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Dietary Supplementation Strategies to Enhance Gut Microbiota Resilience and Intestinal Health in Young Pigs

Laia Ferreres Serafini

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Dietary Supplementation Strategies to Enhance Gut Microbiota Resilience and Intestinal Health in Young Pigs

Tesi doctoral presentada per:

LAIA FERRERES SERAFINI

Sota la direcció de les doctores:

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Per accedir al grau de Doctor dins el programa de Doctorat en Producció Animal del Departament de Ciència Animal i dels Aliments

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La present memòria de tesi ha sigut realitzada gràcies a la concessió de la beca pre-doctoral FI-SDUR 2020, per l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) de la Generalitat de Catalunya, i al finançament proporcionat per les empreses: Laboratoris Ordesa S.L. a través del projecte TOLERA (IDI-20170870), finançat pel Centre de Desenvolupament Tecnològic Industrial (CDTI); Tereos S.A.; i ANH-Innovation.

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Resum

L'objectiu principal d'aquesta tesi ha sigut el d'avaluar el potencial de diferents estratègies biòtiques així com l'efectivitat de nanoanticossos dissenyats per neutralitzar diferents factors de virulència d'*Escherichia coli* enterotoxigènica (ETEC) $F4^+$, per millorar la resposta del garrí davant els desafiaments sanitaris de les primeres edats. La tesi investiga l'efecte d'aquests additius sobre el procés de colonització intestinal, la resposta immunitària i la funció digestiva, intentant aportar més llum sobre els possibles mecanismes d'acció implicats.

Per assolir aquests objectius, es van realitzar quatre estudis. El primer d'ells (Capítol 4) es va dur a terme en garrins lactants de pocs dies alimentats amb llet artificial. En aquest estudi es va avaluar l'impacte de suplementar les fórmules làcties amb diferents additius sobre el desenvolupament de la microbiota intestinal, la resposta metabòlica de l'animal i el desenvolupament de la funció digestiva. En concret, es va avaluar l'efecte de la combinació sinbiòtica de *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosacàrids (GOS) i oligosacàrids de la llet humana (HMO), combinada o no amb un pèptid bioactiu, l'osteopontina.

En aquest estudi van participar 96 garrins de 4-5 dies de vida, distribuïts en 32 corrals al llarg de quatre períodes (8 corrals/tractament). Després de 12 i 14 dies rebent el tractament, es va eutanasiar un animal per corral per avaluar la població microbiana, l'activitat fermentativa, la resposta immunitària i la morfologia intestinal. Aquesta combinació d'additius va aconseguir modificar la població microbiana dels garrins lactants orientant-la cap a un perfil més beneficiós. Va aconseguir una major disminució d'espècies potencialment disbiòtiques, com *Helicobacter* o *Campylobacter*, i

una major activitat fermentativa, amb un augment de la concentració d'àcids grassos de cadena curta. Les tres dietes suplementades van promoure, a més, canvis en l'expressió gènica, suggerint una major funció hormonal i enzimàtica, una major funció de transport de nutrients i una disminució del to inflamatori intestinal.

En un segon estudi (Capítol 5), es va avaluar el potencial d'una combinació sinbiòtica en garrins recentment deslletats desafiats oralment amb ETEC $F4^+$. En aquest cas, els tractaments experimentals incloïen la suplementació amb *Saccharomyces cerevisiae* Sc 47, amb fructo-oligosacàrids de cadena curta (scFOS) o bé la combinació sinbiòtica d'ambdós.

En aquest segon estudi, 96 garrins de 21 dies de vida van ser distribuïts en 32 corrals (8 corrals/tractament). Després d'una setmana d'adaptació, tots els animals van ser inoculats oralment, i 4 i 8 dies després es va eutanasiar un animal per corral per avaluar el recompte de patògens, l'activitat fermentativa, la resposta immunitària i la morfologia intestinal. Les tres dietes suplementades van promoure una disminució de la incidència de diarrea en els dies posteriors al desafiament. Lligat a aquesta millora, també es van trobar menors recomptes del patogen en els diferents compartiments intestinals, suggerint un perfil microbiològic més beneficiós.

En un tercer i quart estudi (Capítol 6), es va avaluar l'efectivitat de diferents nanoanticossos dissenyats específicament per neutralitzar els factors de virulència F4 (fímbria), LT i Stx2e (toxines) d'ETEC mitjançant diferents dissenys experimentals: mitjançant un model de desafiament per ETEC $F4^+$ similar al del capítol 5 i mitjançant una prova de camp en granja comercial.

En el model de desafiament, 96 garrins de 21 dies de vida van ser distribuïts en 32 corrals i assignats a tres tractaments experimentals que incloïen un tractament control, suplementat amb nanoanticossos o bé amb ZnO com a

control positiu (10-11 corrals/tractament). El protocol de mostreig i analítiques va ser similar al del segon estudi. Els nanoanticossos van promoure una millora en el creixement i els signes clínics dels animals després del desafiament, així com una reducció en l'excreció fecal del patogen.

L'estudi de camp es va realitzar en una granja comercial. En aquest cas, un total de 528 garrins de 21 dies de vida van ser distribuïts en 32 corrals repartits entre un grup control, suplementat amb nanoanticossos o bé amb ZnO com a control positiu (16 corrals/tractament). L'additiu es va suplementar durant els 42 dies d'estudi, en els que es va avaluar el rendiment productiu, la clínica de diarrees i la presència de factors de virulència fecals. Els nanoanticossos van disminuir la incidència de diarrea i van disminuir numèricament les toxines excretades fecalment. A més, l'additiu va incrementar el pes dels animals, principalment degut a un millor creixement dels garrins de poc pes.

En conclusió, les estratègies testades (sinbiòtiques i nanoanticossos), malgrat actuar mitjançant mecanismes d'acció diferents, van demostrar efectes beneficiosos sobre l'ecosistema intestinal i la resposta dels garrins als desafiaments sanitaris de les primeres edats. De cara a la seva aplicabilitat, un possible ús complementari podria resultar en una estratègia eficaç davant la problemàtica de les diarrees post-deslletament.

Resumen

El objetivo principal de esta tesis ha sido evaluar el potencial de diferentes estrategias bióticas, así como la efectividad de nanoanticuerpos diseñados para neutralizar diferentes factores de virulencia de *Escherichia coli* enterotoxigénica (ETEC) $F4^+$, para mejorar la respuesta del lechón frente a los desafíos sanitarios de las primeras edades. La tesis investiga el efecto de estos aditivos sobre el proceso de colonización intestinal, la respuesta inmunitaria o la función digestiva intentando poner algo más de luz sobre los posibles mecanismos de acción involucrados.

Para lograr estos objetivos, se realizaron cuatro estudios. El primero de ellos (Capítulo 4), se llevó a cabo con lechones lactantes de pocos días alimentados con leche artificial. En este estudio se evaluó el impacto de suplementar las fórmulas lácteas con diferentes aditivos sobre el desarrollo de la microbiota intestinal, la respuesta metabólica del animal y el desarrollo de la función digestiva. En concreto, se evaluó el efecto de la combinación sinbiótica de *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosacáridos (GOS) y oligosacáridos de la leche humana (HMO), combinada o no con un péptido bioactivo, la osteopontina.

En este estudio participaron 96 lechones de 4-5 días de vida, distribuidos en 32 corrales a lo largo de cuatro periodos (8 corrales/tratamiento). Después de 12 y 14 días recibiendo el tratamiento, se eutanasió un animal por corral para evaluar la población microbiana, la actividad fermentativa, la respuesta inmunitaria y la morfología intestinal. Esta combinación de aditivos logró modificar la población microbiana de los lechones lactantes orientándola hacia un perfil más beneficioso. Logró una mayor disminución de especies potencialmente disbióticas, como *Helicobacter* o *Campylobacter*, y una mayor actividad fermentativa, con un aumento de la concentración de ácidos

grasos de cadena corta. Las tres dietas suplementadas promovieron además cambios en la expresión génica, sugiriendo una mayor función hormonal y enzimática, mayor función de transporte de nutrientes y una disminución del tono inflamatorio intestinal.

En un segundo estudio (Capítulo 5), se evaluó el potencial de una combinación sinbiótica en lechones recién destetados desafiados oralmente con ETEC $F4^+$. En este caso, los tratamientos experimentales incluyeron la suplementación con *Saccharomyces cerevisiae* Sc 47, con fructo-oligosacáridos de cadena corta (scFOS) o bien la combinación sinbiótica de ambos.

En este segundo estudio, 96 lechones de 21 días de vida fueron distribuidos en 32 corrales (8 corrales/tratamiento). Después de una semana de adaptación, todos los animales fueron inoculados oralmente, y 4 y 8 días después se eutanasió un animal por corral para evaluar el recuento de patógenos, la actividad fermentativa, la respuesta inmunitaria y la morfología intestinal. Las tres dietas suplementadas promovieron una disminución de la incidencia de diarrea en los días posteriores al desafío. Ligado a esta mejora, también se encontraron menores recuentos del patógeno en los diferentes compartimentos intestinales, sugiriendo un perfil microbiológico más beneficioso.

En un tercer y cuarto estudio (Capítulo 6), se evaluó la efectividad de diferentes nanoanticuerpos diseñados específicamente para neutralizar los factores de virulencia F4 (fimbria), LT y Stx2e (toxinas) de ETEC mediante diferentes diseños experimentales: mediante un modelo de desafío por ETEC $F4^+$ similar al del capítulo 5 y mediante una prueba de campo en granja comercial.

En el modelo de desafío, 96 lechones de 21 días de vida fueron distribuidos en 32 corrales y asignados a tres tratamientos experimentales que incluyeron un tratamiento control, suplementado con nanoanticuerpos o bien con ZnO como control positivo (10-11 corrales/tratamiento,). La pauta de muestreo y analíticas fueron similares a los del segundo estudio. Los nanoanticuerpos promovieron una mejora en el crecimiento y los signos clínicos de los animales después del desafío, así como una reducción en la excreción fecal del patógeno.

El estudio de campo se realizó en una granja comercial. En este caso un total de 528 lechones de 21 días de vida fueron distribuidos en 32 corrales repartidos entre un grupo control, suplementado con nanoanticuerpos o bien con ZnO como control positivo (16 corrales/tratamiento). El aditivo se suplementó durante los 42 días de estudio, en los que se evaluó el rendimiento productivo, la clínica de diarreas y la presencia de factores de virulencia fecales. Los nanoanticuerpos disminuyeron la incidencia de diarrea y disminuyeron numéricamente las toxinas excretadas fecalmente. Además, el aditivo incrementó el peso de los animales, principalmente debido a un mejor crecimiento de los lechones de poco peso.

En conclusión, todas las estrategias testadas (sinbióticas y nanoanticuerpos), a pesar de actuar mediante mecanismos de acción diferentes, demostraron efectos beneficiosos sobre el ecosistema intestinal y la respuesta de los lechones a los desafíos sanitarios de las primeras edades. De cara a su aplicabilidad, un posible uso complementario podría resultar en una estrategia eficaz frente a la problemática de las diarreas post-destete.

Summary

The main objective of this thesis was to evaluate the potential of various biotic strategies and the effectiveness of nanobodies designed to neutralise different virulence factors of enterotoxigenic *Escherichia coli* (ETEC) $F4^+$, aiming to improve piglet response to health challenges during early stages. The thesis explores the effects of these additives on intestinal colonisation, immune response, and digestive function, shedding light on the possible mechanisms of action involved.

To achieve these objectives, four studies were conducted. The first study (Chapter 4) involved young suckling piglets fed artificial milk. This study assessed the impact of supplementing milk formulas with different additives on the development of intestinal microbiota, the animal's metabolic response, and the development of digestive function. Specifically, it evaluated the effect of a synbiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosaccharides (GOS), and human milk oligosaccharides (HMO), with or without the bioactive peptide osteopontin.

In this study, 96 piglets aged 4-5 days were distributed into 32 pens over four periods (8 pens/treatment). After 12 and 14 days of treatment, one animal per pen was slaughtered to evaluate microbial population, fermentative activity, immune response, and intestinal morphology. This combination of additives successfully modified the microbial population of suckling piglets towards a more beneficial profile, achieving a greater reduction of potentially dysbiotic species such as *Helicobacter* and *Campylobacter*, and increased fermentative activity with higher concentrations of short-chain fatty acids. All three supplemented diets also promoted changes in gene expression,

suggesting greater hormonal and enzymatic function, increased nutrient transport function, and reduced intestinal inflammatory tone.

The second study (Chapter 5) evaluated the potential of a synbiotic combination in newly weaned piglets challenged orally with ETEC $F4^+$. Experimental treatments included supplementation with *Saccharomyces cerevisiae* Sc 47, short-chain fructo-oligosaccharides (scFOS), or their synbiotic combination.

In this study, 96 piglets aged 21 days were distributed into 32 pens (8 pens/treatment). After a week of adaptation, all animals were orally inoculated, and 4 and 8 days later, one animal per pen was slaughtered to evaluate pathogen counts, fermentative activity, immune response, and intestinal morphology. All three supplemented diets promoted a reduction in the incidence of diarrhoea in the days following the challenge. This improvement was linked to lower pathogen counts in the different intestinal compartments, suggesting a more beneficial microbiological profile.

The third and fourth studies (Chapter 6) evaluated the effectiveness of different nanobodies specifically designed to neutralise the virulence factors F4 (fimbria), LT, and Stx2e (toxins) of ETEC through different experimental designs: an ETEC $F4^+$ challenge model similar to that in Chapter 5, and a field trial on a commercial farm.

