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EXPLORING INTESTINAL MICROBIOTA IN PIGS: VALIDATION AND
APPLICATION OF A NOVEL NON-INVASIVE SAMPLING DEVICE

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Abstract

The study of the temporal dynamics and individual variability of microbiota continues to pose challenges for researchers. Advances in technology and computational tools have recently offered more profound insights into the factors that influence variability in host-associated microbial communities. However, there have been limited advancements in developing new, non-invasive sampling tools to directly study the small intestine (SI) microbiota. This Ph.D. research aimed to validate a novel non-invasive sampling device for collecting gut microbiota samples from pigs. Current microbiota sampling methods are either invasive and non-repeatable (e.g., ileal cannulation and post-mortem sampling) or non-invasive and repeatable but inaccurate for studying the SI microbiome (e.g., fecal swabs and feces sampling). In this thesis, a new sampling device called the Capsule for Sampling (CapSa) was validated in postweaning and growing pigs. Alongside the validation of its sampling mechanism and site, a standard operating procedure (SOP) was designed for administering CapSa to pigs. Our validation studies showed that it was possible to retrieve CapSas from pigs of all sizes, except those lighter than 12 kg, as their stomach size made retrieval impossible. The sampling site was located in the first part of the SI for postweaning pigs and in the middle part of the SI for growing pigs. In both scenarios, the microbial composition of the sample taken by CapSa was always different from the microbial composition of the large intestine and feces. We also demonstrated the feasibility of administering CapSa repeatedly to the same pigs throughout their lifespan. This proved it to be a tool that can facilitate longitudinal studies of the gut microbiota using the same pigs, overcoming the need for fecal samples or slaughtering the pigs. Administering CapSa five times during the pig's life showed the evolution of gut microbiota under normal conditions. After an ETEC infection, it revealed long-lasting effects on the gut microbiota sampled at slaughter. While the SOP should be refined to make it even less invasive and stressful for the animals, CapSa is a valid tool for studying SI microbiota in pigs from the postweaning age. It could also be used to study other gut biomarkers in the future.

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*No sabe a dónde va
Pero sí sabe que algún día llegará
No sabe a dónde va
Pero no va olvidar de dónde viene,
Porque es todo lo que tiene
Mientras ella aprende a caminar
- Amaral*

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List of original manuscripts included in the thesis

Manuscript I: Method: Standard operating procedure for the administration of swallowable devices to study pig's gut content in a non-invasive way. *Published in Animal Open Space.*

<https://doi.org/10.1016/j.anopes.2024.100076>

Manuscript II: A non-invasive tool to collect small intestine content in post weaning pigs: validation study. *Published in Scientific Reports.* <https://doi.org/10.1038/s41598-024-59950-3>

Manuscript III: Sampling Intestinal Microbiota in Growing Pigs: Evaluation of CapSa, an Ingestible Capsule. *Published in Italian Journal of Animal Science.*

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Manuscript IV: Dynamic picture of the pig gut's microbiota under normal and pathological conditions. *Under revision by Animal Microbiome.*

Abbreviations

ATP: Adenosine triphosphate

BW: Bodyweight

CapSa: Capsule for Sampling

DNA: Deoxyribonucleic acid

F. Model: F-test value

FE: Feed Efficiency

GIT: Gastrointestinal Tract

IgA: Immunoglobulins A

IUGR: Intrauterine growth restricted

L: liquid phase

LBW: Low birthweight

L_{g-t} : Length of the pig from the groin to the base of the tail

L_o : Length of the oesophagus

NF: Not found.

$p = p$ value

$p_{adj} = p$ values adjusted for multiple comparison using the Bonferroni correction.

PCoA: Principal Coordinate Analysis

PERMANOVA: Permutational

Multivariate Analysis of Variance

PVA: polyvinyl alcohol

r^2 : r-square value

SCFA: short-chain fatty acids

SEM: Standard error of the mean

SI: Small Intestine

SIMBA: Small Intestine MicroBiome
Aspiration

So: solid phase

SOP: Standard Operating Procedure

SumsOfSqs: Sum of square reflecting total variance

Background

The study of microbiota, particularly the temporal dynamics and individual variability of microbial communities in the gastrointestinal tract (GIT), has garnered significant interest in recent years. This is because the gut microbiome plays a crucial role for the host. It not only aids in digestion and nutrient production but also acts as a biological barrier against pathogens and can modulate the immune system (Frick & Autenrieth, 2013). Advances in technology and computational tools have enabled researchers to gain deeper insights into the factors influencing these communities (Galloway-Peña & Hanson, 2020). However, the challenge of non-invasively sampling the small intestine (SI) microbiota remains unresolved (Tang et al., 2020). Traditional methods like ileal cannulation and post-mortem sampling, while precise, are invasive and raise ethical concerns; ileal cannulation can be performed repeatedly, whereas post-mortem sampling cannot be repeated. On the other hand, non-invasive techniques like fecal swabs and fecal sampling, while repeatable, fail to accurately represent the SI microbiome (Choudhury et al., 2019). In recent years, advances have been made in developing non-invasive devices, such as ingestible wireless capsules, to overcome these challenges and constraints (Amoako-Tuffour et al., 2014; Finocchiaro et al., 2021). In today's society, there is a growing demand for research that is more welfare-friendly, aimed at reducing the number of animals used and improving compliance with the 3Rs principles (Replacement, Reduction, and Refinement) (Balls et al., 2024; Herrmann & Jayne, 2019). Non-invasive devices designed for this purpose can enhance research efficiency by minimizing animal usage and improving study methods. These devices could be particularly useful for investigating the dynamics between gut microbiota and their host, providing more accurate and reliable results.

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1. Gut microbiota

1.1. Definition

The gut microbiota is a complex ecosystem comprising bacteria, archaea, and eukarya that colonize the gastrointestinal tract (GIT). Over millennia, these microorganisms have developed a complex relationship with their host species, significantly influencing various physiological processes. (Thursby & Juge, 2017). This diverse microbial community thrives within the digestive tracts of animals and engages in interactions with their host that can be commensal, pathogenic, or mutualistic (Tan et al., 2021).

Trillions of microbes inhabit the mammalian intestinal tract, collectively forming the microbiota and providing essential functions that the host cannot perform independently (Bäckhed et al., 2005). These functions include the digestion of dietary fibres and the extraction of energy from otherwise inaccessible nutrients (Niu et al., 2019; Pandey et al., 2023). The ecological dynamics within the gut microbiota play a critical role in the overall health and well-being of pigs, influencing processes such as digestion (Gardiner et al., 2020; Liao et al., 2024), nutrient metabolism (Stevens & Hume, 1998), immune system development (Duarte & Kim, 2022), and resilience to stressors (Mancin et al., 2024). Understanding and managing the composition and balance of the gut microbiota have significant implications for animal nutrition, health management and production efficiency (Chen et al., 2022).

In addition to exploring sampling methods, this thesis will provide an overview of the various factors influencing the composition and dynamics of the gut microbiota in pigs.

1.2. Factors influencing gut microbiota in pigs

1.2.1. *Intrinsic factors*

1.2.1.1. *Weight at birth and prenatal medical conditions*

Birth weight significantly reflects conditions that shape the gut microbiota colonization and metabolic health of piglets, particularly during the neonatal suckling periods (Li et al., 2018; Riddersholm et al., 2021).

Low birthweight (LBW) piglets exhibit a distinct faecal bacterial community compared to normal birth weight piglets, characterised by reduced abundance of short-chain fatty acids (SCFA)-producing bacteria such as *Prevotella* spp. and *Faecalibacterium* spp. (Li et al., 2019; Li et al., 2018). In addition, the abundance of the genera *Lactobacillus* and *Ruminococcus* remains consistently lower in LBW piglets during the first 35 days after birth (Li et al., 2018; Trevisi et al., 2023; Zhang et al., 2019).

This effect is particularly pronounced in Intrauterine growth restricted (IUGR) pigs, which are defined by impaired foetal growth during gestation (Ruggeri et al., 2024; Tang et al., 2022). Differences in gut microbiota between IUGR and normal piglets are influenced by several maternal factors during gestation and the suckling period. These factors include sow malnutrition, unequal nutrient distribution among foetuses, and variations in vertical microbial transmission from sow to piglets, possibly due to altered intrauterine environments and immature intestinal barriers in IUGR piglets during gestation and lactation (Jiang et al., 2019). Restricted nutrient supply to foetuses during gestation may hinder intestinal development (Kim et al., 2013) and contribute to postnatal alterations in GIT microbiota colonization (Jiang et al., 2019). These alterations often result in the intestinal microbiota of IUGR piglets having lower diversity and altered taxonomic abundances compared to control piglets

during lactation (Zhang et al., 2019). Notable differences include decreased abundances of Bacteroidetes, Firmicutes, and Spirochaeta (Zhang et al., 2019). These changes potentially affect nutrient digestion and absorption, underscoring the importance of specific bacterial groups in maintaining gut health and influencing piglet growth (Mahmud Md et al., 2023). Moreover, higher proportions of Proteobacteria in IUGR piglets are linked to inflammation and increased susceptibility to disease (Xiong et al., 2020; Zhang et al., 2019).

1.2.1.2. *Developmental stage*

As pigs progress through different developmental stages, from birth to maturity, significant changes occur in their gut microbiota composition (Li et al., 2020; Slifierz et al., 2015). However, it is challenging to disentangle the effects of growth stage from the concomitant environmental and dietary changes that pigs experience during their development (Szabó et al., 2023).

During farrowing, piglets are exposed to the microbiome from their mother, primarily from the vaginal tract and the udder, as well as the surrounding environment (Frese et al., 2015). This exposure begins the colonization of the gut microbiota. Over time, these microbes metabolize the oxygen in the GIT, creating anaerobic niches (de Vries & Smidt, 2020; Jost et al., 2012). The gut microbiota of suckling piglets initially mirrors that of their mother, which serves as the primary source of their gut bacteria (Chen et al., 2018; Han et al., 2019). This maternal transfer establishes a robust microbiota crucial for intestinal function and immune development (Li et al., 2018). Additionally, the intake of colostrum and milk significantly impacts GIT colonization, fostering a milk-oriented microbiome and establishing dominant microbial populations early in life (Looft, Allen, Cantarel, et al., 2014; Looft et al., 2012).

Weaning, typically occurring in farms between 21 to 28 days of age, marks a significant transition in the piglets' diet and environment. Abrupt changes, including separation from the sow, introduction to solid feed, and environmental stressors, trigger dramatic shifts in gut microbiota composition (Choudhury et al., 2021). This transition often leads to intestinal dysbiosis, decreased microbial diversity, and increased susceptibility to infections, such as post-weaning diarrhea (PWD) (Frese et al., 2015; Heo et al., 2013).

As pigs enter the adult phase, their gut microbiota matures and stabilizes, exhibiting increased diversity and richness (Zhao et al., 2015). The stability of the gut microbiota plays a crucial role in nutrient metabolism, immune system modulation, and overall health, contributing to improved growth performance and disease resistance in adult pigs (Luo et al., 2022).

1.2.1.3. *Sex*

The influence of sex on the GIT microbial profile is often overlooked, as its impact on microbiota composition becomes significant only after puberty, primarily mediated by hormonal factors (Yurkovetskiy et al., 2013).

Sex has a considerable influence on gut microbiota composition, with different microbial compositions observed between males and females (He et al., 2019). These differences are partly attributed to sex steroid hormones, such as testosterone, oestradiol, and progesterone (García-Gómez et al., 2013). These hormones play crucial roles in modulating various functions in mammals, including immune responses and the metabolic pathways of both pathogenic and commensal bacteria, thereby influencing the gut microbiota composition (Bolnick et al., 2014; Tetel et al., 2018).

Although the initial weeks of life may not show significant sex-related differences in gut microbiota, pronounced disparities emerge post-puberty (Yurkovetskiy et al., 2013; Zhou et al., 2015). Notably, females tend to exhibit higher microbial diversity compared to males (Wang et al., 2020). Studies have identified specific bacterial taxa that are more abundant in boars, such as Veillonellaceae, Roseburia, Bulleidia, and Escherichia, while others, like Treponema and Bacteroides, are more prevalent in gilts (He et al., 2019).

Interestingly, castrated males display an intermediate microbial diversity, suggesting a nuanced impact of hormonal alterations on gut microbiota (Wang et al., 2020). Furthermore, castration in boars leads to a shift in microbiota composition towards that typically found in gilts (He et al., 2019).

1.2.1.4. *Breeds*

Genetics is strongly associated with the host's microbiota diversity and characteristics (Yang et al., 2014). Lot of the research available today is being done in different breeds of pigs, like for instance, Large White (Zhao et al., 2015), Laiwu (Yang et al., 2017), Gloucestershire Old Spot (Kelly et al., 2017), Iberian pigs (Crespo-Piazuelo et al., 2018), Meishan (Jiang et al., 2023), Jinhua and Landrace (Xiao et al., 2018), Shanxi Black (P. Gao et al., 2019), Duroc and Hampshire (Xiao et al., 2017). It has been proven that the microbial profile of gut microbiota varies between the different breeds (Cheng et al., 2017). For example, despite their different origins, breeds like Landrace, Yorkshire, and Duroc share similar production traits such as high growth rates and high lean meat percentages. They also have dominant microbial communities in common. However, these breeds (lean type) show low similarities in microbial diversity when compared to Chinese breeds (relatively obese type) such as Meishan and Eurhalian. These microbial differences are associated

partially, but not entirely, with their conformation, growth characteristics and feed efficiency (Yang et al., 2014).

1.2.1.5. *Segments of the gastrointestinal tract*

The intestinal microbiota can vary in animals based on the location within the GIT (Maradiaga et al., 2018). Various segments of the GIT perform distinct roles in digesting or absorbing nutrients and maintaining immune homeostasis, with microbiota playing a key role in these processes (Szabó et al., 2023). The gut microbiota inhabiting different regions of the GIT are uniquely adapted to thrive in their respective environments (Martinez-Guryn et al., 2019), this results in a spatial heterogeneity in the distribution of gut microbes (Mu et al., 2017; Zhao et al., 2015).

1.2.1.5.1. Stomach

The pig's stomach consists of a single chamber divided into four distinct functional and structural regions: the pars oesophagea, cardia, fundus, and pylorus (Lærke & Hedemann, 2012). The fundus region is primarily responsible for producing gastric juice, which plays a crucial role in regulating pH, lubricating and protecting the mucosal surface, and aiding in the digestion of proteins and triglycerides (Lærke & Hedemann, 2012). Due to the presence of gastric juice, the pH in the stomach is significantly lower compared to other parts of the gastrointestinal tract, creating an environment that favours acid-tolerant bacteria such as *Lactobacillus* spp. (van Winsen et al., 2001). *Lactobacillus* spp. are also found throughout the gastrointestinal tract, indicating that the population in the stomach may contribute to microbial communities in

other regions (Tannock, 2004). Other genus found in the microbiota of this organ are *Acinetobacter* and *Helicobacter* (Holman et al., 2017).

1.2.1.5.2. Small intestine

The pig's small intestine consists of three main sections: the duodenum (4-4.5%), jejunum (88-91%), and ileum (4-5%) (Lærke & Hedemann, 2012). Stretching up to 20 meters in length, it occupies about one-third of the total volume capacity of the GIT (Moran, 1982). The primary functions of the small intestine are to digest and absorb nutrients and to serve as a vital organ for host immunity (Cheng et al., 2023; Weström et al., 2020). Fluids and solids transit through the small intestine typically within 3-4 hours (Henze et al., 2021). This rapid movement creates conditions unfavourable for bacterial growth, resulting in relatively low microbial activity in these segments, as evidenced by low ATP concentrations in the digesta samples (Bach Knudsen et al., 1991; Jensen & Jørgensen, 1994).

1.2.1.5.3. Large intestine

The large intestine, particularly the cecum and colon, is the primary site for the absorption of fluids and electrolytes and the microbial digestion of undigested feed residues. Microbial fermentation produces short-chain fatty acids (SCFAs) that are subsequently absorbed (Nakatani et al., 2018), serving as an important energy source for the animal. The large intestine functions as an

anaerobic fermentation chamber promoting bacterial growth due to its low oxygen concentration and flow rate (Russell Edward, 1979).

1.2.1.5.4. Spatial heterogeneity in microbial composition and diversity across the gastrointestinal tract

These anatomical and environmental distinctions result in notable differences in microbiota between the upper and lower GIT segments. Microbes in different regions of the GIT are uniquely adapted to thrive in their respective environments. Lower GIT samples exhibiting significantly higher diversity, richness, and evenness compared to upper GIT samples (Holman et al., 2017). This finding aligns with the faster transit time in the upper GIT, which limits bacterial adhesion and colonization (Heo et al., 2013).

1.2.2. *Extrinsic factors*

1.2.2.1. *Diet*

While the above-mentioned intrinsic factors are inherent to the individual pig, the offered diet is a crucial lever to directly impact the gut microbiota.

Research indicates that macronutrients such as protein (Burkey, 2020), carbohydrates (Rist et al., 2013), fat and dietary fiber (Sonja N. Heinritz et al., 2016) have significant influence on the gut microbiota of pigs. For instance, fermentable carbohydrates can promote the growth of beneficial bacteria like *Lactobacillus* and *Bifidobacteria* species (Rist et al., 2013), whereas an excessive protein intake can stimulate the proliferation of potentially harmful species, leading to dysbiosis and diarrhea (J. Gao et al., 2019). As for the quantity of fat and dietary fiber in a diet,

evidence suggests that a low-fat/high-fiber diet promotes the growth of beneficial bacteria, such as *Lactobacillus* and *Faecalibacterium*, and the production of SCFA in pigs (S. N. Heinritz et al., 2016).

Numerous reviews on the effect of the diet on the gut microbiota have been published already (Jha & Berrocoso, 2015; Liao et al., 2024; Srikanthithasan et al., 2024). Since this PhD thesis is not about nutrition, I will not extend this discussion further here.

1.2.2.2. *Antibiotics and alternatives to antibiotics*

Over the past two decades, numerous alternatives to antibiotics previously used in animal feed, particularly during the weaning period to prevent postweaning diarrhea, have emerged and been incorporated into feed formulations (Thacker, 2013; Turner et al., 2001). Evidence had already shown that administering antibiotics orally to pigs could have both short-term and long-term effects on their gastrointestinal microbiota (Schokker et al., 2015), potentially diminishing effectiveness and promoting the selection of antibiotic-resistant bacterial strains (Monger et al., 2021).

Antibiotics generally cause a decrease in gut microbial diversity (Abeles et al., 2016). Many antimicrobial classes are non-specific, wiping out a wide range of resident gastrointestinal microbiota and leading to ecosystem disturbances (Looft et al., 2014; Neuman et al., 2018). For example, carbadox, used to control swine dysentery and enteritis, also affects other gram-positive bacteria such as *Slackia*, *Peptococcus* and *Blautia* (Lourenco et al., 2021). Similarly, administering amoxicillin early in pigs' lives impacts the microbiota, reducing host genes involved in short-chain fatty acid signaling and pancreatic development (J. Li et al., 2017). These changes affect multiple locations within the GIT, as demonstrated by a study where a mixture

of amoxicillin and colistin sulfate administered to post-weaning piglets resulted in varied digestive microbiota profiles along the entire GIT (K. Li et al., 2017).

Contrary to antibiotics, which kill bacteria, prebiotics, probiotics, and postbiotics support gut health in different ways. Prebiotics are additives fermented by specific bacteria, promoting a healthy gut microbiome. Probiotics are living microorganisms naturally found in the gut, and postbiotics are non-living microorganisms or their metabolites. (Salminen et al., 2021). These alternatives have been shown to effectively alter the composition of gut microbiota, resulting in improvements in gut health and growth parameters (Shin et al., 2019; Zhang et al., 2024) . The most common probiotic used in the swine industry bacteria from the genus *Lactobacillus*, a gram-positive bacteria naturally present in pigs' GIT since the first colonization (Lim et al., 2023). Administering *Lactobacillus* enhances intestinal health by reducing inflammation, crypt cell proliferation, and diarrhea in nursery pigs (Konstantinov et al., 2008; Yi et al., 2018; Zhang et al., 2010). Its beneficial effects have also been observed when used as a postbiotic, yielding similar positive results in pigs (Xu et al., 2022). The most commonly used prebiotics in swine are dietary fiber (Azad et al., 2020). The soluble dietary fiber inulin, a particularly effective prebiotic, has been shown to increase the population of probiotic *Lactobacillus* spp. and *Bifidobacterium* spp., promote the production of lactic acid and SCFA, and suppress the growth of pathogenic bacteria (van der Aar et al., 2017).

1.2.2.3. *Environment*

Understanding the interaction between the environment and gut microbiota is limited and challenging. Numerous factors influence this interaction simultaneously, including the type and design of the farm, animal management practices, hygiene of the facilities and environmental parameters (Bidot et al., 2018; Mulder et al., 2009).

Many environmentally-acquired microbes play a crucial role in establishing gut microbiota from early life (Schmidt et al., 2011).

The type and design of a farm significantly influence the gut microbiota of animals. For example, pre-weaned pigs raised outdoors (such as in an organic production system) exhibit a notable reduction in the diversity and richness of ileal-mucosa-adherent microbes compared to those raised indoors (Mulder et al., 2009). Surprisingly, *Lactobacillus* spp., known for its health-promoting effects and ability to limit the prevalence of several intestinal pathogens (Blomberg et al., 1993), was the predominant taxon found in outdoor pigs (Mulder et al., 2009; Schmidt et al., 2011).

Stress and welfare enrichment on farms can also affect gut microbiota. Stress can influence microbiota composition, and microbiota can modulate stress responses (Correa et al., 2023; Molina-Torres et al., 2019). A previous study examining the impact of an enriched environment on the gut microbiota of pigs found that the fecal microbiota structure differed significantly between the enriched and control groups as early as 12 days of age. Pigs in enriched pens showed an increase in bacteria involved in the degradation of SCFAs (Wen et al., 2021).

1.3. Connection between gut microbiota, gut health and growth parameters in pigs

The relationship between gut microbiota and gut health is complex due to the lack of consensus on the definition of 'gut health' and what constitutes a 'healthy gut.' When discussing gut health, we typically refer to a state of balance within the individual, characterized by the absence of gastrointestinal illness, efficient digestion and nutrient absorption, and a robust immune system, all while maintaining a normal and stable microbiota (Bischoff, 2011; Lallès et al., 2007).

Indeed, the intestine hosts the majority of the immune cells, making it the largest compartment of the immune system (Mowat & Agace, 2014). The mucosa-associated microbiota directly interacts with intestinal immune cells, thereby modulating immune responses (Belkaid & Hand, 2014). This interaction triggers several protective mechanisms: it defends mechanism against pathogens through competitive exclusion (Belkaid & Hand, 2014), influences the production of immunoglobulins A (IgA) (Gutzeit et al., 2014), and metabolizes toxins from feed while synthesizing vitamins essential for the host's intestinal epithelium (Yang et al., 2016).

As a result, the intestinal microbiota indirectly promotes optimal growth in pigs by allowing them to reach their full potential since no energy is wasted on restoring a healthy state. It also improves bodyweight (BW) gain and feed efficiency (FE) through microbial fermentation (Gardiner et al., 2020). For instance, previous studies demonstrated that the abundance of *Bacteroides* in faeces was negatively correlated with BW (Han et al., 2017; Mach et al., 2015). This negative correlation might be due to some *Bacteroides* species being opportunistic pathogens, which could trigger an immune response, thereby diverting energy away from growth and towards maintaining health. Conversely, beneficial bacteria that aid in nutrient processing and energy harvest are more prevalent in pigs with higher FE (Gardiner et al., 2020).

1.4. Comparison of gut microbiota sampling methods

1.4.1. *Current sampling methods*

Since the beginning of gut microbiota research in pigs, sampling methods have primarily relied on two approaches: fecal sampling and post-mortem sampling (Zhao et al., 2015). While fecal sampling is non-invasive, easily repeatable, and provides sufficient content for DNA extraction, it does not accurately represent the microbiota in

the small intestine (Zhao et al., 2015). In contrast, post-mortem sampling, although more accurate for studying gut microbiota, is a one-time procedure and cannot support longitudinal studies. Additionally, extracting intestines post-mortem carries a risk of cross-contamination, because of the potential introduction of external microbes during the dissection process and the mixing of contents from different sections of the gut.

Other methods have been used over time but have been abandoned due to practical, economic, or welfare concerns. For instance, the use of ileal-cannulated pigs, where a T-cannula is surgically fitted at the distal ileum (Opriessnig et al., 2024), is controversial and highly invasive (Stein et al., 1998).

Rectal swabs offer a more sophisticated and less invasive alternative to fecal samples, especially when dealing with smaller pigs. These swabs can better represent both luminal and mucosa-adherent microbiota, particularly when taken before defecation (Choudhury et al., 2019). However, the limited amount of material obtained often leads to technical issues, such as low DNA yield.

To the best of our knowledge, methods used in human studies, such as biopsy, endoscopy, luminal brush, laser capture microdissection, or catheter aspiration (Tang et al., 2020), have not been evaluated or applied in swine research.

1.4.2. *Novel capsule sampling methods*

Traditional sampling methods have significant limitations, highlighting the need for new approaches (Tang et al., 2020). An ideal sampling device should be ingestible, able to transit safely through the digestive tract, and retrievable. Moreover, it should collect sufficient gut content for high-quality DNA extraction while preventing cross-contamination (Amoako-Tuffour et al., 2014).

1.4.2.1. *Robotic or magnetic devices*

In the early 2000s, researchers in human medicine began developing miniature robotic capsules for exploring the GIT. These capsules were designed to perform various tasks, including endoscopy, drug delivery, autonomous or guided movement through the GIT to specific spots, localization of specific parts or structures within the GIT, measure of parameters such as pH levels and temperature, and tissue biopsy (Kalantar-Zadeh et al., 2017; Nguyen et al., 2019; Zhou & Alici, 2019). In recent years, several capsules with electrical or magnetic components have been tested for sampling gut microbiota. Rehan et al. (2020) designed a shape memory alloy spring-based capsule for microbial content collection from the small intestine, capable of sampling 500 μ l on average. Although this device has been tested *ex vivo* in pigs and other animals, its actual performance *in vivo* and potential tissue interaction need further validation (Rehan et al., 2020). Similarly, Park et al. (2022) proposed a capsule capable of wirelessly moving within the GIT and performing up to three samplings, with minimal cross-contamination. Despite these advantages, it has only been tested *ex vivo* in small intestine segments from pigs, and its larger size (11 mm diameter, 26 mm length) might pose challenges *in vivo* (Park et al., 2022). Shokrollahi et al. (2021) introduced a magnetically actuated capsule encapsulated in soft elastomer, controlled externally by a magnet. This device avoids electronics, relying on passive magnetic control, making it safer. However, the external magnetic control is complex and poses a risk of tissue damage. Additionally, it requires multiple capsules for sampling at different GI locations, limiting its practicality in clinical settings. This device was tested in live pigs, where it was controlled blindly by moving an external magnet around the pig's abdomen (Shokrollahi et al., 2021). Finocchiaro et al. (2021) developed a capsule using an operating system with passive components and an external permanent magnet for wireless activation and *in situ* sampling via mechanical

brushing (Finocchiaro et al., 2021). Lastly, Ding et al. (2021) created a magnetically controlled sampling capsule endoscope (MSCE) for non-invasive and accurate digestive bioinformation acquisition. It boasts good sealing characteristics, preventing contamination post-sampling (Ding et al., 2021).

However, ingesting capsules containing electrical or magnetic components raises safety concerns, as they can cause strain and damage to GI tissues during movement (Min et al., 2020). Many of these movement mechanisms rely on electromechanical actuators that consume too much power for a typical coin cell battery (Min et al., 2020). Additionally, external magnetic locomotion can exert significant forces on body tissues and become stuck in collapsed regions of the intestine (Liu et al., 2015).

1.4.2.2. *Passively engine devices*

To address the issues associated with electronic or magnetic devices, a more straightforward approach involves using the natural peristaltic movements of the GIT to assist with capsule locomotion. Simplifying sampling mechanisms to rely on passive collection methods eliminates the need for power supplies, allowing for more compact and basic designs (Amoako-Tuffour et al., 2014).

Over the past five years, several devices have been designed to collect microbiota from the human gut using this principle. These devices use a pH-based autonomous sampling method, which makes them resistant to the gastric environment and triggers sampling once the outer layer dissolves at a pH higher than the gastric pH.

Salem et al., 2018 developed and published a first 3D-printed capsule composed by three key components: a shell constituting the main body of the capsule with an inlet orifice for fluid entrance, a polyvinyl alcohol (PVA) sponge, and a bistable mechanism to close the capsule. The capsule's opening is regulated by the dissolution

of a biodegradable coating, allowing fluid to enter, while the closing is enabled by the volume increase of the sponge (Salem et al., 2018). Despite this innovative approach, the solution does not allow for specific location collection or proper sample localization.

Nejad et al. (2019) introduced an ingestible osmotic pill designed for in vivo sampling of gut microbiomes. This device relies on the peristaltic motion of the intestine and can perform sampling only once when submerged in gut fluid. However, it excludes the possibility of collecting multiple samples from different points in the GI tract (Rezaei Nejad et al., 2019a). Waimin et al. (2020) introduced another 3-D printed gut-sampling capsule featuring a highly absorbent hydrogel for microbiome sampling (Waimin et al., 2020). Similar to Nejad's design, it depends on intestinal peristalsis for movement and can only perform a single sampling when submerged in gut fluid. The capsule, measuring 9 mm x 15 mm, also faces limitations in collecting multiple samples from various GI locations. Folz et al. (2023) and Shalon et al. (2023) developed ingestible sampling devices consisting of a collapsed collection bladder capped by a one-way valve in a capsule with a pH-sensitive coating (Folz et al., 2023; Shalon et al., 2023). The four device types differ only in their enteric coating, which dissolves at different pH levels, enabling sampling at specific intestinal locations after gastric emptying. These devices, devoid of electronics beyond a passive RFID chip for tracking, collect up to 400 μ l of luminal contents through vacuum suction once the coatings dissolve. However, the exact location of sample collection within the intestines cannot be clearly defined or validated. More recently, a study using Small Intestine MicroBiome Aspiration (SIMBA) capsule was published (Menard et al., 2023; Wang W, 2023), a pH-based autonomous sampling capsule designed to open and close while transiting the small intestine. It features a pH-dependent coating that keeps it sterile through the oral and gastric regions before sloughing off. The capsule

has large sampling ports for collecting varying consistencies in the small intestine, timed non-electronic sealing to avoid colonic contamination, and embedded microbial DNA preserving agents to maintain sample quality. Tested in humans and dogs, SIMBA capsule measures 23.4 mm x 8.6 mm and collects an average of 80 μ L of intestinal fluid. One of the latest advancements in this field comes from Tronel et al. (2024), who designed a medical device specifically targeting the small intestine, developed to be easily recovered from feces. It consists of a porous polymer protected by two walls, with the outermost layer being gastro-resistant. This size 00 gastro-resistant pharmaceutical capsule passes through the stomach causing no tissue damage, degrading in the distal jejunum/proximal ileum. The device contains three modules for multi-sampling of the small intestinal liquid, with a minimum sample volume of 25 μ L (Tronel et al., 2024). The most recent device designed for sampling the microbiota of the small intestine in humans, and also tested in vivo in pigs, is a soft elastic capsule (Del-Rio-Ruiz et al., 2024). According to its creators, this capsule is believed to be safer than previous rigid prototypes. It represents an advancement from an earlier model developed by Rezaei Nejad et al. in 2019. The capsule features an enteric coating that protects it in the oesophagus and stomach, but dissolves in the more alkaline pH of the small intestine. Unlike its predecessor, it includes multiple side wall inlets for collecting luminal content. After sampling is complete, two beads inside the capsule expand, triggering two inner valves to close it. This device has undergone testing in pigs, with a 5-day delay from administration to recovery in faeces and was proved to collect content that closely resembles to the post-mortem content sampled from duodenum and jejunum. These passively engineered devices leverage the natural movements of the GIT for sampling, offering compact and straightforward designs without the need for power supplies. However, they still face challenges such

as the inability to collect multiple samples from different GIT locations and ensuring accurate sample localization and quality.

2. Objectives and hypothesis

2.1. Objectives

The primary overall objective of this thesis was to **validate a novel non-invasive sampling device for collecting gut microbiota samples from pigs**. Such a device would eliminate the need to slaughter pigs for direct gut content sampling. As a secondary objective, the research aimed to **demonstrate the feasibility of repeatedly administering the CapSa device to the same pigs**, thus enabling the collection of intestinal samples over time and providing a dynamic picture of their gut microbiota throughout their lifespan. By achieving these objectives, the device could allow longitudinal studies on gut microbiota and other gut biomarkers. Furthermore, due to the similarities between the GIT of pigs and humans, this tool could be useful in using pigs as animal models for human research, thereby broadening its applications in both the veterinary and medical fields.

2.2. Hypothesis

The first hypothesis of this PhD thesis is that the **newly developed in vivo Capsule for Sampling (CapSa) tool, will effectively and accurately collect small intestine content from living pigs, providing a more precise representation of the intestinal compared to the fecal microbiota**. The second hypothesis is that the **gut microbiota undergoes distinct and measurable changes from post-weaning to slaughter, reflecting the different stages of the pig's life cycle**. Finally, we hypothesize that the **gut microbiota composition of individual pigs will exhibit significant short- and/or long-term alterations in response to pathophysiological conditions**, caused by enterotoxigenic (EPEC) infection. These changes will be detectable and characterized using the previously validated in vivo sampling tool.

2.3. Specific objectives and hypothesis

2.3.1. *Validation of Capsule for Sampling (CapSa)*

2.3.1.1. *Validation of administration protocol*

Before addressing the first hypothesis, we worked on a Standard Operating Procedure (SOP) to optimize and standardize the capsule administration and retrieval processes in pigs, addressing their unique anatomical challenges. The pig's GIT is significantly larger with a longer small intestine and colon, and certain anatomical structures, like the acute “C”-shape of the stomach and the presence of the *torus pyloricus*, can impede capsule passage. These differences necessitate additional measures such as prokinetics for capsule administration. Additionally, successful retrieval of CapSa requires specific modifications, such as altering the slatted floor, providing liquid feed before administration, and performing rectal lavages, to ensure the safe passage through the GIT. The detailed methodology on how to administer and retrieve the CapSas is detailed in **Manuscript I**.

2.3.1.2. *Validation in postweaning pigs and fattening pigs*

After developing the SOP, we proceeded to test the first hypothesis, aiming to **validate that CapSa effectively collects small intestine content from both post-weaning and fattening pigs**. The challenge was to determine the exact sample location, which may vary with the pig's size, but to ensure that the sample would be consistently collected from the small intestine. This validation step aimed to demonstrate that increasing pig weight does not impact the device's functionality and to identify the specific part of the small intestine from which the sample was collected. Detailed results of these experiments are provided in **Manuscripts II and III**.

