samples were rarefied to the lowest sample read depth to avoid bias linked to different sampling efforts. Differences for alpha diversity indices (Chao1, Shannon, and Simpson diversity) between samples within the same or different time points of sampling and within groups were tested at ASV level using the Wilcoxon test. The *p*-values were then adjusted for multiple comparisons using the Bonferroni-Holm correction. Differences in the taxonomic composition between samples (Studies 1 and 2) were tested using linear discriminant analysis (LDA) effect size (LEfSe), aggregating the data at the genus level. The LDA score cut-off of 3 was used to discriminate bacterial taxa. For all statistical analyses, significance was declared if *p* < 0.05, and a trend was considered when 0.05 < *p* < 0.10.

Results

Growth parameters, fecal score and health status

All piglets in Studies 1 and 2 weighed over 1 kg at birth; the mean BW was 1.58 ± 0.24 kg and 1.70 ± 0.20 kg, respectively. None of the piglets experienced health issues before weaning. In Study 2, prior to infection, the mean BW of the N-INF and INF pigs was 9.94 ± 0.82 kg and 9.96 ± 1.13 kg, respectively. Differences in mean BW between the two groups became apparent two days after infection (30 days of age), with the N-INF group weighing 10.34 ± 0.73 kg and the INF group weighing 9.83 ± 1.05 kg. This difference persisted throughout the experiment, culminating in the N-INF group pigs being heavier at slaughter (147 days of age) than the INF pigs (117.38 ± 5.89 kg vs. 111.83 ± 8.10 kg, respectively).

Regarding ETEC count, the N-INF piglets responded accordingly to their group. For the INF group, we observed a standard peak of infection starting on day 1, followed by a collapse in the number of ETEC excreted from day 6 (Fig. 1A). The highest ETEC counts were recorded on day 1 and day 2 post-infection, with two pigs shedding at rates of 3.20 and 3.10 × 10⁷ CFU/mL, respectively.

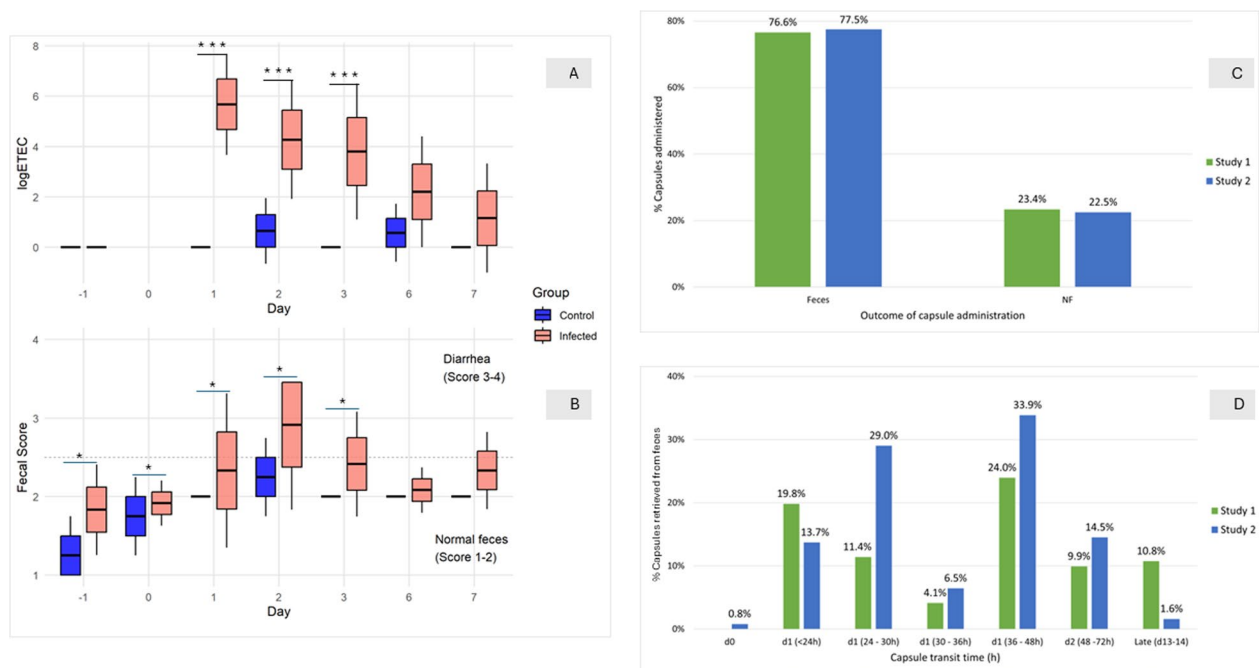


Fig. 1 ETEC infection and Capsule administration and retrieval: **A** Bacterial counts and **B** faecal scores over time in the non-infected (N-INF) and infected (INF) groups. The boxplots represent bacterial counts in colony-forming units (CFU) per millilitre (ml) and faecal scores for each group by day. **C** Fate of the capsule after administration in Study 1 and Study 2. The percentage of capsules retrieved from the faeces or not found (NF) was calculated based on the number of capsules in each category divided by the number of capsules administered. **D** Capsule transit time for capsules retrieved from the faeces in Study 1 and Study 2. The capsule's transit time was calculated as the time between capsule administration and capsule retrieval in the faeces

In Study 1, all pigs had a fecal score of 2 or less. However, in Study 2, after infection, mean fecal scores of N-INF pigs consistently remained below 3, whereas several pigs in the INF group exhibited scores of 4 on days 1, 2, and 3 after infection (Fig. 1B). Throughout the rest of the study, fecal scores for both groups remained within the range of 1 to 2, without exceeding a score of 2. No other health issues related to the administration and passage of CapSa were detected at slaughter. We considered the infection resolved after 7 days because fecal scores normalized and ETEC counts approached baseline levels, even though a few piglets still exhibited minor diarrhea.

CapSa administration and retrieval

In Study 1, all CapSas were administered as planned, except for one pig at T5, which did not receive CapSas due to respiratory distress. Out of the total CapSas administered, 121 (76.6%) were retrieved from the feces, while 23.4% were categorized as “not found” (NF). In Study 2, all capsules were successfully administered at each time point. A total of 124 CapSas were retrieved, equating to a 77.5% retrieval rate, with 22.5% marked as NF (Fig. 1C). Of the retrieved CapSas in

Study 1, 59.3% transited to the GIT within 48 h. In Study 2, 83.9% of the CapSas were recovered within 48 h post-administration (Fig. 1D).

Study 1

Microbiota analysis at weaning

From the eight animals euthanized at weaning, we were able to extract DNA from all samples ($n = 24$). The main relative abundances of the eight most abundant phyla, family, and genera in all SI segments together are shown in Fig. 2A–C, respectively. The three main phylum in every segment of the SI at weaning was Firmicutes ($98.93 \pm 6\%$), followed by *Actinobacteria* ($0.8 \pm 0.34\%$) and *Proteobacteria* ($0.07 \pm 0.02\%$). The most abundant families in all three SI segments were *Lactobacillaceae* ($57.83 \pm 7.6\%$), *Clostridiaceae* ($21.97 \pm 11.69\%$), *Streptococcaceae* ($11.29 \pm 2.46\%$), *Peptostreptococcaceae* ($4 \pm 3.18\%$), and *Veillonellaceae* ($2.12 \pm 2.98\%$). The most abundant genera were *Lactobacillus* ($35.12 \pm 12.33\%$), *Sarcina* ($21.22 \pm 16.79\%$), *HT002* ($14 \pm 4.27\%$), *Streptococcus* ($11.29 \pm 2.46\%$), *Limosilactobacillus* ($5.35 \pm 1.48\%$), *Ligilactobacillus* ($3.01 \pm 1.95\%$), *Veillonella* ($2.11 \pm 3.06\%$), *Terrisporobacter* ($2.06 \pm 2.31\%$), and *Romboutsia* ($1.87 \pm 5.65\%$).