In the challenge model, 96 piglets aged 21 days were distributed into 32 pens and assigned to three experimental treatments: a control treatment, supplementation with nanobodies, or ZnO as a positive control (10-11 pens/treatment). Sampling and analysis followed similar protocols to those in the second study. Nanobodies promoted improvements in growth and clinical signs of the animals after the challenge, as well as a reduction in faecal pathogen excretion.

The field trial was conducted on a commercial farm. In this case, a total of 528 piglets aged 21 days were distributed into 32 pens and divided into three treatments: a control treatment, supplementation with nanobodies, or ZnO as a positive control (16 pens/treatment). The additive was supplemented for 42 days, during which productive performance, diarrhoea incidence, and the presence of faecal virulence factors were evaluated. Nanobodies reduced the incidence of diarrhoea and numerically decreased faecal toxin shedding. Additionally, the additive increased the weight of the animals, primarily due to enhanced growth in low-weight piglets.

In conclusion, all tested strategies (synbiotics and nanobodies), despite acting through different mechanisms of action, demonstrated beneficial effects on the intestinal ecosystem and the response of piglets to early-stage health challenges. For practical application, a complementary use of these strategies could prove to be an effective approach to addressing post-weaning diarrhoea issues.

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Abbreviations

AA	Amino acid	**MUC4**	Mucin 4 gene
ADFI	Average daily feed intake	**NRC**	National Research Council
ADG	Average daily gain	**NO**	Nitric oxide
ANOVA	Analysis of variance	**OPN**	Osteopontin
AGP	Antimicrobial growth promoters	**OS**	Oxidative stress
ASVs	Amplicon sequence variant	**OTUs**	Operational taxonomic units
BCFA	Branched-chain fatty acids	**PBS**	Phosphate buffered saline
CAT	Catalase	**PCA**	Principal component analysis
CD	Crypt depth	**PCoA**	Principal Coordinate Analysis
CF	Colonization factors	**PCR**	Polymerase chain reaction
CFU	Colony forming units	**PI**	Post-inoculation
CTR	Control	**Pig-MAP**	Pig major acute-phase protein
DAO	Diamine oxidase	**PRAN**	*Plan Nacional Resistencia Antibióticos*
DM	Dry matter	**PRRSV**	Porcine reproductive and respiratory syndrome virus
EAST1	Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1	**PRE**	Prebiotic
EFSA	European Food Safety	**PRO**	Probiotic
ELISA	Enzyme-linked immunosorbent assay	**PS**	Pre-starter
EMA	European Medicines Agency	**PUAVM**	Prudent use of antimicrobials in veterinary medicine
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption	**PWD**	Post-weaning diarrhoea
ETEC	Enterotoxigenic *Escherichia coli*	**RONAFA**	Reduction of the need to use antimicrobials in food-producing animals
EU	European Union	**ROS**	Reactive oxygen species
FCR	Feed conversion ratio	**RSE**	Residual Standard error
FOS	Fructo-oligosaccharides	**SBOS**	Soybean oligosaccharides
FM	Fresh matter	**SCFA**	Short-chain fatty acids
GALTS	Gut-associated lymphoid tissues	**scFOS**	Short-chain Fructo-oligosaccharides
GC	Goblet cells	**SGiCE**	Servei de Granges i Camps Experimentals
G:F	Gain to feed ratio	**SOD**	Superoxide dismutase
GI	Gastrointestinal	**STR**	Starter
GLC	Gas-liquid chromatography	**ST**	Heat-stable toxin
GSH-PX	Glutathione peroxidase	**SYN**	Synbiotic
GOS	Galacto-oligosaccharides	**TcdB**	*Clostridium difficile* toxin B
HMO	Human milk oligosaccharides	**TFRC**	Transferring receptor gene
IBD	Intestinal bowel disease	**TNF-α**	Tumour necrosis factor-α
IEL	Intraepithelial lymphocytes	**TOS**	Transgalacto-oligosaccharides
IMO	Isomalto-oligosaccharides	**UAB**	Universitat Autònoma de Barcelona
LAB	Lactic acid bacteria	**USA**	United States of America
LPS	Lipopolysaccharides	**VH**	Villus height
LW	Live weight	**VH:CD**	Villus height : crypt depth
LT	Heat-labile toxin	**WHO**	World Health Organization
M	Mitosis	**XOS**	Xylo-oligosaccharides
MCFA	Medium-chain fatty acids	**YEA**	Yeast
MDA	Malondialdehyde	**ZnO**	Zinc oxide
ME	Metabolizable energy		

*Gene abbreviations are provided in Table S4.8.

Chapter 1. General introduction



General introduction

Representing around the 36% of the meat eaten globally (data from 2023), pork is one of the main consumed meat in the world (Pork Consumption by Country 2023; worldpopulationreview.com). Millions of tons are produced annually, being China the largest producer and the European Union (EU) the second one. Within the EU, after successive years of growth, Spain is now the first producer country, closely followed by Germany (Eurostat, 2023).

The main production system used in Spain is the intensive one, as it can meet the high product demand of the sector. This production system requires maximum efficiency and optimization of the sow's physiology, aiming the maximum possible weaning piglets per year. To prevent possible infections and ensure high growth performance, the prophylactic use of antimicrobials was common, particularly during the post-weaning phase, when diarrhoea frequently occurs contributing to the problem of post-weaning diarrhoea (PWD). It was discovered that at low levels (subtherapeutic quantities of 1/10 or 1/100 the amount of therapeutic dose), antimicrobials enhance growth rate, feed efficiency, reduce mortality and morbidity and improve reproductive parameters, thus earning the term antimicrobial growth promoters (AGPs) (Cromwell, 2002; Marshall & Levy, 2011). By the 1970s-1980s, it was estimated that more than half of the feed used in swine production in the USA contained antimicrobial agents (Cromwell, 2002). Commonly used antibiotics effective in treating PWD included amoxicillin, apramycin, neomycin, gentamicin, trimethoprim-sulfamethoxazole, and colistin sulfate, among others (Luppi, 2017).

For an extended period, the negative consequences were undetected. Nonetheless, microbiologists and infectious disease experts began complaining due to its contribution to the antimicrobial resistance, now

recognized as a paramount global health concern of the 21st century (Marshall & Levy, 2011). For instance, antimicrobial resistance in pathogens causing PWD, such as resistance to apramycin, neomycin, trimethoprim-sulphamethoxazole, and colistin sulphate, became increasingly prevalent (Luppi, 2017). Subsequently, global regulatory actions were initiated, including the prohibition of antimicrobial growth promoters in EU in 2006 (Dibner & Richards, 2005), and later restrictions in the USA in 2017 (Centner, 2016), and in China in 2020 (Hu & Cowling, 2020).

In Europe, the European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) introduced guidelines, known as the EC PUAVM Guidelines (Murphy et al., 2017), aiming the Reduction Of the Need to use Antimicrobials in Food-producing Animals (RONAFA). These guidelines offer practical advice to member states on formulating and executing strategies to encourage judicious use of antimicrobials, especially antibiotics in veterinary practice (*Guidelines for the Prudent Use of Antimicrobials in Veterinary Medicine*, n.d.).

Following updates, the prevailing European guidelines on the prudent use of antimicrobials in veterinary medicine entail regulations 2019/4 (*Regulation - 2019/4 - EN - EUR-Lex*, n.d.), which prohibit the use of medicinal antibiotics in medicated feed for prophylactic purposes, and 2019/6 (*Regulation - 2019/6 - EN - EUR-Lex*, n.d.), allowing the use of antibiotics for disease prevention but under strict limitations, such as targeting single or small groups of animals when the risk is deemed high (Simjee & Ippolito, 2022).

In Spain, adopted measures include the ESVAC-ES project, which involves maintaining a registry of antibiotics sold by pharmacies and dispensers, as well as those administered by farmers and veterinarians, aimed at enhancing awareness and control of the situation. Additionally, the PRAN initiative (*Plan*

Nacional Resistencia Antibióticos| PRAN, n.d.) aims to reduce antibiotic usage in livestock production.

In the pursuit of strategies to manage PWD, the adoption of Zinc Oxide (ZnO) supplementation emerged as a popular choice due to its effectiveness in reducing the incidence of diarrhoea and promoting growth (Bonetti et al., 2021). However, after a few years of practice, this approach was not exempt to apprehensions regarding the escalation of antimicrobial resistance. ZnO was found to favor the proliferation of zoonotic methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Escherichia coli* strains (Bednorz et al., 2013; Ciesinski et al., 2018; Slifierz et al., 2015). Additionally, its application resulted in adverse environmental effects, stemming from substantial accumulation in slurry and subsequent dissemination into the soil. Consequently, the EMA imposed restrictions on high dietary supplementation of ZnO in swine by June 2022 (*Zinc Oxide | European Medicines Agency*, n.d.). This regulatory intervention instigated a significant shift in the swine sector's nutritional practices, prompting a continued search for new strategies to combat these infections.

The implementation of reduction measures in antimicrobial uses has led to a significant decline in antibiotic usage in animal production in Spain in recent years, particularly, in the pig industry. The “REDUCE” program, a PRAN initiative to decrease polymyxin use in the swine sector, has successfully moved Spain from being one of the countries with the highest antibiotic use to one of the lowest in Europe (*Programa REDUCE Porcino | PRAN*, n.d.). However, the figures for milligrams per livestock unit (mg/CPU) sold remain notable. Following rabbits, pigs are the species with the second-highest antibiotic usage, accounting for approximately 20% of the total antibiotics sold for veterinary purposes in Spain in recent years (*Consumos Antibióticos En Veterinaria | PRAN*, n.d.).

Lacking in antimicrobial additives to aid piglet growth, the need to provide integral alternative strategies with this goal has increased. Various approaches are required to maintain performance without relying on antibiotics. These include enhancements in management programs, environmental control, biosecurity, sanitary control and nutritional strategies based on refined dietary formulas. Nevertheless, this thesis focuses on those strategies where additives are supplemented in the diet to mitigate or prevent PWD detrimental effects. There is a long list of additives tested in piglets for this purpose: essential oils, organic acids, amino acids, fatty acids, polyphenol-rich extracts, antimicrobial peptides. However, among the available options, biotic strategies have awaked a more recent interest (Gresse et al., 2017) considering the increasing knowledge regarding the key role that microbiota play in the animal health with long-term benefits (Canibe et al., 2022). These strategies include prebiotics, probiotics, and their combination known as synbiotics.

In addition to biotic strategies, novel alternatives are emerging, like the use of nanobodies to combat infections. Nanobodies possess the same efficacy as antibodies but offer more favourable characteristics in terms of dosage and storage (Wilken & McPherson, 2018) and can be also provided via the oral route.

In this thesis, different biotic strategies, including also the use of nanobodies, are explored in lactating or weaned piglets with the aim of evaluating their potential to improve piglet adaptation and response during these early phases, trying to elucidate their mechanism of action. The results regarding their efficacy and mechanism of action are examined and discussed.

Chapter 2. Literature review



Literature review

2.1. Weaning at current swine farming

Intensive farming systems aim to enhance sow productivity. In these systems, early weaning techniques are frequently employed as it increases the annual litter size of sows, improves the efficiency of breeding equipment utilization, and generates increased economic gains for breeding enterprises (Campbell et al., 2013; Tang et al., 2022). In nature, the separation of piglets from sows occurs as a gradual process, typically between 10 to 12 weeks of age, coinciding with the maturation of gastrointestinal tract. In commercial pig farms, particularly in Europe, weaning is abruptly forced between three to four weeks of age (Modina et al., 2019). At that moment, the digestive and immune systems of these animals are not fully developed, and passive immunity derived from the sow's milk is declining (Campbell et al., 2013). Jointly, all these circumstances result to a limited capacity for piglets to adapt to their intricate environment (Lallès et al., 2007; Su et al., 2022; Tang et al., 2022).

2.1.1. A challenging phase

Piglets have to cope with a critical phase, marked by substantial physiological, environmental, and social challenges. For instance, a combination of psychosocial stressors, including transportation, mixing, fighting, the establishment of new social hierarchies and maternal separation; along with immunological stressors such as vaccination and increased exposure to pathogens; and a rapid transition from easily digestible

milk to more complex solid feed, make this phase the most stressful for piglets (Campbell et al., 2013). Collectively, these stressors disrupt the host physiological and biochemical functions, as well as intestinal digestion and absorption capacity, gut microbiota balance and mucosal inflammatory tone (Tang et al., 2022; Upadhaya & Kim, 2021).

2.1.2. Alterations in piglets

Weaning, in general, is characterized by decreased feed intake, the onset of PWD, and compromised growth performance (Campbell et al., 2013; Lallès et al., 2007; Upadhaya & Kim, 2021).

The intake of metabolizable energy (ME) is reduced by 30-40% of pre-weaning milk intake, and it takes 2 weeks to fully recover the pre-weaning ME intake level (Upadhaya & Kim, 2021). The decline in feed intake has a detrimental impact on the intestinal structure, inflammation tone and gut microbiota.