2.3.2. *Study of the dynamic picture of pig's microbiota*

2.3.2.1. *Evolution of the small intestine microbiome in pigs from post weaning to slaughter under normal*

Using the knowledge acquired from the validation studies, we aimed to tackle the second hypothesis: to show that the **gut microbiota undergoes distinct changes from post-weaning to slaughter, reflecting different stages of the pig's life cycle.** The results of this study are detailed in **Manuscript IV.**

2.3.2.2. *Evolution of the small intestine microbiome in pigs from post weaning to slaughter under pathological conditions*

Regarding the third hypothesis, we anticipated that the **gut microbiota composition of individual pigs would exhibit significant alterations following an infection.** Post-weaning ETEC infections are a common challenge, and we utilized this infection model to study the evolution of the gut microbiota after infection. Our aim was to understand the compositional changes during dysbiosis and determine whether these changes are short-term or long-term. The results of this study are detailed in **Manuscript IV.**

3. Manuscript I

Method: Standard operating procedure for the administration of swallowable devices to study pig's gut content in a non-invasive way

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Abstract

Due to the evolution of welfare laws and the search for novel methods to study pig microbiota, the development of precise and non-invasive sampling methods is key to studying the microbial communities that inhabit the guts of pigs. Administering swallowable devices to pigs is always a challenge due to factors such as anatomy, the requirement for specific materials, and the need to restrain the animals. In this study, we describe a step-by-step protocol on how to administer Capsule for Sampling (CapSa), a biocompatible non-invasive device to study pig's microbiota without harming the animals. The validation of the protocol was done through two different studies. In Study 1, 92 Swiss Large White pigs (Bodyweight (BW): 6.45–71.3 kg) were administered two capsules each and monitored for the following three days for capsule retrieval. On day 3, all pigs were euthanised to locate the missing capsules directly from their gastrointestinal tracts. In Study 2, 16 Swiss Large White pigs were selected at weaning and administered CapSas at five different timepoints (T1: 52 ± 3; T2: 70 ± 3; T3: 83 ± 3; T4: 110 ± 3; T5: 126 ± 3 days of age). To retrieve the capsules in the faeces, pigs were monitored three days post-administration. At T5, the pigs were slaughtered, and CapSas that were not found in the faeces, termed as missing CapSas, were retrieved from their gastrointestinal tracts. The protocol entails acclimation of the animals, housing

modifications, administration of a prokinetic agent (prucalopride) to facilitate gastric emptying, and oesophageal intubations to overcome challenges related to administration, gastric blockage, and retrieval of the capsules. In Study 1, 46.74% of the administered CapSas were found in the faeces within 72 h post-administration, with 47.67% retrieved within the first 24 h, and 28.26% were located in the stomach. The CapSa retrieval was lowest in light pigs (<12 kg). In Study 2, 75.6% of CapSas were recovered in the faeces within 72 h post-administration, with 51.23% retrieved within the first 24 h. The CapSa retrieval rates varied depending on the administration time point being lowest at T1 and T3 and highest at T2 with intermediate values at T4 and T5. In both studies, the pH levels were affected by transit time ($P < 0.01$), resulting in a more acidic content when capsules were expelled after 36-40 h. To the contrary, the volume of the CapSa content was never affected by transit time ($P < 0.05$). In both studies, post-mortem observations showed no health-related issues except one pig from Study 2 excluded due to respiratory distress. The present study describes a valid procedure for administering CapSa or any other swallowable devices in pigs. Moreover, this procedure is applicable to singular and repetitive administrations over the lifespan of pigs.

Keywords: microbiota, sampling methods, ingestible, welfare, small intestine

Implications

The gut microbiota's impact on pig health and performance is of great importance for both research and the pig industry. Recognising the limitations of faecal microbiome studies due to variations in the small intestine's microbiome, we introduce CapSa. This biocompatible, non-invasive capsule, designed for oral administration, collects chyme from the small intestine, ensuring animal welfare. A unique standard operating procedure addresses pigs' anatomical challenges, facilitating CapSa's administration and faeces retrieval. This innovation advances accurate gut microbiota and chyme content research in swine, marking a significant step forward in the field.

Specification table

Subject	<i>Physiology and Functional Biology</i>
Type of data	Table, graph
How data were acquired	Data were gathered from two studies involving the administration of the Capsule for Sampling (CapSa). In Study 1, 92 pigs, with a BW ranging from 6.45 to 71.3 kg, each received two CapSas once. These pigs were monitored for three days and then slaughtered on the third day. Study 2 involved 16 pigs, each receiving two CapSas at five different ages (T1: 52 ± 3, T2: 70 ± 3, T3: 83 ± 3, T4: 110 ± 3, T5: 126 ± 3 days). In both studies, pigs were observed for three days following each administration, with slaughter occurring three days post-administration (Study 1) or at T5 (Study 2). The volume and pH of every retrieved capsule were measured.
Data format	Raw data, pre-processing data
Parameters for data collection	A total of 318 CapSas were orally administered. A total of 208 CapSas were retrieved in the faeces, 52 in the gastrointestinal tract, and 80 were not found. From the retrieved CapSas, 40–580 µl of chyme were collected. The pH ranged from 1 to 8.
Description of data collection	The number of CapSas administered and retrieved either in the faeces or post-mortem in the gastrointestinal tract was determined. From the CapSas retrieved in the faeces, the chyme volume and pH were determined.

Data source location	<p>Institution: Agroscope</p> <p>City/Town/Region: Posieux, Fribourg Canton</p> <p>Country: Switzerland</p> <p>Latitude and longitude (and GPS coordinates, if possible) for collected samples/data: 46°46'07.50" N, 7°06'17.90" E</p>
Data accessibility	<p>Data and supplementary materials used for this paper can be obtained from the repository.</p> <p>Access:</p> <p>https://zenodo.org/records/13131997?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjJkNmMDViLTk2YWEtNGZiNC05NDI2LTRkOjVWjNjNhN2QyYSIsImRhdGEiOnt9LCJyYW5kb20iOiIzNGM2MTI5OTc0YzNhN2Y4NGJjNjg5MTg2YTlhOWU4MCI9.v6vDLR1pYaMc59LgeHUeVOCx2dJ6DxTw9juTez2RktvK-LpL1PWaYHWx2MESPXT27w6wucxpjYIfwo3TW3U8Q</p>
Related research article	<p>García Viñado, I., Correa, F., Trevisi, P., Bee, G., Ollagnier, C., 2024. A non-invasive tool to collect small intestine content in post weaning pigs: validation study. Scientific Reports 14, 9964. doi:10.1038/s41598-024-59950-3.</p>

Introduction

Microbiota, the diverse community of microorganisms residing in the gastrointestinal tract (GIT) of animals, play a crucial role in host metabolism and in maintaining host health and overall well-being (Isaacson & Kim, 2012; Luo et al., 2022). Therefore, accurate and efficient microbiota sampling methods are essential for studying the composition and evolution of the microbial community within the porcine gut.

Traditional microbiota sampling methods in pigs have primarily relied on either invasive techniques, such as cannulation or post-mortem sampling, or non-invasive methods, such as collecting faecal samples and rectal swabs (Zhao et al., 2015). Although these methods provide valuable insights, they pose several challenges regarding accuracy and limitations in repeated sampling over time. Thus, there is a growing demand for non-invasive, repeatable, and less stressful sampling methods that can allow researchers to study intestinal microbiota (Amoako-Tuffour et al., 2014; Tang et al., 2020).

A novel approach to addressing these challenges is the development and implementation of swallowable devices designed to collect chyme samples at a determined location in the GIT and then transit the digestive tract. Some of these devices have already been used in humans (Folz et al., 2023; Rezaei Nejad et al., 2019a; Shalon et al., 2023) and dogs (Menard et al., 2023). These devices offer a less invasive and more animal-friendly alternative for microbiota sampling. However, only one of these devices has been tested in pigs (Rezaei Nejad et al., 2019a) demonstrating a lower rate of retrieval. This underscores the need for an administration procedure tailored specifically to pigs.

The capsule for sampling, referred to as **CapSa**, is a size 0 capsule that collects intestinal content directly from the pigs' guts. It is administered orally, and its sampling mechanism is based on the physicochemical properties of the environment. Its sampling mechanism has already been validated in vitro (García Viñado et al., 2022) and in vivo (Inés García Viñado et

al., 2024), specifically for collecting gut microbiota from the upper portion of the small intestine. However, the successful implementation of swallowable devices in pigs requires standardised administration protocols. Standardisation and refinement of these protocols are imperative to ensure consistent and reliable results. Factors such as administration procedure, materials and housing, and acclimation conditions must be optimised to overcome challenges due to the anatomy and physiology of the pig. In this study, a standardised administration protocol applicable to singular and repetitive administrations of CapSa in pigs was validated.

Materials and methods

Design and operating principle of the CapSa

The CapSa measures 21.7 mm in length with a diameter of 7 mm, corresponding to a rigid size 0 capsule. Its movement along the digestive tract is purely passive, and its transit speed depends entirely on intestinal peristalsis. The capsule can collect a maximum of 400 μ L of GIT content and is engineered to follow a specific sequence of actions: once ingested, it passes through the stomach to the small intestine, where it opens to collect a sample. Within 10 s after sampling, CapSa seals shut and continues its journey through the large intestine, ultimately being expelled with the faeces. The opening mechanism for sample collection is pH dependent (García Viñado et al., 2022).

Study design

Study 1: Validation of capsule administration protocol in pigs of various ages

A total of 92 Swiss Large White pigs from 6.45 to 71.3 kg BW were selected; 57% were females and 43% were castrated male pigs. (Table 1). The pigs originated from 10 different farrowing batches. All pigs were orally administered two capsules that were retrieved in the following three days from the faeces or directly from the animal after euthanasia on day three.

Table 1. Numbers of pigs, their BW and sex distribution across the 10 runs in Study 1.

Run#	N=	BW \pm SD, kg	Castrates, %	Females, %
1	3	17.9 \pm 2.59	100.0	0.0
2	6	13.7 \pm 1.40	50.0	50.0
3	6	13.45 \pm 2.02	50.0	50.0
4	12	7.79 \pm 0.98	50.0	50.0
5	9	13.6 \pm 0.96	22.2	77.8
6	12	34.27 \pm 1.62	8.3	91.7
7	12	41.45 \pm 3.00	50.0	50.0
8	12	58.98 \pm 2.51	50.0	50.0
9	12	61.15 \pm 4.12	41.7	58.3
10	8	60.41 \pm 7.27	50.0	50.0

BW = Bodyweight; SD = Standard Deviation.

Study 2: Tracking capsule retention and recovery across key developmental stages in Swiss

Large White pigs

For this second study, a total of 16 Swiss Large White pigs were selected at weaning: 50% were females and 50% were castrated male pigs. The pigs came from the same farrowing batch, and from 4 litters (4 pigs per litter). All pigs were orally administered two capsules at five different time points: T1: 52 \pm 3, T2: 70 \pm 3, T3: 83 \pm 3, T4: 110 \pm 3, and T5: 126 \pm 3 days of age (Table 2). For the three days following each administration, the pigs were monitored five times daily to ensure the collection of the capsules from their faeces. At 140 \pm 5 days of age, all pigs were slaughtered, and we searched for any missing CapSas.

Table 2. BW and age of pigs at the different administration timepoints in Study 2.

Capsule administration	Age, d	BW \pm SD, kg
T1	52 \pm 3	13.92 \pm 1.79
T2	70 \pm 3	21.48 \pm 2.39
T3	83 \pm 3	30.43 \pm 3.13
T4	110 \pm 3	59.16 \pm 5.83
T5	126 \pm 3	82.04 \pm 8.47

BW = Bodyweight; SD = Standard Deviation.

Pre-administration preparation: Housing, enrichment, and dietary adjustments for pigs prior to capsule administration

To administer CapSa, pigs from both studies were allocated in individual pens (total surface area of 4.47m²) 3 days prior to the administration. The straw was removed two days prior to the CapSa administration, and plastic toys, rope, and softwood were introduced as enrichment material. The pigs were accustomed to liquid meals two days before capsule administration. Pigs were provided with a half-liquid meal one day prior to CapSa administration (day -1) and had no access to food 12 h before capsule administration.

Prokinetic and CapSa administration by oesophageal sondage

A prokinetic was administered 40 min before the administration of the CapSas. The administration of both the prokinetic agent and CapSas was facilitated via oesophageal sondages. For this procedure, pigs were placed into a pig sling adapted to their BW, and a trained individual conducted the oesophageal sondage using a sonde (see Supplementary Figure S1) and mouth gag (Mouth bite bar – small 20mm, Ellegaard Goettingen minipigs, Dalmoose, Denmark). For intubation, the sonde was gently inserted through the mouth gag and placed in the back of the throat. When the pig was inhaling, the sonde was gently pushed

further, so that it was swallowed and reached the oesophagus. Afterwards, the sonde was pushed to the end of the oesophagus and then backed up a few centimetres. Attention was paid to aligning the pig's head with the rest of the body to ease the intubation, especially for the swallowing phase. When the sonde was in place, the CapSa or the prokinetic were delivered through the sonde. The procedure concluded with the careful removal of the sonde. For the administration of the prokinetic, prucalopride (Resolor ®, Takeda Pharma AG, Glattpark, Switzerland) at a dose of 0.15 mg/kg BW was dissolved in 10 ml of water and delivered through the sonde, which was subsequently rinsed with water to ensure full delivery of the dose of the prokinetic agent. For the CapSa administration, two CapSas were delivered via the sonde, followed by 10 ml of orange juice. Each CapSa was uniquely labelled with the pig ID and the capsule ID, that is, 1 or 2. All pigs were fed ad libitum 4 h after capsule administration with a standard diet formulated to meet the nutritional requirements of their production stage (Agroscope, 2005).

Capsule retrieval and collection of capsule's contents

Several modifications were made to the housing system to ensure the retrieval of the CapSas. In each box (4.47 m²), the slatted floor area was reduced to 1.73 m². The openings of the slatted floor were narrower than CapSa's diameter. Furthermore, the faeces of the pigs were examined for the presence of the CapSas five times daily from day 1 until day 3 post administration. On day 1 post administration, a rectal lavage was conducted twice, utilising 50 mL of warm water combined with hand soap. Performing rectal lavages 24 h post-administration enables us to expedite the retrieval of CapSas from faeces, ensuring their safe retrieval.

Immediately after the retrieval of the CapSa, the outside of the capsule was cleaned with 70° alcohol to avoid contamination of the content. After the CapSa was opened, the content was extracted using a micropipette and sterile and DNA-free tips while measuring the volume.

The content was then put in a sterile 0.5 ml Eppendorf (Eppendorf SE, Hamburg, Germany), snap frozen in liquid nitrogen, and stored at -80°C until analysis. The pH of the sample was measured using litmus paper (Merck KGaA, Darmstadt, Germany) by immersing it on the inside of the empty capsule.

Post-mortem observations

All pigs from Study 1 were euthanised 3 days after capsule administration. Meanwhile, pigs from Study 2 were sent to the slaughterhouse at 140 ± 5 days of age. In instances where the CapSas were not recovered from the faeces, the chyme of the pigs' GIT was examined. Additionally, all GITs were checked for macroscopic lesions potentially linked to the administration protocol of the CapSa (for example, gastric ulcers, intestinal perforations, etc).

Calculations and statistical analysis

All statistical analyses were performed in R (v 4.3.1). For all statistical analyses, a difference was declared significant if the P-value < 0.05 and a trend was considered when $0.05 < P < 0.10$.

Study 1

For the pigs used in Study 1, four BW categories were defined: XS: < 12 kg BW; S: ≥ 12 – 20 kg BW; M: ≥ 20 – 40 kg BW; L: ≥ 40 – 70 kg BW (Table 3). The percentage of CapSas retrieved from the faeces, stomach, or not found was calculated based on the number of CapSas in each category divided by the number of CapSas administered. The capsule's transit time was calculated as the time between administration and retrieval from the faeces of the pigs. Percentages were analysed using linear regression with BW category, sex, and their interaction as fixed effects. An ANOVA was performed to check the effect of the BW category and sex on the outcome of the CapSas. The volume and pH of the CapSas were analysed using linear regression with BW category, sex, and the BW category \times sex

interaction as fixed effects and transit time as covariant. An ANOVA was performed to assess the effects of the BW category, sex, and time of retrieval on the pH and volume of the sample of the retrieved CapSas. Interactions were removed from the final model if not significant ($P > 0.05$). Type 3 ANOVA was used if the interaction was significant and type 2 ANOVA if not. Post-hoc tests, such as least squares means, were performed when ANOVA detected an effect of BW category or sex on capsule result, transit time, volume and pH.

Table 3. BW and sex distribution of pigs across the four BW categories in Study 1.

BW category	N=	BW \pm SD, kg	Castrates, %	Females, %
XS (< 12 kg)	14	8.31 \pm 1.57	50.0	50.0
S (\geq 12 – 20 kg)	21	14.07 \pm 1.41	66.7	33.3
M (\geq 20 – 40 kg)	17	34.34 \pm 4.02	82.4	17.6
L (\geq 40 – 70 kg)	40	56.76 \pm 7.99	52.5	47.5

BW = Bodyweight; SD = Standard Deviation.

Study 2

The percentage of CapSas retrieved and transit time was calculated as described in Study 1. Percentages were analysed using linear regression with administration time, sex, and their interaction as fixed effects. An ANOVA was performed to check the effect of administration time and sex on the outcome of the CapSas. The pH and volume of the CapSas were analysed using linear regression with administration time, sex, transit time, and the administration time \times sex interaction as fixed effects. An ANOVA was performed to assess the effects of administration time, sex, and time of retrieval on the pH and volume of the sample of the retrieved CapSas. Interactions were removed from the final model if not significant ($P > 0.05$). Type 3 ANOVA was used if the interaction was significant and type 2 ANOVA if not. Post-hoc tests, such as least squares means, were performed when ANOVA detected an effect of administration time point or sex on capsule result, transit time, volume and pH.

Results

Study 1

Capsule retrieval and sample extraction

Of the 184 CapSas administered, 86 (46.74%) were found in the faeces within 72 h post-administration. Regarding the transit time of these 86 CapSas, 47.67% passed through the GIT within the first 24 h, an additional 48.84% were retrieved within the following 48 h post-administration, and the remaining 3.49% of CapSas were retrieved 72 h post-administration (Figure 1). Furthermore, 28.26% of CapSas were found in the stomach, and 22.28% of CapSas were classified as “not found”. Additionally, due to animal handling issues, five CapSas were not administered to three pigs: two with a low BW (6.91 and 7.2 kg) and one pig with a high BW of 41.8 kg. Independent of sex, the lowest percentage of retrieved CapSas was observed in the faeces of XS pigs, whereas the highest percentage was found in S pigs ($P < 0.05$). Intermediate values were observed for the M and L pigs. By contrast, the percentage of retrieved CapSas in the stomach was higher in the XS category compared to all the other BW categories ($P < 0.05$). Neither age nor sex had an impact on the percentage of capsules ($P \geq 0.30$). Out of the 86 capsules retrieved from faeces, 71 (82.5%) had a pH > 5 . The pH of the retrieved capsules was not affected by BW category ($P = 0.10$) or sex ($P = 0.38$). The mean values of pH ranged between 6.5 and 7 across all BW categories (Table 4). However, the pH of the samples was affected by the capsule transit time increase ($P < 0.01$, Figure 2), which could mean that the content becomes more acidic the longer the capsule takes to exit the stomach, possibly due to contamination with gastric chyme or bacterial fermentation within the capsule. The volume of the collected digesta samples was affected by the bodyweight category ($P < 0.01$) and sex ($P = 0.02$) but not by the time of retrieval ($P > 0.05$) (Figure 2). The highest sampled volumes were found in capsules from animals in BW category M and XS (246 and 242 μL , respectively), while lower volumes were found in S and L pigs (158 and

172 μL , respectively). In the case of sex, capsules from castrated males had higher volumes (226 versus 183 μL) (Table 4).

Figure 1. Time (h) of transit of capsules found in faeces of pigs in Study 1. Time is calculated as the difference between the time of administration and the time of recovery.

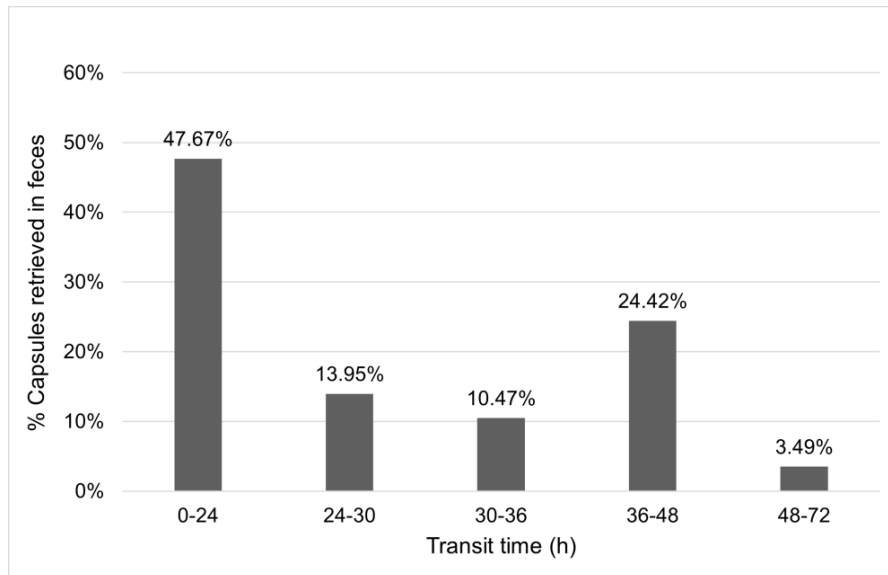


Figure 2. pH and volume of capsule's content from pigs in Study 1 depending on capsule's transit time. The pH and volume of the capsule were analysed using a linear regression with transit time as fixed effect. Only p-values that are significant (<0.05) or tend to be significant ($0.05 < P < 0.10$) are shown.

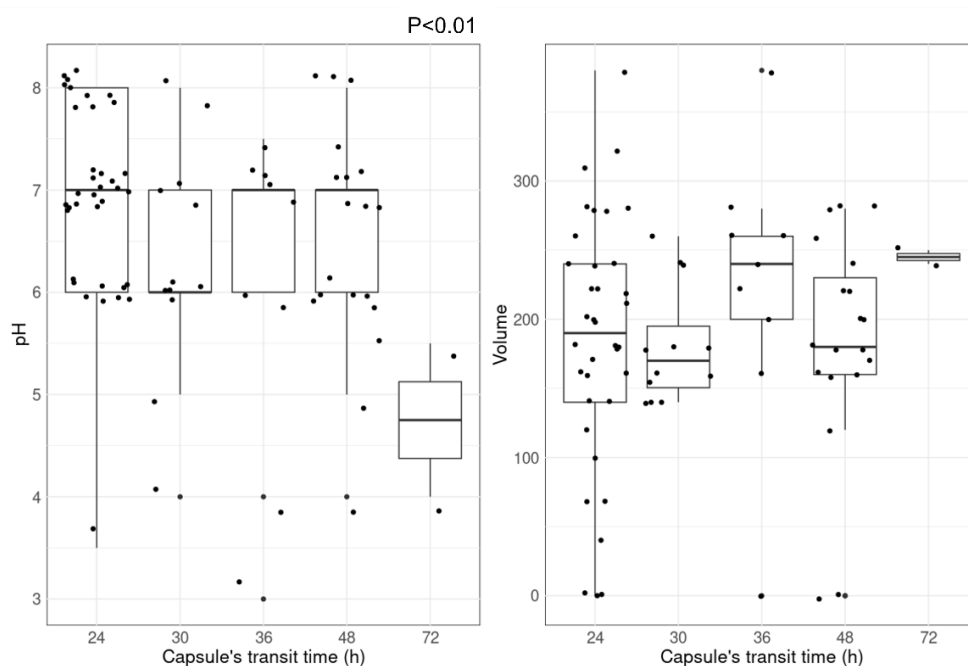


Table 4. Summary of capsule retrieval rate, and characteristics (volume and pH) by BW category and sex of pigs in Study 1. Mean % calculated by linear models.

	BW category ¹						Sex ²			
	XS	S	M	L	SEM	P ³	C	F	SEM	P ⁴
% Capsules ⁵										
in faeces	2.2 ^a	59.6 ^b	62.1 ^{ab}	57.4 ^{ab}	16.10	0.03	50.7	40.0	9.89	0.42
in stomach	97.7 ^a	21.1 ^b	8.2 ^b	10.8 ^b	9.49	< 0.01	34.3	34.6	5.84	0.97
not found	0.00	19.3	29.7	31.8	14.04	0.30	15.0	25.4	8.64	0.37
Transit time, h ⁵	70.3 ^a	29.8 ^b	34.6 ^b	33.8 ^b	11.67	< 0.01	43.8	40.5	3.77	0.24
Volume, µl ⁶	242.0 ^{ab}	158.0 ^a	246.0 ^b	172.0 ^a	81.1	< 0.01	226.0 ^a	183.0 ^b	25.1	0.02
pH ⁶	6.7	6.0	6.7	6.8	1.18	0.10	6.67	6.42	0.37	0.38

Post-mortem observations

There were no abnormal health observations, and none of the pigs had to be euthanised for health-related issues. There was no tissue damage linked to the capsule administration and/or the capsule passage observed after euthanasia in any of the pigs. Every capsule retrieved on day 3 after administration was found in the stomach.

^{ab} Values within a row with different superscripts differ ($P < 0.05$).

¹ XS: < 12 kg BW; S: ≥ 12–20 kg BW; M: ≥ 20–40 kg BW; L: ≥ 40–70 kg BW

² C. castrate; F: female

³ P-value for the effect of BW category

⁴ P-value for the effect of sex

⁵ Percentages were analysed using linear regression with BW category, sex and their interaction as fixed effects. An ANOVA was performed to check the effect of BW category and sex on the outcome of the CapSas and transit time.

⁶ Volume and pH were analyzed using linear regression with BW category, sex and the BW category × sex interaction as fixed effects and transit time as covariant.

Study 2

Capsule retrieval and sample extraction

A total of 158 CapSas were administered, and within 72 h of administration, 121 (75.6%) were recovered in the faeces. However, 23.1% were classified as “not found”, and 2 capsules could not be administered to one pig (1.2%) on T5. For the CapSas retrieved from faeces, 51.23% transited throughout the digestive tract within 24 h, and 38.02% in the following 48 h post-administration (Figure 3). Only 10.74% were retrieved later in the following days after administration. Regardless of sex, the lowest CapSa retrieval rate was observed at T1 and T3, and the highest at T2, with intermediate values at T4 and T5 ($P < 0.05$) (Table 5). Only at T1 CapSas were retrieved after ≥ 312 h after administration. Age had an impact ($P < 0.05$) on the outcome of capsules (found in faeces, found late or not found) but not sex ($P > 0.05$) (Table 5). Out of the 121 capsules retrieved from faeces, 84.2% had a $\text{pH} > 5$.

The pH of the retrieved capsules was not affected by the administration time point ($P = 0.10$), or by sex ($P = 0.12$). The mean values of pH ranged between 5.8 and 6.5 across all administration time points (Table 5). The pH of the CapSa samples was highly affected when the capsule transit time increased ($P < 0.01$, Table 5). In fact, capsules with a $\text{pH} < 5.5$ are only retrieved after 36 hours (Figure 4). The volume of the collected digesta samples tended to be affected by the bodyweight category ($P = 0.06$) but not by sex ($P = 0.21$) or the time of retrieval ($P = 0.97$) (Figure 4). The highest sampled volumes were found in capsules from T2 (243 μL), followed by samples from T1 and T4 (219 and 217 μL , respectively), while lower volumes were found in T3 and T5 (184 and 192 μL , respectively) (Table 5).

Figure 3. Time (h) of transit of capsules found in faeces of pigs in Study 2. Time is calculated as the difference between the time of administration and the time of recovery.

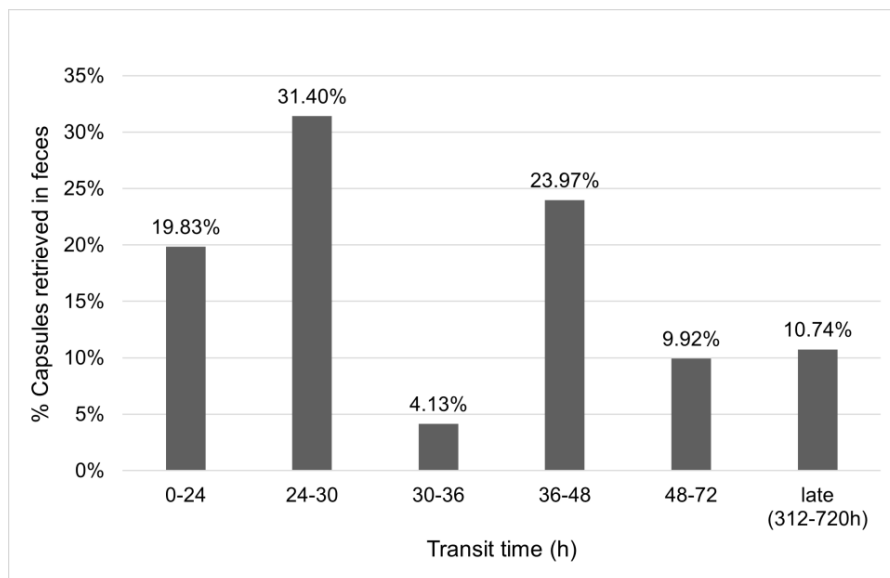


Figure 4. pH and volume of capsule's content from pigs in Study 2 depending on capsule's transit time. The pH and volume of the capsule were analysed using a linear regression with transit time as fixed effect. Only p-values that are significant (<0.05) or tend to be significant ($0.05 < P < 0.10$) are shown.

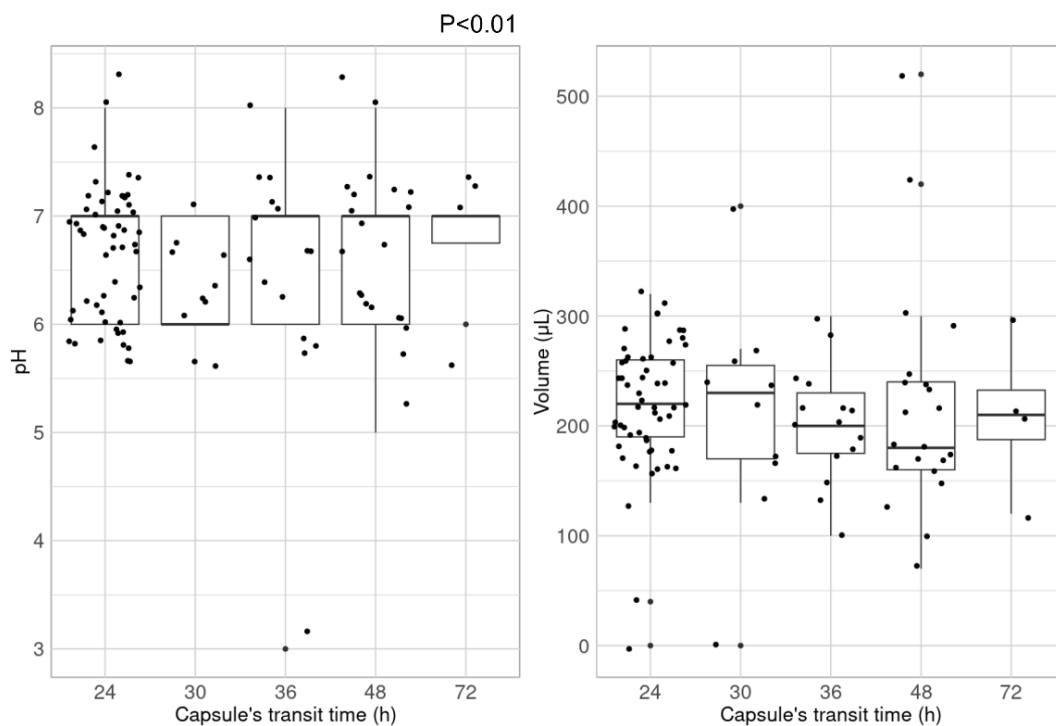


Table 5. Summary of capsule retrieval rate, and characteristics (volume and pH) by BW category and sex of pigs in Study 2. Mean % calculated by linear models.

	Timepoint of administration ⁵							Sex ⁶			
	T1	T2	T3	T4	T5	SEM	P	C	F	SEM	P
% Capsules ⁴											
in faeces	56.2 ^a	93.8 ^b	59.4 ^a	71.9 ^{ab}	62.5 ^{ab}	4.74	0.02	67.5	70.0	3.00	0.58
in faeces “late” ⁷	34.0 ^a	0.0 ^b	0.0 ^b	0.0 ^b	0.0 ^b	1.40	< 0.01	7.5	6.3	0.88	0.37
not found	9.4 ^{ab}	6.2 ^a	40.6 ^b	28.1 ^{ab}	37.5 ^{ab}	5.13	0.02	25.0	23.8	3.25	0.79
Transit time, h ⁴	169.7 ^a	39.4 ^b	62.1 ^b	30.7 ^b	40.6 ^b	27.7	< 0.01	64.4	72.6	15.7	0.70
Volume, µl ⁵	219.0	243.0	184.0	217.0	192.0	17.8	0.06	220.0	202.0	10.25	0.21
pH ⁵	5.8	6.4	6.4	6.4	6.5	0.195	0.10	6.4	6.2	0.11	0.12

Post-mortem observations

None of the pigs had to be euthanised for health-related issues. Only one pig was excluded from capsule administration due to respiratory distress, which upon post-mortem examination revealed upper oesophageal damage, probably due to an incorrect intubation procedure. As for the remaining pigs, there was no tissue damage linked to the capsule administration and/or the capsule passage observed after slaughter. There were no capsules found in the stomach

^{ab} Values within a row with different superscripts differ ($P < 0.05$).

⁵ T1: 52 ± 3 d; T2: 70 ± 3 d ; T3: 83 ± 3 d; T4: 110 ± 3 d; T5: 126 ± 3 d

⁶ C: castrate; F: female

⁷ In faeces “late”: capsules retrieved more than 3 days after administration (e.g. capsules from T1 retrieved in faeces after administration in T2)

⁴ Percentages were analysed using linear regression with time point of administration, sex and their interaction as fixed effects. An ANOVA was performed to check the effect of time point of administration and sex on the outcome of the CapSas and transit time.

⁵ Volume and pH were analysed using linear regression with time point of administration, sex, transit time, and the time point of administration × sex interaction as fixed effects.

after slaughter; probably, the capsules were expelled between administrations when the pig's faeces were not monitored.

Author's point of views

The present study showed a valid procedure for administering CapSa in pigs. To address gastric blockage in a pig's stomach and ensure successful CapSa administration and retrieval, several strategies were implemented. First, to facilitate the process, straw was removed from the pig's enclosure two days prior to the administration of the CapSas, and the pig's diet was switched from solid to liquid. This dietary modification and straw removal were aimed at shortening the digestive transit time and enhancing gastric emptying. According to the literature (Henze et al., 2021), gastric emptying is highly variable and can range from 20 to 233 h. By allowing the stomach to be emptier, we hoped for a faster and more efficient capsule passage through the GIT. Furthermore, on the day preceding CapSa administration, pigs were provided with a semi-liquid meal and fasted for at least 12 h before the capsule was given. Again, this allowed us to have an emptier GIT that would allow the capsule to pass through easier and faster. As has been proven in previous studies (Ochia, 1973), the rate of gastric emptying for pigs is very rapid at first and later slows down after fasting. This sudden initial gush is due to the latent period between duodenal distension by gastric contents and its response in regulating gastric emptying.