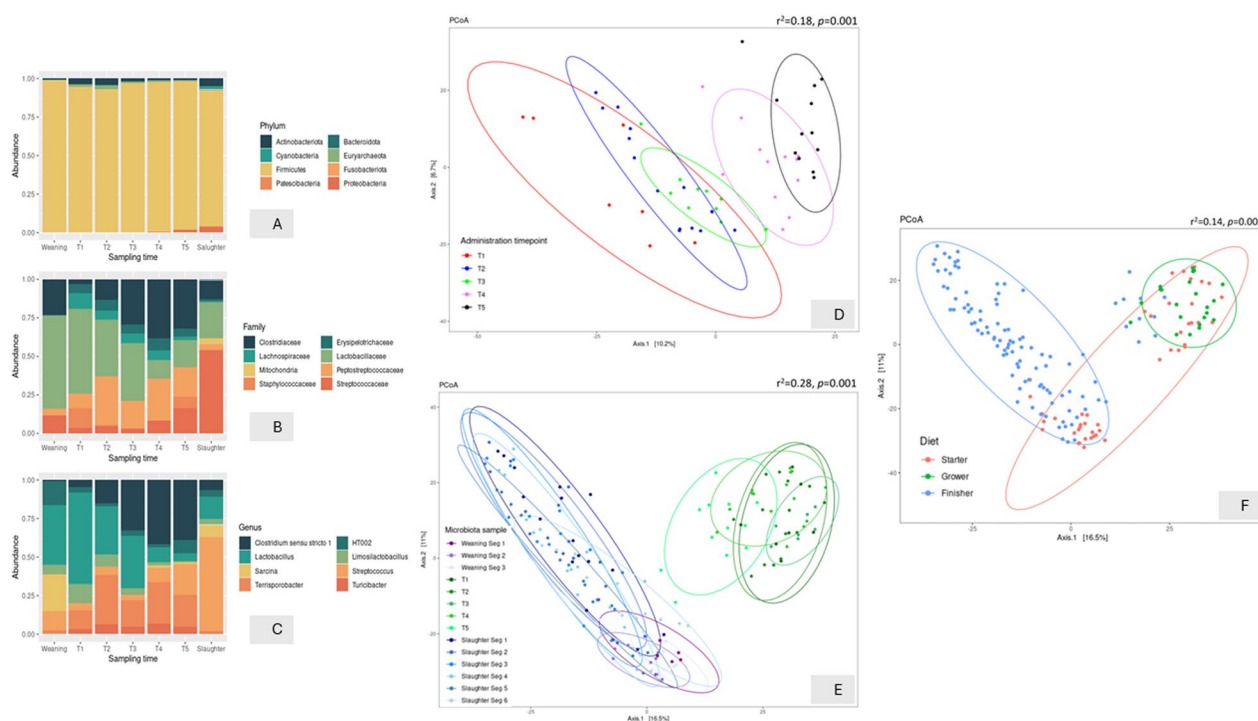


Fig. 2 Mean relative abundance and PCoA in study 1: Mean relative abundance of the eight most abundant phyla (A), family (B), and genera (C) in the small intestine microbiota content at weaning (28 days of age), at T1 (52 ± 3 days of age), T2 (70 ± 3 days of age), T3 (83 ± 3 days of age), T4 (110 ± 3 days of age), and T5 (126 ± 3 days of age), and in the small intestine at slaughter (140 ± 5 days of age) of pigs. **D** PCoA plot generated using a Euclidean distance matrix based on capsule sampling timepoints using the centred log ratio (clr) transformed data of samples from CapSas. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data, such as microbiome profiles. Samples are coloured based on the administration time of the capsules. T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age. **E** PCoA plot generated using a Euclidean distance matrix based on study sampling timepoints using the centred log ratio (clr) transformed data of all samples. **F** PCoA plot generated using a Euclidean distance matrix based on diet using the centred log ratio (clr) transformed data of samples. Samples are coloured based on diet: weaner diet offered from 25 ± 3 to 72 ± 3 days of age; grower diet offered from 73 ± 3 to 112 ± 3 days of age; finisher diet offered from 113 ± 3 days of age to slaughter

Microbiota analysis of CapSa samples

For each CapSa administration time point, only one capsule per pig was chosen for microbiota analysis. The selected CapSa needed to meet specific criteria, including a pH greater than 5.5, sufficient volume for DNA extraction, and ideally retrieved within 48 h post-administration. In cases where a pig expelled both CapSas, the capsule adhering better to these conditions was selected for analysis. From the 16 animals that were administered CapSas five times until slaughter, we were able to extract DNA from 53 of the 61 CapSas. Figure 2A–C display the predominant relative abundance of the eight most common phyla, families, and genera for each administration time.

The main phylum in every capsule, independent of administration time, was Firmicutes (95.52 ± 2.49%), followed by Actinobacteriota (2.25 ± 0.38%) and Euryarchaeota (1.30 ± 0.76%). The most abundant families in every capsule, independent of the administration time, were *Lactobacillaceae* (25.42 ± 4.27%), *Clostridiaceae*

(21.26 ± 6.52%), *Peptostreptococcaceae* (19.42 ± 6.8%), *Streptococcaceae* (6.53 ± 2.32%), *Erysipelotrichaceae* (5.54 ± 1.44%), *Lachnospiraceae* (5.03 ± 0.2%), *Ruminococcaceae* (3.33 ± 0.63%), and *Staphylococcaceae* (3 ± 5.55%). The most abundant genera in every CapSa, independent of administration time, were *Clostridium sensu stricto 1* (20.84 ± 6.73%), *Lactobacillus* (17.72 ± 6.62%), *Terrisporobacter* (17.07 ± 8.7%), *Streptococcus* (6.52 ± 2.35%), *Turicibacter* (4.04 ± 3.07%), *Limosilactobacillus* (3.51 ± 2.04%), *Staphylococcus* (2.84 ± 6.9%), and *HT002* (2.74 ± 2.87%).

Regarding the phyla, we observed that the abundance of Firmicutes, Bacteroidota, and Cyanobacteria remained constant. The abundance of Actinobacteriota decreased over time, as did that of Euryarchaeota after peaking at T2. However, Proteobacteria tended to increase from T4 to slaughter (Figs. 2A–C). As for the families, the abundance of *Lactobacillaceae*, *Staphylococcaceae*, *Lachnospiraceae*, and *Ruminococcaceae* decreased over time, while the abundance of *Peptostreptococcaceae*, *Streptococcaceae*, and *Clostridiaceae* increased. For the genera,

we have a switch in bacterial abundance from T1 to T5. At T1, the main genus was *Lactobacillus*, and its abundance steadily decreased over time, eventually replaced in T5 by *Clostridium sensu stricto 1* (see detail in Additional File 1, Supplementary Table 4).

Regarding beta diversity, the PCoA plot shows a partial clustering of the samples based on the administration time of the CapSas (Fig. 2D). A partial first cluster is formed by the content of CapSas from T1, T2, and T3, and a partial second cluster is formed by the content of CapSas from T4 and T5. The Adonis test showed a significant impact of the time point of administration on bacterial composition ($r^2=0.18$, $p=0.001$; Fig. 2D), indicating variations in bacterial composition among capsules administered at different times. Furthermore, we conducted a pairwise Adonis test to evaluate differences in bacterial composition between capsules based on administration time (Table 1). The results highlighted distinct variations in bacterial composition among CapSas administered at various time points.

Microbiota analysis at slaughter

At the time of slaughter (140 ± 5 days of age), gut contents were collected from six equally divided segments of the SI of all pigs. We were able to extract DNA from all

abundance of the eight most abundant phyla, families, and genera for SI content at slaughter.

The main phylum in every segment of SI at slaughter was Firmicutes ($88.35 \pm 3.99\%$), followed by Actinobacteriota ($4.82 \pm 0.78\%$), Proteobacteria ($3.75 \pm 1.29\%$), and Cyanobacteria ($2.11 \pm 1.6\%$). The most abundant families in all six SI segments were *Streptococcaceae* ($47.26 \pm 7.67\%$), *Lactobacillaceae* ($20.84 \pm 3\%$), *Clostridiaceae* ($11.40 \pm 4.63\%$), Mitochondria ($3.15 \pm 2.90\%$), *Peptostreptococcaceae* ($2.99 \pm 3.56\%$), *Bifidobacteriaceae* ($1.82 \pm 2.09\%$), *Gemellaceae* ($1.66 \pm 1.47\%$), and *Micrococcaceae* ($1.13 \pm 0.39\%$). The most abundant genera were *Streptococcus* ($47.25 \pm 7.71\%$), *Lactobacillus* ($11.74 \pm 4.78\%$), *Sarcina* ($6.37 \pm 6.86\%$), *Clostridium sensu stricto 1* ($5.03 \pm 3.49\%$), *HT002* ($3.61 \pm 2.32\%$), *Limosilactobacillus* ($2.71 \pm 0.93\%$), *Bifidobacterium* ($1.75 \pm 2.51\%$), and *Weissella* ($1.55 \pm 2.55\%$).