Intestinal Physiology

A correlation has been documented between reduced feed intake post-weaning and a decline in villus height (Pluske et al., 1997). Wang et al. (2022) reported a significant decrease in duodenal, jejunal, and ileal villous height immediately after weaning, reaching a reduction of 25-35% within the first 24 hours (Wang et al., 2022b). This diminished height may persist for about 5 days post-weaning, reaching roughly half of the initial height (Hampson, 1986) with full recovery noted around day 14. The decreases in villus height are attributed to either increased cell loss or a temporary reduction in the crypt cell population. Elevated cell shedding in the jejunum from 1 to 7 days post-weaning, accompanied by lower proliferation was reported, which could

be the cause of decline villus height (Wang et al., 2022b). Crypts also undergo alterations, exhibiting hyperplasia (Tang et al., 2022).

Another notable change involves a significant decrease in enzymatic activity at the brush border, including enzymes like lactase, sucrase, and maltase, primarily due to dietary changes (Xiong et al., 2019).

Collectively, these changes might influence the functional capabilities of the small intestine in terms of digestion, absorption, and secretion (Campbell et al., 2013; Xiong et al., 2019).

Inflammatory tone

Changes experienced during weaning have an impact on intestinal inflammation. Stress triggers oxidation processes, leading to a heightened release of free radicals and reactive oxygen species (ROS). The excess ROS presence can alter specific cellular proteins, activating the upregulation of pro-inflammatory cytokines. The mRNA expression of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukins (IL-1β, IL-6, and IL-8), was observed to be upregulated in the mid-jejunum of weaning piglets (McCracken et al., 1999; Pié et al., 2004; Upadhaya & Kim, 2021). Pro-inflammatory environment, in turn, may adversely affect the expression of tight junction proteins, resulting in increased gut permeability (Xiong et al., 2019).

Microbiology

The initial pig gut microbiota at birth is influenced by the sow's colostrum and milk, and it is characterized by a higher abundance of lactic acid bacteria (LAB). Upon weaning and the transition to solid, less-digestible, fiber-rich feed, there is a decline in *Lactobacillus* abundance and a reduction in microbial diversity. *Lactobacillus* is a crucial contributor to disease prevention, then its sudden decline can contribute to increase the risk of

infection (Konstantinov et al., 2006). The decrease in gut microbial diversity increases the accessibility of glycans which compose the mucus layer protecting the gut epithelium, making them more available to pathogenic microorganisms. The degradation of mucus polysaccharides by commensals can release sugars, fucose, galactose or mannose, thereby fostering the growth of pathogenic species (Baümler & Sperandio, 2016). Altogether with the diminished in feed intake during this period, limit the nutrients available for the survival and proliferation of the microbiota. Pathogenic bacteria, including *Clostridium*, *Prevotella*, *Sutterella*, *Proteobacteria*, and *Escherichia coli*, experience increased abundance (Upadhaya & Kim, 2021; Xiong et al., 2019). Although the gut microbial ecosystem is typically resilient, the newborn gut microbiota's composition is relatively simple, resulting in lower flexibility (Su et al., 2022). These pathogenic bacteria inflict damage on intestinal villi, prompting accelerated cell division in the small intestine's epithelium, leading to peeling. The cells fail to attain full maturity, hindering their essential functions. Consequently, piglets encounter difficulties digesting carbohydrates that pass into the large intestine (Knecht et al., 2020).

An inflamed gut seems to create a favourable setting for the expansion of *Enterobacteriaceae* (Zeng et al., 2017). The host-response to gut inflammation generates reactive species like nitric oxide (NO), known for its antimicrobial properties. However, NO released into the intestinal lumen swiftly converts into nitrate. The nitrate-enriched environment in the inflamed gut provides a growth advantage for strains of *Escherichia coli* possessing nitrate reductase genes (Spees et al., 2013; Wei et al., 2017; Winter et al., 2013).

Hence, the sequence of events following weaning in piglets may initiate a vicious cycle, ultimately resulting in enteric infections (Figure 2.1.).

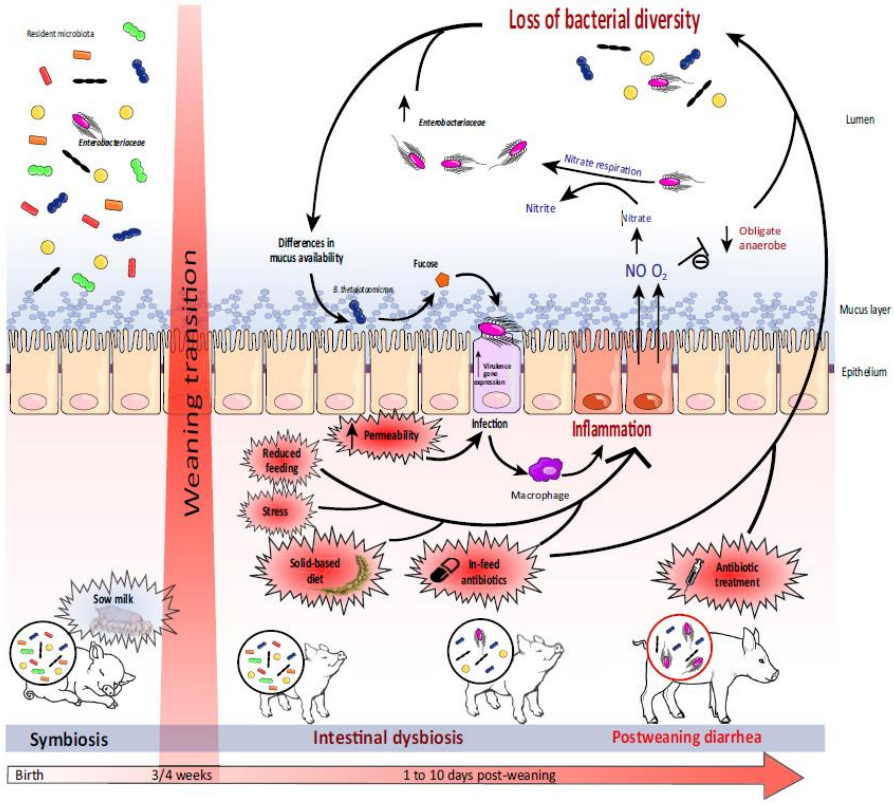


Figure 2.1. Detrimental vicious cycle of PWD.
Figure from Gresse et al. (2017).

2.2. Post-weaning diarrhoea

Such alterations of intestinal environment (damaged intestinal architecture, pro-inflammatory tone, microbial dysbiosis) can dramatically increase the risk of gastrointestinal (GI) disease.

In recent years, there has been a notable decline in porcine diarrhoea in developed countries, attributed to significant advancements in pig housing, management, sanitation, vaccination, and biosecurity measures (Dubreuil et al., 2016). However, despite these improvements, the problems that this disease cause to swine industry remains high. The two predominant pathogens causing piglets' diarrhoea are *Salmonella enterica* serovar *Typhimurium* and *Escherichia coli* (Gresse et al., 2017). Animals can be

infected at early age (3-5 days of age), manifesting as neonatal diarrhoea, and later after weaning, leading to PWD. In the first case, passive colostrum and lactogenic immunity can prevent the problem (Dubreuil et al., 2016; Luppi, 2017). The following chapters of this introduction will focus particularly on enterotoxigenic *E. coli* (ETEC) as one of the main causes of PWD.

2.2.1. Pathogeny of post-weaning diarrhoea

Within pathogenic *E. coli* strains, *Enterotoxigenic E. coli* (ETEC) stands out as the primary infectious agent causing PWD in piglets, accounting for 50% of the global piglet deaths annually (Gresse et al., 2017). ETEC enters animals via oral route. Then, it initiates its pathogenesis by bacterial attachment to the host intestinal epithelial cells (Dubreuil, 2017). This attachment is crucial for ETEC to exert its virulence and is facilitated by colonization factors (CFs), mainly fimbria or fimbria-related extracellular filamentous protein polymers known as pili or fimbriae (Zhang et al., 2022). In swine, frequently encountered fimbrial adhesins include F4 (K88), F5 (K99), F6 (987), F41, and F18 (Dubreuil, 2017), and their recognition is dependent on specific receptors present on the small intestine (Kopic & Geibel, 2010). Once attached, ETEC establishes colonization within the habitat, exhibiting rapid multiplication, being able to reach concentrations of up to 10^9 bacteria per gram of intestine. The extent of colonization plays a crucial role in determining the likelihood of disease manifestation. The presence, modification, or absence of specific receptors in the mucus or brush borders exhibit age-dependent variation, elucidating the age-specific nature of intestinal colonization. For instance, newborn and weaned piglets manifest F4 receptors in their small intestine mucus, while these receptors are scarcely detectable in the mucus of 6-month-old pigs (Dubreuil et al., 2016). Upon ETEC establishment, the

release of enterotoxins commences. ETEC is characterized by its capacity to produce high-molecular-weight heat-labile toxin (LT) and low-molecular-weight heat-stable toxins (ST). Collectively, these enterotoxins do not result in pathological lesions or morphological changes in the intestinal mucosa (Dubreuil et al., 2016), but are associated with the induction of intestinal water and electrolyte secretion, culminating in host diarrhoea (Zhang et al., 2022). The production of LT is influenced by environmental conditions, with an alkaline pH acting as a signal for production and secretion, along with the presence of glucose, in appreciable concentrations in the small intestine. Conversely, the abundance of short-chain fatty acids in the colon impedes LT production (Dubreuil et al., 2016). Among the ST enterotoxins, STa (also known as STI), STb (STII), and EAST1 are recognized. STa, a potent toxin with rapid action but short duration, targets the villi of the jejunum and ileum brush border of intestinal epithelial cells. This enterotoxin not only induces water secretion but also leads to intestinal barrier dysfunction by reducing barrier integrity. STb, with rapid action but moderate potency, promotes electrolyte imbalance and water secretion. It can also act directly on the muscle cells of the ileum, increasing spontaneous motility of the intestine and resulting in contractions (Dubreuil et al., 2016). While EAST1 alone does not appear capable of inducing disease, in conjunction with LT, it efficiently contributes to the production of diarrhoea (Berberov et al., 2004). In summary, ETEC exhibits a dual virulence factor capability, producing adhesins (fimbriae) and enterotoxins (LT and ST, including STa, STb, and EAST1) (Dubreuil et al., 2016).

2.2.2. The role of microbiota in post-weaning diarrhoea

Nonetheless, PWD is not exclusively associated with ETEC infection (Eriksen et al., 2021). Alterations in the intestinal microbiota are increasingly

recognized as key contributors to PWD (Gresse et al., 2017). It has been demonstrated that the gut microbiota profile in pre-weaning piglets can influence the pig's future health, including detrimental signs of PWD (Dou et al., 2017). Dou et al. 2017 reported that healthy and diarrhoeic piglets at post-weaning were distinguishable based on their microbiota composition at post-natal day 7. Since that moment, healthy piglets exhibited lower evenness and higher abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae*. In contrast, future diarrhoeic piglets showed lower diversity, higher evenness and a decreased relative proportion of dominant bacterial species (Dou et al., 2017). In another study, researchers correlated the gut microbiota present in pre-weaning (day 26) and post-weaning (day 35) with the animal's average daily gain (ADG) post-weaning, using growth as an indicator of health. Piglets from different farms with higher relative abundances of *Prevotella*, *Coprococcus*, and *Lachnospira* were associated with higher growth rates 7 days post-weaning. The presence of this microbial signature across individuals from various farms underscores the robustness of this observation (Luise et al., 2021). Further evidence suggesting a correlation between microbiota and performance was reported by Ramayo-Caldas et al. (2016). This study identified two distinct clusters in healthy 60 days old pigs. One cluster, characterised by a higher prevalence of *Prevotella*, was associated with a higher abundance of SCFA, increased body weight and greater ADG in piglets, compared to the other cluster (Ramayo-Caldas et al., 2016).

Even in growing-finishing pigs, microbiota appears to influence performance, though not directly related to PWD. Verschuren et al. (2018) reported that different operational taxonomic units (OTUs) sequenced in faecal samples were associated with higher or lower feed efficiency in growing-finishing pigs at slaughter (Verschuren et al., 2018).

Therefore, addressing PWD should involve not only targeting the ETEC pathogen but also restoring or reinforcing the overall microbial balance in piglets.

2.3. Possible strategies to fight post-weaning diarrhoea

There are various stages and actions to consider for preventing the adverse effects of PWD. Firstly, selecting genetically resilient individuals to ETEC $F4^+$ adhesion at the brush border of the jejunum (MUC4, MUC13, MUC20 and transferring receptor gene (TFRC) involved) (Luise et al., 2019b; Sinha et al., 2019). In terms of management, numerous options exist. Actions supporting proper sow milk production (such as farrowing assistance (Kirkden et al., 2013), pain control (Arnaud et al., 2023a; Homedes et al., 2014; Mainau et al., 2016; Navarro et al., 2021), and dietary strategies during lactation (Wensley et al., 2021)) can positively impact piglet outcomes (Arnaud et al., 2023b). Additional management interventions, like split-suckling (Donovan & Dritz, 2000) or cross-fostering (Bandrick et al., 2011), can also enhance colostrum and milk intake. Moreover, reducing pen density and socialization pre-weaning can mitigate piglet stress (Saladrigas-García et al., 2021b).