Second, to assist the capsule in bypassing the gastric blockage, administration of a prokinetic agent was necessary to increase gastric contractions, facilitating passage through the pylorus and reducing the capsule's transit time through the GIT. The pig's stomach, characterised by its pronounced "C" shape and the close proximity of the gastric cardia to the pylorus, presents challenges for gastric emptying (Henze et al., 2021). The presence of a transverse pyloric fold, known as the "*torus pyloricus*" (Bal & Ghoshal, 1972; Kopáčová et al., 2010), in the

pyloric aperture further complicates the process. This anatomical feature, designed to prevent the passage of unprocessed solid gastric contents into the small intestine, contributes to slower gastric emptying and can result in particles larger than 1 centimetre being retained in the stomach for extended periods (Davis et al., 2001; Hossain et al., 1990). Among the prokinetic agents evaluated, prucalopride, a “last generation” serotonergic 5-HT₄ agonist, has demonstrated efficacy in enhancing peristalsis within both the stomach and colon, thus promoting gastric emptying. Prucalopride’s effectiveness, as noted in several studies (Michael Camilleri & Jessica Atieh, 2021; Priem et al., 2012), distinguishes it from other prokinetics, particularly in maximising capsule retrieval rates. This attribute makes prucalopride a preferred choice for facilitating the passage of the capsule through the pig’s GIT.

Third, to counteract the fasting-induced rise in gastric pH prior to administering the CapSa, orange juice was co-administered with the CapSas. The acidity of orange juice helps maintain an acidic environment around the capsule as it reaches the stomach, a crucial step because the capsule’s collection mechanism is triggered at a pH > 6 (García Viñado et al., 2022), a condition typically found beyond the stomach, in the GIT.

Sondes were adapted to the size of the pig (see Supplementary Figure S1 for more detail). According to our measurements, the length of the oesophagus (L_o) of the pig is approximately 60% of the length of the pig from the groin to the base of the tail (L_{g-t}). The estimation of the size of the sonde was done using the following equation:

$$L_o = 0.6 \times L_{g-t}$$

To ensure at least the retrieval of one capsule per pig, all pigs were administered two CapSas. In the current study, the described protocol successfully enabled the recovery of 86 capsules (46.74%) in Study 1 and 121 capsules (75.6%) in Study 2. These findings are highly promising, indicating the efficacy of the administration protocol in both singular and repeated applications.

In Study 2, our results demonstrated that the efficacy of the administration protocol was maintained even with repeated application. Although there is a significant effect of administration time on the percentage of capsules recovered from faeces ($P = 0.02$), the retrieval rate consistently exceeded 50%, ranging from a minimum of 56.2% to a maximum of 93.8%. Notably, our findings indicate that the protocol's effectiveness is not compromised by repetition. We have also observed that the repeated administration of prucalopride does not appear to reduce the prokinetic effect of prucalopride in pigs; these findings are consistent with previous *in vitro* results in pigs (De Maeyer et al., 2009). Furthermore, regardless of sex, the protocol yielded consistent results, suggesting its applicability to both females and castrated males.

Transit time is strongly influenced ($p < 0.01$) by both body weight category and time of administration (Tables 4 and 5). Indeed, the smallest pigs and the first time of administration had a longer transit time than the rest of the body weight categories and the following time of administration, respectively. In Study 1, we observed that the longest transit time for the capsules occurred in XS pigs, averaging 70.3 h, while for other weight categories, it ranged between 29.8 and 34.6 h. This discrepancy may stem from a size disproportion between the capsule (size 0) and the GIT passage in small piglets. However, we cannot definitively confirm this hypothesis, since only one pig from the XS bodyweight category expelled the capsule. In Study 2, the longest mean transit time was observed at the first administration time point (169.7 h), which significantly decreased in subsequent administration time points. This is attributed to the consistent effectiveness of the protocol in subsequent capsule administrations, which facilitated the expulsion of capsules from previous administrations.

This protocol could prove advantageous for other ingestible devices. A recent study utilising a non-invasive capsule to investigate microbiota was used in pigs (Rezaei Nejad et al., 2019a) and encountered difficulties retrieving all capsules after administration. However, successful

retrieval was achieved when administered to humans and macaques. The establishment of a standardised administration protocol could pave the way for utilising pigs as models for pharmacological and drug delivery studies.

Despite the overall promising results of the administration protocol, a notable limitation in our investigation was the inability to retrieve capsules from pigs with a light BW (category XS: < 12 kg). As shown in Study 1, 97.7% of CapSas administered to pigs belonging to the XS category were retrieved in the stomach post-mortem. This block in the stomach is due to the presence of the *torus pyloricus*, which nearly entirely obstructs the pyloric exit for solid particles in small piglets.

Another limitation of this administration procedure is the precision of the required materials (sling, sondes, mouth gag, etc.) that have to be adapted to be used in pigs, as well as the training of the person executing the oesophageal intubation. Certain protocol specifications, such as the absence of straw in the box around the administration time or the liquid diet two days before administration, even though they are essential for the procedure to work, might have consequences for the gut microbiota and should be investigated in future studies.

In conclusion, this is a valid procedure for administering CapSa in pigs, overcoming the challenges of administration, gastric blockage, and retrieval. In the future, this standard operating procedure could serve to use CapSa to study the dynamic picture of the small intestine microbiota in pigs using the same pigs.

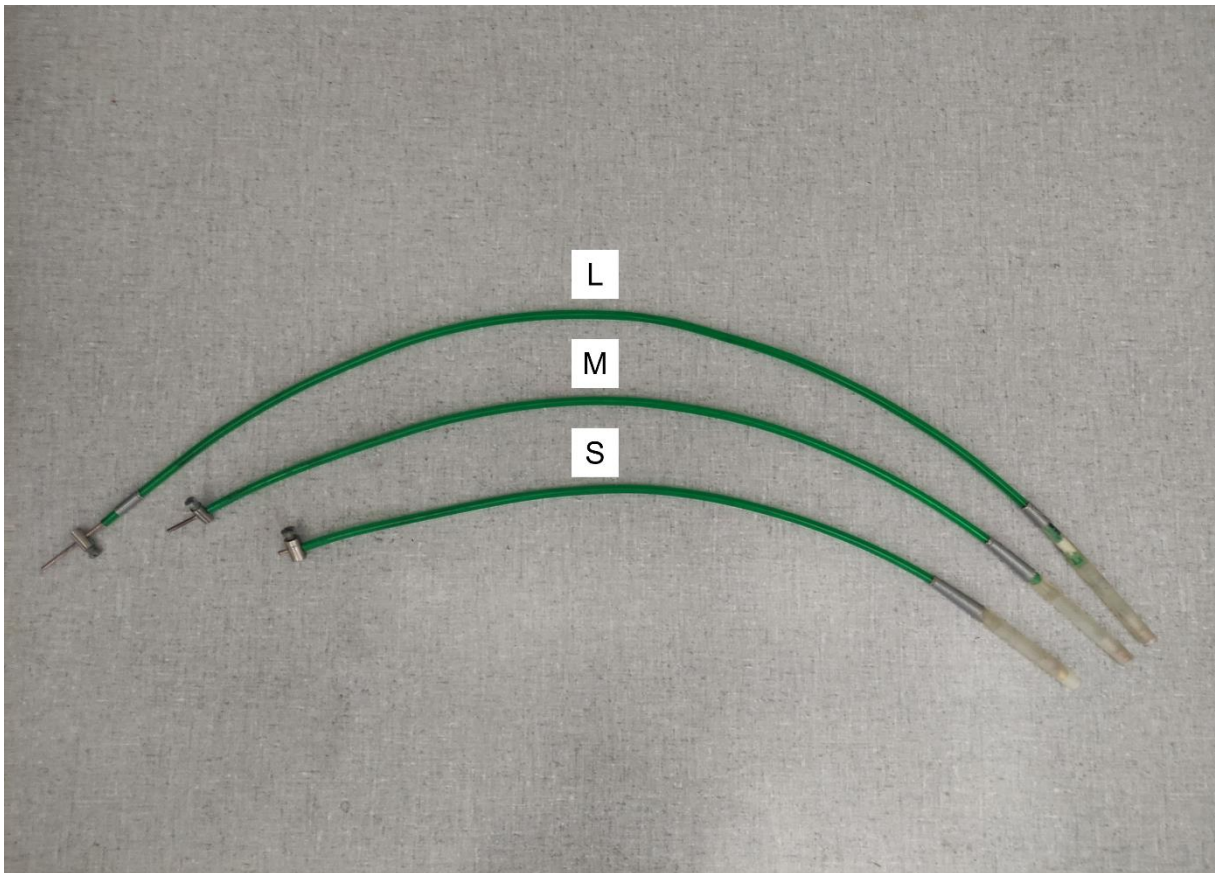
Ethics approval

All experimental procedures were in compliance with Swiss animal welfare guidelines and were approved (No. 2021-39-FR and No. 2022-26-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.

Supplementary information

Figure 1. Sondes used for oesophageal intubation of pigs



Sondes were customized to fit different sizes of pigs, with three versions developed at Agroscope Posieux: Sonde S (54.5 cm long), Sonde M (67 cm long), and Sonde L (80.5 cm long). Each sonde consists of a green flexible tube with an exterior diameter of 5.42 mm and an interior diameter of 3.58 mm, connected to a 3D-printed flexible end piece measuring 10.13 mm in exterior diameter and 8.13 mm in interior diameter. Capsules were inserted into the end piece before the sondes were inserted into the pigs and were pushed out by a metal "pusher" once the sonde was in place.

4. Manuscript II

A non-invasive tool to collect small intestine content in post weaning pigs:

Validation study

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Abstract

The Capsule for Sampling (CapSa) is an ingestible capsule that collects small intestine content while transiting through the natural digestive pathway. In this study, 14 Swiss Large White pigs weighing less than 12 kg (Category <12 kg) and 12 weighing between 12 and 20 kg (Category [12-20 kg]) were given two CapSas and monitored for three days. The animals were euthanized for post-mortem sampling, allowing us to directly obtain gut microbiota samples from the gastrointestinal tract. This post-mortem approach enabled a direct comparison between the microbial content from the gut and the samples collected via the CapSas, and it also facilitated precise identification of the CapSas' sampling sites within the gastrointestinal tract. For the category under 12 kg, only 2.3% of the administered CapSas were recovered from the feces. In contrast, in the 12-20 kg category, 62.5% of the CapSas were successfully retrieved from the feces within 48 h. Of these recovered CapSas, 73.3% — equating to 11 capsules from eight pigs — had a pH > 5.5 and were therefore selected for microbiome analysis. Bacterial composition of the CapSas was compared with that of the three segments of the small intestine, the large intestine and feces of the corresponding pig. The results were tested using a PERMANOVA model (Adonis) including sample type as a

factor, and then pairwise comparisons were made. The bacterial composition found in the CapSas differed from that of the large intestine and feces ($P < 0.01$), while it did not differ from the first segment of the small intestine ($P > 0.10$). This study provides evidence that the CapSa effectively samples the intestinal microbiota from the upper section of the small intestine in post-weaning pigs. Furthermore, it was found that the collection of CapSas could only be successfully achieved in pigs classified within the heavier weight category.

Introduction

The link between gut microbiota and pig health is well-documented (Jang et al., 2020; Schokker et al., 2015), with gut microorganisms playing crucial roles in immunity and nutrient digestion (Fouhse et al., 2016; Luo et al., 2022). While existing studies predominantly examine fecal microbiota (Maltecca et al., 2020), these do not accurately reflect the microbiota in other digestive tract segments like the small intestine (Gresse et al., 2019; Zhao et al., 2015), where the composition varies by location and pig age (Li et al., 2020; Slifierz et al., 2015). This variation is significant, especially in the small intestine, for nutrient digestion and immune system development (Duarte & Kim, 2022). Understanding host-microbiota interactions requires studying the gut microbiota's spatio-temporal changes, emphasizing the need for precise, repeatable sampling methods.

There are several methods for sampling gut microbiota. Post-mortem sampling gives access to all segments of the digestive tract, but can only be performed once on the same individual. Fecal sampling, on the other hand, is non-invasive and can be repeated several times on the same individual, but only represents the fecal microbiota (Choudhury et al., 2019). Additionally, there are other sampling methods that are considered highly invasive like endoscopy, biopsies and cannulated animals. Endoscopy allows multiple sampling of intestinal contents and tissues (biopsies), but can only be performed under general

anaesthesia. Finally, some studies have used cannulated animals for repeated sampling of the small intestine content (Nielsen et al., 2017; van der Wielen et al., 2023). For ethical and practical reasons, these last two methods are not always easily feasible. In 2020, Tang *et al.* highlighted the need for new sampling methods that would allow multiple, non-invasive samplings of intestinal contents (Tang et al., 2020).

Recent advancements in ingestible medical devices designed to traverse the digestive tract have enabled the detailed sampling and examination of the human gut microbiota (Folz et al., 2023; Rezaei Nejad et al., 2019b; Shalon et al., 2023). These devices are either an osmotic pill (Folz et al., 2023; Rezaei Nejad et al., 2019b; Shalon et al., 2023) or an enteric-coated bladder (Folz et al., 2023; Shalon et al., 2023). Their sampling mechanism is activated when they reach the small intestine. Existing devices have been developed for humans and none have been successfully tested in pigs.

The aim of this study is to validate a new capsule prototype (CapSa) for non-invasive sampling of the intestinal microbiota in pigs.

Methods

Animals and rearing conditions

For the study, 26 Swiss Large White pigs with BWs ranging from 6.4 to 20.0 kg were used. Fourteen weighed less than 12 kg (category < 12kg) and 12 weighed between 12 and 20 kg (category [12-20 kg]) (Table 1). Pigs of Category < 12kg were housed in groups of four and pigs of category [12 - 20kg] were housed in groups of two. All pigs had *ad libitum* access to a standard starter diet formulated to meet the nutritional requirements of post-weaning pigs (Agroscope, 2005) (see Supplementary Table S1). Water was available *ad libitum* and distributed via nipple drinkers. The pens (total surface area of 4.47m²) were specially

designed to collect the CapSas by minimizing the slatted area and reducing the openings of the slatted area to a size smaller than the CapSa diameter.

Table 1. Characteristics of pigs included.

Weight category	N=	BW \pm SD	%M	%F
<12kg	14	8.3 \pm 1.6	50.0	50.0
[12-20kg]	12	14.4 \pm 2.6	58.3	41.7

BW = Body Weight; SD = Standard Deviation; %M = Percentage of castrated males; %F = Percentage of females.

Description of the capsule (CapSa)

The capsule studied is 21.7 mm long with a diameter of seven mm, corresponding to a size 0 hard capsule. CapSa 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. CapSa can collect a maximum of 400 μ L.

CapSa is designed to operate as follows: Once ingested, it moves through the stomach and reaches the small intestine, where it opens to collect a sample. After collection, the CapSa closes, continues its journey through the large intestine, and is finally expelled with the stool. This single-use device is specifically designed to collect fluid samples from the intestines for later *ex vivo* analysis. As illustrated in Figure 1, the CapSa is composed of two main components: a dissolvable exterior with an enteric coating and a 3D printed bottom part. The CapSa is engineered to open when it encounters a pH level greater than 6, as the upper part dissolves, permitting intestinal fluids to enter. The entering intestinal content triggers the plunger to expand, which draws luminal content into the CapSa's inner chamber. The CapSa is designed to automatically seal once the plunger mechanism is fully extended. In vitro results show that CapSa can withstand two hours in an acidic-aqueous medium (pH<3), and

then samples within an hour of being transferred to an aqueous medium at pH=7 (García-Viñado, 2022).

Figure 1. Capsule for Sampling (CapSa) device a rule is included for size comparison.



Preparation of the animals and administration of CapSa

Two days prior to CapSa administration, three measures were taken to reduce intestinal load and shorten transit time. Firstly, two days before administering the CapSa (-2d), pigs were fed the starter diet in liquid form (ratio 1 kg of starter diet mixed with 2 L of water) and straw was removed from the pens. Secondly, one day before administering the CapSa (-1d) pigs had access to only half of their ration of feed and the feed was removed 12 h before capsules administration. Thirdly, to increase gastric emptying and thus facilitate CapSa transit through the stomach, 0.16 ± 0.015 mg/kg BW of prucalopride (Resolor[®], Takeda Pharma AG, Glattpark, Switzerland) was administered orally via an oesophageal probe 40 minutes prior to administration. Prucalopride is a 5-HT₄ serotonin agonist, which stimulates gastrointestinal peristalsis and increases gastric emptying (Briejer et al., 2001; M. Camilleri & J. Atieh, 2021; De Maeyer et al., 2008; De Ponti, 2004).

On the day of administration (0d), each pig received 2 CapSas. The capsules were administered by oesophageal sondage, while the pigs were kept in a sling adapted to their weight. A 10 mL bolus of orange juice was then administered to flush the capsule in the stomach. Every CapSa was assigned a unique number, linking it to the pig ID.

CapSa recovery and sample processing

From 0d to three days after administration (3d), pens were inspected five times a day to look for CapSas expelled in the feces. The specifically designed slatted area of the pens allowed the capsule searches by sieving feces with water over the slatted surface. When CapSas were retrieved from the feces, they were directly transported to the laboratory. The outside was cleaned with 70% alcohol to avoid contamination after the opening. The identification of the CapSa was recorded and its content was extracted and the volume of the content determined. The content was transferred to a 0.5 mL Eppendorf (Eppendorf SE, Hamburg, Germany) immersed in liquid nitrogen and stored at -80°C until analysis. The pH of the content was measured using Litmus paper (Merck KGaA, Darmstadt, Germany) by cleaning the inside of the capsule after extraction.

Post-mortem sampling

Three days after CapSa administration, all pigs were euthanised by electronarcosis. The gastrointestinal tract was extracted and starting right after the stomach the small intestine was divided into three segments (Segment 1, 2 and 3) of equal size. Samples of the three segments as well as the contents of the large intestine and feces were collected in sterile 2 mL Eppendorfs. The Eppendorfs were immersed in liquid nitrogen and stored at -80°C until analysis. The entire digestive tract was then inspected for capsules that had not yet been retrieved.

Evaluation of CapSa Innocuity

To assess the innocuity of CapSa administration, passage and retrieval, various parameters were considered to ensure the well-being of the pigs. Post-mortem macroscopic observations were conducted to assess the presence of any tissue damage related to CapSa administration and/or passage (e.g.; gastric ulcers, intestinal perforations, etc). The fecal score was determined using a 4-level scoring scale, as follows: 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). Throughout the study duration, the overall health of the pigs was monitored continuously.

Microbiota analysis

Only CapSa samples with a pH > 5.5 and recovered within 48 h of administration were used for microbiome analysis.

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 (Termo Fisher Scientific, Italy) and the universal primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNBGCASCAG-3' and Pro805R:5'-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3' (Takahashi et al., 2014). 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' (Takahashi et al., 2014). Amplicons were then sequenced by Illumina MiSeq 300 × 2 bp with the MiSeq®

V3-V4 reagent kit on the MiSeq-Illumina[®] platform. Microbiota analysis was performed using the DADA2 pipeline (Callahan et al., 2016) according to the Silva database taxonomy, version 138.1 (Quast et al., 2013). 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 position 280 and 260. All other DADA2 parameters were left with their default settings.

Statistical analysis

All statistical analyses were performed in R (v 4.3.1). The percentage of CapSas recovered in feces, stomach or not found was calculated based on the total number of CapSas administered. Percentages were analysed using linear regression with BW category, sex and their interaction as fixed effects. An ANOVA was performed to check the effect of BW category and sex on the outcome of the CapSas. CapSa transit time was calculated as the time between capsule administration and recovery in the feces. CapSa pH and volume were analysed using linear regression with BW category, sex, transit time and the BW category x sex interaction as fixed effects. An ANOVA was performed to check the effect of BW category, sex and time of retrieval on the pH and volume of the sample of the retrieved CapSas. Interactions were removed if not significant at a $P > 0.05$.

Statistical analysis of alpha and beta diversity, as well as taxonomic analysis was performed using "phyloseq" (McMurdie & Holmes, 2013) v1.38, "vegan" v2.6 (Dixon, 2003) and "microbiomeutilities" v1.0. For the alpha diversity samples were rarefied to the lowest sample depth, to avoid bias linked to different sampling efforts. Differences for alpha diversity indices (Chao1, Shannon, and Simpson diversity) between CapSa samples and other samples were assessed using the Wilcoxon test. For beta diversity, a dissimilarity matrix using Euclidean distances from the centred log-transformed (clr) data was constructed and the results represented using the PCoA plot. Differences were tested using a PERMANOVA

model (Adonis) with 9,999 permutations, including sample type as a factor. Pairwise contrasts between sample types were performed using the pairwise Adonis function included in the "PairwiseAdonis" package (Martinez Arbizu, 2020). P-values were then adjusted for multiple comparisons using the Bonferroni correction.

For all statistical analyses, a difference was declared significant if the P-value < 0.05 and a trend was considered when $0.05 < P < 0.10$.

Results

CapSa innocuity

All pigs remained healthy throughout the study. No macroscopic tissue damage was observed following euthanasia related to CapSa administration and/or passage. Administration of the CapSas had no impact on the fecal scores or the occurrence of diarrhea, with none of the pigs exhibiting a fecal score higher than 1. Moreover, all CapSas recovered on the day of euthanasia were found in the stomach.

CapSa administration and recovery

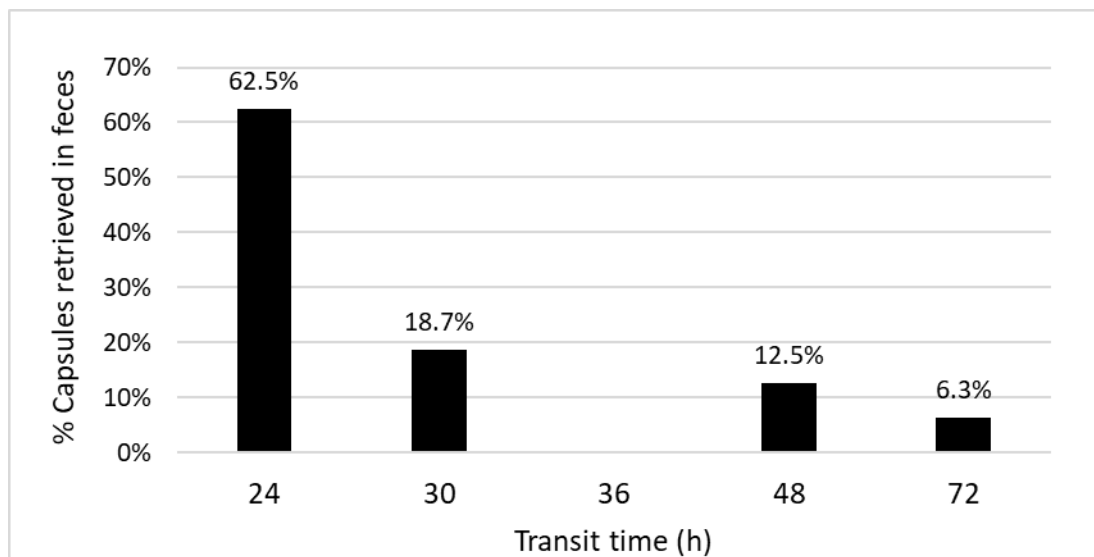
Except for two pigs weighing less than 12 kg, pigs could successfully be administered with two CapSas. Weight category strongly influenced ($p < 0.05$) whether CapSa could be retrieved in the feces or in the digestive tract after slaughter. For pigs weighing less than 12 kg, 97.7% of the CapSas (24 capsules) were located in the stomach, 2.3% (1 capsule) were found in the feces (Table 2). In pigs weighing between 12 and 20 kg, 63.4% (15 capsules) were recovered from the feces, 17.6% (4 capsules) from the stomach, and 18.9% (5 capsules) were unaccounted for. In 62.5% of cases, CapSas found in feces transited within 24 hours (Figure 2). The percentage of CapSas not found was not affected by the weight category ($P > 0.05$). Sex had no impact on CapSa fate ($P > 0.05$).

Table 2. Outcomes of capsule administration by weight category.

	Weight category		SEM	P
	<12kg	12-20kg		
Number of capsules found in feces	1	15	10.71	<0.05
Number of capsules found in stomach	24	4	9.31	<0.05
Number of capsules not found	0	5	13.60	0.34

SEM = Standard error of the mean; P = P-value of the fixed effect.

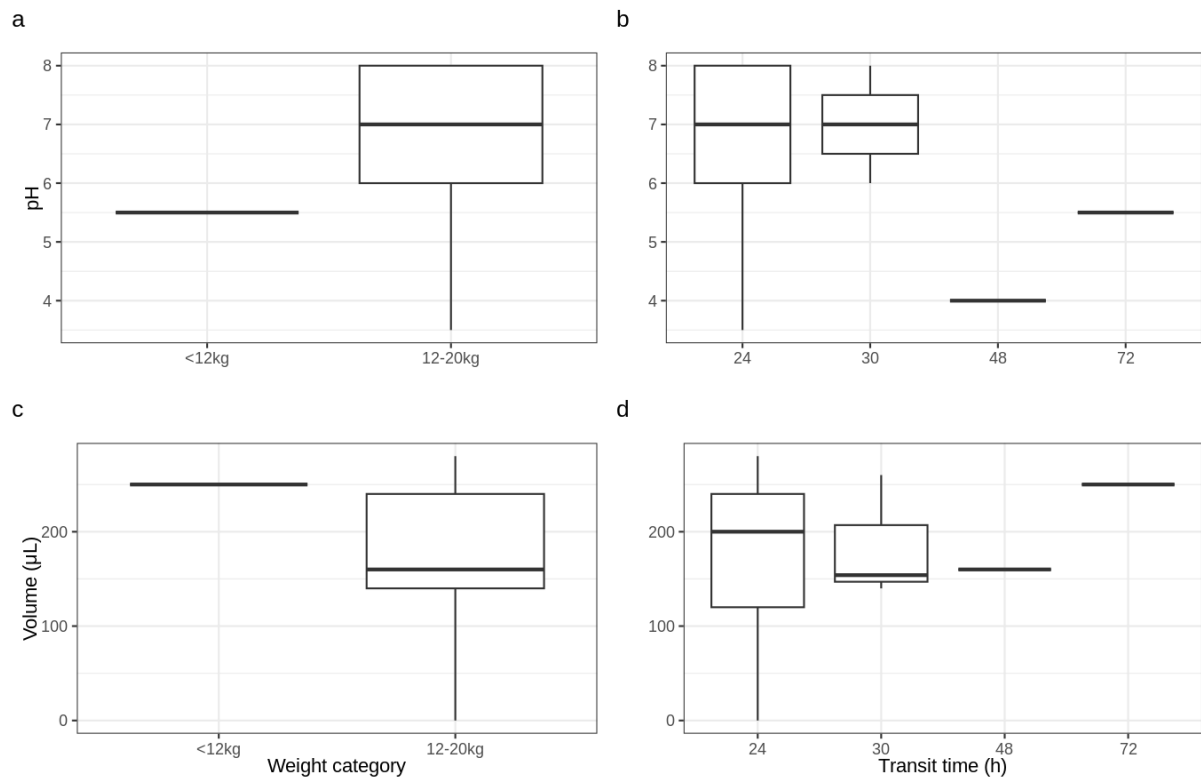
Figure 2. Time (h) of transit of CapSas found in feces. Time is calculated as the difference between the time of administration and the time of recovery.



Characteristics of CapSas recovered from feces

Of the 16 CapSas recovered from the feces, two were broken and 12 (81.2%) had a pH > 5.5. The pH of the CapSa content was not affected ($P > 0.05$) by the body weight (BW) category (Figure 3), but by the sex of the pigs ($P = 0.048$) and the transit time ($P = 0.038$). The pH of the CapSa content was < 6, when the transit time exceeded 48 hours. The volume of digesta samples collected was not affected by the weight category, sex or transit time ($P > 0.05$). All CapSas recovered from feces within 48 hours after administration and having a pH > 5.5 were considered for microbiota analysis.

Figure 3. pH and volume (μL) of CapSa contents as a function of transit time (h) and pig weight category. Weight category had no effect ($P = 0.14$) on the pH of the contents of capsules found in feces (a), but transit time strongly influenced pH ($P = 0.038$), with pH falling below six when transit time exceeded 48 h (b). The volume samples collected was not affected by weight category ($P = 0.44$), sex ($P = 0.11$) or transit time ($P = 0.59$) (c, d).



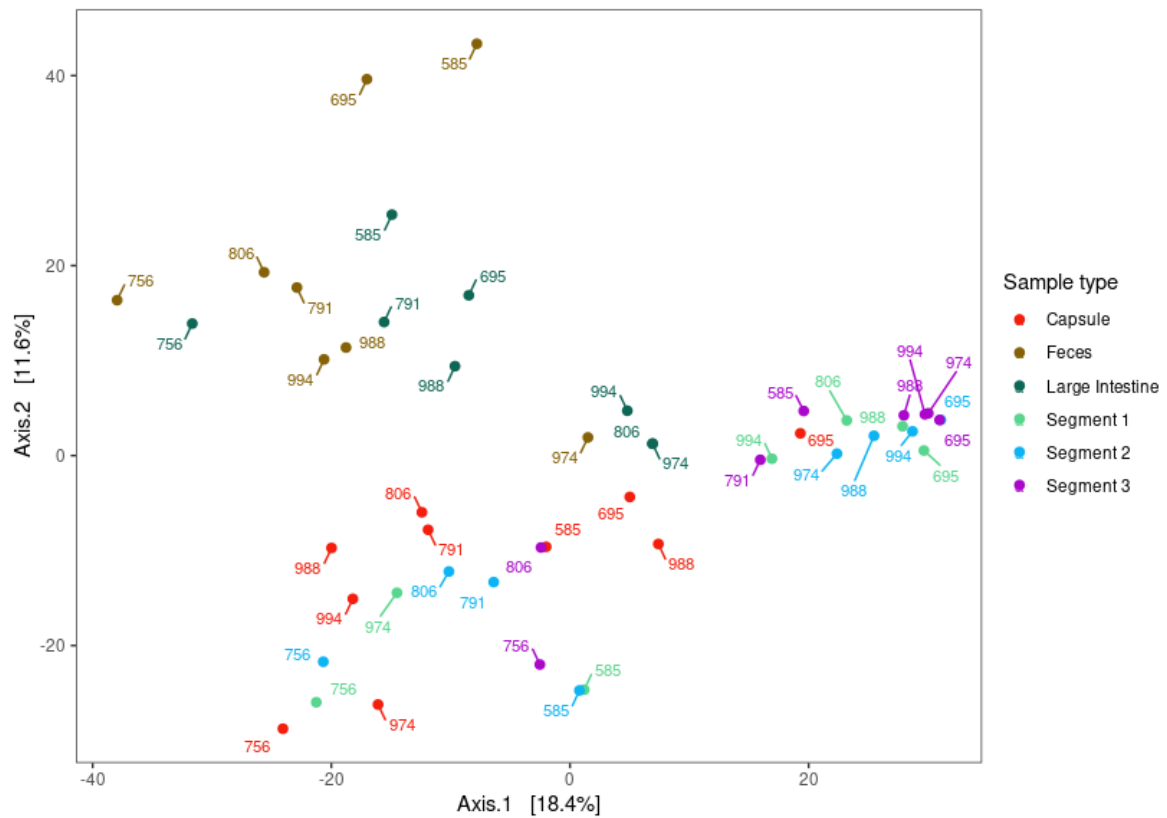
Microbiota analysis

A total of eleven CapSas collected from eight pigs weighing between 12 and 20 kg were sent for microbiota analysis. From these 11 samples, bacterial DNA was successfully extracted and amplified. Thereafter, DNA sequencing was performed for all but one CapSa that did not contain sufficient DNA.

Analysis of β -diversity by the Principal Coordinate Analysis (PCoA) demonstrated a clustering of microbiota according to sample type (Figure 4). The large intestine and feces microbiota clustered together in a first cluster, while the microbiota from samples of the

CapSas and the three segments (Segment 1, 2 and 3) of the small intestine tended to form a second cluster.

Figure 4. PCoA of Euclidean distance matrices of clr-transformed data. Samples are coloured based on sample type and labelled according to the subject.



Based on the Adonis test, sample location and origin influenced the bacterial composition ($P=0.001$). Pairwise comparisons detected differences in the bacterial composition between the CapSa contents, the contents of the three intestinal segments, the contents of the large intestine and feces (Table 3). According to this analysis, the bacterial composition of the three segments of the small intestine did not differ. There was also no difference between the bacterial composition of the large intestine and that of the feces. The microbial composition of the CapSa contents was different from that of the feces and large intestine, but not from that of Segment 1 ($P = 0.32$) and there was a tendency towards similarity with Segment 2 ($P = 0.06$) of the small intestine. It did, however, differ from that of Segment 3 ($P = 0.01$). A list of

the ten main abundance Phylum, Family and Genera (expressed as % of total with the standard deviation) can be found as Supplementary Table S2.

Table 3. Comparisons of the bacterial compositions of the capsule contents, the three segments of the small intestine (Seg 1, 2 and 3), the large intestine and feces, using the Adonis test of Euclidean distances of clr-transformed data.