Microbiota analysis across the pig's life

To obtain a general view of the temporal shift of the microbiota through the pig's life, we performed a beta diversity analysis and an Adonis test of all samples collected during the experiment. For beta diversity, the PCoA plot shows two clusters. One cluster groups the microbiome samples from the SI segments collected at slaughter and weaning. The other clusters group the microbiome samples collected with the CapSas (Fig. 2E). The Adonis test revealed a significant impact of sample type on bacterial composition ($r^2=0.28$, $p=0.001$; Fig. 2E).

A pairwise Adonis test was used to assess bacterial composition differences in SI segments at weaning, CapSas samples across five time points, and upper (segments 1, 2, and 3) and lower (Segments 4, 5, and 6) SI segments at slaughter (Table 2). The microbiota composition differed significantly across all sample types, except for the capsules from the first two administration time points (T1 vs. T2), which were similar ($r^2=0.08$, $p=0.14$; Table 2).

We also tested the effect of diet on bacterial composition. For beta diversity, the PCoA plot shows two separated clusters based on the diet eaten at sample collection: one for the grower diet, and another for the finisher diet. The last cluster representing pigs fed with a starter diet overlaps both other clusters ($r^2=0.14$, $p=0.001$; Fig. 2F). Moreover, we performed a pairwise Adonis test to test for differences in bacterial composition between samples of the SI content of pigs fed the different diets (Table 3). The results revealed diverse microbiota compositions across the various sample types within each diet (Table 3). However, the LefSe test did

Table 1 Adonis pairwise comparisons of microbiota composition in capsule contents across administration time point in Study 1, calculated using the Euclidian distances

Comparisons between administration time points	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
T1 vs. T2	4411.61	1.82	0.08	<0.01	0.03
T1 vs. T3	5107.47	2.13	0.11	<0.01	<0.01
T1 vs. T4	7488.97	3.20	0.15	<0.01	<0.01
T1 vs. T5	9279.67	4.43	0.19	<0.01	<0.01
T2 vs. T3	4900.76	2.13	0.08	<0.01	<0.01
T2 vs. T4	6904.76	3.05	0.11	<0.01	<0.01
T2 vs. T5	8788.63	4.22	0.14	<0.01	<0.01
T3 vs. T4	4316.48	1.95	0.08	<0.01	0.01
T3 vs. T5	7164.07	3.60	0.15	<0.01	<0.01
T4 vs. T5	4875.00	2.46	0.10	<0.01	<0.01

T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age

^a SumsOfSqs = sum of square reflecting total variance

^b F.Model = F-test value

^c r^2 = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

^d p = p-value

^e p adj = p-values adjusted for multiple comparisons using the Bonferroni-Holm correction

samples ($n=93$). Figure 2A–C illustrate the main relative

Table 2 Adonis pairwise comparisons of microbiota composition between small intestine segments at weaning, capsule contents across five different administration time points and lower and upper segments of the small intestine at slaughter in Study 1, calculated using the Euclidian distances

Comparisons between samples	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
SI at weaning vs. T1	17,170.80	11.40	0.27	< 0.01	< 0.01
SI at weaning vs. T2	23,977.24	14.57	0.28	< 0.01	< 0.01
SI at weaning vs. T3	22,721.34	15.26	0.32	< 0.01	< 0.01
SI at weaning vs. T4	24,679.73	16.12	0.32	< 0.01	< 0.01
SI at weaning vs. T5	19,143.41	13.89	0.29	< 0.01	< 0.01
SI at weaning vs. upper SI at slaughter	32,285.07	18.04	0.21	< 0.01	< 0.01
SI at weaning vs. lower SI at slaughter	21,108.38	13.89	0.16	< 0.01	< 0.01
T1 vs. T2	4983.04	1.78	0.08	< 0.01	0.14
T1 vs. T3	5768.83	2.08	0.11	< 0.01	< 0.01
T1 vs. T4	8413.35	3.11	0.14	< 0.01	< 0.01
T1 vs. T5	10,435.42	4.32	0.19	< 0.01	< 0.01
T1 vs. upper SI at slaughter	20,635.74	9.03	0.15	< 0.01	< 0.01
T1 vs. lower SI at slaughter	18,467.90	9.68	0.15	< 0.01	< 0.01
T2 vs. T3	5553.35	2.09	0.08	< 0.01	< 0.01
T2 vs. T4	7787.09	2.98	0.11	< 0.01	< 0.01
T2 vs. T5	9848.06	4.11	0.14	< 0.01	< 0.01
T2 vs. upper SI at slaughter	28,808.27	12.57	0.18	< 0.01	< 0.01
T2 vs. lower SI at slaughter	25,898.15	13.27	0.18	< 0.01	< 0.01
T3 vs. T4	4896.48	1.91	0.08	< 0.01	0.02
T3 vs. T5	8104.20	3.52	0.15	< 0.01	< 0.01
T3 vs. upper SI at slaughter	25,313.75	11.27	0.17	< 0.01	< 0.01
T3 vs. lower SI at slaughter	22,761.76	12.08	0.17	< 0.01	< 0.01
T4 vs. T5	5598.64	2.44	0.10	< 0.01	< 0.01
T4 vs. upper SI at slaughter	24,253.22	10.81	0.16	< 0.01	< 0.01
T4 vs. lower SI at slaughter	22,620.38	11.94	0.17	< 0.01	< 0.01
T5 vs. upper SI at slaughter	17,080.03	7.94	0.12	< 0.01	< 0.01
T5 vs. lower SI at slaughter	15,523.90	8.60	0.12	< 0.01	< 0.01
Upper SI at slaughter vs. lower SI at slaughter	7581.34	3.88	0.040	< 0.01	0.01

T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age; slaughter age: 140 ± 5 days of age

^a SumsOfSqs = sum of square reflecting total variance^b F.Model = F-test value^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences^d p = p-value^e p adj = p-values adjusted for multiple comparisons using the Bonferroni-Holm correction

not identify any clear microbial biomarkers among the samples.

Study 2

Microbiota analysis at weaning

From the four animals euthanized at weaning, we successfully extracted DNA from all samples (n = 12). The main relative abundances of the eight most abundant phyla, family, and genera in the SI content at weaning are shown in Fig. 3A–C, respectively. The main phylum in every SI segment at

weaning was Firmicutes (98.65 ± 6.05%), followed by Proteobacteria (0.70 ± 0.82%) and Actinobacteriota (0.55 ± 0.14%). The most abundant families in all three SI segments were *Lactobacillaceae* (71.71 ± 8.79%), *Clostridiaceae* (12.61 ± 4.71%), *Peptostreptococcaceae* (7.21 ± 3.99%), *Streptococcaceae* (5.29 ± 1.41%), *Enterobacteriaceae* (0.51 ± 1.72%), *Lachnospiraceae* (0.35 ± 0.09%), *Erysipelotrichaceae* (0.34 ± 0.29%), and *Micrococcaceae* (0.34 ± 0.23%). The most abundant genera were *Lactobacillus* (42.61 ± 14.98%), *HT002* (14.99 ± 3.63%), *Limosilactobacillus* (9.90 ± 3.33%), *Sarcina* (8.83 ± 8.01%), *Streptococcus* (5.21 ± 1.43%),

Table 3 Adonis pairwise comparisons of microbiota composition between samples from pigs fed either the starter, grower, or finisher diets at various growth stages in Study 1, calculated based on Euclidean distances

Comparisons	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
Starter vs. grower	19,009.27	7.36	0.10	< 0.01	< 0.01
Starter vs. finisher	37,132.41	16.30	0.09	< 0.01	< 0.01
Grower vs. finisher	37,037.44	16.46	0.11	< 0.01	< 0.01

Weaner diet offered ad libitum from 25 ± 3 to 72 ± 3 days of age; grower diet offered ad libitum from 73 ± 3 to 112 ± 3 days of age; finisher diet offered ad libitum from 113 ± 3 to 140 ± 5 days of age (slaughter)

^a SumsOfSqs = sum of square reflecting total variance

^b F.Model = F-test value

^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

^d p = p-value

^e p adj = p-values adjusted for multiple comparisons using the Bonferroni-Holm correction

Terrisporobacter (5.21 ± 4.16%), *Clostridium sensu stricto 1* (3.78 ± 1.67%), and *Romboutsia* (1.88 ± 5.52%).