Concerning nutritional strategies, several options can be employed during lactation to increase piglet feed intake and achieve higher weaning weights. Creep feeding, using solid or liquid options, has been widely implemented (Carstensen et al., 2005; Luo et al., 2022a; Sulabo et al., 2010; Wolter et al., 2002). At post-weaning, adjusting diet composition can help reduce piglet diarrhoea incidence. High protein levels in post-weaning diets have been associated with adverse effects on intestinal mucosa structure and barrier function, as well as promoting pathogenic proteolytic bacteria growth and reducing beneficial microbiota (Gao et al., 2022; Marchetti et al., 2023; Ren

et al., 2022; Xia et al., 2022). Therefore, reducing crude protein levels can mitigate these effects (Heo et al., 2010, 2015; Marchetti et al., 2023; Yue & Qiao, 2008). Additionally, increasing fiber content can promote the growth of beneficial bacterial populations (Niu et al., 2015) and enhance intestinal morphology (Pascoal et al., 2015).

Vaccination protocols offer another viable option. Oral vaccines administered to sows or piglets target various ETEC $F4^+$ virulence factors. However, their effectiveness may be hindered by the timing of administration and the limited spectrum of strains targeted (Ntakiyisumba et al., 2022).

2.3.1. Additives

In addition to the strategies mentioned earlier, another approach to mitigate the negative effects of PWD involves the use of dietary additives administered during these early stages. Various compounds, when incorporated into the diet as additives, can elicit beneficial responses. This work will delve further into this particular strategy.

Additives can be categorized based on their mechanism of action, as proposed by some authors (Canibe et al., 2022). In this introduction, they are divided into non-biotic and biotic additives, with biotic additives being those capable of altering the composition of the intestinal microbiota.

2.3.1.1. Non-biotic additives

Organic acids

Traditionally, organic acids have been widely used in food preservation due to their acidifying properties. However, numerous studies have highlighted their antimicrobial properties, leading to their recognition not just as acidifiers, but also as growth promoters and potential substitutes for antibiotics (Upadhaya & Kim, 2021). These compounds lower stomach pH, facilitating digestion and nutrient utilization, and enhancing the antimicrobial barrier (Bonetti et al., 2021; Canibe et al., 2022).

A substantial body of research has evaluated the effects of various combinations of organic acids in the diet of weaning piglets. Despite variations in compounds, doses, administration methods, and other conditions across studies, the general consensus is that supplements such as propionic, lactic, formic, malic, citric and fumaric acids improve growth performance by increasing average daily feed intake (ADFI) and ADG. They also reduce harmful intestinal coliform bacteria and decrease the incidence and severity of diarrhoea caused by ETEC (Dębski, 2016; Kovanda et al., 2023; Luise et al., 2020; Ma et al., 2021; Namkung et al., 2004; Tsiloyiannis et al., 2001). Additionally, some studies have reported an enhancement of the immune response (Ferrara et al., 2017).

Microencapsulation can enhance efficacy, and combining it with other strategies, such as essential oils, fatty acids, and phenolic compounds, has shown promising results (Choi et al., 2020; Diether et al., 2023; Rodrigues et al., 2020; Xu et al., 2020).

Phytochemicals, plant or plant extracts

This group of additives includes several natural bioactive compounds derived from plants. They can be used in solid, dried and ground form or as extracts. Their benefits are attributed to their antimicrobial and antioxidant properties (Lillehoj et al., 2018).

One major group of phytochemicals is essential oils. Essential oils are hydrophobic, aromatic and volatile compounds, such as terpenes (carvacrol, thymol and eugenol), aldehydes (cinnamaldehyde), alkaloids (capsaicin), and sulfur compounds (allicin). These oils positively affect animal growth and health due to their antioxidant, anti-inflammatory and antimicrobial properties. The antimicrobial efficacy is linked to their hydrophobic nature, which allows them to penetrate lipid cell membranes, disrupt membrane integrity and enhance membrane permeability. Additionally, they may inhibit toxin production. Given their diverse chemical constituents, multiple mechanism could contribute to their antimicrobial activity (Canibe et al., 2022; Liang et al., 2023; Yan et al., 2024). Beneficial effects of essential oils on piglets include enhanced growth performance and feed intake, modulation of the immune response via different cell signaling pathways, reduction of ROS and TNF-α levels, increased antioxidant enzyme activity, promotion of beneficial microbiota like lactobacilli, reduction of *E. coli* and total coliforms, and improved intestinal morphology with increased absorption surface in the small intestine (Omonijo et al., 2018). However, literature reports inconsistent results, likely due to variation in dosage, trial conditions, and the lack of proper characterization of the active compound involved (Hernández-García et al., 2024; Omonijo et al., 2018; Tian & Piao, 2019; Zeghib et al., 2017). Encapsulation has been found to improve results by alleviating unpleasant odors and enhancing stability (Mo et al., 2022). Combining a blend of essential oils has also shown greater benefits (Grando

et al., 2023; Tian & Piao, 2019). Additionally, combining essential oils with other components, such as organic (Liu et al., 2022; Ma et al., 2022), fibers (Liang et al., 2023), and biotic components (Yan et al., 2024), has reported positive outcomes.

Another significant group of phytochemicals are extracts rich in polyphenols, such as resveratrol, quercetin and phenolic acids. These compounds have demonstrated antimicrobial activity against pathogens like ETEC $F4^+$ and have been shown to inhibit LT toxin (Reggi et al., 2020; Verhelst et al., 2010). Their antioxidant activity is beneficial in detoxifying ROS in the inflamed intestines of weaning piglets. Additionally, their ability to bind proteins and metals is linked to their antidiarrhoeal effects (Bonetti et al., 2021). When tested in weaning piglets, these additive reduced diarrhoea incidences, increased digestive and antioxidative enzyme activity, and improved the intestinal villi structure (Liu et al., 2020a).

Lastly, tannins form another batch in this category. They are part of the defence system of plant species and have the ability to interact and precipitate macromolecules like proteins, polysaccharides and alkaloids (Girard & Bee, 2020). In the context of ETEC $F4^+$ interaction, tannins can potentially bind and block extracellular bacterial proteins, such as F4 and F18 fimbria, as well as the secreted LT and ST enterotoxins. When tested in weaning piglets challenged with the pathogen, tannins have shown effects in reducing PWD clinical signs, such as lower faecal scores and a reduced percentage of piglets with diarrhoea (Girard et al., 2018, 2020).

Functional amino acids

The supplementation of certain amino acids (AAs) in post-weaning piglets offers a variety of beneficial effects. These AAs serve as substrates for protein synthesis and play a crucial role in regulating gene expression and

intracellular signaling pathways, thereby improving health, growth and development (Wu, 2013; Xiong et al., 2019). These compounds are known as functional AAs (Wu, 2013). When arginine, glutamine, methionine, and cysteine are added to weaned piglets' diet, they help maintain appropriate intestinal integrity, permeability, epithelial renewal, and alleviate morphological damage. Regarding the immune response, these amino acids improve gut mucosal immunity and modulate inflammation and oxidative stress (Canibe et al., 2022; Wu, 2013; Zhou et al., 2023).

It has been reported that tryptophan and threonine requirements increase when the immune system is challenged, such as during the weaning stage. It is estimated that 60% of dietary threonine is used for the synthesis of intestinal mucosal proteins, such as mucins (Mou et al., 2019). Therefore, a deficiency in threonine increases paracellular permeability and the incidence of diarrhoea in piglets. However, an excess can decrease protein synthesis in the small intestine, possible because threonine shares the same transport system with neutral amino acids, leading to reduced uptake of these amino acids (Zhou et al., 2023). Balanced threonine supplementation was reported to improved intestinal mucin synthesis (Ren et al., 2014; Zhang et al., 2019) and enhanced intestinal morphology (Ren et al., 2014) in piglets.

Dietary supplementation of tryptophan in weaning piglets has been linked to significant alterations in microbial composition, increasing the relative abundances of *Prevotella* (Rao et al., 2021), *Lactobacillus* (Liang et al., 2018a; Rao et al., 2021) and *Ruminococcaceae* (Rao et al., 2021), while decreasing potential pathogens such as *Sutterella* (Rao et al., 2021) and *Clostridium* (Liang et al., 2018b, 2018a). Additionally, mRNA expression of mucosal integrity proteins is upregulated (Liang et al., 2018a, 2018b). Overall, this results in a decreased diarrhoea rate and increased ADG and ADFI in the animals (Capozzalo et al., 2015; Jayaraman et al., 2017; Zhou et al., 2023).

Short and medium-chain fatty acids

Short-chain fatty acids (SCFAs) serve as a major fuel source for colonocytes and influence the expression of genes involved in gut motility, host defense and inflammatory responses. They stimulate the formation of the intestinal barrier, protect against intestinal barrier disruption, and affect the composition of gut microbiota (Xiong et al., 2019).

Medium-chain fatty acids (MCFAs) exhibit strong antibacterial activity due to their ability to penetrate bacterial membrane and improve intestinal integrity during inflammatory conditions. When tested under challenging conditions, SCFA and MCFA supplementation reduced the incidence of diarrhoea in weaning piglets (Lei et al., 2017; Li et al., 2018a).

Antimicrobial peptides

These compounds are polypeptides naturally produced by various organisms as a self-defense mechanism. Most are classified into either defensin or cathelicidin families. They possess a strong, broad-spectrum activity against gram-negative and gram-positive bacteria, fungi, parasites and viruses (Canibe et al., 2022; Xiong et al., 2019). When supplemented in the diets of weaned piglets, they resulted in improved growth performance, optimal intestinal structure, and modulation of the immune system (Cao et al., 2021; Feng et al., 2020; Wu et al., 2012; Xiong et al., 2019; Zhang et al., 2021a).

The list of possible additives is even longer. Other supplements such as algae, egg yolk antibodies, and spray-dried plasma have also shown benefits in piglets (Bonetti et al., 2021; Canibe et al., 2022).

2.3.1.2. Biotic additives

Regulation of gut microbiota

As highlighted earlier, there is mounting evidence underscoring the pivotal role of the microbiota in animal health and performance from early life to the end of its productive life. During the initial stages, gut microbiota exhibits considerable adaptability, presenting a window of opportunity for the effective introduction of biotic compounds (Hashemi et al., 2016). The early administration of biotic additives is believed to enhance resilience in the newly weaned pig, thereby increasing their chances of overcoming challenges associated with weaning (Hansen et al., 2022a). Consequently, it is recommended that this supplementation be initiated as soon as possible after birth.

These biotic additives are known for their ability to facilitate the early maturation of the gut, stimulate the growth of beneficial bacteria, modulate immune response, enhance the animal's intestinal barrier mechanisms (Bezkorovainy, 2001; Canibe et al., 2022; Hansen et al., 2022a), and serve as a source of functional components (Ali et al., 2023) .

The most commonly used additives capable of robustly modulating intestinal microbiota are probiotics, prebiotics and their combination, synbiotics. However, other biotic additives are emerging, such as postbiotics and proteobiotics, also reviewed here.

2.3.1.2.1. Probiotics

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host”, as defined by the ISAPP (Hill et al., 2014). Among the most commonly used strains are *Lactobacillus* spp., *Bifidobacterium* spp. (Linares et al., 2017), bacteria from the genera *Bacillus*, *Enterococcus*, *Streptococcus*, and yeast such as *Saccharomyces cerevisae* (Ali et al., 2023). The array of commercially available probiotic strains is extensive. Here, we delve deeper into *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces cerevisiae* strains, exploring their application in weaning piglets.

Their advantages arise from their ability to enhance intestinal population profile, protection of intestinal barrier integrity, regulation of immune responses (Su et al., 2022), antioxidant properties (Li et al., 2023) and provision of functional components (Ali et al., 2023). These different aspects will be reviewed below.

Microbiota-related effects

Early maturation, increased biodiversity and ecosystem resilience

Newborn piglets have a less stable microbial community in their GI tract (Cheng et al., 2019). During the suckling period, the predominant phyla in the hindgut are Firmicutes, Bacteroidetes, Proteobacteria and Fusobacterium, although there is a significant individual variability (Chen et al., 2017; Saladrigas-García et al., 2022). This early microbial profile is associated with a milk-oriented microbiome. When solid feed is introduced, there is a substantial shift in bacterial populations (Bian et al., 2016), leading to an initial dysbiosis (Chen et al., 2017). Subsequently, the microbiota profile becomes more diverse and richer, favoring carbohydrate fermentation, with

an increase in Bacteroidetes (Li et al., 2018b). This increased diversity and richness add redundancy to specific functions, resulting in a more mature, resilient and stable gut ecosystem (Saladrigas-García et al., 2022).

Evidence suggests that increasing microbial richness and biodiversity at early ages can lead to better adaptation during weaning, reduced incidence of diarrhoea and improved performance (Cheng et al., 2019; Gaukroger et al., 2020). Taking advantage of the "window of opportunity" offered by early ages (Torow & Hornef, 2017), probiotic supplementation enhances the richness of gut microbiota and promote the growth of beneficial functional microbes, thereby increasing resistance to pathogenic infections (Su et al., 2022; Tang et al., 2020). For instance, increased relative abundance of beneficial functional microbiota, such as *Lactobacillus, Prevotella, Lachnospira*, contribute to gut robustness, intestinal health and mucosal immunity (Luise et al., 2021; Qi et al., 2021).