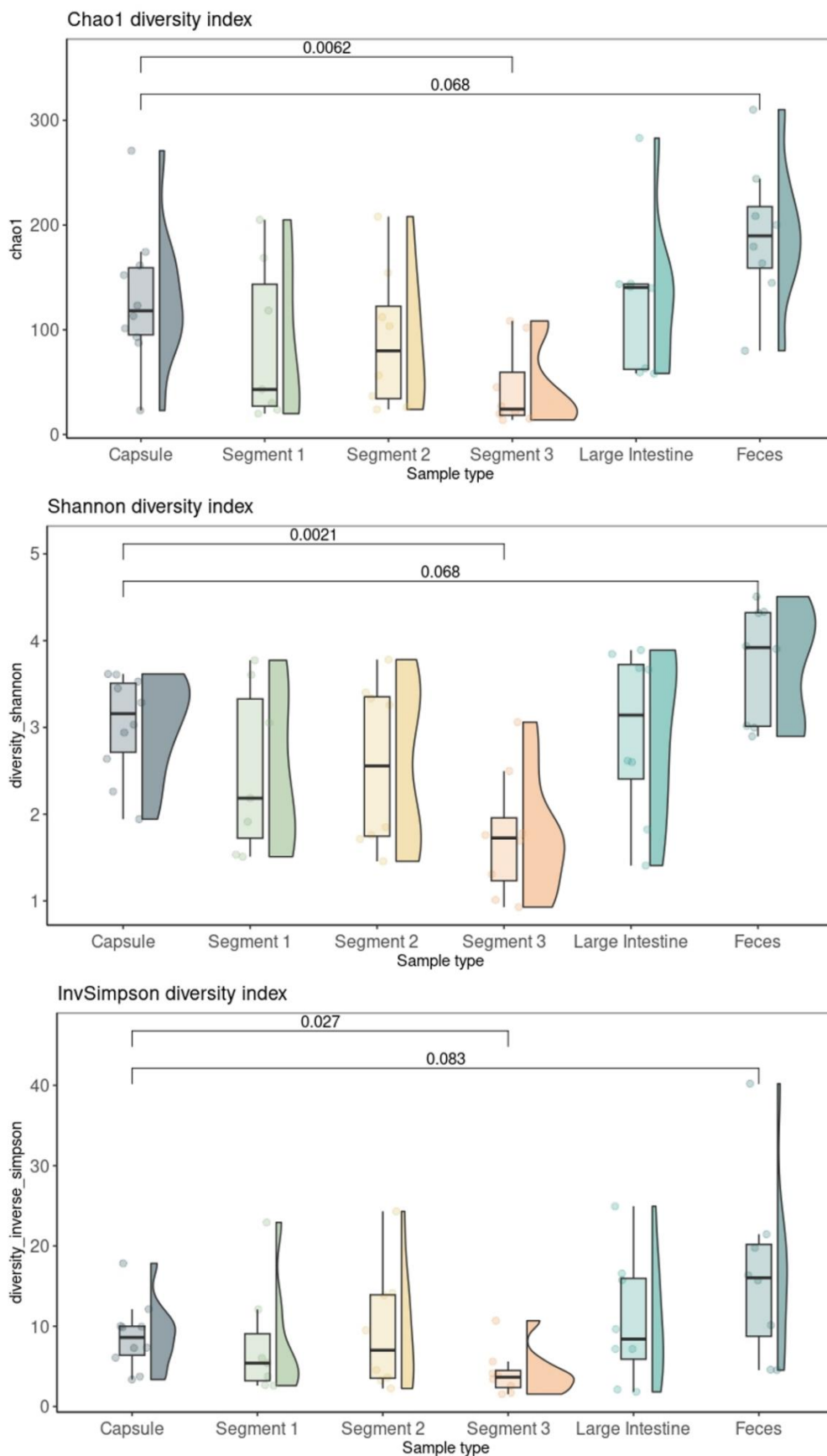
Pairwise comparisons	SumsOfSqs	F. Model	r²	P	P adj
Capsule vs Feces	7921.83	3.33	0.14	0.00	0.00
Capsule vs Large intestine	5007.93	2.56	0.11	0.00	0.00
Capsule vs Segment 3	6514.44	4.51	0.19	0.00	0.01
Capsule vs Segment 1	3623.75	2.06	0.10	0.02	0.32
Capsule vs Segment 2	4464.66	2.65	0.12	0.00	0.06
Feces vs Large intestine	3026.24	1.15	0.06	0.23	1.00
Feces vs Segment 3	9863.60	4.88	0.23	0.00	0.00
Feces vs Segment 1	8160.77	3.35	0.18	0.00	0.00
Feces vs Segment 2	8615.03	3.73	0.18	0.00	0.00
Large intestine vs Segment 3	4902.91	3.23	0.16	0.00	0.01
Large intestine vs Segment 1	3982.20	2.09	0.12	0.00	0.14
Large intestine vs Segment 2	4257.28	2.36	0.12	0.00	0.08
Segment 3 vs Segment 1	1280.95	1.02	0.06	0.32	1.00
Segment 3 vs Segment 2	1490.14	1.24	0.07	0.18	1.00
Segment 1 vs Segment 2	972.36	0.62	0.03	0.82	1.00

SumsOfSqs = Sum of square reflecting total variance; F. Model = F test value; r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences; P = P value; P adj = P values adjusted for multiple comparison using the Bonferroni correction.

Figure 4 shows the alpha diversity values for Chao1, Shannon and InvSimpson indices for each sample. We compared the alpha diversity of the CapSa samples to that of other sample types. The overall bacterial richness (Chao1) was significantly higher in CapSa samples compared to Segment 3 (P<0.10), tended to be lower compared to fecal samples (P=0.06) and no differences were observed for all other comparisons. Similarly, Shannon diversity was

higher in CapSa samples compared to Segment 3 ($P < 0.01$) and tended to be lower if compared with feces ($P = 0.06$), while no differences were observed for all other comparisons. The InvSimpson diversity was higher in CapSa compared to Segment 3 ($P = 0.02$) and tended to be lower if compared with feces ($P = 0.08$), while no differences were observed for all other comparisons.

Figure 4: Box plots showing alpha diversity values for Chao1, Shannon and InvSimpson for each sample. Only p-values < 0.10 are shown.



Discussion

This study validates the first non-invasive device to collect small intestinal content for microbiota analysis in post-weaning pigs. CapSas administered orally and then retrieved in the feces collected digesta with a pH > 5.5. As the pH of the fasting stomach does not exceed 5.5 (Reynaud et al., 2020), CapSa's sampled the contents of a segment after the stomach. In addition, the *in vitro* studies showed that the majority of CapSas sample within one hour of being placed in an aqueous medium at pH=7 (García-Viñado, 2022). The *in vivo* sampling site is dependent on the increase of pH after passing through the stomach and its location depends on the speed of transit of the CapSa through the small intestine. Henze et al. (Henze et al., 2021) reported that it took the SmartPill® , an indigestible capsule of a slightly larger size (26 mm × 13 mm) 2.3 to 4 hours to pass through the small intestine of male Landrace pigs weighing 15 to 17 kg. Together with the *in vitro* finding we conclude that CapSa sampling site is located in the small intestine.

The stomach appears to be the only site where CapSa can get trapped as no CapSas were found outside the stomach when examining the entire digestive tract *post-mortem*. In addition, prolonged transit time was associated with lower pH of the sampled content. We hypothesize that due to a prolonged retention time in the stomach, CapSa fail to resist acidic conditions which activates the sampling mechanism. Therefore, we conclude that content of CapSa with a pH < 5.5 could contain gastric content. Our findings align with the conclusions of the study by Rezaei Nejad et al. (Rezaei Nejad et al., 2019b), which found that capsules retrieved from the stomachs of pigs had a bacterial composition similar to that of the stomach content.

Expanding on this, we considered additional factors influencing the pH of the retrieved CapSa contents like the metabolization of the microbiome inside the capsule and the aggregation of SCFAs. Short-chain fatty acids, recognized as weak acids, are acknowledged contributors to pH modulation within the gastrointestinal tract. Notably, Hadinia et al. (Hadinia et al., 2022)

demonstrated a pH-dependent production of SCFA, with the highest and lowest amounts observed at pH 6 and pH 5, respectively. Another study by Henze et al. (Walker Alan et al., 2005) further underscored the influence of pH on SCFA production, particularly noting that a mildly acidic pH (5.5) stimulates specific SCFA production. Although this study did not directly assess fermentation within the CapSas, the existing literature suggests that the involvement of SCFAs in acidifying the CapSa contents cannot be ruled out.

Therefore, to ensure that CapSa contained digesta from the small intestine, only CapSas with a pH > 5.5 and a transit time of < 48 h were sent for DNA extraction and subsequent microbiota analysis. In our study, 11 CapSas from eight pigs met these requirements.

We then proceeded to validate the CapSa based microbiota composition. Applying the PCoA and the Adonis test, we demonstrated that the microbiota composition of the CapSa content is similar to that of Segment 1, tends to diverge from Segment 2, and is markedly distinct from that in Segment 3, large intestine and fecal samples. Previous studies have shown that the composition of microbiota varies significantly between different gastrointestinal segments and feces. Zhao *et al.* (Zhao et al., 2015) concluded that microbial profiles in feces were different from those in the small intestine. In addition, the microbial composition in the large intestine was more similar to feces than the small intestine, even across different pigs' age (Zhao et al., 2015). Similarly, Adhikari *et al.* (Adhikari et al., 2019) demonstrated the difference in microbiota composition between digesta samples from jejunum and colon on weaned piglets (Adhikari et al., 2019; Zhao et al., 2015). In the present study, the comparisons of the bacterial compositions of the three segments of the small intestine and feces showed a completely different composition. This further confirms that feces is not representative of the small intestinal microbiota.

Overall, it can be observed how bacterial richness and diversity tend to increase going from the small intestine to feces, these results are in line to what was observed in other studies

(Gresse et al., 2019; Mu et al., 2017). In all three indices, the alpha diversity of the CapSa content did not differ from that observed in Segment 1 and 2 but was significantly higher than that in Segment 3. The similarity in alpha diversity between the CapSa content and Segment 1 indicates that the CapSa sample accurately mirrors the species diversity observed in Segment 1.

As the bacterial density in the lumen is higher than at or within the mucosa (Earle et al., 2015), most mucosa-associated bacteria are represented in the luminal contents (Zmora et al., 2018) and many metabolites of interest are in the lumen. Due to its opening and closing mechanism and its movement via natural peristaltic motions, we hypothesized that CapSa samples luminal bacteria. These capsules therefore provide a non-invasive alternative to sample the content of the small intestine in pigs, which can be used for any laboratory analysis that can be performed with ~150µL.

To date, only a few sampling capsules were able to collect intestinal content. The sampling mechanism of the pill developed by Rezaei Nejad *et al.* (Rezaei Nejad et al., 2019b) was tested in weaned pigs and in macaques. They validated their sampling device *in vivo* by comparing the microbial composition profile of the capsule's sample to that of the matrix from which the capsule was retrieved. In their study, the bacterial composition of pills found in pigs stomach clustered with the stomach contents, while those found in feces clustered with the fecal microbial profile. The results in macaques were quite different, and the bacterial microbiome collected by the pill retrieved in feces was clearly different from feces. The authors concluded the pill was able to sample the regions of the gut upstream of the colon with quite distinct microbiome populations compared with the feces. Shalon *et al.* (Shalon et al., 2023) developed pill prototypes that can sample four different sites in human small intestine, from the duodenum to jejunum. To validate their device as a sampling tool to collect small intestine content, they attached one pill to a capsule endoscope and visualized

successful video sampling in one human. They further confirm their results by observing differences between pills and saliva/stool samples, specifically in microbiota composition, prophage induction, protein abundance and bile acids profile.

With the aim of using CapSa for pigs, a standardised protocol was necessary to administer the capsules. Indeed, pig's gastric emptying is very slow, and only small amounts of stomach content leave the stomach at once (Kvetina et al., 2015). The speed of gastric emptying is highly variable between pigs, and large solid particles (>1 cm) can remain in the stomach for several days (Henze et al., 2021). The reason for this delayed emptying is anatomical. Indeed, pig's stomach has a very pronounced "C" shape, and the gastric cardia is very close to the pylorus (Kvetina et al., 2015). In addition, a transverse pyloric fold, called the *torus pyloricus*, is located right before the pylorus and serves as a "gate-keeper" to prevent any large particle to enter the small intestine (Kopáčová et al., 2010). To overcome the anatomical limitations, the administration protocol consisted in providing a liquid feed to limit gastrointestinal load, and a prokinetic to reinforce gastric contractions. A prokinetic is a substance that amplifies and coordinates the gastrointestinal muscular contractions to facilitate the transit (Michael Camilleri & Jessica Atieh, 2021). Despite this protocol, pig's BW still affected the percentage of CapSas found in the stomach, and consequently the percentage of CapSas recovered from feces. The smaller the piglet, the more likely the CapSa became stuck in the stomach. This explains why only pigs over 12 kg could be successfully sampled. As a consequence, CapSa is not an effective device in pigs with a bodyweight below 12kg since its retrieval in feces is impossible due to anatomical reasons.

The transit time did not distort bacterial composition. Indeed, CapSa content still had similar bacterial composition to Segment 1 of the small intestine, despite <48h of transit under body temperature (38°C). Similarly, Shalon *et al.* (Shalon et al., 2023) demonstrated that there were no major changes in microbiota composition if transit (incubation) did not exceed 58 h.

This study validates the first non-invasive device for the collection and analysis of intestinal microbiota in post-weaning pigs with a bodyweight above 12 kg. A standardised protocol has also been established for successful deployment of the CapSas in pigs. This new tool opens new perspectives to study the gut physiology.

Further studies will be conducted to validate CapSa in fattening pigs as well as the effect of the protocol on the small intestine microbiota.

Ethics approval

All experimental procedures were in compliance with Swiss animal welfare guidelines and were approved (No. 2021-39-FR) by the Cantonal Veterinary Office of Fribourg (Switzerland). All methods are reported in accordance with the ARRIVE guidelines.

Data availability statement

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

Raw sequences are available at NCBI sequence read archive (SRA under the accession number PRJNA1049758).

Supplementary material

Supplementary Table S1. Ingredients and gross chemical composition of the post-weaning, starter.

	Starter diet ¹
Ingredients (%)	
Barley, ground	6,426
Oats, ground	10,000
Oats flakes	2,000
Maize	3,442
Wheat, kernels	45,096
Wheat starch	0,688
Cerolac 15-2175	5,000
Rapeseed oil	2,500
Potato protein	8,459
Soya extract	2,011
Wheat bran	0,506
Beet pulp	6,585
Apple pomace, dried	4,000
L-lysine HCl	0,401
DL-methionine	0,013
L-threonine	0,086
Monocalcium phosphate	0,476
Salt	0,390
Ca-formate	1,000
Pellan ²	0,300
ALP-S 463 Piglets	0,400
Luctarom ³	0,010
Greencab-70-C ⁴	0,200
Natuphos 5000 G ⁵	0,010

¹ Diet for the piglets from 15 days after birth to 14 days post-weaning, formulated according to the Swiss feeding recommendations for pig.

² Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany.

³ Luctarom, Lucta; Montornès del Vallès, Spain.

⁴ Coated calcium butyrate: Greencab 70-c, Brenntag; Denmark.

⁵ Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 µmol P from 5 mM phytate/min at pH 5.5 and 37°C.

	Starter diet ¹
Gross chemical composition analysed (g/kg as fed)	
Dry matter	887
Crude protein	170
Fat	45
Crude fibre	44
Digestible energy (MJ/kg)	14
Lysine	11
Methionine	3.1
Threonine	7.5
Tryptophan	1.9
Ca	5.8
P	4.5
Na	1.9
Vitamin A (IE/kg)	8000
Vitamin D3 (IE/kg)	1000
Vitamin E	25
I (mg/kg)	0.15
Mn (mg/kg)	10
Cu (mg/kg)	6
Zn (mg/kg)	75
Se (mg/kg)	0.20

Supplementary Table S2. Relative abundance (expressed as % of total) and standard deviation of the main Phyla, Family and Genera present in the different microbiota samples

	Sample type					
	Capsule	Segment 1	Segment 2	Segment 3	Large intestine	Feces
Main Phyla (%) ± SD¹						
<i>Firmicutes</i>	93.25 ± 6.10	96.65 ± 6.1	97.57 ± 2.61	98.87 ± 2.36	97.33 ± 2.73	92.27 ± 6.41
<i>Euryarchaeota</i>	3.88 ± 3.92	1.00 ± 3.92	0.83 ± 1.18	0.30 ± 0.72	0.26 ± 0.28	1.37 ± 1.07
<i>Proteobacteria</i>	1.32 ± 4.27	0.43 ± 4.27	0.17 ± 0.39	0.02 ± 0.03	0.43 ± 0.79	0.12 ± 0.15
<i>Actinobacteriota</i>	1.02 ± 1.28	1.68 ± 1.28	1.17 ± 1.85	0.72 ± 2.23	0.07 ± 0.10	0.11 ± 0.17
<i>Bacteroidota</i>	0.40 ± 1.34	0.07 ± 0.16	0.03 ± 0.04	0.02 ± 0.02	1.50 ± 2.08	4.98 ± 4.31
<i>Patescibacteria</i>	0.07 ± 0.23	0.05 ± 0.11	0.06 ± 0.14	0.02 ± 0.09	0.07 ± 0.13	0.12 ± 0.14
<i>Cyanobacteria</i>	0.04 ± 0.05	0.10 ± 0.19	0.08 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.13
<i>Desulfobacterota</i>	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.02	0.06 ± 0.17	0.11 ± 0.12
<i>Spirochaetota</i>	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.11	0.77 ± 2.13
<i>Campylobacterota</i>	0.00 ± 0.01	0.02 ± 0.08	0.09 ± 0.47	0.05 ± 0.24	0.19 ± 0.45	0.07 ± 0.11
Main Family (%) ± SD¹						
<i>Lactobacillaceae</i>	30.48 ± 18.17	76.63 ± 25.98	79.03 ± 23.39	73.87 ± 34.39	63.02 ± 22.45	47.79 ± 5.59
<i>Clostridiaceae</i>	12.72 ± 10.76	3.58 ± 5.46	1.74 ± 3.02	10.41 ± 18.79	3.10 ± 7.45	5.09 ± 7.33
<i>Lachnospiraceae</i>	11.24 ± 5.97	3.24 ± 5.34	5.38 ± 10.58	1.20 ± 2.95	11.63 ± 7.90	15.60 ± 8.85
<i>Peptostreptococcaceae</i>	11.09 ± 7.80	1.70 ± 1.07	0.86 ± 1.47	3.52 ± 8.11	2.51 ± 3.00	1.86 ± 2.03
<i>Streptococcaceae</i>	7.11 ± 7.73	5.77 ± 1.51	5.65 ± 10.42	6.50 ± 16.38	0.52 ± 1.06	0.68 ± 1.20
<i>Erysipelotrichaceae</i>	5.04 ± 4.12	0.71 ± 0.66	0.68 ± 1.01	1.60 ± 5.22	1.87 ± 2.11	1.92 ± 1.47
<i>Ruminococcaceae</i>	4.34 ± 3.22	1.11 ± 1.51	1.41 ± 2.76	0.40 ± 0.98	6.00 ± 4.07	6.28 ± 4.45
<i>Enterococcaceae</i>	4.27 ± 15.87	0.14 ± 0.66	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
<i>Methanobacteriaceae</i>	3.88 ± 3.92	1.00 ± 1.68	0.83 ± 1.18	0.30 ± 0.72	0.26 ± 0.28	1.37 ± 1.07
<i>Aerococcaceae</i>	2.19 ± 4.04	1.92 ± 4.07	1.13 ± 2.54	0.46 ± 1.52	0.00 ± 0.00	0.00 ± 0.00
Main Genera (%) ± SD¹						
<i>Lactobacillus</i>	19.63 ± 14.4	38.96 ± 23.69	27.33 ± 20.61	44.28 ± 26.7	51.81 ± 22.32	38.22 ± 21.98

<i>Clostridium sensu stricto 1</i>	12.03 ± 9.99	1.81 ± 2.78	0.80 ± 1.45	9.60 ± 17.89	3.05 ± 7.06	5.00 ± 7.25
<i>Terrisporobacter</i>	9.41 ± 7.05	1.04 ± 1.69	0.65 ± 1.28	1.98 ± 5.00	2.04 ± 2.47	1.35 ± 1.41
<i>HT002</i>	8.81 ± 6.65	28.26 ± 18.79	43.90 ± 25.17	25.48 ± 21.15	8.89 ± 5.92	8.50 ± 6.26
<i>Streptococcus</i>	7.11 ± 7.72	5.77 ± 12.41	5.65 ± 10.42	6.50 ± 16.38	0.52 ± 1.06	0.68 ± 1.20
<i>Blautia</i>	4.30 ± 2.42	1.58 ± 3.18	3.48 ± 7.82	0.67 ± 1.71	5.61 ± 6.30	5.30 ± 6.32
<i>Enterococcus</i>	4.27 ± 15.87	0.14 ± 0.66	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
<i>Methanosphaera</i>	3.42 ± 3.89	0.89 ± 1.44	0.73 ± 1.02	0.27 ± 0.64	0.17 ± 0.20	0.96 ± 0.82
<i>Subdoligranulum</i>	2.81 ± 2.29	0.59 ± 1.03	1.00 ± 2.10	0.26 ± 0.63	3.34 ± 2.36	2.58 ± 2.35
<i>Turcibacter</i>	2.77 ± 3.16	0.21 ± 0.38	0.10 ± 0.33	1.46 ± 5.25	0.53 ± 1.16	0.23 ± 0.38

¹ SD: Standard Deviation

5. Manuscript III

Sampling Intestinal Microbiota in Growing Pigs: Evaluation of CapSa, an Ingestible Capsule

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Abstract

This study aims to investigate Capsa, an ingestible capsule designed to collect the contents of the small intestine as it passes through the gastrointestinal tract. Eight Swiss Large White pigs weighing between 52.5 and 71.3 kg were administered two capsules each and monitored for three days before euthanasia for post-mortem sampling. Samples were collected from six equally divided segments of the small intestine, along with separate sampling of the solid and liquid contents of each segment when feasible. Samples were also obtained from the large intestine and faeces to determine CapSa's sampling location. Fifteen capsules were retrieved from faecal samples (93.75%), with 87.5% recovered on the first day post-administration. Only one capsule was not recovered. Comparative analysis of the bacterial composition of the capsules and post-mortem samples was conducted using a Permutational Multivariate Analysis of Variance (PERMANOVA) model (Adonis), with sample type as a factor. The results revealed significant differences in bacterial composition between capsules and samples from the large intestine and faeces ($p < 0.01$). However, no significant difference was observed between capsule content and the liquid and solid parts of the fourth segment of the

small intestine ($p > 0.05$). This study provides evidence that CapSa can effectively sample the intestinal microbiota of the middle part of the small intestine in growing pigs.

Keywords: intestinal microbiota, small intestine sampling, ingestible capsule

Highlights

- CapSa effectively collects the contents of the small intestine as it passes through the gastrointestinal tract.
- Comparative analysis shows significant differences in bacterial composition between CapSa capsules and large intestine/faeces samples.
- CapSa exhibits the capability to sample the intestinal microbiota of the median part of the small intestine in growing pigs.

Introduction

In recent years, significant research has highlighted the intricate relationship between gut microbiota and pig health (Jang et al., 2020; Schokker et al., 2015). Microbiota play crucial roles in diverse physiological processes, such as immunity and nutrient digestion (Fouhse et al., 2016; Luo et al., 2022), indicating their indirect impact on animal growth (Mahmud et al., 2023).

Despite a steady increase in pigs' feed intake and body weight, the composition of faecal microbiota during the growing period remains very stable under normal physiological conditions (Luo et al., 2022). This stability reduces susceptibility to infectious enteric diseases and optimises the animals' growth potential (Luo et al., 2022). Research has identified a relationship between enterotypes and feed intake in growing-finishing pigs (Yang et al., 2018), and different gut microbiota compositions have been linked to feed efficiency

(McCormack et al., 2017; Tan et al., 2017). For instance, an Operational Taxonomic Unit (OTU)-based analysis of faecal samples revealed 31 OTUs associated with dietary polysaccharide metabolism that could potentially affect feed efficiency in 140-day-old finisher pigs (Yang et al., 2017), suggesting that modulating microbial composition could improve feed efficiency. However, faecal microbiota analysis is imperfect as a proxy for studying gut microbiota, as its profile differs significantly from that of the small intestine (Gresse et al., 2019; Zhao et al., 2015).

Alternative approaches to faecal sampling, such as animal cannulation or the collection of chyme samples subsequent to slaughter, face ethical concerns (Castillo & Hernández, 2021) and sampling limitations of a single collection per animal (Choudhury et al., 2019). A new generation of non-invasive devices for sampling gut microbiota has emerged in human studies (Park et al., 2022; Shalon et al., 2023; Waimin et al., 2020) and in studies with dogs (Menard et al., 2023). However, there are currently no devices specifically tailored for studying pig gut microbiota. Only two devices, which were originally designed for human use (Nejati et al., 2022; Rezaei Nejad et al., 2019a), have been tested in pigs. However, in one of the studies (Rezaei Nejad et al. 2019), the capsules were not retrieved after administration, while the second study tested the devices in only two pigs, with the objective of collecting samples in the colon.

The aim of this study is to test and validate a capsule prototype (CapSa) for non-invasive sampling of the intestinal microbiota of large pigs in the grower finisher stage. CapSa has already been successfully used to sample gut microbiota in small pigs in the post-weaning stage (Inés García Viñado et al., 2024).

Materials and methods

Animals and rearing conditions

For the study, eight Swiss Large White pigs with bodyweights (BW) ranging from 52.5 to 71.3 kg were used. Pigs were housed in groups of four. All pigs had *ad libitum* access to a standard starter diet formulated to meet the nutritional requirements of fattening pigs (Agroscope, 2005) (Supplementary Table 1). Water was available *ad libitum* and distributed via nipple drinkers. As previously described by García Viñado et al. (2024), pens with a total surface area of 4.47 m² were specifically designed to collect the capsules by minimising the slatted area and reducing the openings of the slatted area to a size smaller than the capsule diameter of 7 mm.

Description of the capsule (CapSa)

The capsule studied is 21.7 mm long and 7 mm in diameter, corresponding to a size 0 hard capsule. Capsa passively moves along the gastrointestinal tract (GIT) at a speed that depends entirely on intestinal peristalsis. CapSa can collect a maximum of 400 µL (Inés García Viñado et al., 2024). In vitro results show that CapSa can withstand two hours in an acidic-aqueous medium (pH <3), and then samples within 1 h after transfer to an aqueous medium at pH 7 (García Viñado et al., 2022). Moreover, it has been validated in post-weaning pigs (Inés García Viñado et al., 2024).

Preparation of the animals and administration of the capsule

Three measures were taken prior to capsule administration to reduce intestinal load and shorten transit time. First, two days before the CapSa (-2d) was administered, pigs were fed the finisher diet in liquid form (ration of 1 kg of finisher diet mixed with 2 L of water), and straw was removed from the pens. Second, one day before the administration of the CapSa (-1d), the pigs had access to only half of their feed ration, and the feed was removed 12 h before

CapSa administration. Third, to increase gastric emptying and thus facilitate CapSa transit through the stomach, 0.16 ± 0.001 mg/kg BW of prucalopride (Resolor[®], Takeda Pharma AG, Glattpark, Switzerland) was administered orally via an oesophageal probe 40 min prior to administration. Prucalopride is a 5-HT₄ serotonin agonist that stimulates GIT peristalsis and increases gastric emptying (Briejer et al., 2001; M. Camilleri & J. Atieh, 2021).

On the day of administration (0d), each pig received two CapSas. The capsules were administered by oesophageal sondage, while the pigs were kept in a sling adapted to their BW. A 10 mL bolus of orange juice was then administered to flush the capsule in the stomach. Every CapSa was assigned a unique number, linking it to the pig ID.

Capsule recovery and sample processing

From 0d to three days after administration (3d), pens were inspected five times a day to look for CapSas expelled in the faeces. The specifically designed slatted area of the pens allowed searching for the capsule by sieving faeces with water over the slatted surface. Capsules retrieved from the faeces were directly transported to the laboratory. The outside of the capsule was cleaned with 70% alcohol to avoid contamination after opening. The identification of the capsule was recorded, its content was extracted, and the volume of the content was determined. The content was transferred to a 0.5 mL Eppendorf tube (Eppendorf SE, Hamburg, Germany), flash frozen by immersion in liquid nitrogen, and stored at -80°C until analysis. The pH of the content was measured using Litmus paper (Merck KGaA, Darmstadt, Germany) by cleaning the inside of the capsule after extraction.

Post-mortem sampling

Three days after capsule administration, all pigs were euthanised by electronarcosis. The GIT was extracted and samples of the colon and faeces were collected. Immediately after euthanasia, the abdominal cavity was opened, and the viscera were collected and placed on a

table. The gastrointestinal tract was carefully unfolded, beginning just after the stomach. The small intestine was divided into six equal segments (Segments 1, 2, 3, 4, 5 and 6) and each segment was immediately sampled. After delineating a 3-meter segment of the intestine, a sample was taken by concentrating the contents of the small intestine in the central part of the segment. This process was repeated until all six segments were sampled. Additionally, prior to storage in sterile 2 mL Eppendorf tubes, the segment contents were also separated into the solid and liquid phases by sedimentation for 5 min in 50 mL tubes (Ratiolab®, Germany) cooled on ice. Subsequently, all tubes were submerged in liquid nitrogen and stored at -80°C until further analysis. The GIT was carefully examined at the end of the sampling to ensure that all capsules had been retrieved.

Evaluation of Capsule Innocuity

To assess the innocuity of CapSa administration, passage, and retrieval, various parameters were considered to ensure the pigs' well-being. Post-mortem macroscopic observations were conducted to assess the presence of any tissue damage related to CapSa administration and/or passage (e.g., gastric ulcers and intestinal perforations). The faecal score was determined using a 4-level scoring scale, as follows: 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). Throughout the study duration, the overall health of the pigs was monitored continuously.

Microbiota analysis

Only capsule samples with a pH > 5.5 and recovered within 48 h of administration were used for microbiota analysis. 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'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNBGCASCAG-3' and Pro805R:5'-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3' (Takahashi et al., 2014). The PCR reaction conditions for amplification of DNA were as follows: initial denaturation at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension 65°C for 45 s, ending with 1 cycle at 68°C for 7 min (Takahashi et al., 2014). Amplicons were then sequenced by Illumina MiSeq 300 × 2 bp with the MiSeq® V3-V4 reagent kit on the MiSeq-Illumina® platform. Microbiota analysis was performed using the DADA2 pipeline (Callahan et al., 2016) according to the Silva database taxonomy, version 138.1 (Quast et al., 2013). 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 maintained at their default settings.

Statistical analysis

All statistical analyses were performed in R (v 4.3.1). The percentage of CapSas recovered in the faeces and stomach or those not found was calculated based on the total number of CapSas administered. Capsule transit time was calculated as the time between capsule administration and recovery in the faeces. Capsule pH and volume were analysed using linear regression with transit time as fixed effects (function “lm” in package “lm4”) (Bates et al., 2015). An ANOVA was performed to check the effect of time of retrieval on the pH and volume of the sample of the retrieved capsules (function “Anova” in package “car”) (Weisberg & Fox, 2011). Post hoc tests, such as least squares means, were performed when ANOVA detected the effect of time of retrieval on pH and volume.

Statistical analysis of alpha and beta diversity, as well as taxonomic analysis, was performed using “phyloseq” (McMurdie & Holmes, 2013) v1.38, “vegan” v2.6 (Dixon, 2003) and “microbiomeutilities” v1.0. For the alpha diversity analysis, data were rarefied to the lowest sample depth to avoid bias linked to different sampling efforts. The Wilcoxon signed-rank test (procedure in R) was used to test for differences in the alpha diversity indices (Chao1, Shannon, and Simpson diversity) of the CapSas microbiota content and those obtained from the six segments of the small intestine, large intestine, and faeces. For beta diversity, a dissimilarity matrix using Euclidean distances from the centred log-transformed (clr) data was constructed, and the results were represented using a principal coordinate analysis (PCoA) plot. Differences were tested using a PERMANOVA model (Adonis) with 9,999 permutations, including sample type (CapSa, six segments, large intestine, faeces) as the main factor. Pairwise contrasts between capsules and post-mortem samples were performed using the pairwiseAdonis function included in the “PairwiseAdonis” package (Martinez Arbizu, 2020). Bonferroni correction was then applied to adjust the p values for multiple comparisons. Differences in the taxonomic composition between samples 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, a difference was considered significant if $p < 0.05$ and a trend if $0.05 < p < 0.10$.

Results

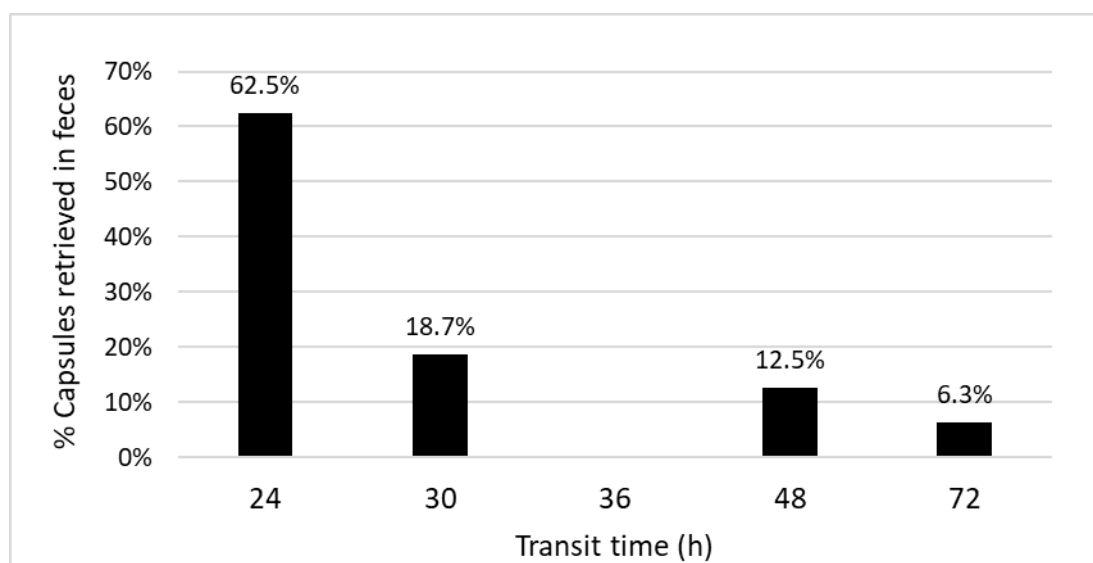
CapSa innocuity

All pigs remained healthy throughout the study. No macroscopic tissue damage was observed following euthanasia related to CapSa administration and/or passage. The administration of CapSa had no impact on the faecal scores or the occurrence of diarrhoea, with none of the pigs exhibiting a faecal score higher than 1.

CapSa administration, recovery, and the volume and pH of the content

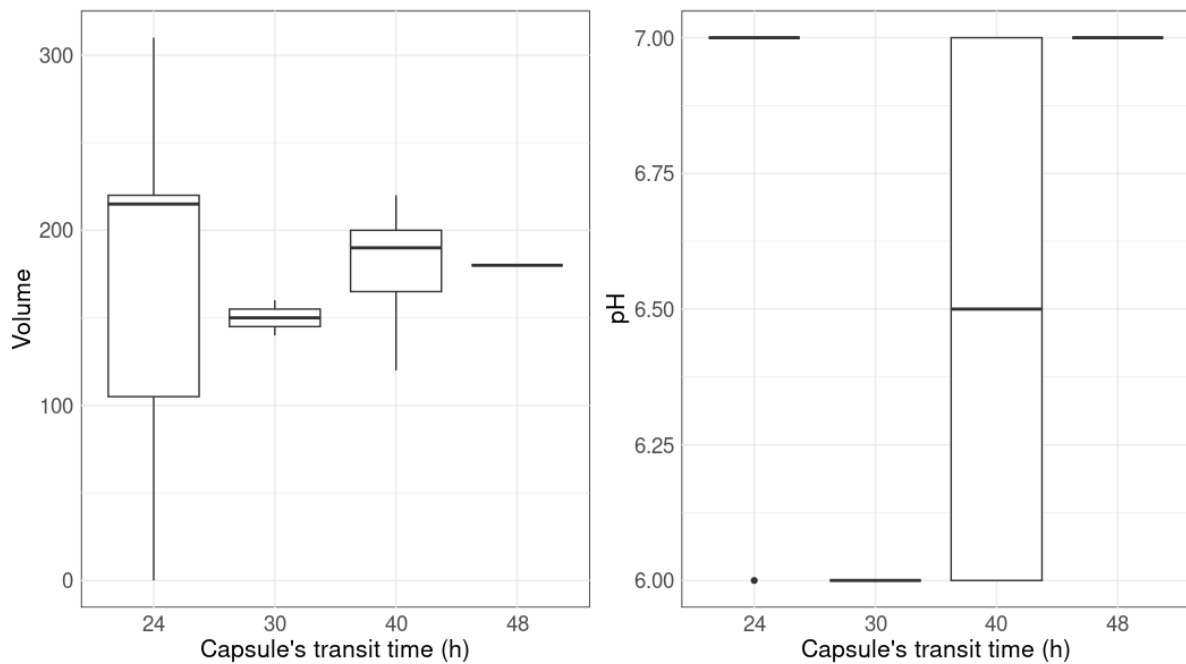
All CapSas were successfully administered to all pigs. A total of 15 capsules were recovered in faeces (93.75%), one of which was found to be empty. Overall, 87.5% of the CapSas were recovered on the first day post administration and 6.25% on the second day. Only one capsule was not found in the faeces or inside the pig after euthanasia. In this study, we recovered at least one capsule from each pig (Figure 1). Of the 15 CapSas recovered within 48 h, 14 had a pH > 5.5 and were therefore included in the microbiota analysis. The pH of the retrieved content was 6.60 ± 0.51 (mean + standard deviation, STD), and the sampled volume was 172.67 ± 72.26 μ L. Neither the volume nor the pH of the CapSa content was affected by transit time ($p > 0.05$) (Figure 2).