Microbiota analysis of CapSa samples

From the 16 animals that were administered capsules five times until slaughter, we were able to extract DNA from all capsules (n = 62) selected for microbiota analysis.

For each administration time, the mean relative abundances of the eight most abundant phyla, family, and genera by group (N-INF and INF) and type of box (infected or mixed) are shown in Fig. 3D–F. Regarding the phyla, the abundance of Firmicutes remained constant over time, independent of the group. As for the families, *Lactobacillaceae* abundance decreased over time, always remaining more abundant in N-INF pigs than in INF, except for T5 (2.24% vs. 5.37%, Additional File 1, Supplementary Table 5). The abundance of *Streptococcaceae* increased with increasing age. Regarding the genera, for the N-INF group, we observed a switch in bacterial abundance from T1 to T5. At T1, the main genera were *Lactobacillus* and *Terrisporobacter*, and they steadily decreased over time (since T2), eventually replaced at T5 by *Streptococcus* and *Clostridium sensu stricto 1* (Fig. 3F). In the INF group, *Lactobacillus* exhibited a lower abundance as

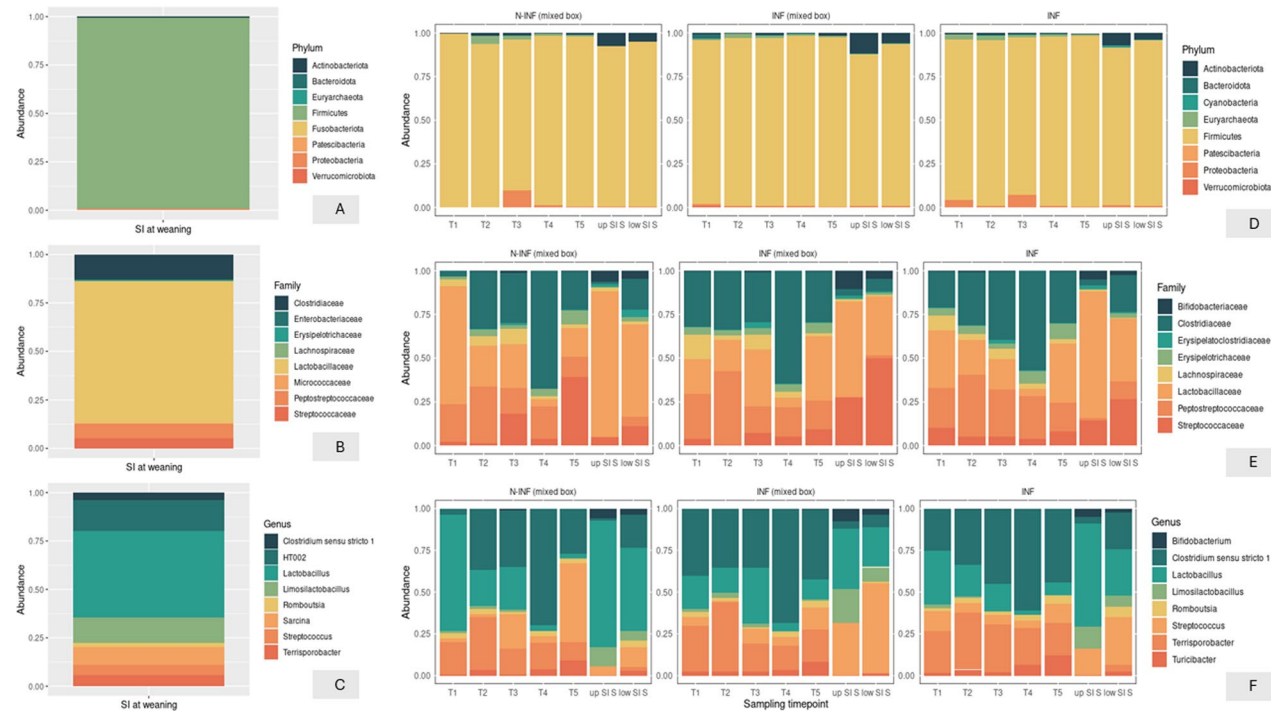


Fig. 3 Mean relative abundance of the eight most abundant phyla, family, and genera in Study 2: **A** in the small intestine microbiota content at weaning, **B** in the capsule samples and in the lower part (low SI) and upper part (up SI) at slaughter. Capsule samples were taken at T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age; low SI and up SI S: lower and upper part of the SI at slaughter at 140 ± 5 days of age; N-INF (mixed box): non-infected group reared in the same pen as infected pigs; INF: infected group; INF (mixed box): Infected pigs reared in the same pen as non-infected pigs

early as T1 and continued decreasing over time, whereas *Terrisporobacter* maintained its abundance after peaking at T2. At T5, the main genera in the INF group were *Clostridium sensu stricto 1* (26.15%) and *Pediococcus* (14.07%); however, *Streptococcus* remained lower compared to its abundance in the N-INF group (7.42% vs. 35.51%) (see details in Additional File 1, Supplementary Table 5).

For beta diversity, the PCoA plot shows a partial clustering of the samples based on the administration time of the capsules (Fig. 4A). There is an overlap between samples from T1, T2, and T3, with samples from T3 also partially clustering with T4 and a separate cluster formed by samples from T5. The Adonis test revealed that bacterial composition was affected by the administration time point ($r^2=0.18$, $p=0.001$; Fig. 4A). In agreement, the pairwise Adonis test revealed that the bacterial composition differed between the different time points (Table 4). By contrast, within sampling time, bacterial composition did not differ, regardless of the pigs' infection status ($r^2=0.01$, $p=0.64$; Fig. 4B). In agreement, the pairwise Adonis test confirmed that regardless of whether pigs were infected or not, the microbiota composition did not differ within the same administration time (Table 5).

Table 4 Adonis pairwise comparisons of microbiota composition in capsule contents across administration time point in Study 2, calculated using the Euclidian distances

Comparisons	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
T1 vs. T2	4396.66	1.89	0.08	<0.01	<0.01
T1 vs. T3	5194.90	2.17	0.09	<0.01	<0.01
T1 vs. T4	6926.28	3.18	0.14	<0.01	<0.01
T1 vs. T5	10,637.02	4.65	0.18	<0.01	<0.01
T2 vs. T3	4899.34	2.47	0.09	<0.01	<0.01
T2 vs. T4	6432.94	3.70	0.14	<0.01	<0.01
T2 vs. T5	10,102.78	5.41	0.18	<0.01	<0.01
T3 vs. T4	4139.22	2.26	0.08	<0.01	<0.01
T3 vs. T5	9030.63	4.65	0.15	<0.01	<0.01
T4 vs. T5	5070.97	2.98	0.11	<0.01	<0.01

T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age

^a SumsOfSqs = Sum of square reflecting total variance

^b F.Model = F-test value

^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

^d p = p-value

^e p adj = p-values adjusted for multiple comparison using the Bonferroni-Holm correction