Control of pathogens

Probiotics can promote the growth of beneficial bacteria able to exclude pathogenic cells in the gut. For instance, Gresse et al. (2021) reported that incubating *Saccharomyces cerevisiae* in an *in vitro* model of the weaning piglet colon resulted in an exclusion of ETEC cells due to competitive settlement. In this study, there was a substantial increase in the relative abundance of non-pathogenic *Escherichia*/*Shigella* genus, which occupied the ETEC ecological niche, thereby protecting the intestinal cells from ETEC colonization (Gresse et al., 2021). In a similar vein, supplementation of *Bifidobacterium* spp. and *Lactobacillus rhamnosus* was able to prevent the attachment of pathogens (*Salmonella*, *Clostridium* and *E. coli)* to the intestinal epithelium by competitively excluding their adhesion (Collado et al., 2007). In addition to promoting the competitive exclusion of pathogens, probiotics have demonstrated the ability to displace pathogens and inhibit

their adhesion as well. Following Collado et al. (2007) work, it was found that *Bifidobacterium* spp. and *Lactobacillus rhamnosus* not only prevented pathogen adhesion but also displaced pathogens when incubated simultaneously and inhibited their already established adhesion when incubated after the pathogens (Collado et al., 2007). In *in vivo* studies, what can be observed is a better control of harmful pathogens and increased growth of beneficial species. For instance, Bai et al. (2022) found that supplementing challenged weaned piglets with *Lactobacillus rhamnosus* increased the relative abundance of beneficial genera such as *Collinsella*, *Lactobacillus*, and *Ruminococcus* in the caecum. This increase in beneficial species competed with and reduced the counts of harmful pathogens (Bai et al., 2022).

By increasing the presence of beneficial SCFA-producing bacteria, higher concentrations of SCFA are found in the gut content (Gresse et al., 2021). Increased SCFA levels help defend against pathogenic invasion by lowering pH and providing energy to intestinal epithelial cells, thus strengthening gut barrier function (Su et al., 2022).

Intestinal inflammation increases oxygen levels, supporting the growth of facultative anaerobes like pathogenic *E.coli*. However, facultative anaerobic probiotics, such as *Saccharomyces cerevisiae*, can rapidly consume oxygen, creating an anaerobic environment that inhibits the growth of aerobic pathogens in the gut (Su et al., 2022).

Inactivation of virulence factors

These probiotic compounds also play a role in toxin inactivation and removal from the body. This can occur through adsorption, where toxins bind to the probiotic cell wall, or via the metabolism of mycotoxins (Markowiak & Ślizewska, 2017). For example, *Bifidobacterium* spp. has been reported to

bind and neutralize lipopolysaccharides (LPS) (Park et al., 2007) or vero cytotoxin from pathogenic *E.coli* (Kim et al., 2001). Additionally, probiotics can secrete antimicrobial substances such as bacteriocins, organic acids, and hydrogen peroxide.

A summary of microbiota related effects of probiotics is showed in Figure 2.2.

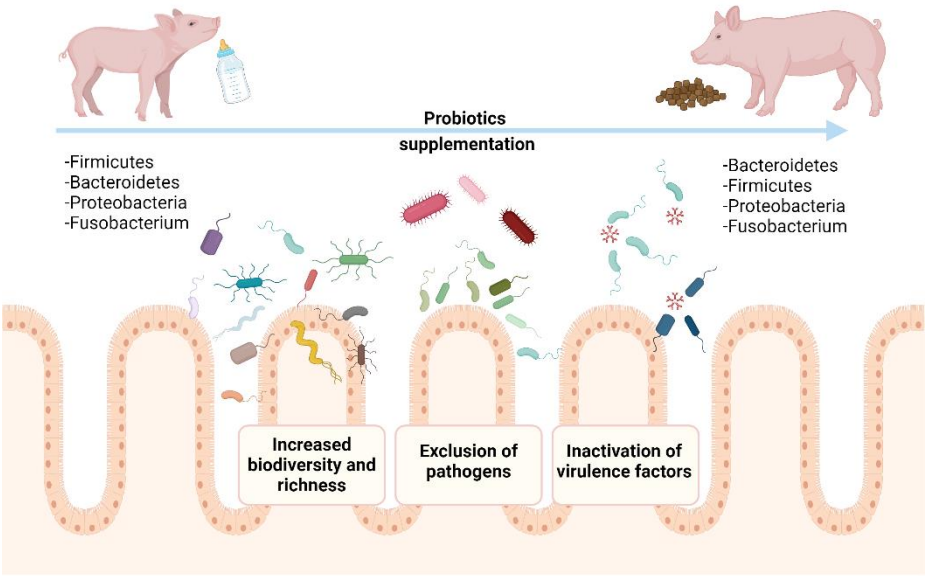


Figure 2.2. Summary of probiotic's effect on gut microbiota population. Created with BioRender.com

Chemical and mechanical barrier

The chemical barrier consists of mucus secreted by the epithelial cells, digestive fluids, and bacteriostatic substances produced by normal gut bacteria (Su et al., 2022).

Kumar et al. (2014) found that ETEC secretes a serine protease (Eat A) that degraded MUC2, protein involved in mucous production (Liu et al., 2020c). This degradation allows the bacteria to penetrate the mucus layer, reach the

epithelium, and trigger an inflammatory response (Kumar et al., 2014). Various probiotics strains have shown beneficial effects on these regards. In an *in vitro* model of weaning piglet colon, *Saccharomyces cerevisiae* (Gresse et al., 2021) and *Lactobacillus reuteri* (Liu et al., 2017) increased the expression of MUC1 gene. In *in vivo* studies, *Lactobacillus rhamnosus* have been reported to enhance intestinal mucosal barrier by boosting the expression of MUC1 and MUC2 in jejunum mucosa of weaning piglets (Mao et al., 2016; Wang et al., 2019).

On the other hand, the mechanical barrier's structure relies on intact intestinal epithelial cells and the tight junctions between these cells. These junctions, which include occludin, claudin-1 and ZO-1 proteins, play a crucial role in regulating the intestinal barrier and cell survival (Su et al., 2022). Supplementation of *Lactobacillus* spp. in piglets has been reported to upregulate tight junction proteins (ZO-1, occludin, and claudin-1 (Bai et al., 2022; Hu et al., 2018; Mao et al., 2016; Sun et al., 2020)), and compete with potential pathogens for intestinal epithelial binding sites to promote the expression of tight junction protein (Li et al., 2019b). When the intestinal barrier is damaged and tight junction proteins are disrupted, diamine oxidase (DAO) is released into the serum (Suzuki, 2013). DAO and D-lactate levels are commonly used indicators to evaluate intestinal barrier function (Yang et al., 2019), while maldonaldehyde (MDA) served as an indicator of oxidative stress (OS), which also contributes to intestinal barrier damage (Del Rio et al., 2005). In studies where the expression of tight junction proteins increased, lower activity of DAO and D-lactate was also observed (Hu et al., 2018; Wang et al., 2019).

A summary of probiotic's effect on chemical and mechanical intestinal barrier is showed in Figure 2.3.

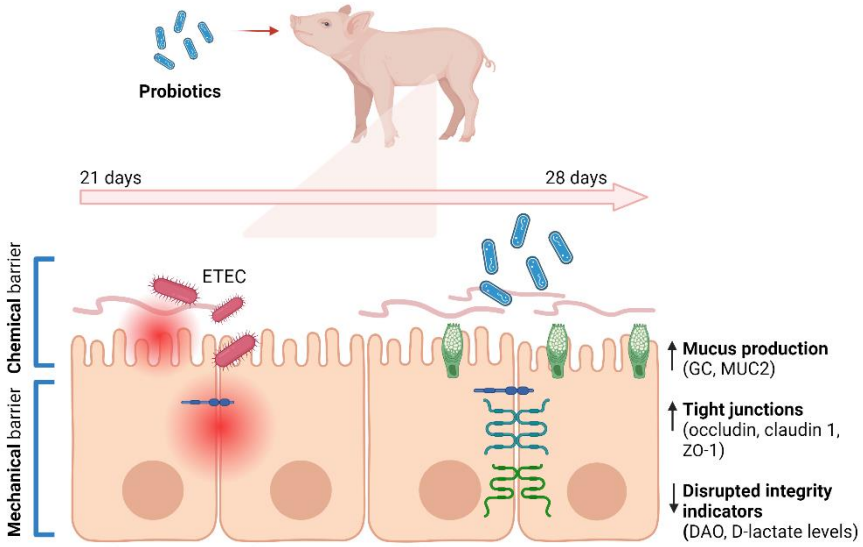


Figure 2.3. Effect of probiotics on chemical and mechanical intestinal barrier. Created with BioRender.com

Immunological function

During the early weaning period, the innate immune system's defenses are more developed compared to the adaptive immune system, making weaning piglets more reliant on innate immunity (Humphrey et al., 2019).

When weaned piglets are not infected by pathogens, probiotics such as *Lactobacillus*, *Bacillus*, and yeast, along with their metabolites, function as immune activators, regulating the activation of extracellular signaling kinase through the TLR signaling pathway, stimulating the proliferation and differentiation of T-lymphocytes and B-lymphocytes, increasing the number of $CD3^+$, $CD4^+$, and leading to a more stable and resilient immune response (Shonyela et al., 2020; Su et al., 2022; Zhu et al., 2014). The cell wall components of LAB enhance macrophage activity enabling rapid microbe destruction. Additionally, glucans in yeast cell walls activate the reticuloendothelial system, favoring the increase of phagocytic cells (Markowiak & Ślizewska, 2017). Probiotics also stimulate the production of

gut antibodies, particularly sIgA, which can prevent pathogen attachment to the epithelial cells (Mao et al., 2016; Su et al., 2022).

Conversely, when weaned piglets are infected with pathogens, the immune system recognizes pathogen-associated molecular patterns, triggering the release of pro-inflammatory mediators and responses (Humphrey et al., 2019). Probiotics can inhibit the expression of pro-inflammatory cytokines and promote the expression of anti-inflammatory cytokines, thereby protecting the intestinal tract from pathogen-induced inflammation (Su et al., 2022). As examples, dietary supplementation of *Lactobacillus rhamnosus* in weaning piglets resulted in a decreased in IL-1β, IL-8 (Wu et al., 2021), as well as IL-6, IL-12 and TNF-α levels (Wang et al., 2019), along with an increase in the anti-inflammatory cytokine TGF-β1 and IL-10 (Wang et al., 2019) and sIgA and IL-4 concentration (Mao et al., 2016). Other *Lactobacillus* strains supplemented in young piglets promoted a downregulation of IL-1β, IFN-γ and TNF-α (Sun et al., 2020), and an increased in sIgA (Hu et al., 2018; Li et al., 2019c). *Bifidobacterium* longum subsp. *infantis* promoted attenuation of the secretion of pro-inflammatory cytokines induced by pathogenic *E. coli* challenged, particularly, IL-6, IL-8, IFN-γ and TNF-α, in cultured intestinal cells of a piglet model (Álvarez-Mercado et al., 2022).

Regarding yeast supplementation, in an *in vitro* model of weaning piglet colon cells, *Saccharomyces cerevisiae* appeared to mitigate the expression of IL-1β, TNF-α, IL-6, IL-8 (Badia et al., 2012b; Gresse et al., 2021; Zanello et al., 2011b).

A summary of the impact of probiotics on immunological barrier is showed in Figure 2.4.

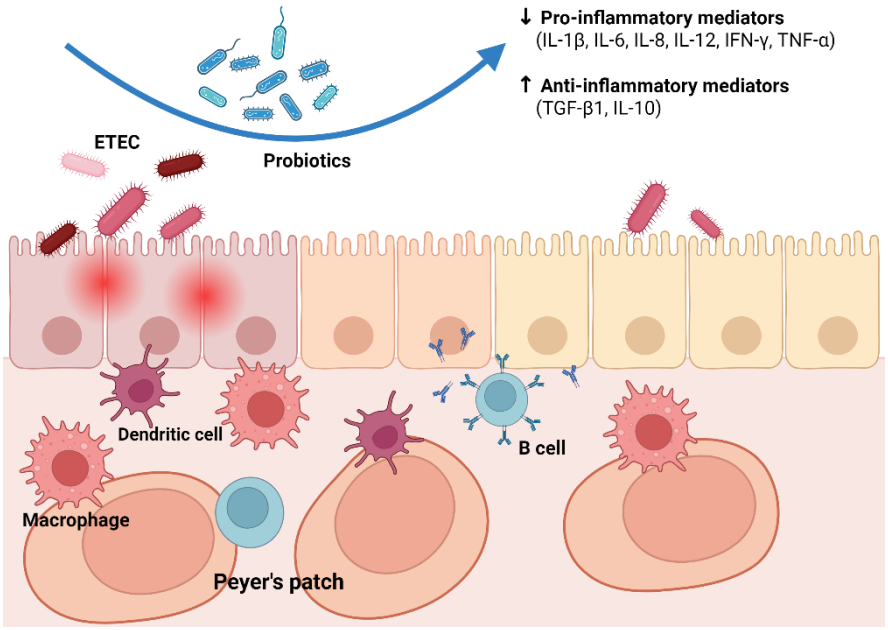


Figure 2.4. Effect of probiotics on immunological barrier.
Created with BioRender.com.

Antioxidant effects

The antioxidant properties of probiotics are attributed to their capacity to scavenge ROS, chelate metals, and regulate host enzyme levels (McCormack et al., 2017). OS arises from an imbalance between oxidants and antioxidants, with ROS being the predominant highly reactive molecules in organisms. Excessive ROS can damage cell biomolecules, particularly membrane lipids, DNA, and proteins (McCormack et al., 2017). To maintain this balance, the organism has physiological antioxidant systems consisting of enzymatic (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX)) and nonenzymatic defenses (ascorbic acid, vitamin E, C, A, metal elements such as zinc, copper, manganese, iron). When ROS levels rise, the intestine is particularly vulnerable, leading to intestinal inflammation, barrier disruption, diarrhoea, and microbial disorders. This increased permeability facilitates pathogen invasion, further exacerbating the

production of proinflammatory factors and ROS, creating a detrimental cycle (Rapozo et al., 2017).