Figure 1. Time (h) of transit of capsules found in faeces. Time was calculated as the difference between the time of administration and the time of recovery. Transit time strongly influenced the number of capsules recovered in faeces, and the majority of the capsules were found within 24 h after administration.



NF = Not found.

Figure 2. Volume (μL) and pH of capsule contents as a function of transit time (h).



Post-mortem sampling

We successfully collected faecal and large intestine samples from all pigs as well as from Segments 1 to 6 of the small intestine. However, due to consistency differences, we could only obtain samples of both the solid and liquid phases from Segments 1 to 4 of the small intestine. In the upper part of the small intestine, particularly in Segments 1 to 4, the content tends to be more liquid in nature. In these segments, sedimentation was fast and efficient in separating the solid phase from the liquid phase. However, in Segments 5 and 6, and occasionally in Segment 4, the liquid phase was limited, making collection impossible.

Microbiota analysis

Except for one CapSa sample, bacterial DNA was successfully extracted, amplified, and sequenced. The PCoA plot showed a clustering of the samples based on sample type, with large intestine and faecal samples tending to cluster together and CapSa and intestinal segment samples from two distinct clusters showing no overlap between them (Figure 3). The Adonis test proved that bacterial composition was affected by sample type ($r^2 = 0.33$, $p =$

0.001). The pairwise Adonis test shows that except for the solid and liquid phases of Segment 4 ($r^2 = 0.26$, $p_{adj} = 0.36$ and $r^2 = 0.26$, $p_{adj} = 0.31$, respectively), the microbial composition of the CapSa content was significantly different from all other sample types (Table 1).

Figure 3. PCoA of Euclidean distance matrices of clr-transformed data. Samples are coloured based on sample type and labelled according to the subject. SI: small intestine sample; L: liquid phase sample; So: solid phase sample.

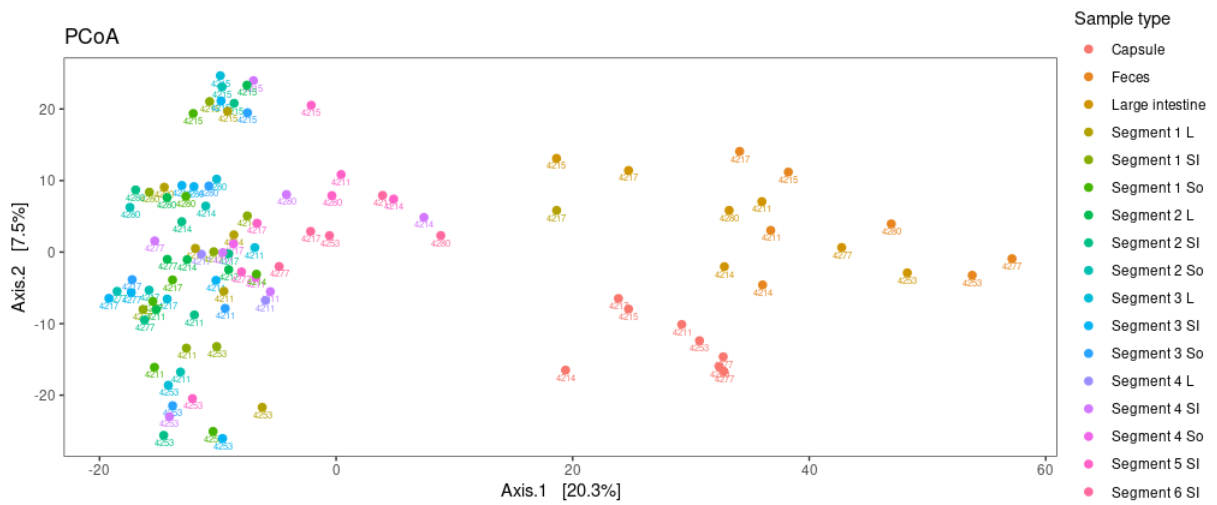


Table 1. Adonis pairwise comparisons of microbiota structure between capsules and gastrointestinal content samples, calculated using Euclidian distances.

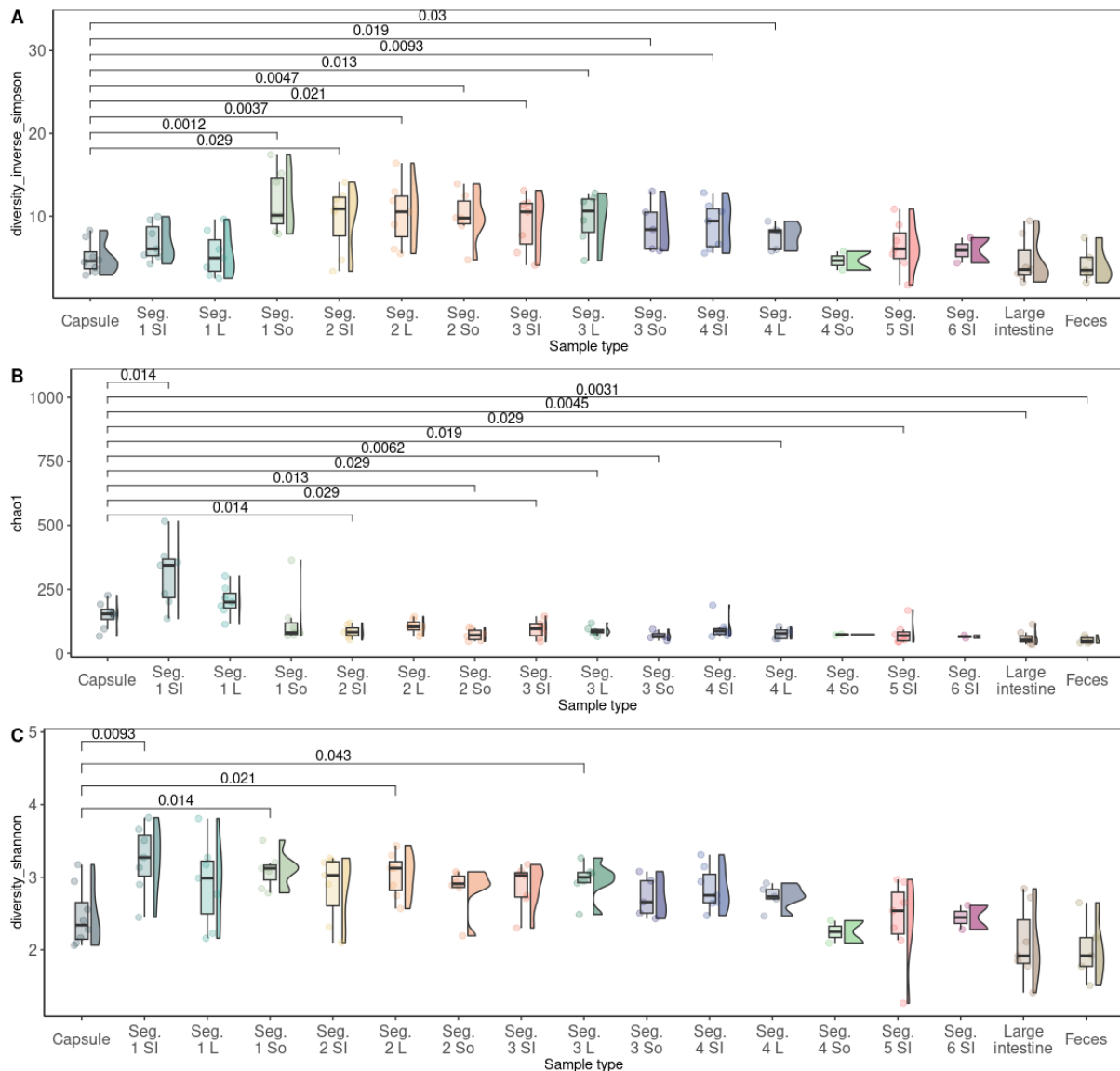
Pairwise comparisons	SumsOfSqs	F. Model	r²	P	P_{adj}
Capsule vs. Segment 1 SI	9525.21	6.77	0.34	<0.01	0.04
Capsule vs. Segment 2 SI	9772.48	6.66	0.33	<0.01	<0.01
Capsule vs. Segment 3 SI	10132.53	6.92	0.34	<0.01	<0.01
Capsule vs. Segment 4 SI	8290.68	5.81	0.30	<0.01	<0.01
Capsule vs. Segment 5 SI	8000.25	6.24	0.32	<0.01	<0.01
Capsule vs. Segment 6 SI	5929.84	4.61	0.29	<0.01	0.01
Capsule vs. Large intestine	7872.01	3.70	0.22	<0.01	<0.01
Capsule vs. Faeces	8154.14	3.18	0.19	<0.01	<0.01
Capsule vs. Segment 1 So	9642.59	6.56	0.33	<0.01	<0.01
Capsule vs. Segment 1 L	8974.71	5.11	0.28	<0.01	<0.01
Capsule vs. Segment 2 So	9677.64	6.77	0.36	<0.01	<0.01
Capsule vs. Segment 2 L	9199.97	6.71	0.35	<0.01	<0.01
Capsule vs. Segment 3 So	8342.67	5.81	0.34	<0.01	<0.01
Capsule vs. Segment 3 L	8101.19	5.61	0.33	<0.01	<0.01
Capsule vs. Segment 4 So	4519.61	2.93	0.26	0.02	0.36
Capsule vs. Segment 4 L	4452.47	2.90	0.26	0.01	0.31

SI = small intestine (without separation in solid and liquid phase); L = liquid phase; So = solid phase; SumsOfSqs = Sum of square reflecting total variance; F. Model = F-test value; r² = r-square value, reflects grouping differences; the higher the value, the higher the grouping differences; *p* = *p* value; *p*_{adj} = *p* values adjusted for multiple comparison using the Bonferroni correction.

Overall alpha diversity (InvSimpson) was lower ($p \leq 0.03$) in the CapSa content compared to the total, liquid, or solid fractions of Segments 2, 3, and 4 (except solid fraction of Segment 4) and solid fraction of Segment 1. No differences in alpha diversity were observed between CapSa and the rest of the samples (Figure 4A). The Chao1 index was higher ($p \leq 0.02$) in CapSa samples compared to the total fractions of Segments 2, 3, and 5, the solid fractions of Segments 2 and 3, the liquid fractions of Segments 3 and 4, large intestine, and faeces. However, it was lower in the CapSa samples compared to the total fraction of Segment 1 ($p = 0.014$) (Figure 4B). The Shannon diversity index was lower ($p \leq 0.04$) in the CapSa samples

compared to the total fraction of Segment 1, the liquid fractions of Segments 2 and 3, and the solid fraction of Segment 1 (Figure 4C). By contrast, microbiota distribution was similar between the CapSa samples and the rest of the samples.

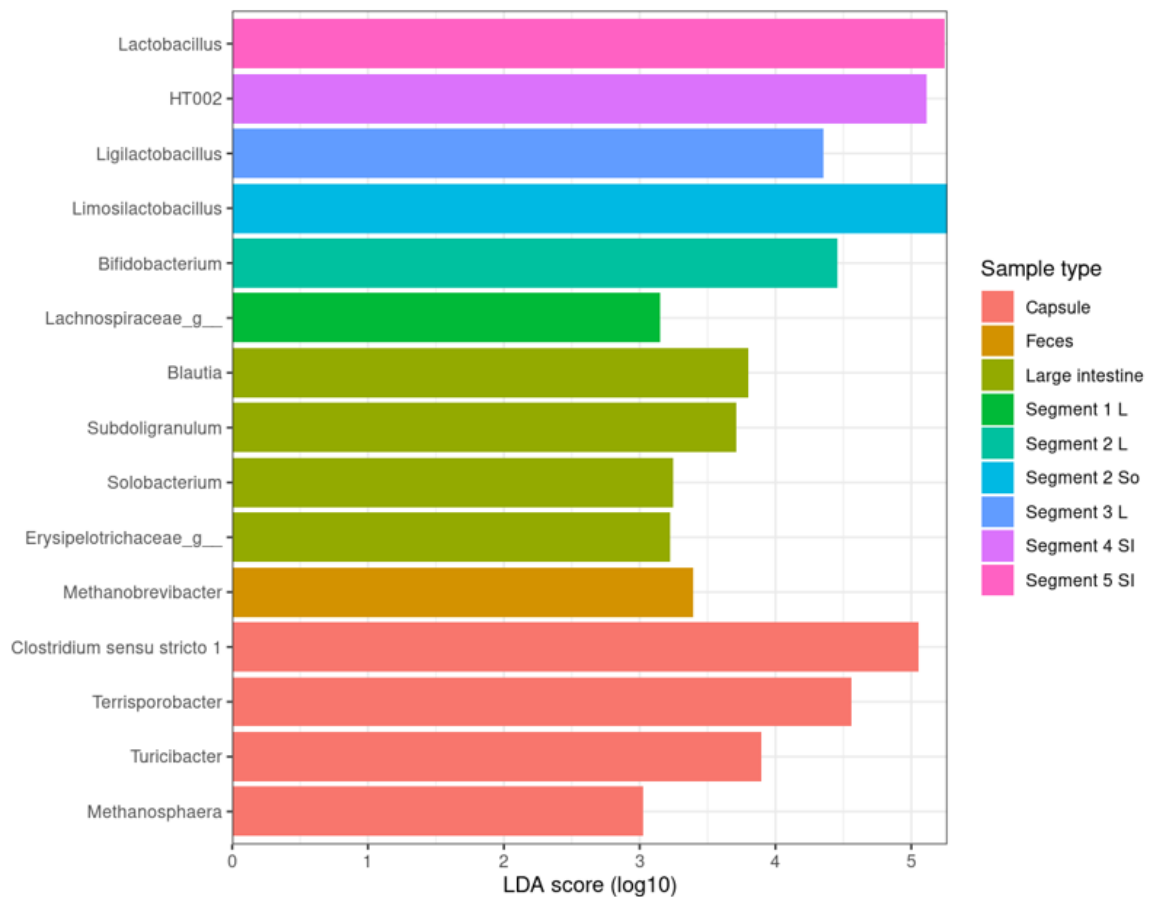
Figure 4. Box plots showing alpha diversity values for inverse Simpson (A), Chao1 (B), and Shannon index (C) for each sample. Only p -values < 0.10 are shown.



LEfSe analysis revealed different biomarkers characterising each sample type (Figure 5). Capsules had four major biomarkers: *Clostridium sensu stricto 1* (LDA score = 5.06, $p < 0.01$), *Terrisporobacter* (LDA score = 4.54, $p < 0.01$), *Turicibacter* (LDA score = 3.92, $p <$

0.01), and *Methanosphaera* (LDA score = 3.00, $p < 0.01$). Faeces had one biomarker: *Methanobrevibacter* (LDA score = 3.37, $p < 0.01$). The large intestine had four biomarkers: *Blautia* (LDA score = 3.84, $p < 0.01$), *Subdoligranulum* (LDA score = 3.72, $p < 0.01$), *Solobacterium* (LDA score = 3.28, $p < 0.01$), and an unidentified genus of the *Erysipelotrichaceae* family (LDA score = 3.22, $p < 0.01$). The liquid phase of Segment 1 had one main biomarker: unidentified genera of the *Lachnospiraceae* family (LDA score = 3.15, $p < 0.01$). The liquid phase of Segment 2 had one biomarker: *Bifidobacterium* (LDA score = 4.47, $p < 0.01$), similar to the solid phase of that same segment, which had *Limosilactobacillus* (LDA score = 5.25, $p < 0.01$). The liquid phase of Segment 3 had 3 biomarkers: *Ligilactobacillus* (LDA score = 4.33, $p < 0.01$). Segment 4 had one main biomarker: HT002 (from *Lactobacillaceae* family, LDA score = 5.12, $p < 0.01$). Segment 5 also had one main biomarker: *Lactobacillus* (LDA score = 5.26, $p < 0.01$).

Figure 5. Bar plot of linear discriminant analysis (LDA) effect size (LEfSe).



SI: small intestine sample; L: liquid phase sample; So: solid phase sample.

Discussion

A standardised protocol was previously designed by (Inés García Viñado et al., 2024) for the administration of CapSa to post-weaning pigs. However, the faecal CapSa recovery rate in heavier pigs was higher in the present study, at 93.75%, demonstrating that the protocol, combined with a high level of expertise in handling the pigs, is highly effective as a non-invasive sampling method for GIT content. The majority of the CapSas were recovered within the first day after administration, indicating efficient passage through the GIT. The shorter transit time and the higher recovery rate observed in this study compared to the previous study (Inés García Viñado et al., 2024) can be attributed to the higher BW, as it seems easier for CapSa to exit the stomach in larger pigs. Indeed, none of the capsules were found in the stomach after euthanasia, contrary to findings in other studies (Inés García Viñado et al., 2024). The CapSa contained a digesta with a pH > 5.5. In agreement with the results obtained with post-weaning pigs (Inés García Viñado et al., 2024), our findings confirm that CapSas sampled the contents of segments beyond the stomach, as the fasting stomach typically does not exceed a pH > 5.5 (Reynaud et al. 2020).

Using PCoA and the Adonis test, we observed notable differences in microbial composition between the CapSa samples and samples from the large intestine and faeces, underscoring the unique microbial ecosystems across the GIT (Crespo-Piazuelo et al., 2018). Although certain phyla, such as Firmicutes, consistently inhabit the entire length of the intestine, variations in factors such as pH and oxygen concentration can cause other phyla to differ along the GIT (Crespo-Piazuelo et al., 2018). Our findings corroborate previous studies (Zhao et al., 2015) demonstrating differences in microbial composition between faecal and small intestine samples (Table 1). The similarity between the microbial content of the CapSa and the liquid and solid fractions of Segment 4 ($r^2 = 0.26$, $p_{adj} = 0.31$ and $r^2 = 0.26$, $p_{adj} = 0.36$, respectively; Table 1) indicates that the CapSas sampled in the midsection of the small intestine. However,

as indicated by the results of the pairwise Adonis test (Table 1), the CapSa content differed significantly from that of Segment 4 when compared as a whole rather than separated into liquid and solid fractions. Our first hypothesis is that the limited volume of the Capsa sample (approximately 170 μ L) hampered the capturing the diversity and richness of Segment 4 when both liquid and solid phases were mixed together. Separating the two phases into liquid and solid reduced the richness and diversity of each sub-sample, which became similar to those found in the Capsa sample.

A previous study demonstrated that CapSa sampled gut content from the first segment of the small intestine in postweaning piglets (Inés García Viñado et al., 2024). However, in this study, the CapSa content differed from the bacterial composition of the first segment, sampling further along the small intestine, specifically in the jejunum (Segment 4), as shown in the pairwise Adonis analysis. It is noteworthy that the age of the pigs not only influences organ size but also transit time. Snoeck et al. (2004) observed that transit time was significantly prolonged immediately after weaning and returned to normal 3 weeks after weaning. The increase in transit time appeared to be related to retention in the stomach and colon (Snoeck et al., 2004). Considering this, we hypothesised that in our study, the capsule opened and closed further along the intestine due to faster transit. We speculate that in young pigs, the smaller size of the stomach and the space occupied by the *torus pyloricus* might result in a less forceful expulsion of intestinal content compared to larger growing pigs with a bigger pylorus and larger small intestine diameter. Moreover, in both post-weaning and growing pig studies, feeding occurred four hours after CapSa administration. Larger pigs may better handle stress, adapting more effectively to handling and intubation, thereby exhibiting a quicker return to feeding post-manipulation. This, coupled with the known peak in transit time immediately after feeding (Krawielitzki et al., 1990), suggests that the sooner they are fed, the faster the capsule advances. Taking into account these factors and previous findings, in post-

weaning pigs, CapSa is observed to open in the first segment of the small intestine, whereas in growing pigs, it is expected to open in a more distal position.

To assess whether CapSa provided a representative sample of Segment 4, we also evaluated various alpha diversity indices. No differences in species richness (Chao 1) were observed between the Capsa samples and the total and solid fractions of Segment 4. The liquid fraction had a lower richness. Indices that simultaneously compared the number of species present (richness) and the relative abundance of each species (evenness) yielded conflicting results. Indeed, the Shannon index showed no difference between CapSa and any of the sample fractions from Segment 4. By contrast, the Capsa samples had lower richness and evenness compared to the total and liquid fractions of Segment 4, while no difference was found compared to the solid fraction. In summary, the assessment of alpha diversity indices suggests that CapSa effectively reflects alpha diversity in Segment 4 of the small intestine.

Additionally, the presence of characteristic genera, such as *Clostridium sensu stricto 1* and *Terrisporobacter*, in capsule samples underscores CapSa's ability to capture representative microbiota from the small intestine. These genera have been proven to be the most prevalent in the jejunum of growing pigs in other studies (Wu et al., 2022). Further, the abundance of *Terrisporobacter* and *Clostridium sensu stricto 1* in the GIT has been positively correlated with adult pig weight gain (Kim et al., 2016; Yu et al., 2024).

Conclusion

After comparing the content of CapSa samples to a large set of microbiota samples from 14 different types, including various segments of the small intestine, feces, large intestine, and both solid and liquid fractions from the small intestine segments, this found that the bacterial composition of CapSa is distinct from that of the large intestine and feces. The capsule sample

more closely resembles the composition found in the solid and liquid phases of the fourth segment of the small intestine. However, although the composition of the capsule did not mirror that of the Segment 4 sample as a whole, it aligned with this segment when its liquid and solid phases were examined separately.

Ethics approval

All experimental procedures were in compliance with Swiss animal welfare guidelines and were approved (No. 2021-39-FR) by the Cantonal Veterinary Office of Fribourg (Switzerland). All methods are reported in accordance with the ARRIVE guidelines.

Data availability statement

The datasets generated during and/or analysed 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 PRJNA1107150).

Supplementary material

Supplementary Table S1. Ingredients and gross chemical composition of the growing diet.

	Growing diet ¹
Ingredients (%)	
Barley	34,00
Oats	10,00
Wheat	39,48
Mixed fat	0,80
Soybean extract	9,78
Dried beet pulp	2,00
L-Lysin-HCl	0,47
DL_methionin	0,06
L-Threonin	0,12
L-Valine	0,15
DCP	0,79
Lime, carbonic acid	1,23
Sodium chloride	0,41
Pellan2	0,30
ALP-S 467 Mast	0,40
Natuphos 5000 G3	0,01

¹ Growing diet was formulated based on the energy and nutrient requirements of pigs with a BW of 40 kg.

² Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany.

³ Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 μmol P from 5 mM phytate/min at pH 5.5 and 37°C.

	Growing diet ¹
Gross chemical composition analysed (g/kg as fed)	
Dry matter	898.48
Ash	52.20
Crude protein	153.18
Fat	30.42
Raw fibre	40.03
Digestible energy (MJ/kg)	13.70
Lysine	9.91
Methionine	2.78
Threonine	6.12

Tryptophan	1.78
Ca	8.06
P	5.20
Na	1.66
Cu (mg/kg)	6.32
Zn (mg/kg)	27.36

6. Manuscript IV

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

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Abstract

Background: The gastrointestinal tract (GIT) of pigs harbors a dense and diverse population of bacteria crucial for nutrient synthesis, digestion, and immune modulation. These microbes play pivotal roles in synthesizing essential vitamins and maintaining gut homeostasis by influencing host immune responses and barrier functions. Despite their importance, understanding the temporal dynamics and inter-individual variability of these microbiota remains challenging for researchers. Recent advancements in technology and computational tools have enabled deeper insights into the factors influencing variability in host-associated microbial communities. 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. The small intestine not only serves as the primary site for nutrient absorption but also houses a substantial portion of the pig's immune system. Study 1 employed CapSa, a non-invasive sampling capsule, to collect microbiota at five distinct administration time points. The aim was to investigate changes in the small intestine microbiome composition throughout the lifespan of grower-finisher pigs. 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: The CapSa sampling method was successful, with a retrieval rate higher than 70% in both studies. In both studies, microbiota analysis of the small intestine 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, significant 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.

Conclusions: CapSa represents a groundbreaking advancement in the non-invasive collection of gut microbiota from pigs, allowing for repeated sampling. This innovation can facilitate comprehensive studies on microbiome evolution from weaning to slaughter, encompassing both healthy and pathological conditions.

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 (Morowitz et al., 2011) and participate in feed digestion (Hansen & Sams, 2018). Furthermore, GIT bacteria create an ecological barrier to pathogenic bacteria by stimulating the host to produce IgA and antimicrobial proteins (Kamada et al., 2013). Intestinal bacteria also influence central physiological

functions, such as lymphatic tissue development, mucosal tolerance induction, angiogenesis, and fat storage (Laukens et al., 2016).

However, understanding fluctuations in microbiota composition over time and the diversity among individuals poses constraints on microbiome experimentation (Tang et al., 2020). Advances in technical 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 (Laukens et al., 2016). The temporal stability of an individual's gut microbiome remains a critical question (Lozupone et al., 2012). Shotgun sequencing has revealed that over a five-year period, an individual conserves on average 60% of ~200 phylotypes (highest taxonomic level) (Faith et al., 2013). At lower taxonomic levels, each individual's microbiome may be as unique as a fingerprint (Schloissnig et al., 2012), although its stability remains unclear and poses methodological challenges (Martínez et al., 2013). 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 (Gaire et al., 2023). By contrast, the development of small intestine (SI) microbiota remains less understood due to the lack of longitudinal sampling methods (Tang et al., 2020). 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 (Kim & Duarte, 2021). Understanding the roles of intestinal microbiota and their interactions with the host is essential in feed formulation (Duarte & Kim, 2022). Manipulating the intestinal microbiota can have both immediate and long-term effects on pig intestinal health (Jang et al., 2020; Schokker et al., 2015). Although most research in this area emphasizes fecal microbiota (Tang et al., 2020; Zhao et al., 2015), 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 (Duarte & Kim, 2022; Mowat & Agace, 2014). Studies show that microbiota from feces contain fewer microbial functions than those from the SI, indicating significant differences between the microbial profiles (Zhao et al., 2015). The pig's gut microbiota undergoes remarkable changes throughout its life, influencing the nutritional and health status of the host (Luo et al., 2022).

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 (Fairbrother et al., 2005). PWD is commonly associated with enterotoxigenic *Escherichia coli* (ETEC), with F4 and F18 being the predominant adhesins (Delisle et al., 2012; Fairbrother et al., 2005; Luppi et al., 2016; Nagy & Fekete, 2005). Recent studies indicate that PWD caused by ETEC has a lasting impact on piglet fecal microbiota (Rhouma et al., 2021). Agroscope has developed and validated a PWD model using ETEC with F4 attachment factors, inducing diarrhea without causing fatalities (Girard et al., 2018).

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 (Inés García Viñado et al., 2024). This device has been validated in vitro (García Viñado et al., 2022) and in vivo (Inés García Viñado et al., 2024). This study details the quantitative and qualitative shifts in the SI's microbiota over time in the same pigs. 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 (I. García Viñado et al., 2024). All pigs were slaughtered at 140 ± 5 days of age.

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 (Hu et al., 2019). 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). At the same five time points (T1 to T5) as in Study 1, CapSa was administered to all pigs in Study 2.

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 ETEC F4ac. 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 (Girard et al., 2018).

During infection and until one week later, 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 (Agroscope, 2005). 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 the finisher diet period, 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 (Inés García Viñado et al., 2024). 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. (I. García Viñado et al., 2024). 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

The fecal score was assessed individually at each fecal sampling time, as well as on day 1 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).

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.

Microbiota analysis

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'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3' and Pro805R:5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3' (Takahashi et al., 2014). 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' (Takahashi et al., 2014). Amplicons were then sequenced by Illumina MiSeq 300 × 2 bp with the MiSeq® V3-V4 reagent kit on the MiSeq-Illumina® platform. Microbiota analysis was performed using the DADA2 pipeline (Callahan et al., 2016) according to the Silva database taxonomy, version 138.1 (Quast et al., 2013). 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” (McMurdie & Holmes, 2013) v1.38, “vegan” v2.6 (Dixon, 2003) and “microbiomeutilities” v1.0. For beta diversity, 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 (Martinez Arbizu, 2020).

For alpha diversity, samples were rarefied to the lowest sample 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 using the Wilcoxon test. The p -values were then adjusted for multiple comparisons using the Bonferroni 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

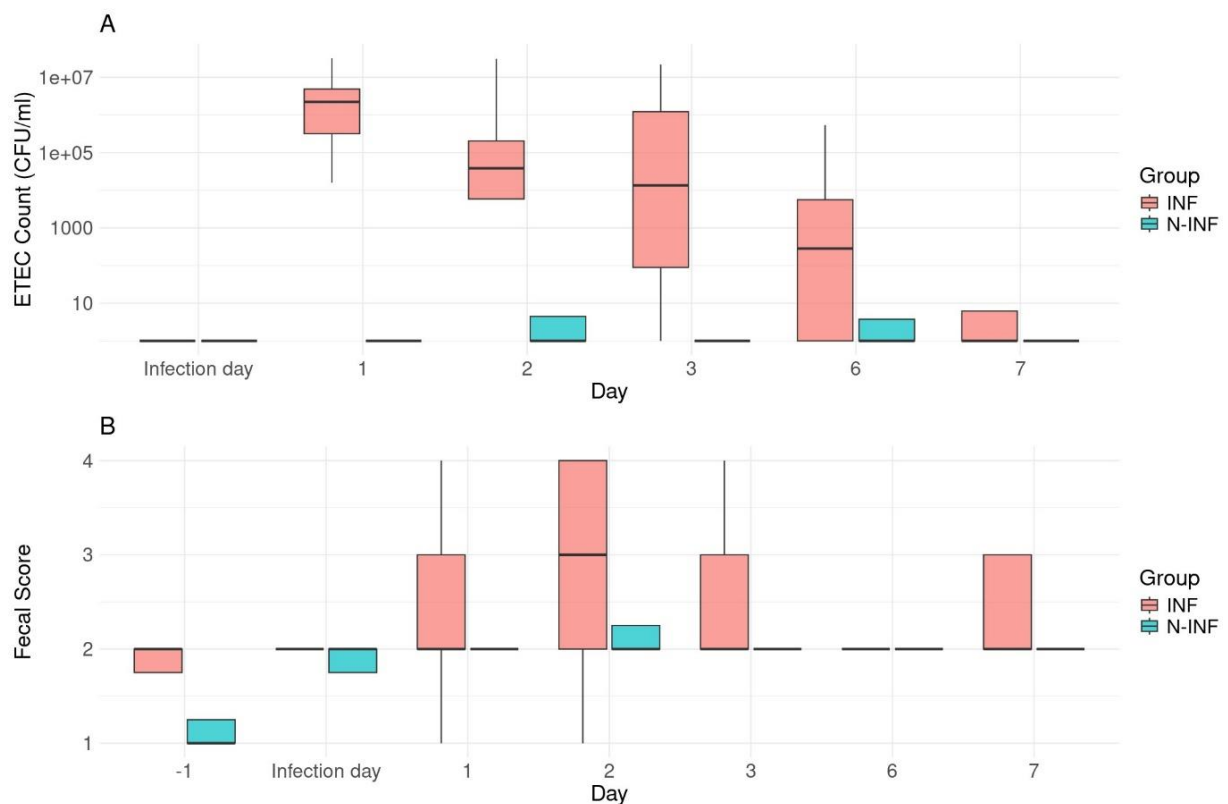
Growth parameters, fecal score and health status

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 (Figure 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 3.10×10^7 CFU/mL, respectively. We considered that the infection had passed after 7 days.

In Study 1, all pigs had a fecal score of 2 or less (data not shown). 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. Even a week after infection (D7), some pigs in the INF group still displayed a fecal score of 3 (Figure 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.

Figure 1. Bacterial counts (A) and fecal scores (B) over time in non-infected (N-INF) and infected (INF) groups. The boxplots represent bacterial counts in colony-forming units (CFU) per milliliter (ml) and fecal scores for each group by day.



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 (Figure 2). 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 (Figure 3)

Figure 2. Fate of the capsule after administration in Study 1 and 2. The percentage of capsules retrieved from the feces or not found (NF) was calculated based on the number of capsules in each category divided by the number of capsules administered.

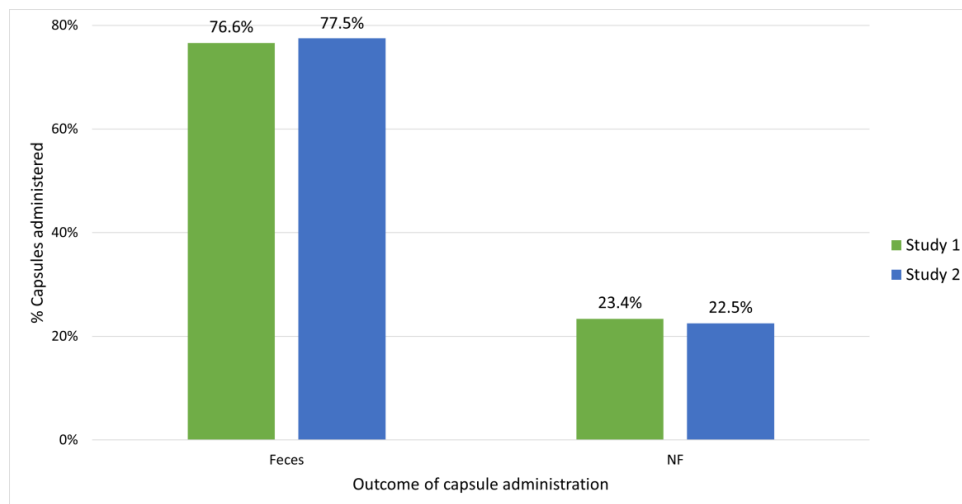
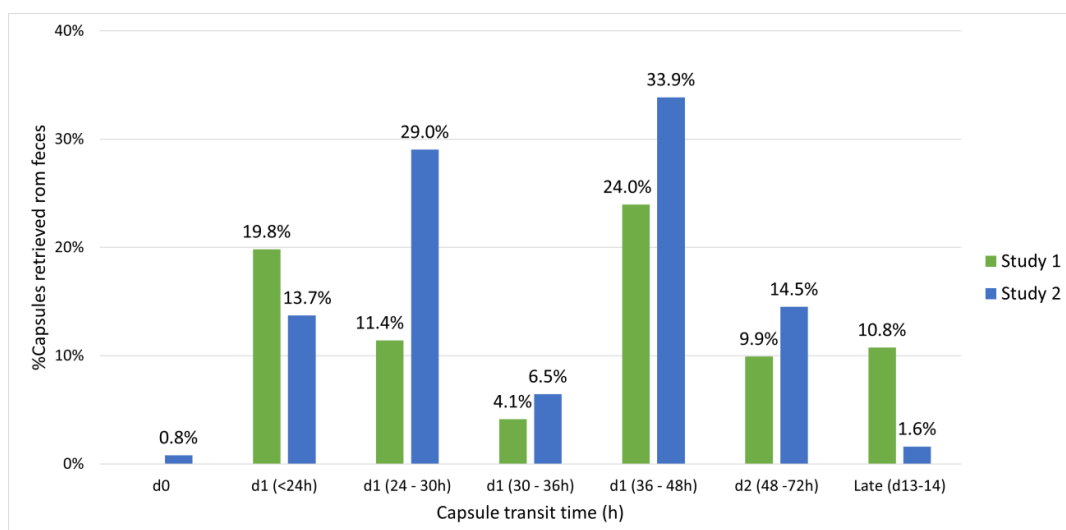


Figure 3. Capsule transit time for capsules retrieved from the feces in Study 1 and 2. Capsule’s transit time was calculated as the time between capsule administration and capsule retrieval in the feces.