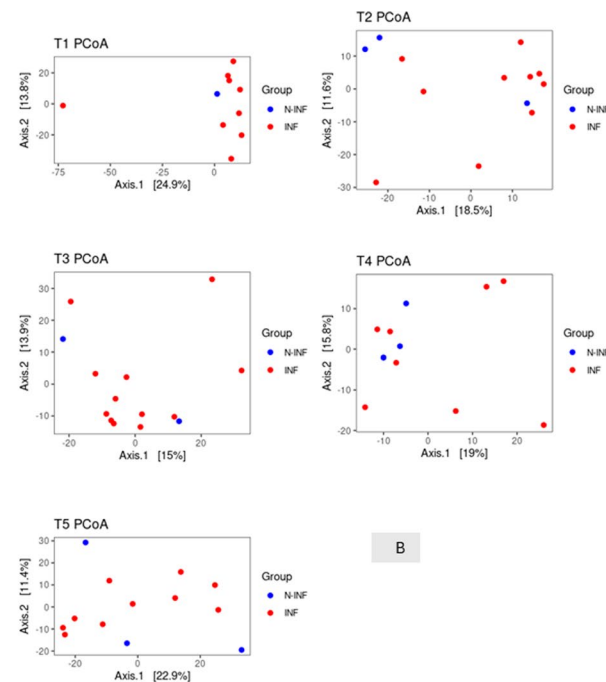
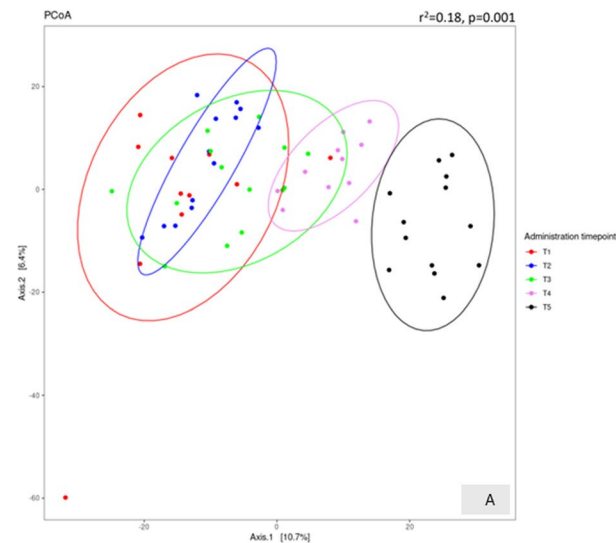


Fig. 4 PCoA plot generated using a Euclidean distance matrix based on **centred log ratio** (clr) transformed data of samples from CapSas in Study 2: **A** based on administration time of the capsules. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data, such as microbiome profiles. Samples are coloured based on the administration time of the capsules. T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age. **B** Based on Infection status. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data, such as microbiome profiles. Samples are coloured based on the group (N-INF or INF) of the pigs in Study 2. T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age

Table 5 Adonis pairwise comparisons of microbiota composition in capsule contents collected from non-infected (N-INF) and infected (INF) pigs across different time points of administration in Study 2, calculated using the Euclidian distances

Comparisons	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
T1: INF vs. N-INF	1615.08	0.57	0.06	1.00	1.00
T2: INF vs. N-INF	1685.02	1.09	0.09	0.27	0.27
T3: INF vs. N-INF	1452.09	0.90	0.07	0.61	0.61
T4: INF vs. N-INF	863.74	0.90	0.09	0.63	0.63
T5: INF vs. N-INF	1483.04	0.84	0.07	0.69	0.69

T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age

^a SumsOfSqs = sum of square reflecting total variance

^b F.Model = F-test value

^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

^d p = p-value

^e p adj = p-values adjusted for multiple comparison using the Bonferroni-Holm correction

The alpha diversity values for Chao1, Shannon, and InvSimpson diversity indexes were determined within samples of the same administration time point to check for differences in bacterial richness between the gut microbiota of N-INF and INF pigs (Figs. 5). It was not possible to check the alpha diversity difference by group from capsules at the first administration time point (T1) because there was only one capsule from the N-INF group. As for the rest, there was no difference in the alpha diversity between N-INF and INF pig's microbiota in any of the indexes.

Microbiota analysis at slaughter

For the upper (Segments 1, 2, and 3) and lower (Segments 4, 5, and 6) segments of the SI at slaughter, the main relative abundances of the eight most abundant phyla, families, and genera by group (N-INF and INF) and type of box (Infected and Mixed) are shown in Fig. 3D–F. The main phylum in every segment of the SI (independent of the group and box type) was Firmicutes (> 85%), followed by Actinobacteria. As for the family, in both the N-INF and INF groups, we observed a decrease in the abundance of *Lactobacillaceae* from the upper to lower segments of the SI; this decrease was more pronounced in the N-INF group (Figs. 3D–F).

For beta diversity, the PCoA plot does not show a clear clustering of the samples based on location on the SI (Fig. 6A), but by group (Fig. 6B). The Adonis test revealed that bacterial composition was significantly affected by the sample type ($r^2 = 0.10$, $p = 0.002$;

Fig. 6A) and by group ($r^2 = 0.04$, $p = 0.001$; Fig. 6B). This indicates a difference between the bacterial composition of the SI from different segments and between the N-INF and INF groups.

We conducted a pairwise Adonis test to assess differences in microbiota composition between the different segments of the SI samples at slaughter, considering group (Table 6) and group within box type (see Additional File 1, Supplementary Table 6). The results showed that only the bacterial composition of Segment 4 of the SI significantly differed between the N-INF and INF pigs ($p = 0.02$), while only a tendency to differ was detected for Segments 3 and 5 ($p = 0.10$ and $p = 0.06$, respectively). No differences were detected in the other segments, suggesting that the group did not have an impact on the microbial composition of these segments of the SI (Table 6). The test for the effect of the group and type of box revealed no difference in microbial composition between any of the groups for each SI segment. There was only a tendency to differ between the N-INF and INF groups in Segments 4 and 5 ($p = 0.07$ and $p = 0.09$, respectively) (see Additional File 1, Supplementary Table 6).

The alpha diversity values for Chao1, Shannon, and InvSimpson were calculated in samples from the lower and upper SI segments to assess differences in bacterial richness between the N-INF and INF groups. Significant differences were found only in the Shannon and InvSimpson indices between the N-INF and INF groups in the upper part of the SI ($p = 0.036$ and $p = 0.0058$, respectively). The INF group exhibited higher bacterial richness in this region (Fig. 7).

Microbiota analysis across the pig's life

For a more general view of the evolution of the microbiota through the pig's life, we performed a beta diversity analysis and an Adonis test of all samples collected during the experiment. For beta diversity, the PCoA plot shows two clusters based on SI segments (from slaughter and weaning) and capsules (Fig. 8). The Adonis test revealed that bacterial composition was affected by the sample type ($r^2 = 0.32$, $p = 0.001$; Fig. 8). Moreover, we performed a pairwise Adonis test to test for differences in bacterial composition between SI segments from weaning, capsules from the five different administration times, and lower an upper part of the SI at slaughter (Table 7) without considering the pig's group. As shown in Table 7, the microbiota compositions of the different types of samples were all different. No microbial biomarkers between samples were detected in the LefSe test of any of the samples.