Research shows that weaning significantly increases ROS levels in the liver, intestine and blood of weaned piglets while inhibiting the activities of GSH-PX and SOD (Novais et al., 2021). *Bifidobacteria* and lactobacilli, in particular, have been reported to mitigate this condition through various mechanisms (Mishra et al., 2015). *Bifidobacterium longum* has been found to effectively remove ROS and improve the host's oxygen resistance, for example, improving intestinal bowel disease (IBD) conditions in mammals (Yao et al., 2021). Due to its high free-radical scavenging properties, *Lactobacillus fermentum* supplementation was analysed in growing-finishing pigs, resulting in increased serum levels of SOD and GSH-Px and decreased levels of MDA (Wang et al., 2009). *Lactobacillus delbrueckii* supplementation during the piglet suckling phase was reported to reverse the increased serum levels of DAO and MDA induced by LPS challenge (Li et al., 2019c). Similarly, Sun et al. (2020) showed that *Lactobacillus salivarius* supplementation at weaning promoted a down-regulation of D-lactate, DAO and MDA levels, accompanied by increased antioxidant levels of CAT, GSH-Px and SOD (Sun et al., 2020). Lastly, *Lactobacillus rhamnosus* supplemented to challenged weaning piglets promoted a decreased DAO and MDA activity, and an increased GSH-Px expression (Wu et al., 2021).

A summary of the antioxidant effect of probiotics is showed in Figure 2.5.

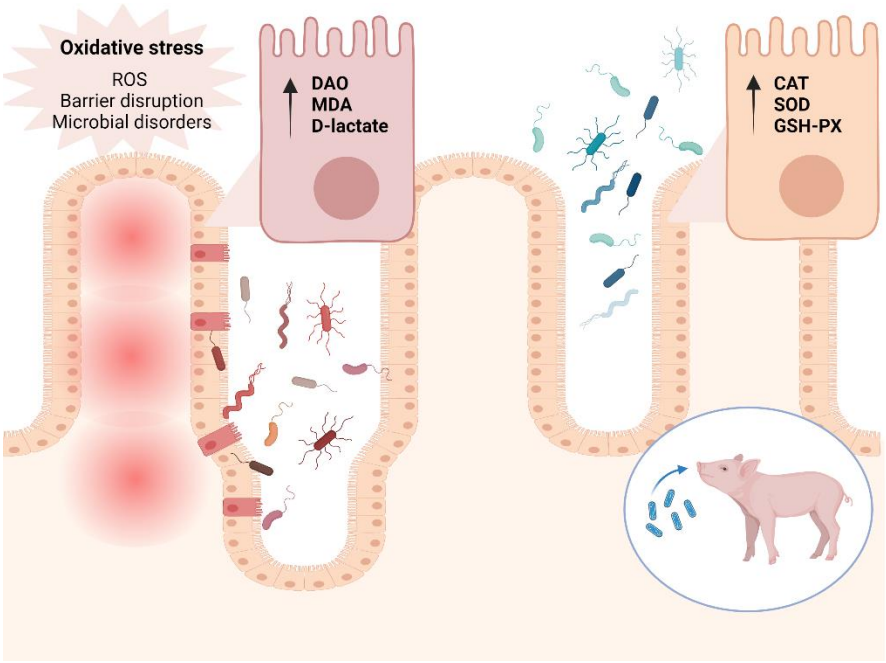


Figure 2.5. Antioxidant effect of probiotics on intestinal environment. Created with Biorender.com.

All these previously reviewed alterations had an undeniable impact on the intestinal health of individuals. A compilation of the effects of probiotics in various *in vitro* and *in vivo* studies are summarise in Table 2.1.

Regarding the observable changes that could be seen in the animals would be a better adaptation to the weaning challenge, including a reduction in diarrhoea incidence and an increase in growth rates. For instance, dietary supplementation of *Lactobacillus rhamnosus* strain on weaning piglets resulted in increased weights, ADG, and decreased diarrhoea incidence (Wang et al., 2019). In challenged conditions, *in vivo* studies showed reduced diarrhoea (Bai et al., 2022; Wu et al., 2021), along with increased body weight, enhanced ADG and ADFI (Bai et al., 2022). Oral supplementation of *Bifidobacterium spp.* to weaning piglets challenged with pathogens led to increased feed intake (Barba-Vidal et al., 2017b), and reduced pathogen excretion (Barba-Vidal et al., 2017a, 2017b). When a combination of these two strains was used, improved weights gains were seen (Rodríguez-Sorrento

et al., 2021). Finally, dietary supplementation with *Saccharomyces cerevisiae* resulted in higher growth, enhanced feed efficiency and lower diarrhoea rates in weaning piglets (Dang et al., 2024).

Table 2.1. Compilation of main probiotic effects on *in vitro* and *in vivo* trials.

Probiotic strain	Model	Challenge	Main effects	References
Microbiota related effect				
Competitive exclusion of pathogens				
Saccharomyces cerevisiae var *boulardii* CNCM I-1079	*In vitro* gut fermentation model of weaning piglet colon (Mpigut-IVM)	ETEC	Reduction of ETEC adhesion to intestinal cells.	(Gresse et al., 2021)
Lactobacillus rhamnosus GG	36, 21-days-old weaning piglets	Deoxynivalenol (DON) mycotoxin	Increased relative abundance of *Lactobacillus*, *Collinsella*, *Ruminococcus* and decresase in relative abundances of harmful bacteria, *Parabacteroides* and *Ruminiclostridium*.	(Bai et al., 2022)
Bifidobacterium lactis Bb12, *Lactobacillus rhamnosus* LGG	*In vitro* adhesion assay on intestinal mucus (from 9-10-months-old piglets) of duodenum, jejunum, ileum, ascending colon, transverse colon and descending colon.	*Salmonella enteric* serovar *Typhimurium*, *Clostridium perfringens*, *Clostridium difficile* and *Escherichia coli* K2	Reduction of all pathogen's adhesion by exclusion in all parts of the intestine. Displacement of all preadhered pathogens in all parts of the intestine. Inhibition of *E.coli, S. Enterica* serovar *Typhimurium* and *C. Perfringens* by competition, in all parts of the intestine.	(Collado et al., 2007)
Inactivation of virulence factors				
Bifidobacterium longum HY8001	Mice	Vero cytotoxin (VT)-producing *Escherichia coli* (VTEC)	*B. longum* supernatant binded VTs.	(Kim et al., 2001)

Ninety bifidobacteria isolates from human faeces	*In vitro* model	*Escherichia coli*	Five bifidobacteria strains showed high levels of LPS binding.	(Park et al., 2007)
Chemical and mechanical barrier				
Saccharomyces cerevisiae var *boulardii* CNCM I-1079	*In vitro* gut fermentation model of weaning piglet colon (Mpigut-IVM)	ETEC	Increased expression of MUC1 gene.	(Gresse et al., 2021)
Lactobacillus rhamnosus GG	36, 21-days-old weaning piglets	Deoxynivalenol (DON) mycotoxin	Increased levels of ZO-1, occludin and claudin-4 in jejunum and ileum.	(Bai et al., 2022)
Lactobacillus reuteri I5007	*In vitro* model of porcine small intestinal epithelial cell line (IPEC-J2), and *in vivo* model with 22, 4-days-old piglets	None	Increased relative abundance of mRNA for MUC1 in colon.	(Liu et al., 2017)
Lactobacillus frumenti JCM 11122	100, 6-days-old piglets	None	Upregulation of tight junction proteins (ZO-1, occludin, claudin-1) in duodenum, jejunum and ileum. Decreased D-lactate serum levels.	(Hu et al., 2018)
Lactobacillus rhamnosus GG	24, 21-days-old piglets	Rotavirus	Increased MUC1 and MUC2 concentrations, and ZO-1 and occludin levels in jejunum mucosa.	(Mao et al., 2016)
Lactobacillus delbrueckii	100, 1-day-old piglets	None	Decreased MDA serum levels.	(Li et al., 2019b)
Lactobacillus rhamnosus GG	24, pre-weaning piglets	None	Increased MUC2 gene expression. Decreased DAO activity.	(Wang et al., 2019)
Lactobacillus salivarius	72, 21-days-old piglets	LPS	Increased claudin -1, occludin, ZO-1 tight junction proteins.	(Sun et al., 2020)
Immunological function				

Saccharomyces cerevisiae var *boulardii* CNCM I-1079	*In vitro* gut fermentation model of weaning piglet colon (Mpigut-IVM)	ETEC	Reduced CCL20, CXCL2, IL-1β, IL6, IL8, TNF-α, TLR4 expression.	(Gresse et al., 2021)
Bifidobacterium longum HY8001	Mice	vero cytotoxin (VT)-producing *Escherichia coli* (VTEC)	Decreased TNF-α, IL-1β levels in serum and expression of their mRNA.	(Kim et al., 2001)
Lactobacillus frumenti JCM 11122	100, 6-days-old piglets	None	Increased IgG, sIgA, IFN-γlevels.	(Hu et al., 2018)
Lactobacillus rhamnosus GG	24, 21-days-old piglets	Rotavirus	Increased sIgA and IL-4 concentration in jejunal mucosa.	(Mao et al., 2016)
Lactobacillus delbrueckii	100, 1-day-old piglets	None	Increased sIgA, IL-12 and IL-6 jejunal concentration.	(Li et al., 2019b)
Bifidobacterium longum subsp. *Infantis* CECT 7210	*In vitro* model of Caco-2 cells, epithelial cell line from colorectal adenocarcinoma, and a co-cultured with human dendritic cells	*Escherichia coli* CECT 515 or LPS	Reduction of IL-12, IL-1β, IFN-γ, IL6, IL-8 levels and TNF-α.	(Álvarez-Mercado et al., 2022)
Lactobacillus rhamnosus GG	24, pre-weaning piglets		Upregulation of TGF-β1. Decreased IL-1β, IL-6, IL-12, IL-8, TNF-α concentrations. Increased IL-10 and TGF-β1 production.	(Wang et al., 2019)
Lactobacillus rhamnosus LB1	64, 7-days-old piglets	ETEC	Reduction of IL-8, IL-1β concentration.	(Wu et al., 2021)
Saccharomyces cerevisiae CNCM I-3856, *S.cerevisiae*	Intestinal epithelial IPI-2I cells cultured	ETEC $F4^+$	Inhibition of TNF-α gene expression. Culture supernatant decreased TNF-α, IL-1β, IL-6, IL-8, CXCL2, CCL20 mRNA expression.	(Zanello et al., 2011b)

boulardii CNCM I-3799				
S. Cerevisiae var. *Boulardii* + β-galactomanann	Porcine small intestine epithelial cell line (IPI-2I)	ETEC $F4^+$	Reduction of IL-1β, IL-6, CCL2, CXCL8 mRNA expression.	(Badia et al., 2012b)
Lactobacillus salivarius	72, 21-days-old piglets	LPS	Downregulation of IL-1β, IL-6, IFN-γ, TNF-α. Increased IL-10 concentration.	(Sun et al., 2020)
Antioxidant effects				
Lactobacillus delbrueckii	100, 1-day-old piglets	None	Decreased MDA serum and hepatic levels. Increased CAT and hepatic GSH-PX activity.	(Li et al., 2019b)
Lactobacillus rhamnosus LB1	64, 7-days-old piglets	ETEC incubation	Decreased DAO and MDA activity, increased GSH-PX expression.	(Wu et al., 2021)
Lactobacillus fermentum	108 pigs	None	Increased SOD and GSH-PX levels. Decreased MDA levels.	(Wang et al., 2009)
Lactobacillus salivarius	72, 21-days-old piglets	LPS	Increased CAT, GSH-PX, SOD expression levels. Decreased D-lactate, DAO and MDA levels.	(Sun et al., 2020)

2.3.1.2.2. Prebiotics

Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Recently, prebiotics have emerged as potential antibiotic substitutes, primarily because they support the viability of probiotics by serving as substrates (Yang et al., 2024). Carbohydrates, such as dietary fibre, are considered potential prebiotics. For a product to be classified as a prebiotic, it must meet specific criteria: resistance to digestion in the upper GI tract, fermentation by intestinal microbiota, and promotion of a beneficial effect on the host’s health (Markowiak & Ślizewska, 2017). Prebiotics can be categorized based on the number of monomers they contain: disaccharides, oligosaccharides (3-10 monomers), and polysaccharides. Among these, oligosaccharides are the most promising and well-studied, including fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), isomalto-oligosaccharides (IMO), xylo-oligosaccharides (XOS), transgalacto-oligosaccharides (TOS) and soybean oligosaccharides (SBOS). Polysaccharides such as inulin, starch cellulose, hemicellulose, or pectin also have potential as prebiotics (Markowiak & Ślizewska, 2017).

The primary purpose of prebiotics is to be fermented by beneficial microbiota, thereby stimulating their growth and activity (Yang et al., 2024). The molecular structure of prebiotics determines their physiological effects and the types of microorganisms that can utilize them as a carbon and energy source in the bowel. However, despite the variety of carbohydrates exhibiting prebiotic activity, the main effect is an increased count of beneficial bacteria, particularly from the *Bifidobacterium* and *Lactobacillus* genus. The final products of carbohydrate fermentation are primarily SCFA, which the host uses as an energy source.