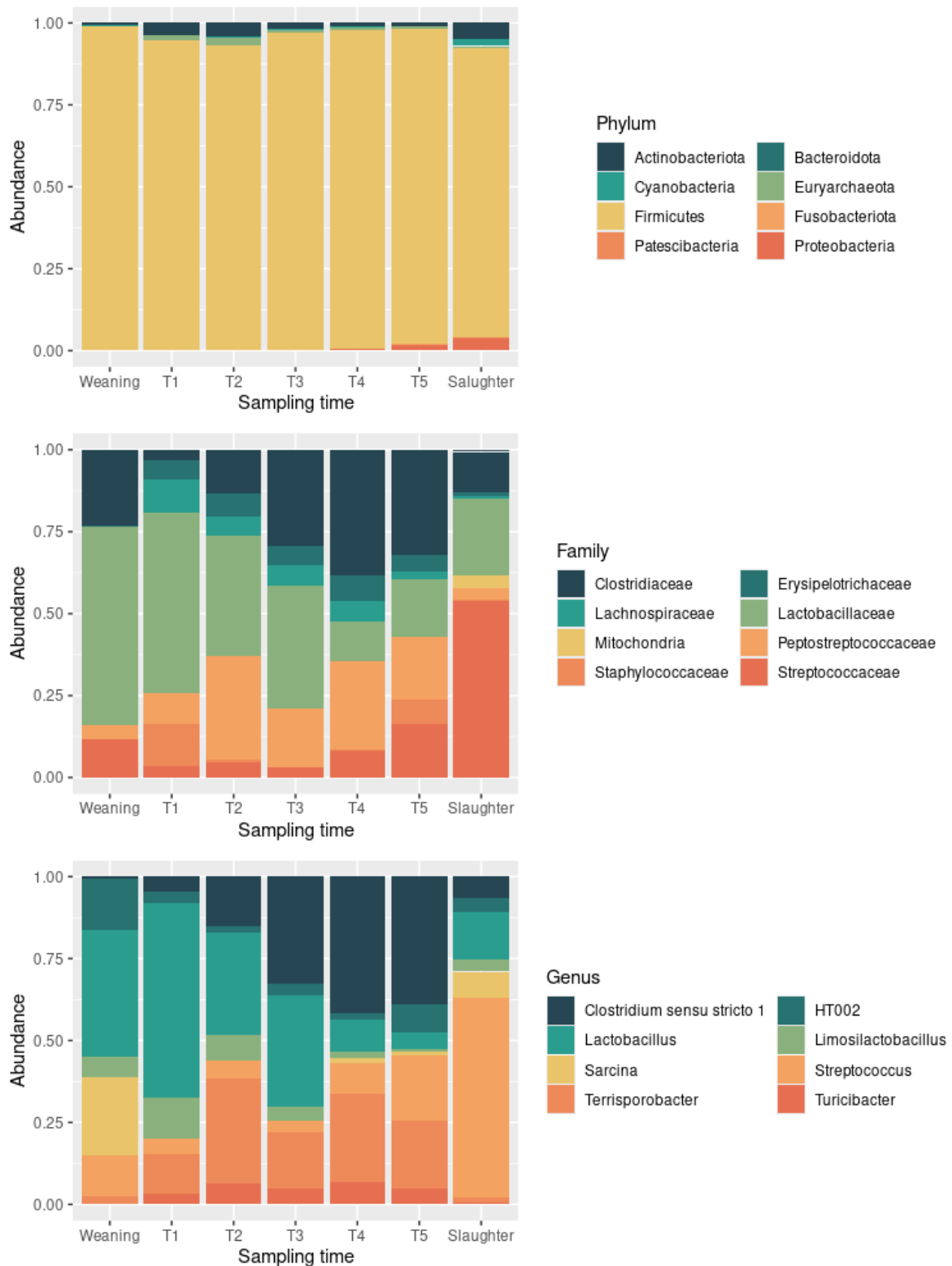


Study 1

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 Figure 5. The main phylum in every segment of the SI at weaning was Firmicutes ($98.93 \pm 6\%$), followed by *Actinobacteriota* ($0.8 \pm 0.34\%$) 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* ($1.87 \pm 5.65\%$).

Figure 4. Bar plots showing the mean relative abundance of the 8 most abundant phyla, family, and genera determined in the SI at weaning (28 days of age), capsules from 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 the SI at slaughter (140±5 days of age) of pigs at weaning in Study 1.



Microbiota analysis from CapSa's 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 4 displays the predominant relative abundance of the eight most common phyla, families, and genera for each administration time.

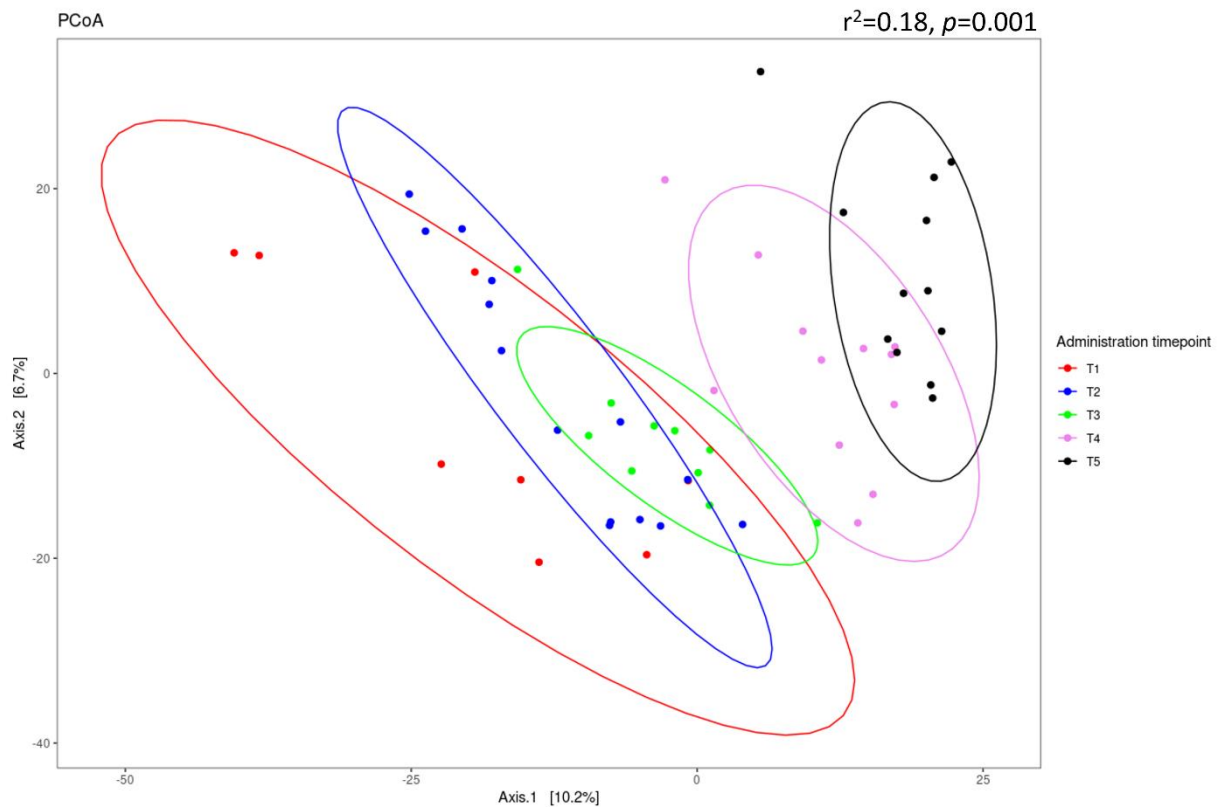
The main phylum in every capsule, independent of administration time, was Firmicutes ($95.52 \pm 2.49\%$), followed by Actinobacteriota ($2.25 \pm 0.38\%$) and Euryarchaeota ($1.30 \pm 0.76\%$). The most abundant families in every capsule, independent of the administration time, were *Lactobacillaceae* ($25.42 \pm 4.27\%$), *Clostridiaceae* ($21.26 \pm 6.52\%$), *Peptostreptococcaceae* ($19.42 \pm 6.8\%$), *Streptococcaceae* ($6.53 \pm 2.32\%$), *Erysipelotrichaceae* ($5.54 \pm 1.44\%$), *Lachnospiraceae* ($5.03 \pm 0.2\%$), *Ruminococcaceae* ($3.33 \pm 0.63\%$), and *Staphylococcaceae* ($3 \pm 5.55\%$). The most abundant genera in every CapSa, independent of administration time, were *Clostridium sensu stricto 1* ($20.84 \pm 6.73\%$), *Lactobacillus* ($17.72 \pm 6.62\%$), *Terrisporobacter* ($17.07 \pm 8.7\%$), *Streptococcus* ($6.52 \pm 2.35\%$), *Turicibacter* ($4.04 \pm 3.07\%$), *Limosilactobacillus* ($3.51 \pm 2.04\%$), *Staphylococcus* ($2.84 \pm 6.9\%$), and *HT002* ($2.74 \pm 2.87\%$).

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 throughout the pig's life (Figure 5). 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 *Streptococcus* (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 (Figure 5). 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 ($r^2 = 0.18$, $p = 0.001$; Figure 5), 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.

Figure 5. PCoA plot generated using a Euclidean distance matrix based on clr transformed data of samples from CapSas in Study 1. 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 colored 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.

Table 1. Adonis pairwise comparisons of microbiota composition in capsule contents across administration time point¹ in Study 1, calculated using the Euclidian distances.

Comparisons between administration time points	SumsOfSqs ²	F.Model ³	r ² ⁴	p ⁵	p adj ⁶
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

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 samples ($n = 93$). Figure 4 illustrates the main relative 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

¹ 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.

² SumsOfSqs = Sum of square reflecting total variance

³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

⁵ p = p-value

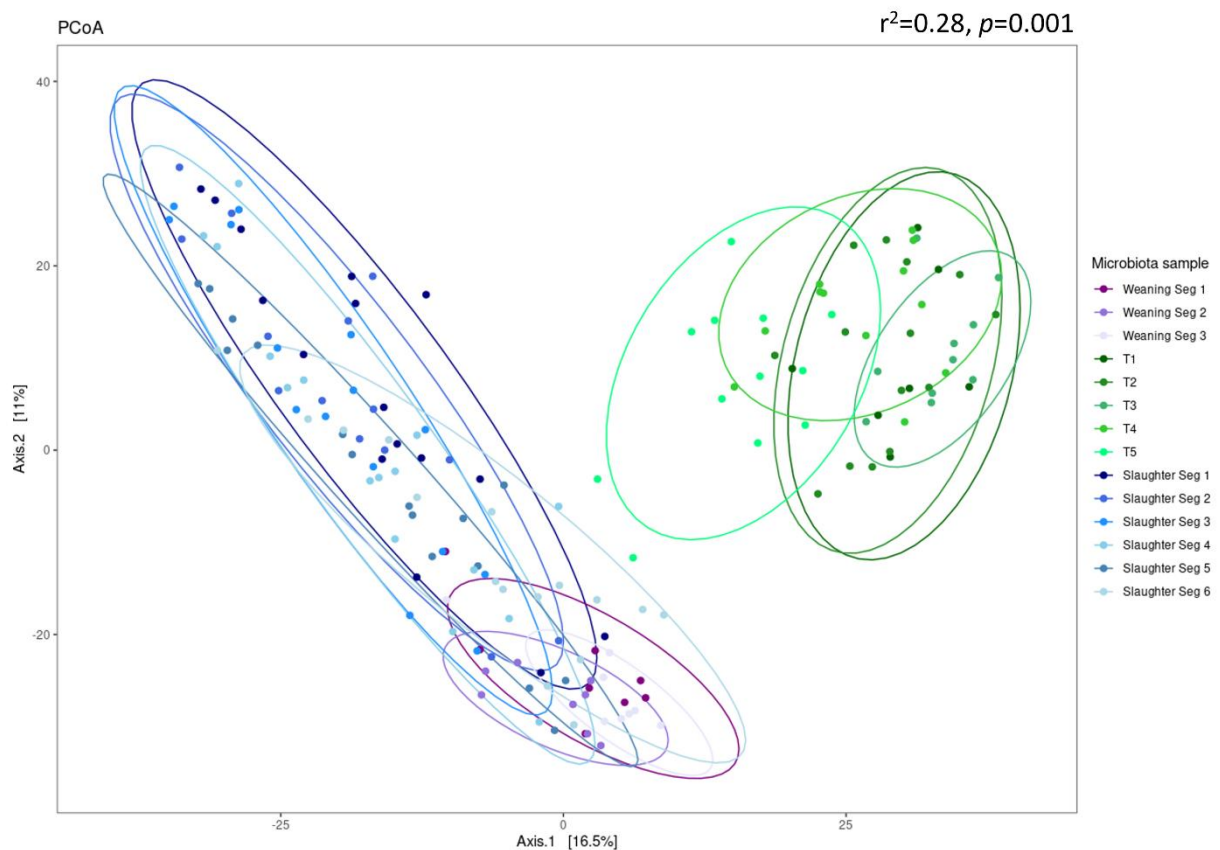
⁶ p adj = p values adjusted for multiple comparisons using the Bonferroni correction.

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 through pig's life

To obtain a 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. 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 (Figure 6). The Adonis test revealed a significant impact of sample type on bacterial composition ($r^2 = 0.28$, $p = 0.001$; Figure 6).

Figure 6. PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data of all samples from Study 1. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data such as microbiome profiles



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 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).

Table 2. Adonis pairwise comparisons of microbiota composition between small intestine segments at weaning, capsule contents across five different administration timepoints¹ and lower and upper segments of the small intestine at slaughter in Study 1, calculated using the Euclidian distances.

Comparisons between samples	SumsOfSqs ₂	F.Model ³	r ²⁴	p ⁵	p adj ₆
SI at Weaning vs T1	17170.80	11.40	0.27	< 0.01	< 0.01
SI at Weaning vs T2	23977.24	14.57	0.28	< 0.01	< 0.01
SI at Weaning vs T3	22721.34	15.26	0.32	< 0.01	< 0.01
SI at Weaning vs T4	24679.73	16.12	0.32	< 0.01	< 0.01
SI at Weaning vs T5	19143.41	13.89	0.29	< 0.01	< 0.01
SI at Weaning vs Upper SI at Slaughter	32285.07	18.04	0.21	< 0.01	< 0.01
SI at Weaning vs Lower SI at Slaughter	21108.38	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	10435.42	4.32	0.19	< 0.01	< 0.01
T1 vs Upper SI at Slaughter	20635.74	9.03	0.15	< 0.01	< 0.01
T1 vs Lower SI at Slaughter	18467.90	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	28808.27	12.57	0.18	< 0.01	< 0.01
T2 vs Lower SI at Slaughter	25898.15	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	25313.75	11.27	0.17	< 0.01	< 0.01
T3 vs Lower SI at Slaughter	22761.76	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	24253.22	10.81	0.16	< 0.01	< 0.01
T4 vs Lower SI at Slaughter	22620.38	11.94	0.17	< 0.01	< 0.01
T5 vs Upper SI at Slaughter	17080.03	7.94	0.12	< 0.01	< 0.01
T5 vs Lower SI at Slaughter	15523.90	8.60	0.12	< 0.01	< 0.01

¹ 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.

² SumsOfSqs = Sum of square reflecting total variance

³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

⁵ p = p-value

⁶ p adj = p values adjusted for multiple comparisons using the Bonferroni correction.

Upper SI at Slaughter vs Lower SI at Slaughter 7581.34 3.88 0.040 < 0.01 **0.01**

We also tested the effect of diet on bacterial composition. For beta diversity, the PCoA plot shows two clusters based on the diet eaten in the period before sample collection: one for the grower diet, and another for the finisher diet and samples of pigs fed with a starter diet that overlaps both clusters ($r^2 = 0.14$, $p = 0.001$; Figure 7). 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 not identify any clear microbial biomarkers among the samples.

Figure 7. PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data of samples from Study 1. 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 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 to slaughter.

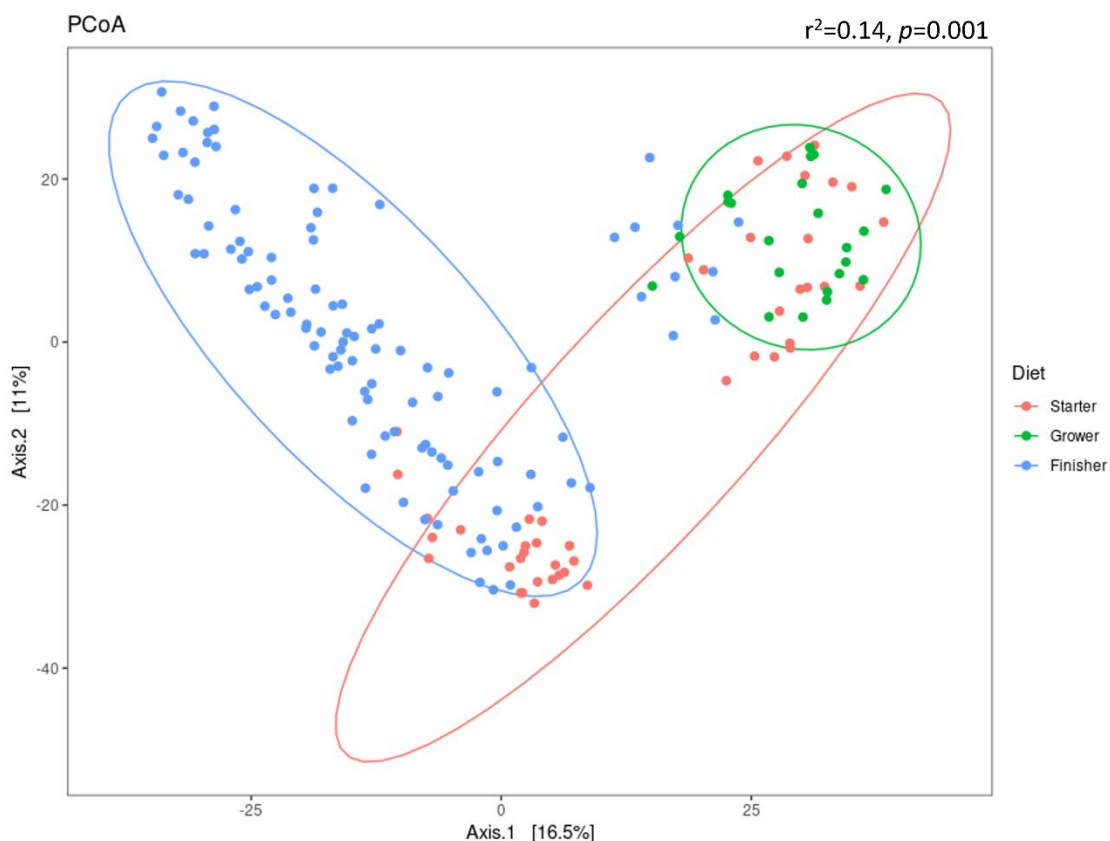


Table 3. Adonis pairwise comparisons of microbiota composition between samples from pigs fed either the starter, grower or finisher diets at various growth stages¹ in Study 1, calculated based on Euclidean distances.

Comparisons	SumsOfSqs ²	F.Model ³	r ²⁴	p ⁵	p adj ⁶
Starter vs Grower	19009.27	7.36	0.10	< 0.01	< 0.01
Starter vs Finisher	37132.41	16.30	0.09	< 0.01	< 0.01
Grower vs Finisher	37037.44	16.46	0.11	< 0.01	< 0.01

Study 2

¹ 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 d113±3 to 140±5 days of age (slaughter).

² SumsOfSqs = Sum of square reflecting total variance

³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

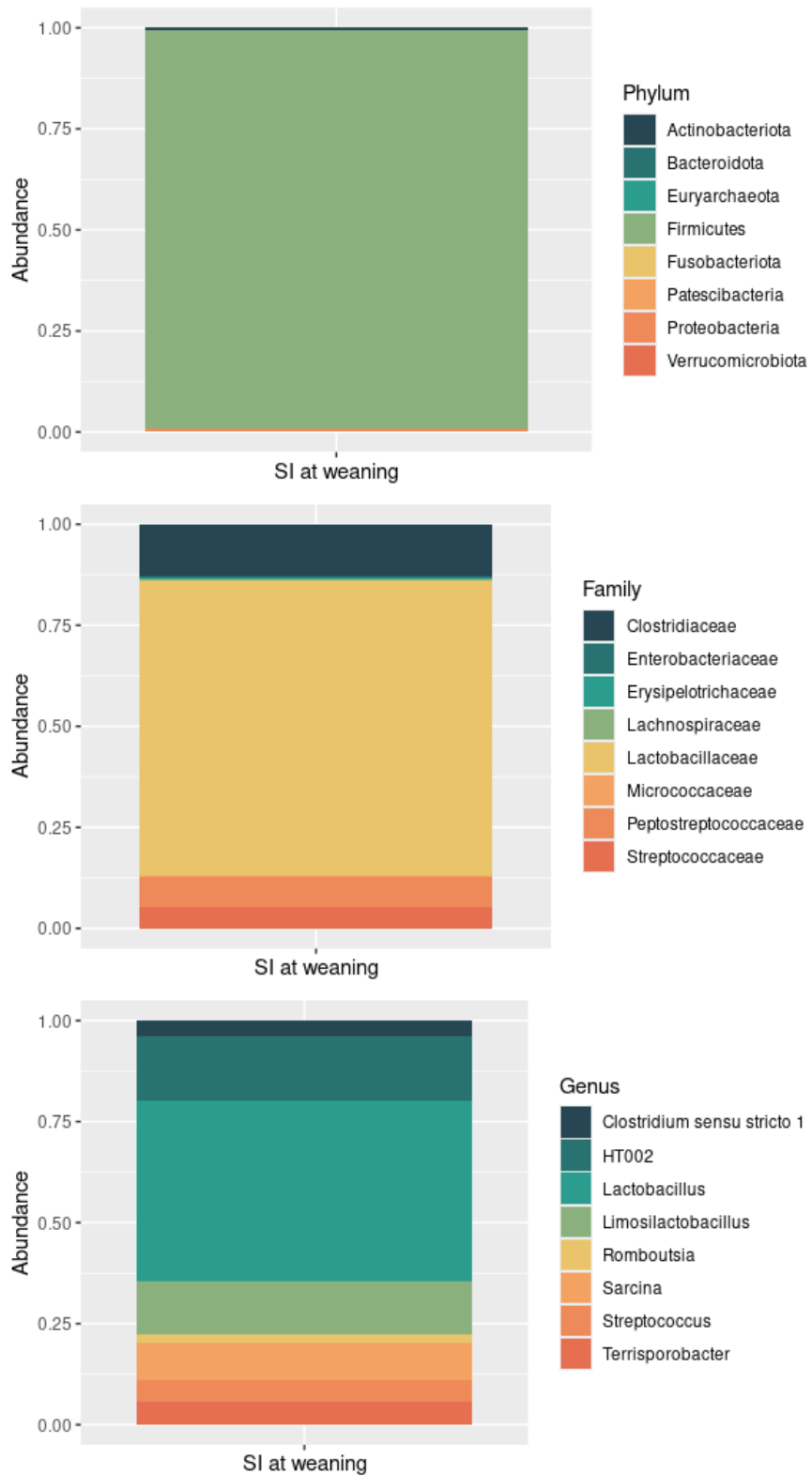
⁵ p = p-value

⁶ p adj = p values adjusted for multiple comparisons using the Bonferroni correction.

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 Figure 8. 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%), *Terrisporobacter* (5.21 ± 4.16%), *Clostridium sensu stricto 1* (3.78 ± 1.67%), and *Romboutsia* (1.88 ± 5.52%).

Figure 8. Mean relative abundance of the eight most abundant phyla, family, and genera in the small intestine microbiota content at weaning in pigs in Study 2 (100% stacked bar plot).



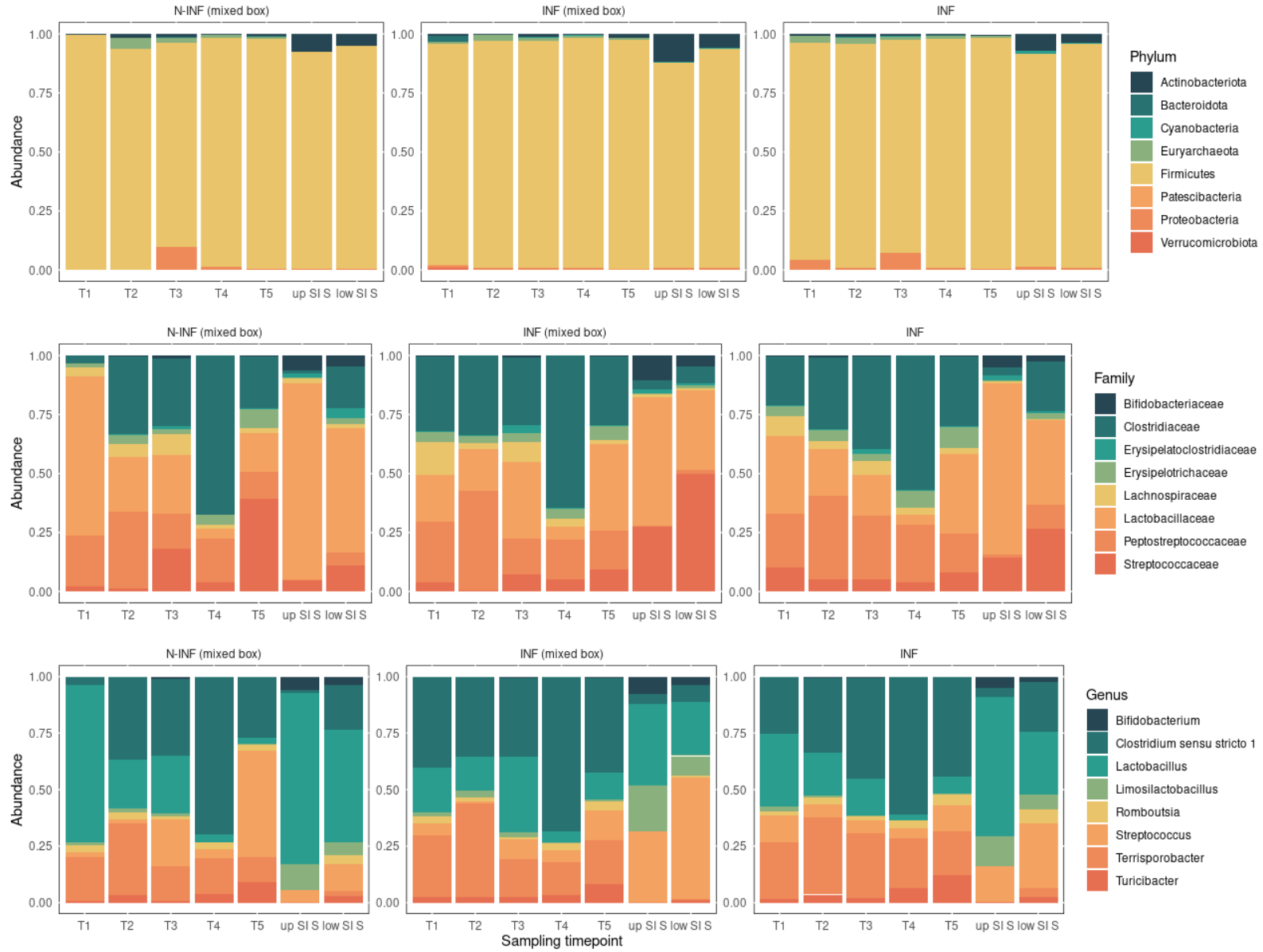
Microbiota analysis from CapSa's samples

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 Figure 9. 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 *Clostridiaceae* and *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* (Figure 9). In the INF group, *Lactobacillus* exhibited a lower abundance as 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).

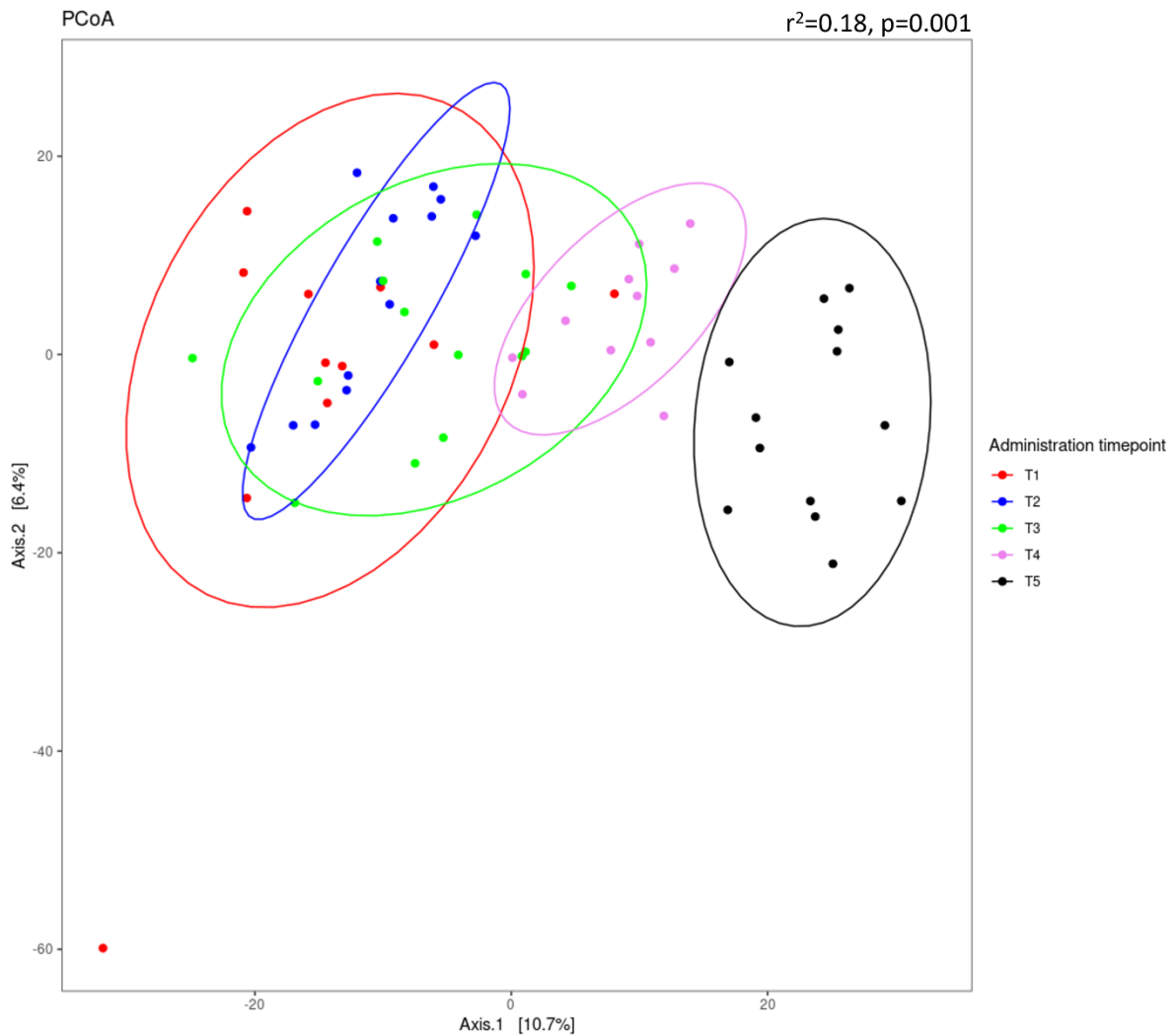
Figure 9: Mean relative abundance of the eight most abundant phyla, family, and genera in the lower part (low SI) and upper part (up SI) of the small intestine by sampling timepoint (Study 2, 100% Stacked Bar Plot) (T1: 52±3 days of age; T2: 70±3 days of age; T3: 83±3 days of age; T4: 110±3 days of age; 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.



For beta diversity, the PCoA plot shows a partial clustering of the samples based on the administration time of the capsules (Figure 10). 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 ($r^2 = 0.18$, $p = 0.001$; Figure 10). 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 ($r^2 = 0.01$, $p = 0.64$; Figure 11). 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). Similarly, the microbiota composition did not differ between the N-INF and INF pigs reared in the mixed pen (data not shown).

Figure 10. PCoA plot generated using a Euclidean distance matrix based on centered log ratio (clr) transformed data of samples from CapSas in Study 2. 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 colored 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.

Table 4. Adonis pairwise comparisons of microbiota composition in capsule contents across administration time point¹ in Study 2, calculated using the Euclidian distances.