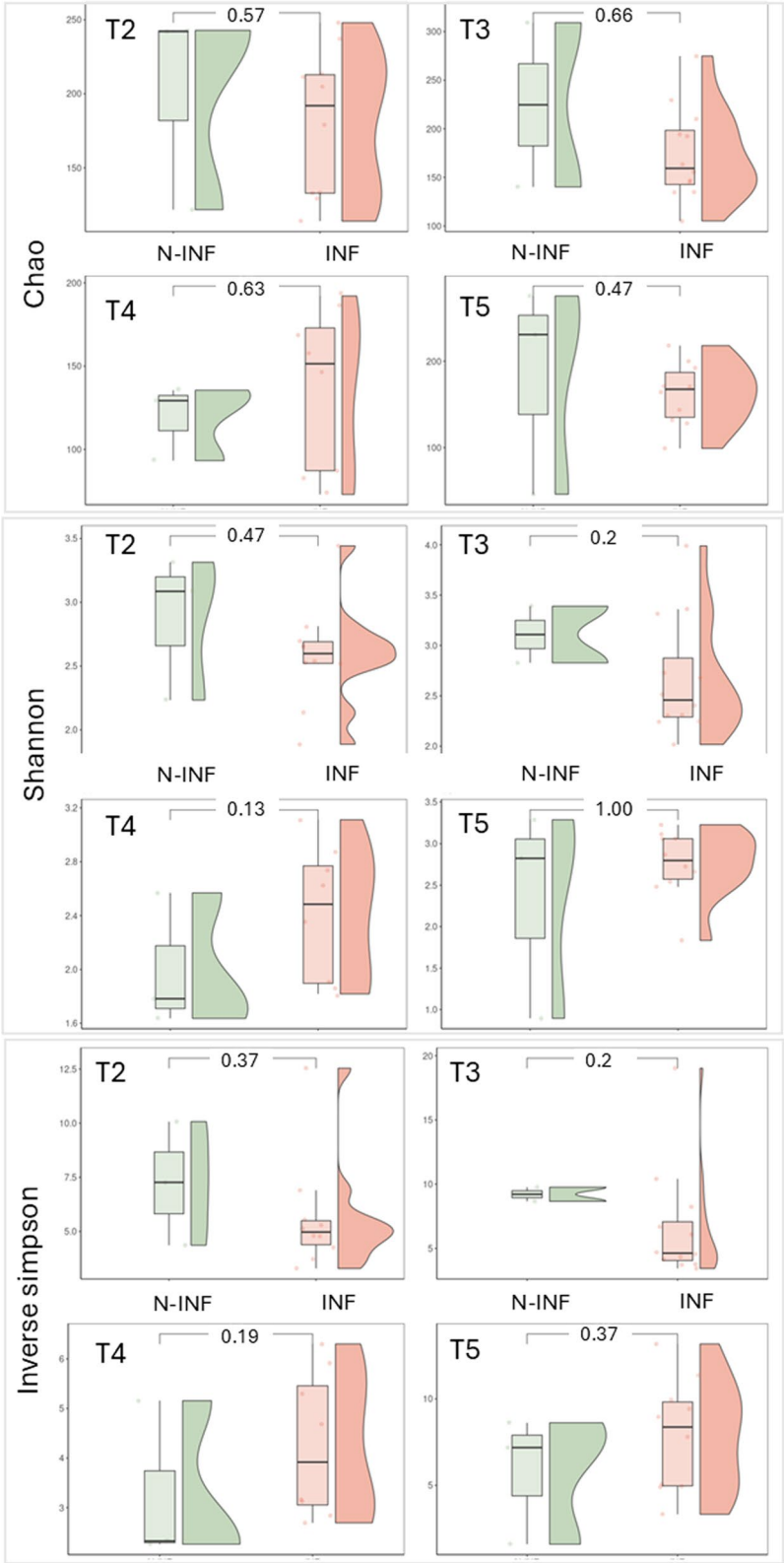


Fig. 5 Box plots showing alpha diversity determined in the capsule contents within administration time points and by group (N-INF or INF) in Study 2: **A** values for the Chao1 index, **B** Shannon index, **C** InvSimpson index. T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age

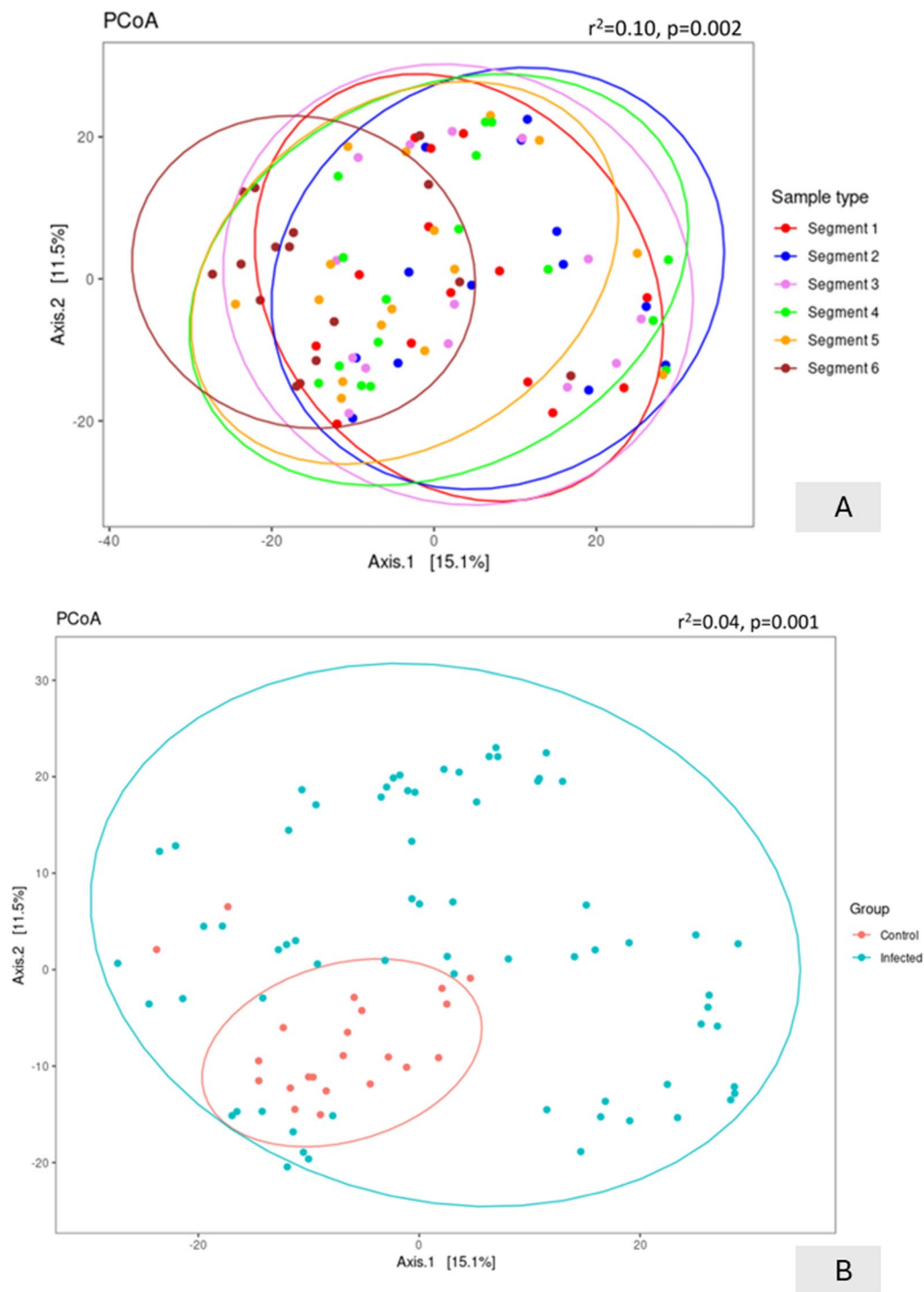


Fig. 6 PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) of transformed data at slaughter (140 ± 5 days of age) in Study 2: **A** analysis based on segments. **B** Analysis based on infection status (N-INF and INF). The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data, such as microbiome profiles

Discussion

This study evaluated the temporal dynamics of the small intestine microbiota using a novel sampling capsule. In

non infected pigs, amplicon sequencing revealed significant shifts in beta diversity from post-weaning to slaughter, indicating microbial succession over time. However,

Table 6 Adonis pairwise comparisons of microbiota composition in six segments of the small intestine collected from non-infected (N-INF) and infected (INF) pigs at slaughter in Study 2, calculated using the Euclidian distances

Comparisons	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
Segment 1:					
N-INF vs. INF	1215.47	1.01	0.07	0.42	0.42
Segment 2:					
N-INF vs. INF	1754.31	1.27	0.10	0.12	0.12
Segment 3:					
N-INF vs. INF	1525.14	1.38	0.10	0.10	0.10
Segment 4:					
N-INF vs. INF	1959.14	1.80	0.11	0.02	0.02
Segment 5:					
N-INF vs. INF	1780.76	1.51	0.09	0.06	0.06
Segment 6:					
N-INF vs. INF	872.30	1.06	0.07	0.32	0.32

^a SumsOfSqs = Sum of square reflecting total variance

^b F.Model = F test value

^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

^d p = p value

^e p adj = p values adjusted for multiple comparison using the Bonferroni-Holm correction

no differences in beta diversity were detected between ETEC-infected and control groups during the capsule sampling period, and only a marginal but significant difference (p = 0.02) was observed at slaughter.