Additionally, prebiotics have been reported to influence immunological functions. These effects are proposed to be linked with the modulation of histone acetylation, increasing the availability of numerous genes for transcription factors; modulation of mucin production; increased counts of lymphocytes and /or leukocytes in gut-associated lymphoid tissues (GALTS) and in peripheral blood; stimulation of phagocytic function of macrophages through the secretion of IgA by GALTs (Markowiak & Ślizewska, 2017).

Several prebiotics are commercially available; however, this work will focus on GOS, FOS and Human Milk oligosaccharides (HMO).

Galacto-oligosaccharides

GOS supplementation in early ages has been linked to the growth of *Bifidobacteria* (Ruiz et al., 2020) and the *Lactobacillus* genus, along with an increased ratio of Firmicutes/Bacteroidetes in the gut (Tian et al., 2019; Wu et al., 2024). The presence of LAB leads to higher lactate and SCFA concentration, reducing the pH of the environment. Since *Escherichia* thrives in a slightly alkaline environment, SCFA partially inhibits its proliferation (Tian et al., 2019). Additionally, GOS has been associated with improved expression of ZO-1 and occludin in the jejunal mucosa, enhancing intestinal barrier function, and promoting an anti-inflammatory environment by increasing TGF-β1 expression, reducing IL-12, IL-1β, TNF-α mRNA expression (Tian et al., 2018, 2022; Wu et al., 2024), and decreasing Pig-MAP serum levels in piglets (Rodríguez-Sorrento et al., 2021). In terms of performance, the supplementation of these prebiotics has been suggested to promote enhanced growth and health in piglets (Rodríguez-Sorrento et al., 2021).

Fructo-oligosaccharides

FOS is selectively fermented by most bifidobacteria strains due to the production of β-fructofuranosidases (Imamura et al., 1994). When administered to weaned piglets resulted in improved nutrient digestibility, a reduction in diarrhoea rates and increased SCFA content in faeces (Ma et al., 2024). Additionally, in challenged piglets, FOS supplementation has been reported to improved antioxidant capacity, by increasing GSH peroxidase and catalase levels in plasma and intestinal mucosa. Intestinal barrier integrity was also enhanced, as marked by improved ZO-1 and plasmatic DAO levels. Moreover, treated animals showed lower levels of inflammatory cytokines and decreased apoptosis of jejunal cells (Luo et al., 2022b).

As seen with other additives, combining different compounds could lead to an enhanced response. Supplementation of GOS, FOS and milk fat globule membrane in neonatal piglets resulted in improved growth performance, increased abundance of *Lactobacillus*, *Christensenella*, *Enterococcus* and *Romboutsia* genera, and higher levels of fermentation products such as acetate and propionate. There was also an upregulation of intestinal tight junction proteins (occludin, claudins and ZO-1), mucins (MUC1, MUC2, MUC4 and MUC20) and cytokines (TNF-α, IL-1β and IL-22), along with a decreased in DAO levels. Correlation analysis revealed a positive relationship between the beneficial microbiota and the modified intestinal barrier genes (Wu et al., 2020).

Human Milk Oligosaccharides

HMO comprises more than 200 different types of non-digestible oligosaccharides found in human milk (Zuurveld et al., 2023). These compounds are crucial for infants as they are the primary prebiotics consumed, shaping their microbiota (Donovan et al., 2012). HMOs promote

the growth of *Bacteroides*, *Lactobacillus* and, particularly *Bifidobacterium* (Okburan & Kızıler, 2023). They possess recognized anti-infective and immunoregulatory activities and are reported to inhibit the binding of pathogens to the intestinal epithelium due to structural similarities to mucus glycans (Martín-Sosa et al., 2002). In most cases, their supplementation is intended in infant formulas, to provide neonates with protection against infections. Nevertheless, there are studies in the literature where they have been tested in the animal model of the young piglet to observe their effect against infections, such as rotavirus (Hester et al., 2013) or intestinal diseases, such as necrotizing enterocolitis (Sodhi et al., 2021). When supplemented in piglets at early ages, HMOs impact inflammatory response, increasing IFN-γ (Comstock et al., 2017) and IL-10 expression, and in microbiota population, promoting an increase in *Lachnospiraceae* abundance (Li et al., 2014).

2.3.1.2.3. Synbiotics

Synbiotics are defined as "mixtures of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements" (Swanson et al., 2020). There are two types of synbiotics that should be distinguished: complementary synbiotics, which combine a probiotic with an independently active prebiotic; and synergistic synbiotics, where the substrate is specifically designed to be used selectively by the co-administered microorganism (Nguyen et al., 2022; Swanson et al., 2020). Although optimal results should be achieved with synergistic approach, demonstrating their efficacy is challenging, thereby most of the commercially available synbiotics used in clinical trials are complementary (Nguyen et al., 2022).

The use of prebiotics in synbiotics helps probiotic microorganisms gain higher tolerance to environmental conditions such as oxygenation, pH, and temperature within the host's intestine (Markowiak & Ślizewska, 2017). The improved outcomes from combining these two products result from either enhanced viability of probiotic microorganisms or the provision of specific health benefits, such as the increase of SCFAs, (Markowiak & Ślizewska, 2017).

This synergy was observed in the study by Girard et al. (2021), where *Enterococcus* and *Saccharomyces cerevisiae* combined with inulin led to improved post-weaning ADG and decreased diarrhoea incidence (Girard et al., 2021). Similarly, the combination of *Saccharomyces cerevisiae*, lactobacilli and *Bacillus subtilis* with β-glucan and mannan oligosaccharide, resulted in greater outcomes, including increased levels of IL-10, IgM and IL-4 levels, decreased DAO serum levels, improved mRNA expression of claudin-1, ZO1, MUC2, IFN-γ and occludin, and downregulation of mRNA expression of TLR3 and TNF-α (Luo et al., 2024).

In some cases, individually these compounds provide benefits, but when used together, these effects are lost. This was reported by Rodríguez-Sorrento et al. (2021), in which a probiotic composed of *Bifidobacterium longum* susp. *infantis* and *Lactobacillus rhamnosus* showed improvement in weight gain after ETEC inoculation, and GOS as a prebiotic improved weight gain and lowered Pig-MAP levels. However, when combined, these effects were not observed (Rodríguez-Sorrento et al., 2021). A similar outcome occurred with *Bifidobacterium longum* susp. *infantis* and *Lactobacillus rhamnosus* combined with oligofructose-enriched inulin, where no synergy was detected (Rodríguez-Sorrento et al., 2020).

2.3.1.2.4. Postbiotics and proteobiotics

A relatively new concept gaining attention is postbiotics, although the term's usage is inconsistent and can be found under alternative names such as paraprobiotics or ghost probiotics in the literature (Dang et al., 2024).

The ISAPP defines postbiotics as "a preparation of inanimate microorganisms and / or their components that confers a health benefit on the host" (Salminen et al., 2021). These include, inactivated microbial cells, inactivated through various methods such as heat treatment, UV radiation or sonication (high frequency sound waves) (Ali et al., 2023), as well as cell components like flagella, pili, surface layer proteins and capsular polysaccharide. (Canibe et al., 2022; Su et al., 2022). To be considered postbiotics, the preparation must contain inactivated microbial components, with or without metabolites. Purified metabolites alone are not considered postbiotics (Salminen et al., 2021) .

Different reported studies have evidenced the potential benefits of postbiotics in piglets. Administering inactivated and stabilized cells of *Saccharomyces cerevisiae* to gestating sows resulted in improved lactation efficiency, higher birth weights and enhanced host defense of piglets towards challenging conditions (Scollo et al., 2023). Moreover, certain secreted protein isolated from *Lactobacillus rhamnosus* was proven to have a similar protective role on intestinal barrier function as the probiotic itself, enhancing intestinal mucin expression and preventing pathogen-induced intestinal barrier injury (Gao et al., 2019).

Although still in the preliminary phases and not yet formally defined by ISAPP, the term "proteobiotics" is emerging in the scientific community. Proteobiotics consist of metabolites derived from probiotics; these products do not contain microbial cells but still confer health benefits (Tarsillo &

Priefer, 2020). They may share similarities with antimicrobial peptides, as both are derived from microorganisms. Proteobiotics have been shown to disrupt pathogen virulence strategies by interfering the bacteria cell-to-cell communication, leading to a downregulation of virulence genes in gram-positive and gram-negative bacteria. *In vitro* studies have demonstrated that proteobiotics from *Lactobacillus* significantly reduce the number of ETEC cells that can attach to the colon, thereby diminishing their virulence (Medellin-Peña et al., 2007). Additionally, cell-free fermentation supernatants, potentially classified as proteobiotics, from various LAB were found to improve the feed-to-gain ratio and serum levels of D-lactate in weaning piglets (Abrehame et al., 2024). Moreover, there is a commercially available proteobiotic for weaning piglets, *Nuvio* from MicroSintesis Inc., which has been reported to decrease clinical signs of infection and reduce the presence of the pathogen in the gut and faecal shedding (Nordeste et al., 2017; Tarsillo & Priefer, 2020).

These emerging compounds offer a distinct advantage over probiotics. Since they are not live, viable organisms, there is no risk of overgrowth or bacteraemia in immunocompromised individuals (Ali et al., 2023).

2.4. Novel strategies

2.4.1. Bacteriophages

Bacteriophages, or just phages, are viruses that specifically target bacteria. They are the most prevalent entities on Earth and play a crucial regulatory role in microbial ecosystems. There are two main types of phages: lytic phages, which destroy their host cells shortly after infection, producing numerous infectious phage particles that can infect nearby bacteria; and

lysogenic phages, which integrate their genetic material into the bacterial genome and coexist with the host as prophages without immediately lysing the cell. For therapeutic purposes, lytic phages are preferred due to their antimicrobial properties (Górski et al., 2018). Because they affect multiple bacterial processes, the risk of developing bacterial resistance is low. Additionally, phages continuously replicate and co-evolve with their bacterial hosts, with mutation rates higher than those of the bacteria (Hampton et al., 2020). Another advantage is their self-replicating nature, eliminating the need for repeated treatments (Canibe et al., 2022). Another point to consider about bacteriophages is that, similar to how the microbiome can influence the susceptibility of piglets to pathogens, the virome can also have a distinctive impact on the health status of piglets. As reported by Z. Wang et al. (2023), there could be notable differences in the virome between healthy and diarrheic piglets. This underscores the importance of considering the entire gut genetic material, including the microbiome, virome and host genome (Wang et al., 2023).

Although bacteriophages were discovered in 1915-1917 (Herelle, 1917; Twort, 1915), they are still considered novel therapies under Regulation (EU) 2019/6 (European Medicines Agency, n.d.). The application of phage therapy has been irregular since its discovery. In the 1920s, the first human therapy took place primarily in Eastern countries such as Georgia, the Soviet Union and Poland, where the main research was conducted. In Europe, some research was also carried out in centers in France, Germany and Belgium. However, in the 1940s phage therapy began to decline due to insufficient knowledge, and the advent of antibiotics, which were easier to handle and more powerful. Around 1990s, the pioneering research in Eastern countries was interrupted due to political and economic reasons following the end of the Soviet Union, putting its evolution on pause (Kutter et al., 2010).

To this day, this research has not yet fully recovered. Although phage therapies were applied to humans for years, the lack of awareness and supporting data from clinical trials makes it difficult to validate these advances (Naureen et al., 2020). Currently, in the EU, bacteriophages are considered medicinal products, and in the USA, they are classified as drugs, falling under these regulations despite their different nature. However, their use is accepted in special cases in countries such as the USA, France and Belgium, under the condition "compassionate use", for instance, when no other treatment is available (Naureen et al., 2020). To date, only one phage-based product against staphylococcal infections is available on EU market under the name Stafal® (Dvořáčková et al., 2019).

Regarding animal application, phage therapy has been used in animals since its discovery, primarily in preclinical experimentation. It was soon observed that a single dose of a specific *E. coli* phage could significantly reduce the number of target bacteria in the guts of calves, lambs and piglets infected with a diarrhoea-causing *E.coli* strain (Williams Smith et al., 1987; Williams Smith & Huggins, 1983). With the growing concern over antimicrobial substances, phage therapy regained attention as an alternative of preventing and treating PWD. Recent studies have shown that administering bacteriophages targeting ETEC $F4^+$ in weaned piglets reduces diarrhoea incidence and improves growth performance parameters (Hosseindoust et al., 2017; Lee et al., 2017; Zeng et al., 2021). Phage treatment also mitigates the harmful effects of ETEC on intestinal morphology (Lee et al., 2017; Zeng et al., 2021) and enhances intestinal barrier integrity by upregulating tight junction proteins and reducing serum D-lactate and DAO levels (Zeng et al., 2021). In terms of immune response, phage supplementation increases IgA, TGF-α and serum IL-10, while decreasing serum IL-1β (Zeng et al., 2021) and TNF-α (Lee et al., 2017; Zeng et al., 2021). Due to their high host specificity, phage therapy does not disturb other bacterial populations, and can even

beneficially modulate the microbiota. For instance, Hosseindoust et al. (2017) reported increased relative abundance of *Bifidobacterium* and *Lactobacillus*, and a reduction in *E.coli* and *Clostridium* in the ileum (Hosseindoust et al., 2017).