Comparisons	SumsOfSqs²	F.Model³	r²⁴	p⁵	p adj⁶
T1 vs T2	4396.66	1.89	0.08	0.00	0.00
T1 vs T3	5194.90	2.17	0.09	0.00	0.00
T1 vs T4	6926.28	3.18	0.14	0.00	0.00
T1 vs T5	10637.02	4.65	0.18	0.00	0.00
T2 vs T3	4899.34	2.47	0.09	0.00	0.00
T2 vs T4	6432.94	3.70	0.14	0.00	0.00
T2 vs T5	10102.78	5.41	0.18	0.00	0.00
T3 vs T4	4139.22	2.26	0.08	0.00	0.00
T3 vs T5	9030.63	4.65	0.15	0.00	0.00
T4 vs T5	5070.97	2.98	0.11	0.00	0.00

¹ 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

² SumsOfSqs = Sum of square reflecting total variance

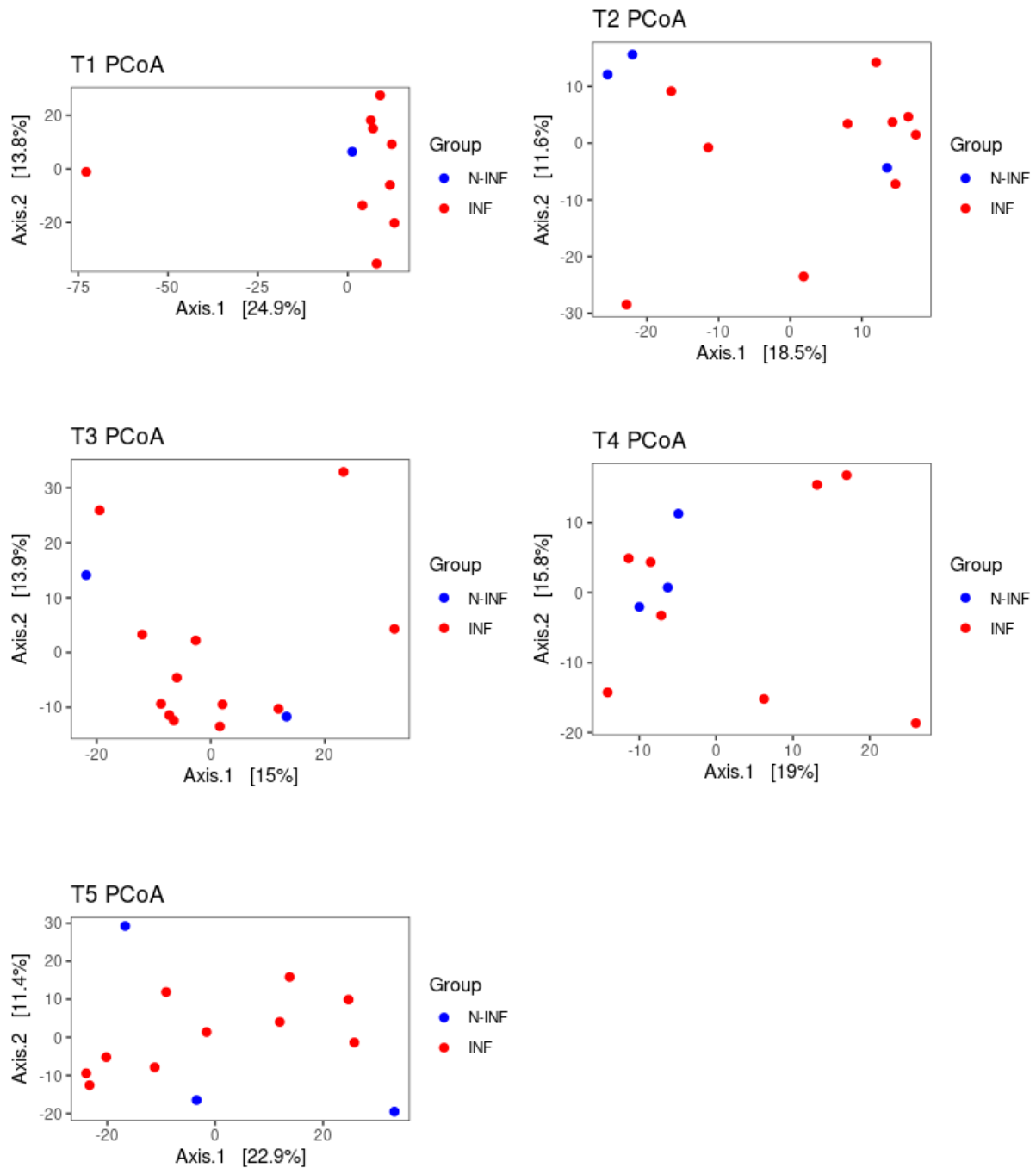
³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

⁵ p = p value

⁶ p adj = p values adjusted for multiple comparison using the Bonferroni correction

Figure 11. PCoA plots generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data of samples from CapSas in Study 2. 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 colored based on 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 capsule contents collected from non-infected (N-INF) and infected (INF) pigs across different timepoints of administration¹ in Study 2, calculated using the Euclidian distances.

Comparisons	SumsOfSqs₂	F.Model³	r²⁴	p⁵	p adj⁶
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

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 (Figures 12, 13, and 14). 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

¹ 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.

² SumsOfSqs = Sum of square reflecting total variance

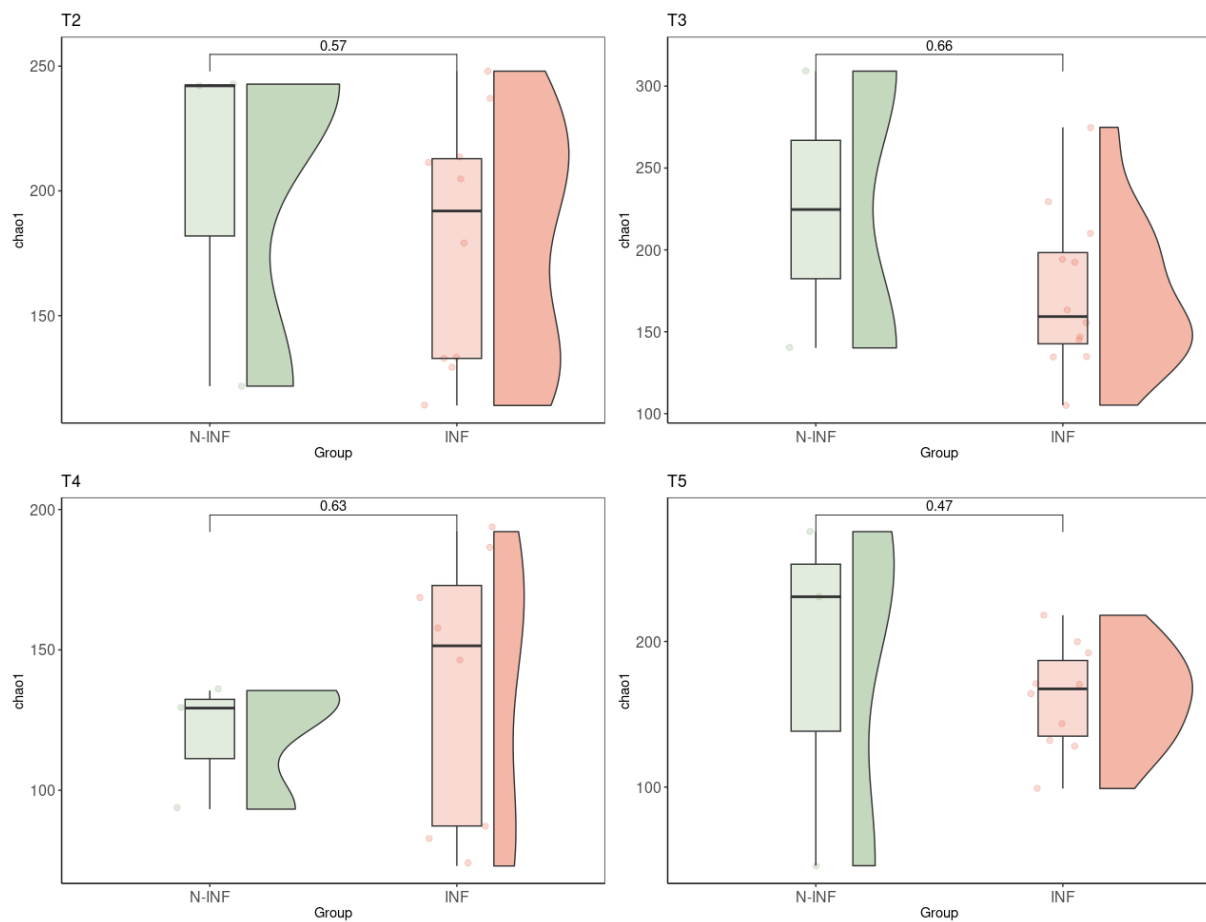
³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

⁵ p = p value

⁶ p adj = p values adjusted for multiple comparison using the Bonferroni correction.

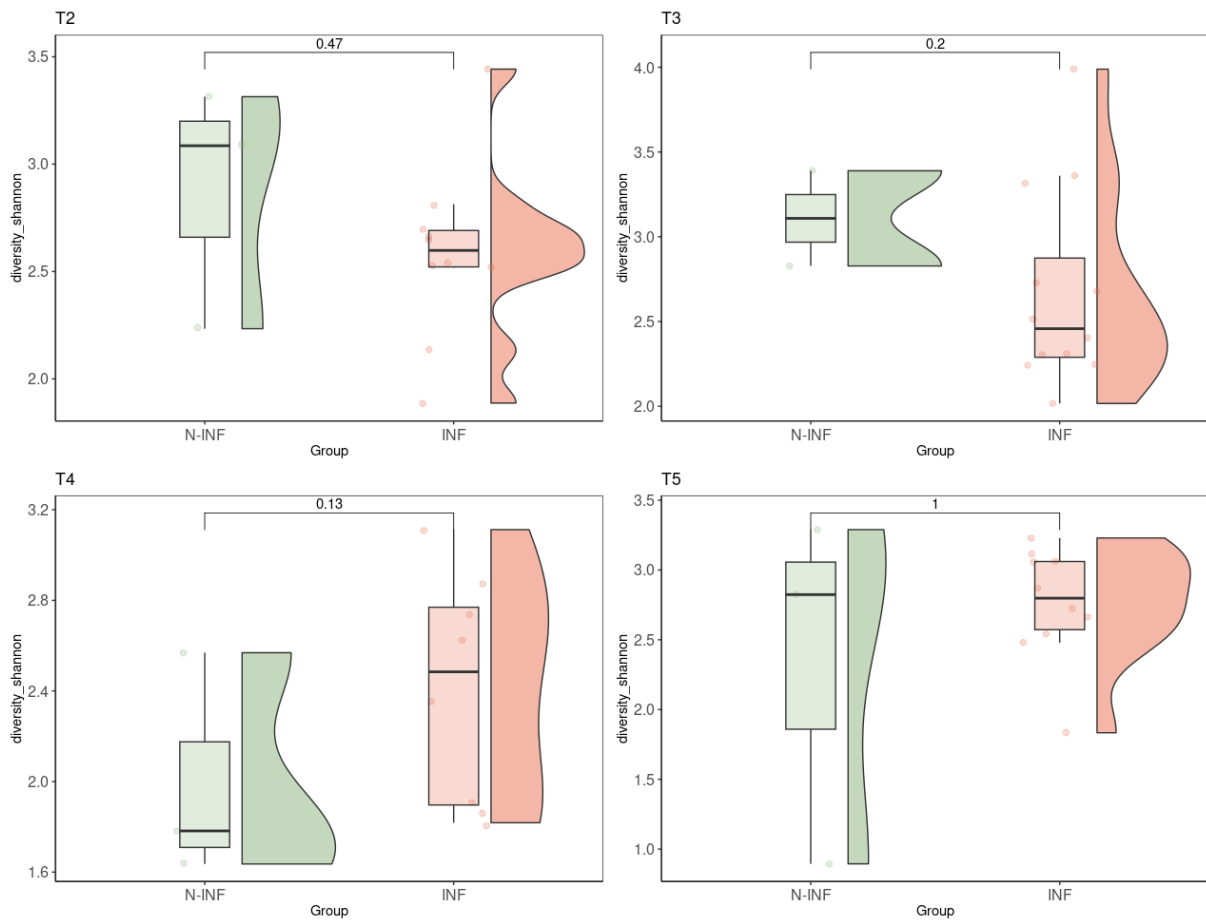
Figure 12. Box plots showing alpha diversity values for Chao1 index determined in the capsule contents within administration timepoint and by group (N-INF or INF) in Study 2.



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

days of age.

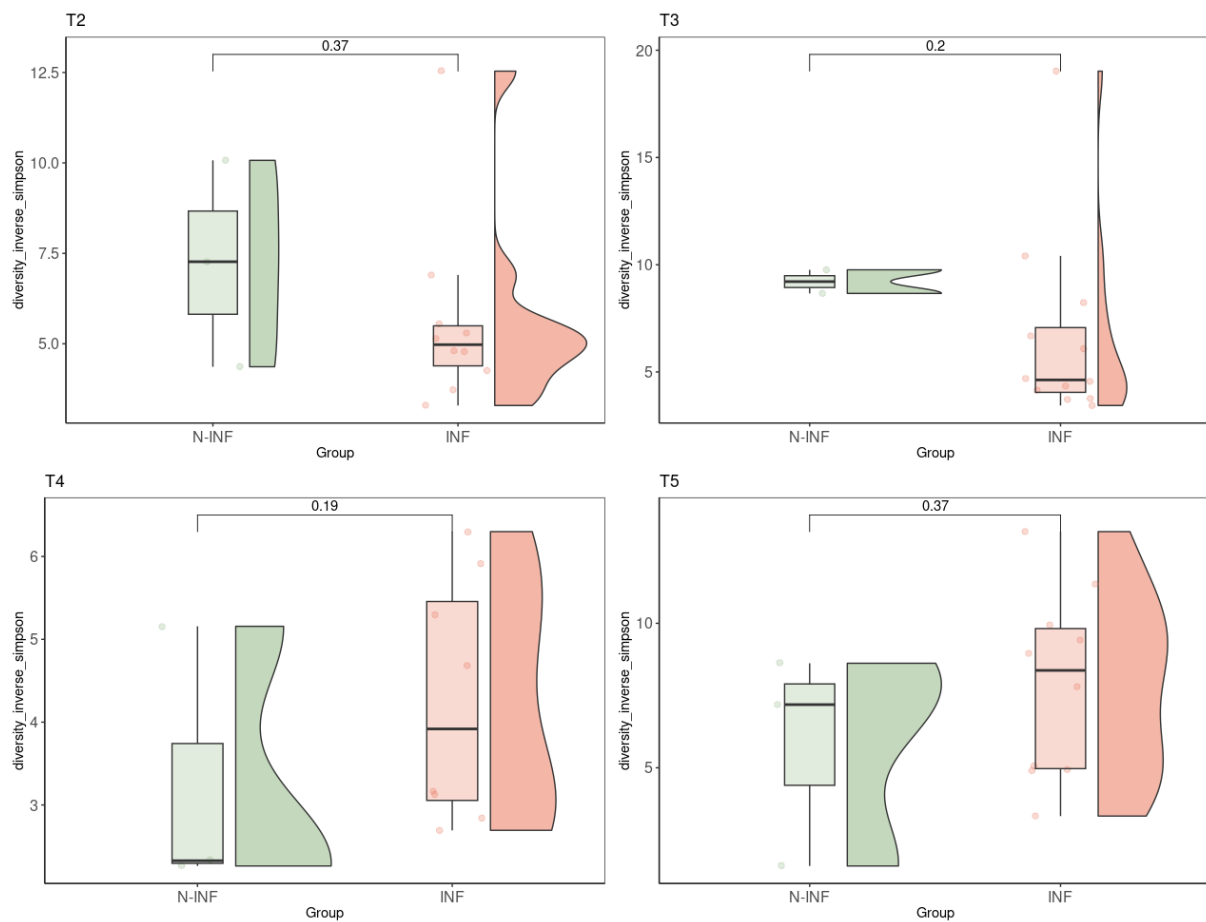
Figure 13: Box plots showing alpha diversity values for Shannon index determined in the capsule contents within administration timepoint and by group (N-INF or INF) in Study 2.



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

days of age.

Figure 14: Box plots showing alpha diversity values for InvSimpson index determined in the capsule contents within administration timepoint and by group (N-INF or INF) in Study 2.



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

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 Figure 9. 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 (Figure 9).

For beta diversity, the PCoA plot does not show a clear clustering of the samples based on location on the SI (Figure 15), but by group (Figure 16). The Adonis test revealed that bacterial composition was significantly affected by the sample type ($r^2 = 0.10$, $p = 0.002$; Figure 15) and by group ($r^2 = 0.04$, $p = 0.001$; Figure 16). This indicates a difference between the bacterial composition of the SI from different segments and between the N-INF and INF groups.

Figure 15. PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data of samples from SI segments at slaughter (140 ± 5 days of age) in **Study 2**. 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 sample type (Segments 1, 2, 3, 4, 5 and 6).

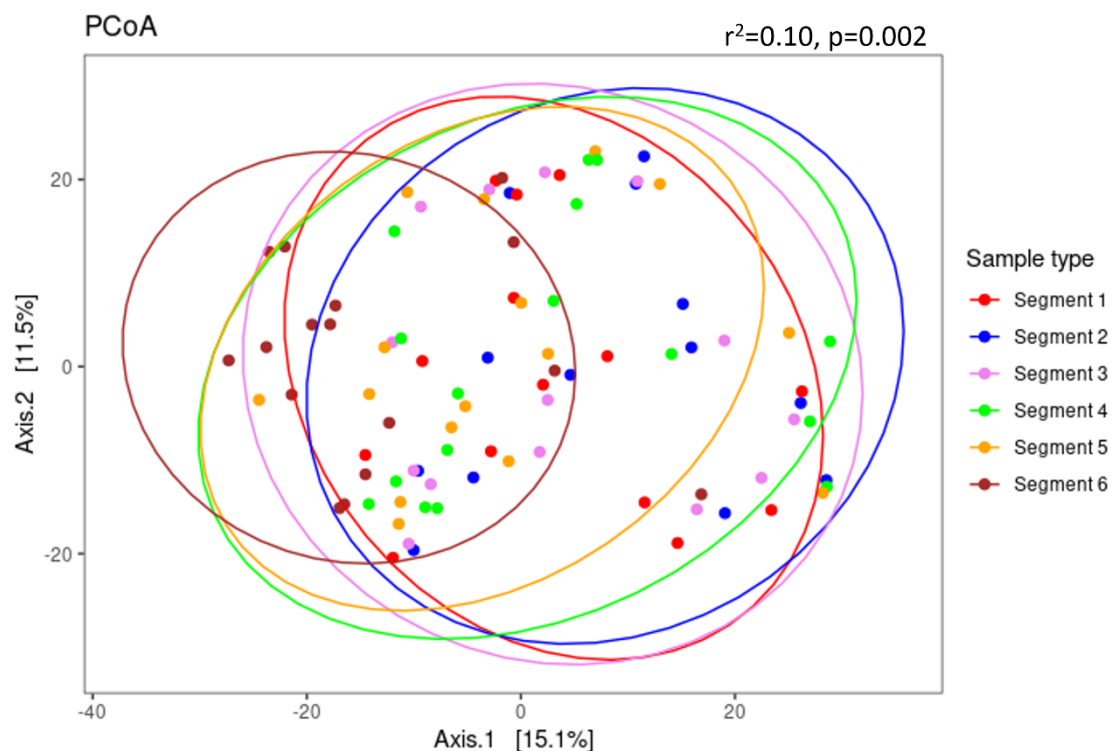
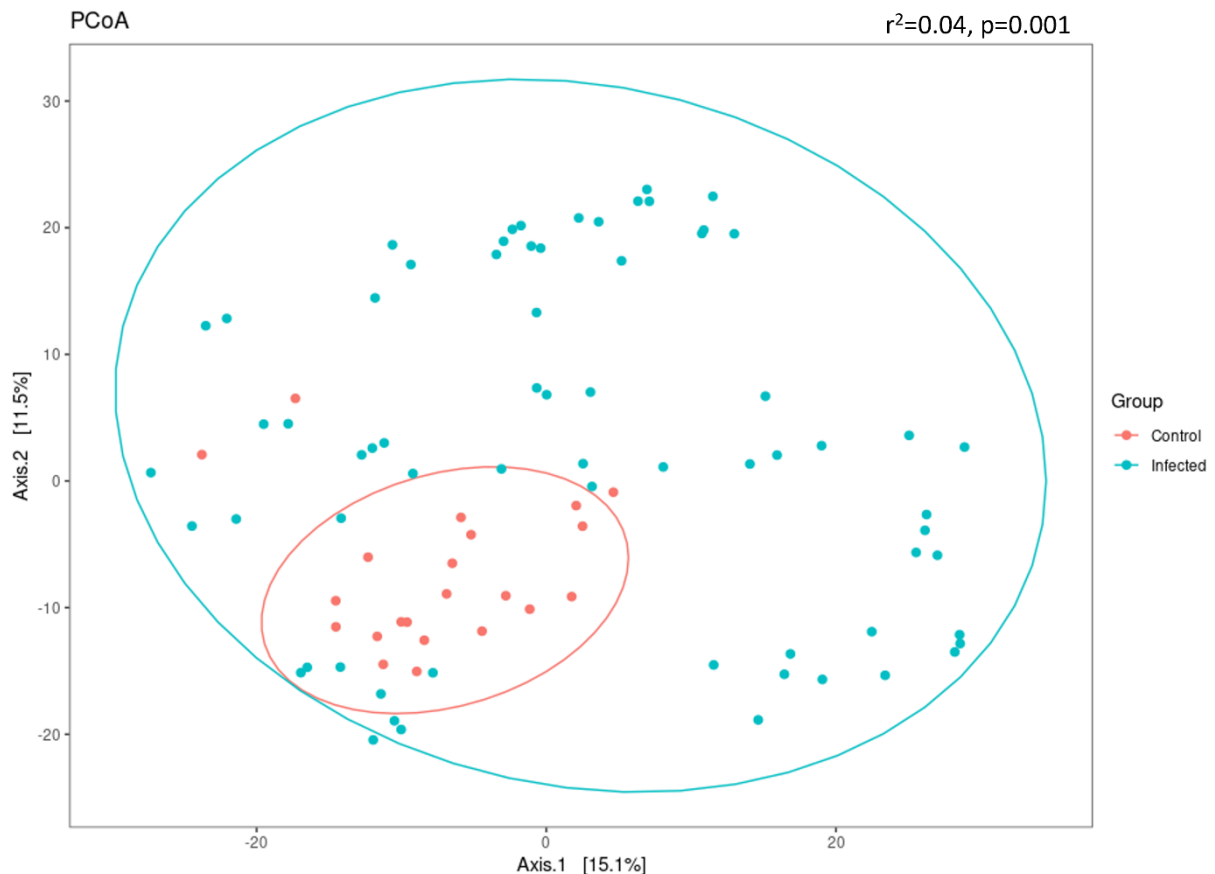


Figure 16. PCoA plot generated using a Euclidean distance matrix based on centred log ratio (clr) transformed data. The distances between samples were calculated based on clr-transformed data, which provides a more suitable representation for compositional data such as microbiome profiles. Samples are coloured based on group (N-INF and INF).



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).

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 ¹	F.Model ²	r ^{2 3}	p ⁴	p adj ₅
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

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 ($p = 0.036$

¹ SumsOfSqs = Sum of square reflecting total variance.

² F.Model = F test value.

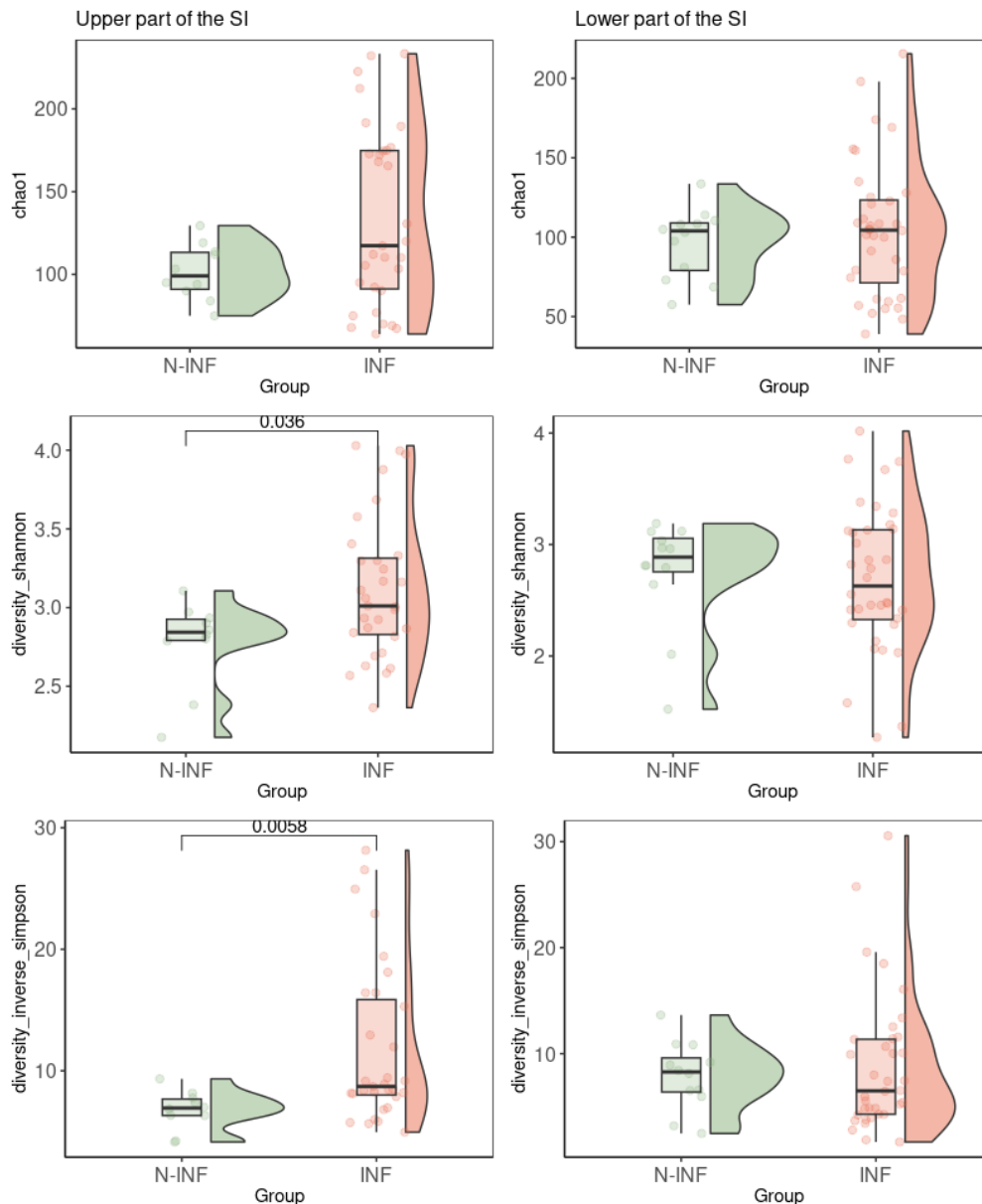
³ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences.

⁴ p = p value.

⁵ p adj = p values adjusted for multiple comparison using the Bonferroni correction.

and $p = 0.0058$, respectively). The INF group exhibited higher bacterial richness in this region (Figure 17).

Figure 17. Box plots showing alpha diversity values for Chao1, Shannon and InvSimpson determined in the 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.

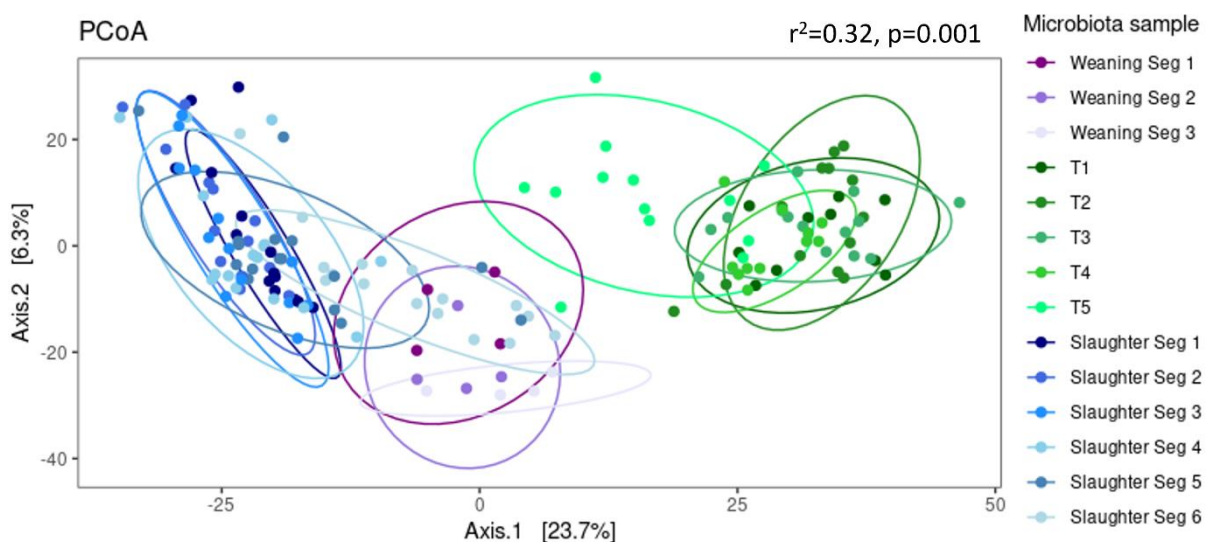


Microbiota analysis through pig's life

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 (Figure 18). The Adonis test revealed that bacterial composition was affected by the sample type ($r^2 = 0.32$, $p = 0.001$; Figure 18). 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.

Figure 18. 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 sampling timepoint 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.

Table 7. Adonis pairwise comparisons of microbiota composition between small intestine segments at weaning, capsule contents across five different administration timepoints¹ and lower and upper segments of the small intestine at slaughter in Study 2, calculated using the Euclidian distances.

Comparisons	SumsOfSqs²	F.Model³	r²⁴	p⁵	p adj⁶
SI at Weaning vs T1	14004.36	6.56	0.24	0.00	0.00
SI at Weaning vs T2	17920.71	10.59	0.32	0.00	0.00
SI at Weaning vs T3	18859.73	10.54	0.31	0.00	0.00
SI at Weaning vs T4	14254.24	9.75	0.32	0.00	0.00
SI at Weaning vs T5	14545.12	8.81	0.28	0.00	0.00
SI at Weaning vs Upper SI at Slaughter	17739.26	12.38	0.19	0.00	0.00
SI at Weaning vs Lower SI at Slaughter	14248.67	10.44	0.15	0.00	0.00
T1 vs T2	4733.13	1.86	0.08	0.00	0.01
T1 vs T3	5514.40	2.12	0.08	0.00	0.00
T1 vs T4	7112.07	2.98	0.13	0.00	0.00
T1 vs T5	10986.61	4.39	0.17	0.00	0.00
T1 vs Upper SI at Slaughter	28844.78	16.07	0.24	0.00	0.00
T1 vs Lower SI at Slaughter	24018.24	14.24	0.20	0.00	0.00
T2 vs T3	5287.22	2.45	0.09	0.00	0.00
T2 vs T4	6968.71	3.67	0.14	0.00	0.00
T2 vs T5	10857.20	5.31	0.18	0.00	0.00
T2 vs Upper SI at Slaughter	33781.81	20.92	0.28	0.00	0.00
T2 vs Lower SI at Slaughter	28255.26	18.48	0.24	0.00	0.00
T3 vs T4	4494.09	2.26	0.09	0.00	0.01
T3 vs T5	9717.98	4.58	0.15	0.00	0.00
T3 vs Upper SI at Slaughter	34522.49	20.81	0.28	0.00	0.00
T3 vs Lower SI at Slaughter	29046.32	18.49	0.24	0.00	0.00
T4 vs T5	5477.95	2.95	0.12	0.00	0.00
T4 vs Upper SI at Slaughter	26570.42	17.51	0.26	0.00	0.00
T4 vs Lower SI at Slaughter	20794.50	14.45	0.20	0.00	0.00
T5 vs Upper SI at Slaughter	22094.56	13.83	0.21	0.00	0.00
T5 vs Lower SI at Slaughter	18239.50	12.05	0.17	0.00	0.00
Upper SI at Slaughter vs Lower SI at Slaughter	5355.11	3.75	0.04	0.00	0.00

¹ 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.

² SumsOfSqs = Sum of square reflecting total variance.

³ F.Model = F test value.

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences.

⁵ p = p value.

⁶ p adj = p values adjusted for multiple comparison using the Bonferroni correction.

Discussion

In these two studies, we used CapSa, a novel device that collects intestinal content from pigs, opening the door to a new way of studying the dynamic picture of the pig's microbiota using the same pigs. Through a standardized protocol of administration (Inés García Viñado et al., 2024), we achieved an overall retrieval rate that reached more than 70% of the CapSas found in feces..

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 exclusively 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 (Crespo-Piazuelo et al., 2021; Inoue et al., 2005; Kim et al., 2011). 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 (Chen et al., 2017; Konstantinov et al., 2006; Petri et al., 2010), and its abundance sequentially decreased as pigs matured, eventually replaced by *Streptococcus* and *Clostridium* as the main genera in the SI microbiota at slaughter (Luo et al., 2022). However, contrary to the publication of Luo et al., 2022 (Luo et al., 2022), *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 (Luo et al., 2022) and shows a positive correlation with BW during this period (Wang et al., 2019). 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 (Kim et al., 2011), with a succession of microbial populations in the SI that carries on through the life of the pig (Zhao et al., 2015). Furthermore, similar to observations in humans, each pig may possess a unique microbiota fingerprint alongside its core and stage-associated microbiota (Holman et al., 2017). 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 (Girard et al., 2018). INF pigs exhibited ETEC shedding in feces for a week post-infection, accompanied by fecal scores higher or similar to 3 (indicating watery diarrhea) in the days following infection (see Figure 1).

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. We considered the challenge of retrieving capsules in pigs weighing less than 12 kg due to the size of the gastric pylorus, a limitation acknowledged in the first validation study of CapSa (Inés García Viñado et al., 2024).

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 (Barros et al., 2023). 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, 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 influence beta diversity at the time of slaughter, 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 (Inés García Viñado et al., 2024), may not accurately reflect the primary site of infection, as ETEC receptors are predominantly found in the lower part of the SI (Jin & Zhao, 2000). 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. Our observation of long-term changes in the microbiota, but not short-term changes, partially

aligns with previous research (Rhouma et al., 2021) 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

CapSa is the first device to successfully and repeatedly collect gut microbiota from pigs without causing harm, enabling the study of microbiome evolution from weaning to slaughter under both normal and pathological conditions. This research highlights the long-term effects of ETEC infection on bacterial composition. Further studies with different approaches, such as transcriptomics, metabolomics, or additional samplings, are necessary to better characterize the microbiota of the SI.

List of abbreviations

BW: Body weight

clr: Centred log ratio

ETEC: Enterotoxigenic *Escherichia coli*

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 diarrhea

SI: Small intestine

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.

Availability of data and material

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

Raw sequences are available at NCBI sequence read archive (SRA under the accession number PRJNA1162443).

Additional Files

Supplementary Table 1. Ingredients and gross chemical composition of the starter diet.