Microbiota study under normal conditions

The primary objective of Study 1 was to investigate the evolution of pig gut microbiota under normal conditions using CapSa as a sampling method. This approach involved direct analysis of gut content from the CapSas, diverging from previous studies that utilized fecal samples as a proxy for studying pig microbiota [35–37]. In the SI microbiota, Firmicutes emerged as the predominant phylum across all developmental stages. As shown in previous studies, *Lactobacillus* was one of the main genera in the SI microbiota at weaning [38–40], and its abundance sequentially decreased as pigs matured, eventually replaced by *Streptococcus* and *Clostridium* as the main genera in the SI microbiota at slaughter [17]. However, contrary to the publication of Luo et al., 2022 [17], *Prevotella* was not one of the main genera detected in the SI microbiota either at T5 or at slaughter. We also observed an increase in *Clostridium* from T2 until slaughter. This genus is recognized to be associated with the finishing stage [17] and shows a positive correlation with BW during this period [41]. The microbial composition differed between sampling points, except for the

microbiota samples obtained from CapSas at T1 and T2. This difference in microbiota composition within samples at different time points could be attributed to the increasing BW and age of the pig. In grower-finisher pigs, these changes are accompanied by dietary shifts, transitioning from a starter diet to grower and finisher diets, which differ in both nutrient and ingredient composition. The microbiome composition changes during the life of the animal [37], with a succession of microbial populations in the SI that carries on through the life of the pig [15]. Furthermore, similar to observations in humans, each pig may possess a unique microbiota fingerprint alongside its core and stage-associated microbiota [42]. This variability is evident in individual gut microbiomes, where the microbiome of each pig exhibits distinct characteristics.

Microbiota study using an ETEC infection model

A total of 16 pigs were involved, with 12 infected at weaning age (28 days old) using Agroscope's ETEC F4 strain, a well-established infection model [23]. INF pigs exhibited ETEC shedding in feces for a week post-infection, accompanied by fecal scores higher or equivalent to 3 (indicating watery diarrhea) in the days following infection.

To establish a baseline for the microbiota study, 4 additional pigs from the same litters as the 16 pigs in the study were euthanized one day before weaning. This allowed us to determine the microbiota composition at weaning. Indeed, it is not possible to use the capsule to sample small intestinal content in pigs weighing less than 12 kg due to the small size of their gastric pylorus, a limitation that was acknowledged in the first validation study of CapSa [24].

The bacterial composition of SI samples at weaning resembled that of Study 1 samples. Although we did not conduct statistical analysis to directly compare Study 1 and Study 2, we observed a consistent pattern with the genus *Lactobacillus*, which was prominent at weaning and progressively replaced by other genera, such as *Clostridium* and *Streptococcus*, as the pigs matured.

Two weeks after infection and seven days after confirming the resolution of clinical signs, indicated by the absence of ETEC shedding in feces, the pigs were segregated into separate boxes. Two pens housed only INF pigs, while the other two pens housed two N-INF and two INF pigs together. The decision to use a mixed group is based on the current literature, which indicates that in modern pig farms, ETEC infections rarely achieve 100% prevalence or total absence [43]. This approach allowed us to investigate whether the interaction between N-INF and INF pigs influenced the evolution of their gut microbiota.

The Firmicutes phylum consistently dominated the SI microbiota throughout various developmental stages,

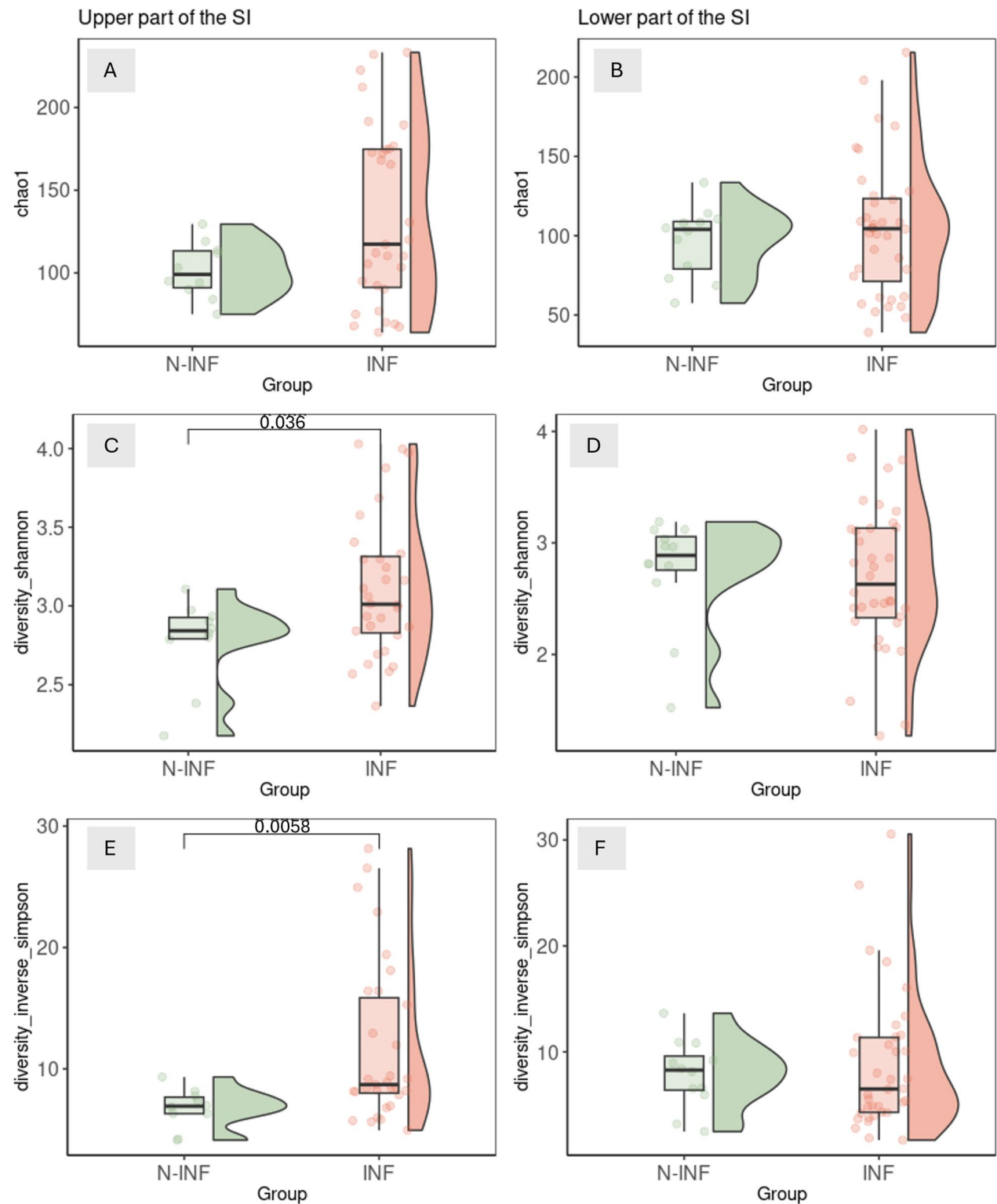


Fig. 7 Box plots showing alpha diversity values for Chao1, Shannon, and InvSimpson determined in the upper and lower sections of the SI at slaughter (140 ± 5 days of age) by group (N-INF and INF) in Study 2. Only significant p-values (< 0.05) are shown

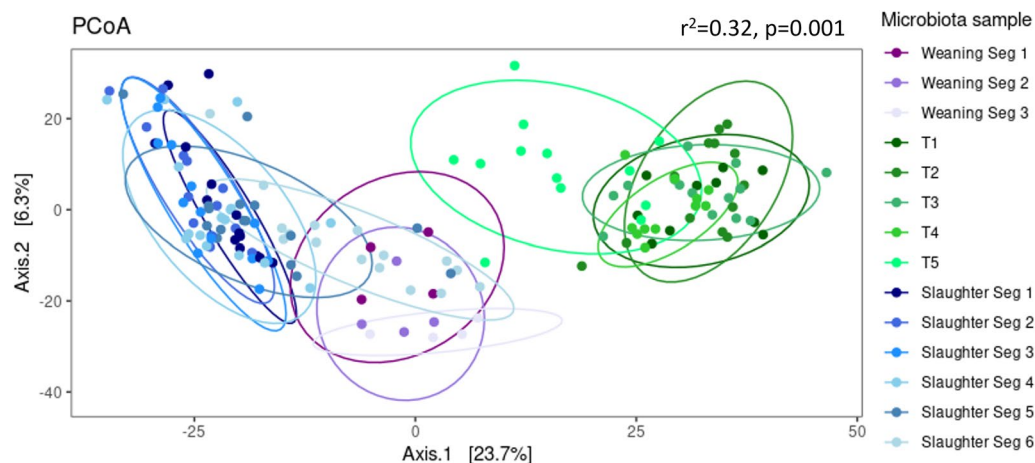


Fig. 8 PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data, such as microbiome profiles. Samples are coloured based on the sampling time point and type of sampling. T1: administration at 52 ± 3 days of age; T2: administration at 70 ± 3 days of age; T3: administration at 83 ± 3 days of age; T4: administration at 110 ± 3 days of age; T5: administration at 126 ± 3 days of age

independent of the animals' group. Although the group and pen allocation (mixed or not) had no discernible impact on beta diversity at T1, T2, T3, T4, and T5, it did slightly influence beta diversity at the time of slaughter ($p=0.02$), with a notable decrease in *Lactobacillus* abundance observed in the microbiota of the INF groups. Based on the Adonis comparisons, only the microbiota composition of Segment 4 of the SI differed between samples from N-INF and INF pigs at slaughter.