	Starter diet ¹
Ingredients (%)	
Barley, ground	6,426
Oats, ground	10,000
Oats flakes	2,000
Maize	3,442
Wheat, kernels	45,096
Wheat starch	0,688
Cerolac 15-2175	5,000
Rapeseed oil	2,500
Potato protein	8,459
Soya extract	2,011
Wheat bran	0,506
Beet pulp	6,585
Apple pomace, dried	4,000
L-lysine HCl	0,401
DL-methionine	0,013
L-threonine	0,086
Monocalcium phosphate	0,476
Salt	0,390
Ca-formate	1,000
Pellan2	0,300
ALP-S 463 Piglets	0,400
Luctarom 3	0,010
Greencab-70-C 4	0,200
Natuphos 5000 G 5	0,010
¹ Diet for the piglets from 15 days after birth to 14 days post-weaning, formulated according to the Swiss feeding recommendations for pig.	
² Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany.	
³ Luctarom, Lucta; Montornès del Vallès, Spain.	
⁴ Coated calcium butyrate: Greencab 70-c, Brenntag; Denmark.	
⁵ Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 µmol P from 5 mM phytate/min at pH 5.5 and 37°C.	
	Starter diet ¹
Gross chemical composition analysed (g/kg as fed)	
Dry matter	887
Crude protein	170
Fat	45

Crude fibre	44
Digestible energy (MJ/kg)	14
Lysine	11
Methionine	3.1
Threonine	7.5
Tryptophan	1.9
Ca	5.8
P	4.5
Na	1.9
Vitamin A (IE/kg)	8000
Vitamin D3 (IE/kg)	1000
Vitamin E	25
I (mg/kg)	0.15
Mn (mg/kg)	10
Cu (mg/kg)	6
Zn (mg/kg)	75
Se (mg/kg)	0.20

Supplementary Table 2. Ingredients and gross chemical composition of the grower diet.

Grower diet ¹	
Ingredients (%)	
Barley	34,00
Oats	10,00
Wheat	39,48
Mixed fat	0,80
Soybean extract	9,78
Dried beet pulp	2,00
L-Lysin-HCl	0,47
DL_methionin	0,06
L-Threonin	0,12
L-Valine	0,15
DCP	0,79
Lime, carbonic acid	1,23
Sodium chloride	0,41
Pellan2	0,30
ALP-S 467 Mast	0,40
Natuphos 5000 G3	0,01
¹ Diet for the pigs from days 73±3 to days 112±3 of age, formulated according to the Swiss feeding recommendations for pigs.	
² Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany.	
³ Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 µmol P from 5 mM phytate/min at pH 5.5 and 37°C.	
Grower diet ¹	
Gross chemical composition analysed (g/kg as fed)	
Dry matter	898.48
Ash	52.20
Crude protein	153.18
Fat	30.42
Raw fibre	40.03
Digestible energy (MJ/kg)	13.70
Lysine	9.91
Methionine	2.78
Threonine	6.12
Tryptophan	1.78
Ca	8.06
P	5.20
Na	1.66
Cu (mg/kg)	6.32
Zn (mg/kg)	27.36

Supplementary Table 3. Ingredients and gross chemical composition of the finisher diet.

	Finisher diet ¹
Ingredients (%)	
Barley	34.00
Oats	10.00
Wheat	44.72
Mixed fat	0.30
Soybean extract	5.94
Dried beet pulp	2.00
L-Lysin-HCl	0.36
DL_methionin	0.03
L-Threonin	0.10
L-Valine	0.15
DCP	0.28
Lime, carbonic acid	1.10
Sodium chloride	0.32
Pellan ²	0.30
ALP-S 467 Mast	0.40
Natuphos 5000 G ³	0.01

¹ Diet for the pigs from **days 113±3 to slaughter at 140±5 days** of age, formulated according to the Swiss feeding recommendations for pig.

² Pellet binding aid: Pellan, Mikro-Technik, Bürgstadt, Germany.

³ Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 µmol P from 5 mM phytate/min at pH 5.5 and 37°C.

	Finisher diet ¹
Gross chemical composition analysed (g/kg as fed)	
Dry matter	897.00
Ash	43.13
Crude protein	139.99
Fat	25.55
Raw fibre	39.98
Digestible energy (MJ/kg)	10.24
Lysine	8.05
Methionine	2.33
Threonine	5.35
Tryptophan	1.62
Ca	6.14
P	4.14
Na	1.33
Cu (mg/kg)	6.04
Zn (mg/kg)	27.13

Supplementary Table 4. Relative abundance (% of total; mean±SD) of mayor phyla, family and genera in microbiota samples collected across different administration time points¹ in Study 1.

	T1	T2	T3	T4	T5
Main Phyla (%)					
<i>Firmicutes</i>	94.66 ± 3.00	93.00 ± 2.81	96.74 ± 2.80	97.11 ± 2.57	96.35 ± 3.61
<i>Actinobacteriota</i>	3.59 ± 0.46	4.13 ± 0.54	1.82 ± 0.27	1.05 ± 0.14	0.83 ± 0.14
<i>Euryarchaeota</i>	1.26 ± 0.67	2.46 ± 1.14	0.91 ± 0.62	1.03 ± 0.50	0.57 ± 0.15
<i>Bacteroidota</i>	0.19 ± 0.03	0.11 ± 0.01	0.08 ± 0.02	0.12 ± 0.02	0.17 ± 0.09
<i>Cyanobacteria</i>	0.14 ± 0.03	0.10 ± 0.04	0.27 ± 0.05	0.15 ± 0.08	0.06 ± 0.02
<i>Proteobacteria</i>	0.06 ± 0.02	0.07 ± 0.03	0.09 ± 0.03	0.44 ± 0.37	1.89 ± 1.21
Main Family (%)					
<i>Lactobacillaceae</i>	46.53 ± 6.60	2.36 ± 0.52	32.70 ± 4.37	10.85 ± 1.49	27.76 ± 7.56
<i>Staphylococcaceae</i>	11.19 ± 10.67	0.19 ± 0.08	0.13 ± 0.07	0.12 ± 0.03	6.43 ± 5.98
<i>Lachnospiraceae</i>	8.51 ± 0.27	4.75 ± 0.19	5.56 ± 0.18	5.57 ± 0.19	2.33 ± 0.14
<i>Peptostreptococcaceae</i>	8.02 ± 2.99	4.00 ± 0.65	15.75 ± 5.61	23.74 ± 6.15	16.37 ± 6.90
<i>Ruminococcaceae</i>	5.74 ± 0.72	0.61 ± 0.09	4.92 ± 0.90	3.61 ± 0.47	1.44 ± 0.40
<i>Erysipelotrichaceae</i>	4.98 ± 0.76	26.65 ± 8.96	5.07 ± 1.41	7.02 ± 1.59	4.08 ± 0.40
<i>Streptococcaceae</i>	2.84 ± 0.36	31.06 ± 4.71	2.70 ± 0.74	7.28 ± 1.64	14.12 ± 4.18
<i>Clostridiaceae</i>	2.76 ± 1.28	0.70 ± 0.86	25.66 ± 7.53	33.65 ± 6.62	27.76 ± 7.56
Main Genera (%)					
<i>Clostridium sensu stricto 1</i>	2.74 ± 1.32	11.15 ± 3.79	25.66 ± 7.62	32.32 ± 6.88	27.19 ± 7.99
<i>Lactobacillus</i>	35.87 ± 11.16	23.07 ± 6.81	26.55 ± 6.26	7.57 ± 2.24	3.68 ± 1.99
<i>Terrisporobacter</i>	7.13 ± 3.95	23.43 ± 11.36	13.47 ± 7.42	20.91 ± 7.61	14.61 ± 8.95
<i>Streptococcus</i>	2.84 ± 0.36	4.00 ± 0.65	2.67 ± 0.74	7.28 ± 1.65	14.07 ± 4.36
<i>Turicibacter</i>	2.01 ± 1.83	4.84 ± 3.20	3.81 ± 2.94	5.17 ± 3.17	3.33 ± 3.19
<i>Limosilactobacillus</i>	7.58 ± 1.68	5.97 ± 3.24	3.42 ± 1.00	1.36 ± 0.68	0.51 ± 0.42
<i>Staphylococcus</i>	11.09 ± 13.30	0.03 ± 0.02	0.11 ± 0.08	0.10 ± 0.02	6.33 ± 7.07
<i>HT002</i>	2.23 ± 0.50	1.36 ± 0.65	2.51 ± 1.19	1.44 ± 0.58	6.16 ± 5.83

¹T1: 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.

Supplementary Table 5. Relative abundance (% of total; mean \pm SD) of mayor phyla, family and genera in microbiota samples collected from non-infected (N-INF) and infected (INF) pigs across different administration time points in Study 2¹.

	T1		T2		T3		T4		T5	
	N-INF	INF	N-INF	INF	N-INF	INF	N-INF	INF	N-INF	INF
Main Phyla (%)										
<i>Firmicutes</i>	99.58 \pm 4.30	91.89 \pm 2.56	93.52 \pm 2.92	95.51 \pm 3.55	86.33 \pm 2.25	92.25 \pm 3.39	96.89 \pm 5.82	97.12 \pm 4.66	97.70 \pm 4.42	97.50 \pm 3.22
<i>Actinobacteriota</i>	0.34 \pm 0.05	0.81 \pm 0.14	1.48 \pm 0.13	0.95 \pm 0.11	1.28 \pm 0.16	0.97 \pm 0.13	0.30 \pm 0.04	0.28 \pm 0.07	0.89 \pm 0.08	1.00 \pm 0.12
<i>Euryarchaeota</i>	0.05 \pm 0.00	2.00 \pm 1.09	4.74 \pm 0.90	2.56 \pm 0.68	2.08 \pm 0.71	1.39 \pm 0.65	1.41 \pm 0.43	1.23 \pm 0.43	0.72 \pm 0.34	0.96 \pm 0.31
<i>Cyanobacteria</i>	0.01 \pm 0.00	0.08 \pm 0.03	0.016 \pm 0.02	0.10 \pm 0.03	0.10 \pm 0.02	0.06 \pm 0.02	0.03 \pm 0.02	0.04 \pm 0.02	0.11 \pm 0.02	0.09 \pm 0.04
<i>Proteobacteria</i>	-	2.93 \pm 2.26	0.06 \pm 0.02	0.73 \pm 0.65	9.88 \pm 4.60	5.12 \pm 5.45	1.29 \pm 0.83	0.95 \pm 0.69	0.27 \pm 0.07	0.40 \pm 0.29
<i>Bacteroidota</i>	-	1.08 \pm 0.08	-	0.09 \pm 0.05	0.22 \pm 0.02	0.15 \pm 0.08	0.07 \pm 0.02	0.32 \pm 0.06	0.20 \pm 0.01	0.01 \pm 0.01
<i>Fusobacteriota</i>	-	0.65 \pm 0.00	-	-	0.06 \pm 0.00	0.01 \pm 0.04	-	-	0.01 \pm 0.00	-
Main Family (%)										
<i>Lactobacillaceae</i>	65.27 \pm 9.49	22.40 \pm 3.89	20.23 \pm 2.94	17.19 \pm 2.89	19.25 \pm 3.34	19.23 \pm 3.69	3.88 \pm 0.90	4.44 \pm 0.74	14.86 \pm 3.91	30.3 \pm 5.33
<i>Peptostreptococcaceae</i>	20.61 \pm 5.59	19.71 \pm 5.49	28.55 \pm 6.44	34.45 \pm 8.33	11.24 \pm 3.39	19.79 \pm 6.39	17.62 \pm 5.59	19.68 \pm 5.37	10.40 \pm 3.26	14.57 \pm 4.05
<i>Lachnospiraceae</i>	3.59 \pm 0.16	8.86 \pm 0.36	4.77 \pm 0.20	2.96 \pm 0.14	6.61 \pm 0.22	5.88 \pm 0.48	1.80 \pm 0.08	2.77 \pm 0.18	2.07 \pm 0.13	1.95 \pm 0.14
<i>Clostridiaceae</i>	3.25 \pm	20.88 \pm	29.09 \pm	28.73 \pm	22.24 \pm	30.51 \pm	63.66 \pm	54.69 \pm	20.02 \pm	26.16 \pm

¹ T1: 52 \pm 3 days of age; T2: 70 \pm 3 days of age; T3: 83 \pm 3 days of age; T4: 110 \pm 3 days of age; T5: 126 \pm 3 days of age.

	1.32	7.88	6.69	8.04	5.46	8.85	13.58	11.89	4.54	6.24
<i>Streptococcaceae</i>	2.19 ±	6.16 ±	1.29 ±	3.01 ±	13.88 ±	4.91 ±	3.46 ±	3.98 ±	35.53 ±	7.44 ±
	0.21	2.64	0.09	1.57	5.75	2.51	1.00	1.30	12.27	1.60
<i>Erysipelotrichaceae</i>	1.60 ±	3.34 ±	3.60 ±	3.33 ±	1.62 ±	2.81 ±	3.82 ±	5.57 ±	7.32 ±	7.10 ±
	0.25	0.46	1.23	1.32	0.14	0.61	1.27	1.83	4.17	2.89
<i>Ruminococcaceae</i>	0.90 ±	2.83 ±	1.93 ±	1.94 ±	3.11 ±	2.45 ±	0.68 ±	1.60 ±	0.71 ±	0.44 ±
	0.03	0.28	0.29	0.25	0.51	0.48	0.12	0.31	0.22	0.08
<i>Enterobacteriaceae</i>	-	2.44 ±	0.11 ±	0.65 ±	9.44 ±	4.90 ±	1.10 ±	0.21 ±	0.10 ±	0.26 ±
		3.85	0.03	0.75	6.84	9.41	1.55	0.29	0.08	0.47
Main Genera (%)										
<i>Clostridium sensu stricto 1</i>	3.25 ±	20.87 ±	28.96 ±	28.72 ±	22.23 ±	30.47 ±	63.50 ±	54.66 ±	19.99 ±	26.15 ±
	1.32	8.01	7.05	8.21	5.56	9.03	13.85	11.99	4.64	6.29
<i>Lactobacillus</i>	62.74 ±	18.08 ±	17.20 ±	14.93 ±	16.84 ±	16.18 ±	3.27 ±	2.83 ±	2.24 ±	5.37 ±
	13.63	5.58	4.37	3.90	5.15	5.23	1.27	0.83	0.61	1.68
<i>Terrisporobacter</i>	17.31 ±	17.32 ±	25.42 ±	31.77 ±	10.17 ±	18.29 ±	14.73 ±	16.44 ±	8.11 ±	11.69 ±
	7.97	7.52	8.73	10.60	5.22	8.11	6.90	6.76	4.15	5.03
<i>Streptococcus</i>	2.19 ±	6.16 ±	1.28 ±	3.01 ±	13.87 ±	4.90 ±	3.46 ±	3.98 ±	35.51 ±	7.42 ±
	0.21	2.64	0.09	1.57	6.00	2.55	1.00	1.31	12.74	1.61
<i>Romboutsia</i>	2.45 ±	1.37 ±	2.44 ±	2.20 ±	0.74 ±	1.15 ±	2.71 ±	2.85 ±	1.99 ±	2.70 ±
	0.00	0.78	1.97	0.95	0.12	0.66	0.57	0.60	2.38	1.90
<i>Turicibacter</i>	0.73 ±	1.20 ±	2.54 ±	2.70 ±	0.41 ±	1.56 ±	3.30 ±	4.64 ±	6.94 ±	6.70 ±
	0.00	0.92	3.60	2.85	0.12	1.30	0.93	3.31	9.05	4.82
<i>Weissella</i>	-	1.19 ±	0.90 ±	0.13 ±	0.23 ±	0.43 ±	0.10 ±	0.52 ±	1.91 ±	9.88 ±
		1.71	0.37	0.12	0.12	0.25	0.10	0.75	1.10	5.89
<i>Pediococcus</i>	-	0.03 ±	0.06 ±	0.07 ±	0.21 ±	1.25 ±	0.11 ±	0.47 ±	9.86 ±	14.07 ±
		0.13	0.01	0.08	0.25	2.10	0.14	1.41	14.51	15.14

Supplementary Table 6. Adonis pairwise comparisons of microbiota composition determined in each segment of the SI at slaughter by health status and box¹ in Study 2, calculated using the Euclidian distances.

Comparisons	SumsOfSqs ²	F.Model ³	r ² ⁴	p ⁵	p adj ₆
Segment 1:					
Control vs Infected (mixed box)	963.08	0.93	0.18	0.70	1.00
Control vs Infected	1356.10	1.15	0.11	0.24	0.72
Infected mixed vs Infected	1226.35	0.94	0.09	0.57	1.00
Segment 2:					
Control vs Infected (mixed box)	1424.65	1.09	0.18	0.22	0.68
Control vs Infected	1807.58	1.36	0.16	0.08	0.26
Infected mixed vs Infected	1144.91	0.74	0.08	0.93	1.00
Segment 3:					
Control vs Infected (mixed box)	1168.27	1.10	0.18	0.31	0.94
Control vs Infected	1570.39	1.51	0.14	0.06	0.19
Infected mixed vs Infected	1025.06	0.83	0.09	0.66	1.00
Segment 4:					
Control vs Infected (mixed box)	1254.16	1.42	0.19	0.08	0.25
Control vs Infected	2136.61	1.95	0.16	0.02	0.07
Infected mixed vs Infected	1081.60	0.89	0.08	0.60	1.00
Segment 5:					
Control vs Infected (mixed box)	1102.57	1.13	0.15	0.28	0.85
Control vs Infected	2044.97	1.71	0.14	0.03	0.09
Infected mixed vs Infected	1217.02	0.96	0.08	0.47	1.00
Segment 6:					
Control vs Infected (mixed box)	677.74	0.94	0.13	0.50	1.00
Control vs Infected	1035.61	1.28	0.11	0.16	0.48

¹ Control: Control pigs in Mixed box, Infected: Infected pigs in non-mixed box; Infected (M): Infected pigs in Mixed box.

² SumsOfSqs = Sum of square reflecting total variance

³ F.Model = F test value

⁴ r² = r-square value, reflects grouping differences, the higher the value, the higher the grouping differences

⁵ p = p value

⁶ p adj = p values adjusted for multiple comparison using the Bonferroni correction.

7. General discussion

7.1. Introduction of CapSa methodology

Introducing new methodologies in research begins with establishing a standardized operating procedure that allows fellow researchers to reproduce the methodology. The CapSa device, designed to sample small intestine content in pigs through a capsule-based tool, is a novel technology that has yet to be standardized in swine research. Similar devices designed for human research have been tested *ex vivo* or *in vivo* in pigs, but their retrieval success rates in feces have shown considerable variability. Rezaei Nejad et al. (2019) were unable to retrieve their device after application, whereas Nejati et al. (2022), using a slightly smaller device than CapSa, successfully retrieved theirs. Using a small number of pigs, these studies primarily aimed to validate the sampling mechanism rather than focusing on the administration and retrieval procedures in pigs, as a preparatory step before moving to human trials.

A key objective when initiating the project was to ensure that any device tested would be non-invasive and cause minimal stress to the animals, with a process that could be repeated without compromising the sampling mechanism or the integrity of the sampled microbiota. The process should be repeatable without compromising the sampling mechanism or the sampled microbiota. Despite pigs being a good model for human research due to their physiological and anatomical similarities (Kobayashi et al., 2012; Walters & Prather, 2013), administering and retrieving CapSa presented several challenges

that needed to be resolved to validate its ability to collect small intestine samples safely and effectively.

7.2. Challenges in CapSa administration and retrieval and solutions

As described in **Manuscript I**, environmental modifications were necessary to facilitate CapSa's passage through the GIT. These adjustments included removing straw, administering a liquid diet, and fasting the pigs for 12 hours prior to capsule administration. Additionally, due to the pigs' gastric anatomy (Bal & Ghoshal, 1972; van Hees, 2022), a prokinetic agent was essential for the passage of CapSas through the pylorus, with prucalopride proving the most effective among the prokinetics tested (Emmanuel et al., 2014). This agent enhances gastric contractions, crucial to overcome the blockage of the capsules in the stomach.

Ensuring accurate administration involved performing two oesophageal sondages per pig: one for the prokinetic and another 40 minutes later to guarantee the capsule's arrival in the stomach during peak contractions (Priem et al., 2012). The CapSa administration was the most invasive and delicate step, requiring modifications like a sling for pig handling, a modified mouth gag, and adapted oesophageal sondes for various BW. Extensive training in animal handling was essential to minimize stress during administration, which could affect CapSa's successful retrieval from feces or its retention in the stomach.

Despite these adaptations and procedures, CapSa could not be retrieved from pigs weighing less than 12 kg, due to anatomical constraints that prevented the capsule from

passing through the stomach. This presents a significant limitation for this sampling method. In contrast, in heavier pigs, retrieval rates exceeded 45% (**Manuscript III**), even with repeated application of CapSas to the same animal, highlighting the potential of CapSa in larger animals (**Manuscript IV**).

7.3. Validation of sampling site

After standardizing the administration procedure, two experiments were conducted, aiming to identify the sampling site in post-weaning (**Manuscript II**) and grower-finisher pigs (**Manuscript III**), while testing whether CapSa can accurately collect a sufficient amount of small intestine content from live pigs (**Hypothesis 1**). In both studies, two capsules were administered to each pig and analysed upon retrieval, along with post-mortem samples from the small intestine, large intestine, and feces. This was done to compare bacterial composition and to identify the sampling site. In post-weaning piglets, the small intestine was divided post-mortem into three segments, while in fattening pigs, it was divided into six segments. Additionally, the small intestine of fattening pigs was further divided into solid and liquid fractions when possible. The greater segmentation of the GIT in older pigs is attributed to the lengthening of the small intestine as they grow. Therefore, maintaining segment sizes of approximately 1.5 meters was considered appropriate to improve the accuracy of locating the CapSa sampling site. Both studies confirmed that CapSa samples the contents of the small intestine in pigs. However, differences in microbiota composition were noted in the precise sampling location. Based

on the microbiota composition, in post-weaning pigs (**Manuscript II**), CapSa collected samples from the first segment of the small intestine, located just after the stomach. In contrast, for grower-finisher pigs (**Manuscript III**), the sampling site was approximately 6 meters downstream, in the fourth segment of the small intestine. Interestingly, in grower-finisher pigs the CapSa microbiota content was similar to both the solid and liquid fractions of the fourth segment but showed a difference when compared to the combined content of the entire fourth segment. A possible reason for the different sampling sites in the GIT between post-weaning and grower-finisher pigs could be the variation in the transit time (or passage rate) as the pigs age. Transit time is longer three days postweaning and shortens from two weeks after weaning (Snoeck et al., 2004). Consequently, the capsule transits faster in older, heavier pigs, sampling at a more distal position. In larger pigs, the sampling site could only be identified when analysing the solid and liquid phases of the small intestine segments separately. This may be due to the limited volume of the CapSa sample, which limits its ability to capture the diversity and richness of the microbiota in the entire segment. Gut microbiota is generally known to stabilize and increase in diversity with age (Niu et al., 2015; Shao et al., 2021). However, one study contradicts this trend, reporting a decrease in microbial diversity in finishing pigs (147 days of age) compared to growing pigs (10–93 days of age) (Han et al., 2018).

The validation studies described in **Manuscripts II** and **III** have revealed significant differences in the microbial composition between faecal and small intestine samples, consistent with previous findings (Zhao et al., 2015). This highlights the importance of

developing tools like CapSa, which provide more accurate data for small intestine microbiota studies. CapSa also facilitates longitudinal studies of microbiota evolution in the same pigs, aligning with the 3Rs principle (Refinement, Reduction, and Replacement) proposed by Russell and Burch in 1959 (Russell et al., 1959; Tannenbaum & Bennett, 2015).

7.4. Dynamic picture of the small intestine microbiota in pigs using CapSa

Current literature relies on faecal samples to study microbiota evolution, documenting changes from post-weaning to slaughter (Inoue et al., 2005; Kim et al., 2011). In **Manuscript IV**, we report about the changes in the gut microbiota composition from the post-weaning period (28 days of age) to slaughter (145 days of age) and how ETEC infection early post-weaning determines microbiota composition later in growth (**Hypothesis 3**). Consistent with previous research performed with fecal samples, we observed a sequential decrease of *Lactobacillus* from weaning to slaughter and its replacement by *Streptococcus* also in the small intestine (Luo et al., 2022; Petri et al., 2010). Families like *Lactobacillaceae* are part of the milk-oriented microbiome found in pre- and post-weaning pigs. However, once the diet shifts to a solid, cereal-based diet, a change in the microbial composition of the small intestine occurs (Saladrigas-García et al., 2022). Another notable change was the increase of *Clostridium* from approximately 70 days of age until slaughter, which coincided with increased BW and its role as a bacterium prevalent during the finishing stage. Additionally, it is noteworthy that the genus

Prevotella was not detected in our studies, aligning with previous findings that indicate its very low abundance in the small intestine (Crespo-Piazuelo et al., 2018). As previously mentioned, these changes in the microbiota composition can be partly attributed to the pigs' age, as CapSa was administered five times throughout their lifespan. However, the impact of diet must also be considered. During the starter and grower diet periods, CapSas were administered at both the beginning and the end of each respective period. In contrast, when the finisher diet was provided, CapSas were administered only at the end of that period. The PCoA and Adonis tests showed that the microbiota composition of the different sample types varied significantly between diet periods. However, the experimental design does not allow determination of the extent to which the observed changes can be attributed to either age progression or changes in diet.

In the same manuscript, we reported an experiment where piglets were infected with an F4 ETEC strain at weaning. The objective was to investigate whether the infection led to significant alterations in microbial composition of the small intestine, like those reported in fecal microbiota following ETEC infection. (Bin et al., 2018; Duarte et al., 2020; Rhouma et al., 2021). To replicate farm conditions, we mixed infected and non-infected pigs in half of the boxes, reflecting the typical epidemiological situation where ETEC infections do not have zero or 100% prevalence within boxes (Barros et al., 2023) and this cohabitation might have an effect on gut microbiota modulation. Surprisingly, health status and type of box did not affect beta diversity at any administration time point, but differences emerged at slaughter. We can hypothesize that the health status has a

significant long-term effect on gut microbiota composition, as evidenced by the differences observed between control and infected pigs at the time of slaughter. The impact of the infection on microbiota composition throughout life cannot be clearly determined due to the limited sample size in both the control and infected groups. Additionally, the influence of housing conditions, specifically the box effect (i.e., the prevalence of infection within boxes), remains unclear. Although a tendency towards a difference reappeared when comparing control pigs to infected pigs housed in separate boxes, this was not statistically significant, likely due to the limited sample size in our study. Therefore, while health status may affect microbial composition, further research with a larger sample size is needed to definitively determine the impact of the infection and box effect on gut microbiota composition and development.

These findings from the second part of **Manuscript IV** suggest that ETEC infection might have a significant long-term measurable effect, rather than a short-term one. It is also important to note that first CapSa were administered only after two weeks post infection, at a time when infection has subsided, as no ETEC could be detected in the faeces. This could indicate that we missed the detection window to identify a difference between microbiota composition of control and infected pigs due to the inability to retrieve capsules from pigs weighing less than 12 kg. Additionally, it's important to consider that the capsules primarily sample the first segment of the small intestine in post-weaning pigs, whereas ETEC predominantly affects the more distal segments of the small intestine (Jin &

Zhao, 2000). Furthermore, only one capsule was retrieved from the control group at the first time point, which limited our ability to make comparisons.

7.5. Limitations and future perspectives

To perform a reliable microbiota analysis, it is essential to have an effective and consistent sampling method that accurately reflects the gut microbiota (Choudhury et al., 2019). The ideal sampling method should be non-invasive and minimize cross-contamination (Tang et al., 2020). CapSa is considered a better sampling method than post-mortem sampling because it can be used multiple times on the same animals. Additionally, it is more accurate than the commonly used fecal sampling if we want to study the small intestine microbiota.

Despite the promising results obtained with the validated CapSa methodology, some of its limitations must be acknowledged. The capsule itself remains harmless to the piglets' health even after repeated applications to the same animal when performed correctly. However, the two required oesophageal intubations are invasive and may raise concerns regarding animal welfare. Future improvements to the CapSa methodology should focus on reducing the number of procedures and the degree of invasiveness, potentially by using a pill “gun” as other studies have done (Del-Rio-Ruiz et al., 2024; Rezaei Nejad et al., 2019a).

In addition, the impact of this administration protocol on the pig's normal gut microbiota has yet to be explored. Changes in the physical form of the diet, the use of a

prokinetic, and the associated stress may all influence the microbiota, highlighting the need for further investigation in the future.

Furthermore, it is important to study the effect that the time lapse between CapSa sampling of the intestinal content and its retrieval in the feces has on gut microbiota composition. Addressing these issues will further confirm that CapSa is a reliable method for studying the small intestine microbiota in pigs.

8. Conclusion

The current methodologies for microbiota studies often fall short in accuracy due to limitations in sampling methods. While fecal sampling is non-invasive and convenient, it fails to accurately represent the microbial environment of the small intestine. Advanced DNA extraction techniques and cutting-edge sequencing technologies cannot compensate for the inaccuracies introduced by current sampling methods.

Our research validates CapSa as an effective tool for sampling small intestine microbiota in pigs without harming them in the process. By standardizing the administration protocol, we have shown that CapSa can be reliably used to obtain samples of the small intestine in pigs. This advancement opens new possibilities for longitudinal microbiota studies in live animals and aligns with the ethical principles of the 3Rs (Refinement, Reduction, and Replacement).

Furthermore, our work indicates that the CapSa administration protocol can be adapted for other encapsulated devices aimed at drug delivery or the measurement of gastrointestinal biomarkers. This adaptability extends its potential applications beyond microbiota research. However, further investigations are recommended to explore the effects of administration protocol parameters on gut microbiota.

In conclusion, our findings underscore the importance of reliable sampling methods for microbiota research. CapSa offers a significant improvement over traditional methods by providing accurate, non-invasive sampling of the small intestine microbiota. This advancement enhances the relevance and reliability of microbiota studies, paving the way for more precise and non-invasive research practices.

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Academic activities

My Ph.D. is part of the MonoGutHealth project, an Innovative Training Network funded by the European Commission under the Horizon 2020 Marie Skłodowska-Curie Action (grant agreement N° 955374). The project aims to identify nutritive, microbial, and disease factors affecting the gut in pigs and chickens prior to birth and/or in the early neonatal period, and to propose innovative technologies and tools to improve their resilience and sustainability.

The primary goal of my doctoral thesis was to validate a novel non-invasive sampling device (Capsule for Sampling, CapSa) for collecting gut microbiota samples from pigs without causing them any harm. Additionally, we aimed to demonstrate that it was possible to administer CapSa repeatedly to the same pigs, providing a dynamic picture of their gut microbiota throughout their lifespan.

In the first month of my Ph.D. program, I conducted an extensive literature review focusing on the gastric anatomy of pigs and novel prokinetics. According to the existing literature, pigs' stomachs are quite different from human stomachs, so devices like CapSa would remain blocked unless we used an effective prokinetic.

In the first animal experiments, performed at Agroscope from November 2021 to March 2022, we administered CapSa to pigs and tested the efficacy of different prokinetic molecules on overcoming the gastric blockage of the capsules. These experiments also served to train the team on handling the animals, performing oesophageal sondages, and perfecting our materials (such as sondages). All the contents of the retrieved CapSas were stored alongside post-mortem intestinal contents. After this first set of experiments and observing the efficacy of prucalopride as a prokinetic agent, we started a new set of experiments from March to August 2022 to validate the location of the sampling site of CapSa in post-weaning and growing pigs. Additional experiments to validate the device in growing pigs were also carried out a year later, in July and September 2023.

From September to November 2022, I did a two-month secondment at the University of Bologna, in the Dipartimento di Scienze e Tecnologie Agro-Alimentari (DISTAL) with Prof. Paolo Trevisi and his team. During this secondment, I learned how to extract DNA from my samples, quantify DNA post-extraction, and perform PCRs. I also learned about bioinformatics for microbiome analysis. After sending the DNA samples for sequencing, we were able to analyse the results in January-February 2023 and locate the sampling site of CapSa in post-weaning pigs.

From January to the beginning of September 2023, I performed two large experiments that involved administering CapSa repeatedly after weaning (up to five times) during the lifespan of the pig, under both normal and pathological conditions. In February of that year, I also conducted another experiment to test if the ETEC strain of Agroscope used in the infection model was still pathogenic. As mentioned earlier, two additional experiments were carried out during this period to validate the sampling site of CapSa in growing pigs.

All samples from the experiments carried out in 2023 were shipped to Bologna for microbiota analysis. For the second time, from September to December 2023, I did a three-month secondment at the University of Bologna, in DISTAL with Prof. Paolo Trevisi and his team. During this time, I improved my laboratory skills, carrying out more DNA extractions, DNA quantifications post-extraction, and PCRs. I also enhanced my bioinformatics skills and analysed the sequencing data.

In November 2023, I submitted my first paper, entitled “A non-invasive tool to collect small intestine content in post-weaning pigs: validation study” to Scientific Reports (Manuscript II).

From January to February 2024, I analysed all my data from the experiments carried out in 2023 and started writing my second paper, “Method: Standard operating procedure for the

administration of swallowable devices to study pig's gut content in a non-invasive way” (Manuscript I).

During March 2024, I did a one-month secondment at TwentyGreen®, a Swiss start-up specializing in next-generation probiotic feed supplements for livestock production, specifically as part of the *M.smithii* project. This project aimed to create a novel postbiotic by supplementing *Methanobrevibacter smithii* to piglets during the pre- and post-weaning phases. The objective of this secondment was to gain insight into the development of customer information and science-based marketing.

From April to September 2024, I have been working on my doctoral thesis and writing my third and fourth papers (Manuscripts III and IV).

In May 2024, my first paper was accepted for publication and made available online, and my second paper was submitted to Animal Open Space.

My third paper (Manuscript III), entitled “Sampling Intestinal Microbiota in Growing Pigs: Evaluation of CapSa, an Ingestible Capsule,” was submitted to the Italian Journal of Animal Science in June 2024 and later accepted for publication and made available online on November 2024.

My fourth paper (Manuscript IV), entitled “Dynamic picture of the pig gut's microbiota under normal and pathological conditions,” was submitted to Animal Microbiome on September 2024. Additionally, my second paper was accepted for publication and made available online.

Since May 2023, I have been a board member of the PIGWEB Junior Community. The PIGWEB Junior Community is a network that is part of the PIGWEB project, dedicated to postdocs, Ph.D. students, and early-career scientists working on pig production. My tasks include organizing and hosting webinars and managing social media.

Throughout the duration of my Ph.D., I periodically attended training schools organized by the MonoGutHealth project and received training on monogastric nutrition, gut biology and health, industry work, communication, and scientific writing. At the same time, I also followed courses from the University of Bologna online whenever possible.

Participation to scientific events

Poster at Journées de Recherche Porcine (JRP), Saint Malo, France, 2025.

Poster at Spring Conference Animal Nutrition, Zürich, Switzerland, 2024.

Oral presentation at 4th MonoGutHealth Project Meeting, Teagasc, Cork, Ireland, 2024.

Oral presentation at Journées de Recherche Porcine (JRP), Saint Malo, France, 2024.

Oral presentation at EAAP, Lyon, France, 2023.

Oral presentation at PIGWEB Junior Community webinar series, online, 2023.

Oral presentation at 3rd MonoGutHealth Project Meeting, KU Leuven, Belgium, 2023.

Oral presentation of a poster at EAAP, Oporto, Portugal, 2022.

Oral presentation at Gut Biology and Health: PhD course and Early-Stage Researcher Training School, Department of Animal Science, Aarhus University, Denmark, 2022.

Oral presentation at 2nd MonoGutHealth Project Meeting, Research Institute for Farm Animal Biology (FBN) Dummerstorf, Germany, 2022.

List of publications

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