The alpha diversity remained unaffected on T2, T3, T4, and T5. However, at slaughter, samples from the SI of N-INF pigs exhibited significantly lower microbial richness than samples from the SI of INF pigs. The assessment of the impact at T1 days of age was inconclusive due to the reduced sample size in the N-INF group. These findings suggest that there is no long-term effect on microbiota richness after an ETEC infection at weaning. Another hypothesis is that the Capsa sample site, located in the upper part of the SI [24], may not accurately reflect the primary site of infection, as ETEC receptors are predominantly found in the lower part of the SI [44]. The capsule's spatial resolution may preclude detection of niche-specific ETEC effects, as it collects only up to 400 μL of small intestinal content, potentially limiting analysis to a localized portion of the lumen. This sampling constraint should also be considered when comparing capsule-derived data to post-mortem samples. Additionally, the strong group size imbalance (Control: $n=4$ vs. ETEC-infected: $n=12$) may have reduced statistical power to detect infection-associated differences. Furthermore, the number of capsules analyzed from the N-INF and INF pigs and the high variability among them might have hindered the detection of these differences if they

exist. Finally, analyzing two capsules per animal (when meeting viability criteria: expulsion within 48h and $\text{pH} > 5.5$) could have improved sensitivity to microbial shifts, though this approach was not implemented in the current design. Our observation of long-term changes in the microbiota, but not short-term changes, partially aligns with previous research [22] noting early changes in fecal microbiota following ETEC infection, followed by a persistent shift in microbiota. However, in that study, fecal samples were used, and measurements were taken only from day 0 of infection until day 36 post-infection.

It is important to note that the conclusion of Study 2 may be biased because we had a larger number of samples at slaughter compared to the number of capsules analyzed from each administration time point. Additionally, due to the pigs' size and the fact that they could not be administered the capsule at a BW less than 12 kg, there was quite a long-time gap between the infection date (28 days of age) and the first capsule administration (52 ± 3 days of age).

Furthermore, microbial composition differed significantly between all sampling points, potentially attributed to growth, development, environmental factors, and dietary changes in pigs.

Conclusion

This study characterizes the temporal dynamics of porcine small intestinal microbiota under both physiological and pathological conditions. We observed progressive microbial succession from post-weaning to slaughter in healthy animals, demonstrating substantial longitudinal restructuring of the microbiota. While ETEC infection induced no observable

Table 7 Adonis pairwise comparisons of microbiota composition between small intestine segments at weaning, capsule contents across five different administration time points and lower and upper segments of the small intestine at slaughter in Study 2, calculated using the Euclidian distances

Comparisons	SumsOfSqs ^a	F.Model ^b	r ^{2c}	p ^d	p adj ^e
SI at weaning vs. T1	14,004.36	6.56	0.24	< 0.01	< 0.01
SI at weaning vs. T2	17,920.71	10.59	0.32	< 0.01	< 0.01
SI at weaning vs. T3	18,859.73	10.54	0.31	< 0.01	< 0.01
SI at weaning vs. T4	14,254.24	9.75	0.32	< 0.01	< 0.01
SI at weaning vs. T5	14,545.12	8.81	0.28	< 0.01	< 0.01
SI at weaning vs. upper SI at slaughter	17,739.26	12.38	0.19	< 0.01	< 0.01
SI at weaning vs. lower SI at slaughter	14,248.67	10.44	0.15	< 0.01	< 0.01
T1 vs. T2	4733.13	1.86	0.08	< 0.01	0.01
T1 vs. T3	5514.40	2.12	0.08	< 0.01	< 0.01
T1 vs. T4	7112.07	2.98	0.13	< 0.01	< 0.01
T1 vs. T5	10,986.61	4.39	0.17	< 0.01	< 0.01
T1 vs. upper SI at slaughter	28,844.78	16.07	0.24	< 0.01	< 0.01
T1 vs. lower SI at slaughter	24,018.24	14.24	0.20	< 0.01	< 0.01
T2 vs. T3	5287.22	2.45	0.09	< 0.01	< 0.01
T2 vs. T4	6968.71	3.67	0.14	< 0.01	< 0.01
T2 vs. T5	10,857.20	5.31	0.18	< 0.01	< 0.01
T2 vs. upper SI at slaughter	33,781.81	20.92	0.28	< 0.01	< 0.01
T2 vs. lower SI at slaughter	28,255.26	18.48	0.24	< 0.01	< 0.01
T3 vs. T4	4494.09	2.26	0.09	< 0.01	0.01
T3 vs. T5	9717.98	4.58	0.15	< 0.01	< 0.01
T3 vs. upper SI at slaughter	34,522.49	20.81	0.28	< 0.01	< 0.01
T3 vs. lower SI at slaughter	29,046.32	18.49	0.24	< 0.01	< 0.01
T4 vs. T5	5477.95	2.95	0.12	< 0.01	< 0.01
T4 vs. upper SI at slaughter	26,570.42	17.51	0.26	< 0.01	< 0.01
T4 vs. lower SI at slaughter	20,794.50	14.45	0.20	< 0.01	< 0.01
T5 vs. upper SI at slaughter	22,094.56	13.83	0.21	< 0.01	< 0.01
T5 vs. lower SI at slaughter	18,239.50	12.05	0.17	< 0.01	< 0.01
Upper SI at slaughter vs. lower SI at slaughter	5355.11	3.75	0.04	< 0.01	< 0.01

T1: 52 ± 3 days of age; T2: 70 ± 3 days of age; T3: 83 ± 3 days of age; T4: 110 ± 3 days of age; T5: 126 ± 3 days of age; slaughter age: 140 ± 5 days of age

^a SumsOfSqs = sum of square reflecting total variance^b F.Model = F-test value^c r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences^d p = p-value^e p adj = p-values adjusted for multiple comparison using the Bonferroni-Holm correction

community-level changes during active infection, the marginal but significant difference at slaughter ($p=0.02$) suggests potential long-term ecological impacts. These findings were enabled by a novel ingestible sampling capsule that permitted repeated, non-invasive access to intestinal contents—overcoming a key limitation in large animal microbiome research. The distinct temporal patterns between physiological development and pathogen challenge highlight the importance of longitudinal sampling when evaluating enteric microbiota. Further studies with different approaches, such as transcriptomics, metabolomics, or

additional samplings, are necessary to better characterize the microbiota of the SI.

Abbreviations

BW	Body weight
clr	Centred log ratio
ETEC	Enterotoxigenic <i>Escherichia coli</i>
GIT	Gastrointestinal tract
INF	Infected
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
N-INF	Non-infected
PCoA	Principal component analysis
PWD	Post-weaning diarrhoea
SI	Small intestine

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42523-025-00424-w>.

Additional file 1.

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Author contributions

IGV, CO, and FC validated the procedure and conducted the main statistical and bioinformatics analyses. IGV and CO conceived the study, and CO and GB secured substantial funding. IGV and CO performed the animal experiments, recorded the data, and collected and processed the capsules' samples. CO, GB, and PT supervised the study. IGV drafted the manuscript, and CO, GB, FC, and PT critically reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Raw sequences are available at the NCBI sequence read archive (SRA) under Accession Number PRJNA1162443.

Declarations

Ethics approval and consent to participate

All experimental procedures were in compliance with Swiss animal welfare guidelines and were approved (No. 2022-26-FR and No. 2022-39-FR) by the Cantonal Veterinary Office of Fribourg (Switzerland). This study was performed at the piggery of the research station Agroscope—Posieux (Switzerland). All methods are reported in accordance with the ARRIVE guidelines.

Consent for publication

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Competing interests

The authors declare no competing interests.

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