However, the efficacy of this therapy is significantly influenced by factors such as the animal's age, transmission route, timing, frequency, and concentration of administration, as well as the viability of the bacteriophages. Digestive enzymes, extreme pH levels, high temperatures, host immunity and other environmental factors can reduce bacteriophage viability. A major limitation of bacteriophage therapy is its narrow host range. Unlike controlled experimental conditions where the pathogen strain is well-defined, field conditions exhibit variability in bacterial strains between farms and even outbreaks, making it difficult to target the therapy precisely (Canibe et al., 2022).

2.4.2. Nanobodies

In 1993, researchers discovered a unique type of antibodies in camelids that lack the light chain (Hamers-Casterman et al., 1993) (Figure 2.5). These antibodies were also found in the serum of sharks and rodents (Greenberg et al., 1995; Rast et al., 1998). The single variable domain of these camelid-type antibodies is now known as "nanobodies", a term originally trademarked by Ablynx in 2003 (Yong Joon Kim et al., 2023). Since their discovery, nanobodies have been extensively studied due to their appealing characteristics similar to conventional antibodies, such as selectivity, antigen specificity and binding affinity. Additionally, they possess unique biochemical properties, including high stability, strong permeability, low immunogenicity and small size (Van Der Linden et al., 1999; Wilken & McPherson, 2018). Nanobodies can be

conveniently administered orally as they resist gastric conditions (Yong Joon Kim et al., 2023), and they can be stored under simpler conditions, compared to antibodies. Consequently, they are considered a promising tool for diagnostics and treatment (Yu et al., 2023).

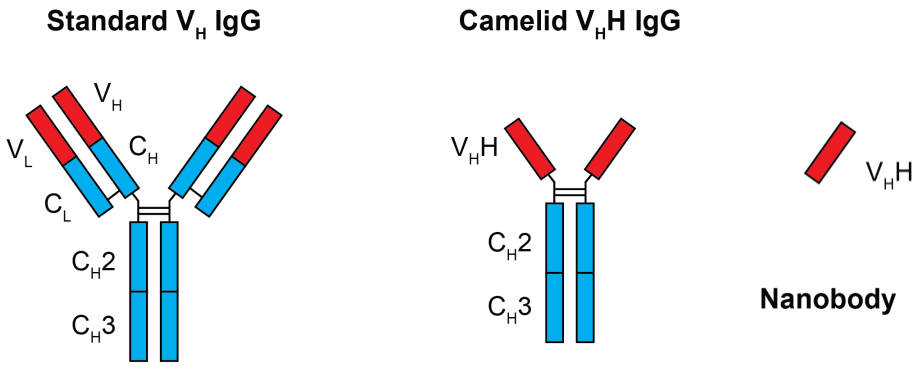


Figure 2.6. Structure of nanobodies.
Figure from https://www.ncdir.org/resources/nanobodies/

These compounds are proving valuable in the diagnosis and treatment of various bacterial, viral (Wilken & McPherson, 2018), and parasitic infectious diseases, as well as in autoimmune disorders, toxins, venoms and cancer therapies(Jin et al., 2023; Yang & Shah, 2020). Currently, due to their similarity to monoclonal antibodies, nanobodies are regulated under the same framework (EMA, 2009). To date, three nanobody-based therapies have been approved (Jin et al., 2023; Yong Joon Kim et al., 2023): Caplacizumab, for the treatment of acquired thrombotic thrombocytopenic purpura (Scully et al., 2019), Ciltacabtagene autoleucel (cilta-cel) for myeloma (Chekol Abebe et al., 2022) and Ozoralizumab, for rheumatoid arthritis, which is currently only approved in Japan (Keam, 2023).

As with other difficult-to-treat infections, nanobodies have been tested for controlling ETEC $F4^+$. In 2005, Harmsen et al. isolated a V_HH construct (nanobody) specifically targeting F4 fimbriae that successfully inhibited attachment of ETEC $F4^+$ to the intestinal brush border. They also tested its efficacy in reducing fluid loss using an *in vitro* perfusion with small intestine piglet segments. However, in an *in vivo* trial on challenged weaning piglets, no improvement in growth performance was observed (Harmsen et al., 2005).

Similarly, Fiil et al. (2022) designed a bivalent V_HH construct, with two identical antigen-binding sites to enhance bacterial agglutination, targeting the F4 fimbriae to interfere with the pathogen's attachment to the intestinal epithelium, thereby reducing its pathogenicity. They examined the impact of GI tract's conditions (temperature, pH, bile acids and physiological conditions) on V_HH to ensure its viability after gastric transit. They proposed a functional dose and conducted an *in vivo* trial with this precise V_HH construct. Despite observing a reduction of ETEC $F4^+$ in faecal samples, there were no improvements in productive parameters or diarrhoea scores in the animals (Fiil et al., 2022).

The lack of efficacy in *in vivo* trials is a common finding in various studies, highlighting several limitations. Firstly, V_HH degradation during GI tract transit is a potential issue (Harmsen et al., 2005; Reilly et al., 1997). Secondly, the monovalent nature (just one antigen-binding site) of the tested V_HH limits its ability to induce bacterial agglutination, an important mechanism in monoclonal antibodies oral immunization. Thirdly, bacteria could have other adherence factors, so targeting only F4 fimbriae virulence factor might be insufficient (Harmsen et al., 2005). Various improvements on V_HH constructs are being developed to address these limitations. For example, researchers are creating bivalent structures, or administering cocktails of V_HH constructs

that target different pathogen structures to interfere with multiple pathogen functions.

The use of nanobodies in pigs has not only been studied for treating and preventing ETEC infections but also for diagnosing and treating other viral infections. Promising results have been shown in the diagnosis of porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhoea virus, African swine fever virus, classical swine fever virus, porcine circovirus type 2, and porcine parvovirus. Additionally, nanobodies have been explored for the treatment of PRRSV and foot-and-mouth disease virus, as illustrated in Figure 2.6. Although no nanobody-based treatment is currently commercialized for the livestock sector, the growing number of studies suggests it is only a matter of time before they become available (Tohidi et al., 2024).

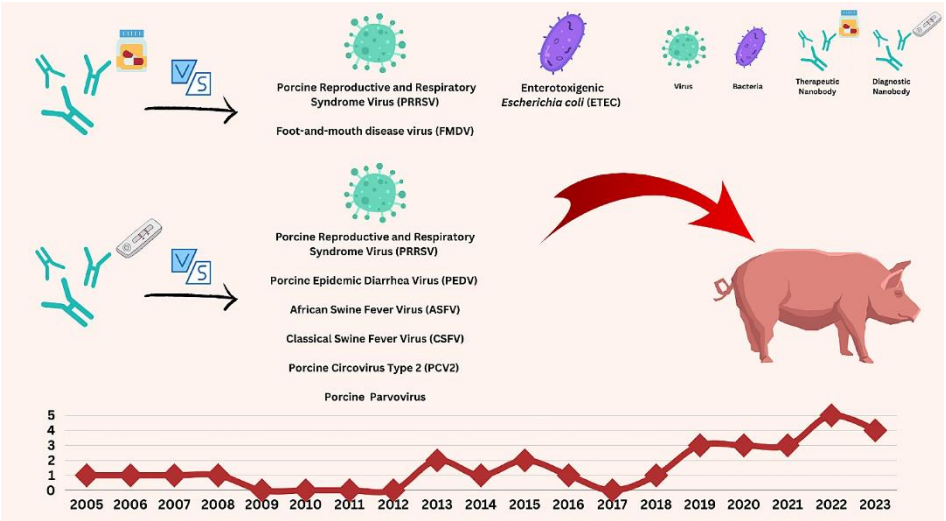


Figure 2.7. Infectious agents impacting pigs for which potential nanobodies have been developed, alongside the number of publications over the years on nanobodies targeting diseases affecting Suidae.
Figure from Tohidi et al. (2024).

A promising administration method gaining attention is producing V_HH inside the host using a probiotic. For example, Andersen et al. (2016) proposed using two engineered strains of lactobacilli to produce V_HH specifically targeting *Clostridium difficile* toxin B (TcdB) (Andersen et al., 2016). Similarly, Gangaiah et al. (2022) developed an engineered strain of *Limosilactobacillus reuteri* to produce V_HH specifically designed to neutralize NetB and α toxin of *Clostridium perfringens* (Gangaiah et al., 2022).

Despite nanobodies are still in their first clinical stages, outcomes achieved look promising, and these constructs are suggested to be one of the fastest growing categories of biologics for clinical applications (Yong Joon Kim et al., 2023).

To summarize, it is important to recognize that PWD caused by ETEC $F4^+$ is a significant issue during the weaning stage, affecting animal health and performance. Several strategies have been studied to address this condition, involving various aspects of swine production. One promising approach, further explored here, is the modulation of microbiota towards a more beneficial profile. This can be achieved using biotic additives, such as probiotics and prebiotics, at early stages of life. These compounds influence intestinal microbial populations, enhance chemical and mechanical barriers, support immune system development and maintain oxidative balance in the host.

Added to biotic additives other novel and promising strategy is the use of nanobodies specifically targeting ETEC $F4^+$ virulence factors. With a completely different mechanism of action, these compounds can interfere with pathogenic processes, reducing their virulence without altering other gut parameters.

Given their potential, further research on the application of these strategies and the optimal timing for their use is warranted.

Chapter 3. Objectives and experimental design



Objectives and experimental design

This present PhD thesis was developed in collaboration with three different companies: Laboratorios Ordesa S.L., through the TOLERA PROJECT (IDI-20170870) funded by the Center for Technological and Industrial Development (CDTI), and different research agreements of UAB with Tereos S.A. and ANH-Innovation. Following the research focus and expertise of our group, this work centers on dietary strategies based in the use of in-feed additives implemented in the early stages of piglet's life, to strengthen their intestinal health and achieve optimal performances, in the frame of reduced antibiotic use in livestock.

The main hypotheses of this PhD dissertation are:

- The rational design of synbiotic combinations administered at early stages of life may improve the intestinal health of piglets by modulating microbial colonization, developing the immune system and enhancing intestinal function.
- An oral administration of nanobody bivalent constructs designed to inactivate pathogen virulence factors may help to prevent and alleviate ETEC $F4^+$ infections and PWD clinical signs.

To assess these hypotheses, this PhD dissertation state as main objectives:

1. To assess the potential of a synbiotic additive combination in suckling piglets, composed of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosaccharide (GOS) and Human Milk Oligosaccharide (HMO), combined or not with the bioactive peptide, osteopontin; to enhance the maturation and development of the intestinal and immune system at early ages. This

evaluation involves clarifying the mechanisms of action of the additives.

2. To assess the potential of a synbiotic combination in weaning piglets, consisting of *Saccharomyces cerevisiae* Sc 47 and short-chain fructo-oligosaccharides (scFOS); to enhance intestinal health and help young animals successfully overcome possible infections. These evaluations also include elucidating the additive's mechanisms of action.
3. To assess the potential of a novel bivalent construct designed against F4 fimbria and LT and Stx2 toxins to prevent and treat postweaning diarrhoea in weaning piglets orally challenged with ETEC $F4^+$, and also in naturally infected animals, highlighting its impact on the dynamic of the disease.

To achieve these objectives, four experimental trials were conducted, described in Chapters 4 to 6.

- Chapter 4. This chapter includes a trial designed to evaluate the effect of including a synbiotic (*Bifidobacterium longum* subsp. *Infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, GOS and HMO) combined or not with osteopontin in a milk formula. The trial was designed to assess the effects of these additives when incorporated into neonates' artificial milk formulas, using a suckling piglet model. The main parameters assessed were animal performance, clinical signs, fermentation profile, immune response, gene expression, intestinal microbiota profile, and intestinal morphology.

- Chapter 5. This chapter includes a trial that evaluates the administration of a prebiotic, a probiotic and their combination as a synbiotic in weaning piglets challenged with ETEC $F4^+$. The main

parameters assessed were animal performance, clinical signs, fermentation profile, immune response, microbiological analysis, pathogen quantification and intestinal morphology.

- Chapter 6. This chapter includes two trials intended to assess the effect of bivalent V_HH constructs specifically targeting F4 fimbria and LT toxin (trial 1) plus Stx2e (trial 2) in weaning piglets challenged with ETEC $F4^+$ under experimental conditions (trial 1) or naturally infected under field conditions (trial 2). The main parameters assessed were the same as in Chapter 5.

Chapter 4. Supplementing infant milk formula with a multi-strain synbiotic and ostepontin enhances colonic microbial colonization and modifies jejunal gene expression in lactating piglets



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4.1. Introduction

The optimal nutrition for newborns is provided by human breast milk, which offers a well-balanced combination of essential nutrients, including proteins, lipids, carbohydrates, minerals, and vitamins. Additionally, breast milk contains trace elements and bioactive components crucial for meeting infants' nutritional requirements and ensuring proper growth and development (Nomayo et al., 2020; Perdijk et al., 2019). Among its various health benefits, breast milk plays a pivotal role in shaping the gut microbiota of newborns. The development of intestinal microbiota is a complex process that commences around birth and persists for the first 2-3 years of life. This process can be influenced by several early-life factors, such as gestational age, delivery mode, maternal weight, stress, and notably, diet, which significantly affects the relative proportion of bacteria (Kongnum et al., 2020; Miqdady et al., 2020; Verkhnyatskaya et al., 2019). Due to its profound impact, breast milk is recognized as the "gold standard" for infant nutrition, recommended by the World Health Organization (WHO) as the exclusive source of nutrition for the first 6 months of life (Li et al., 2019a; Perdijk et al., 2019). However, some infants are unable to receive human breast milk and, milk formula becomes the alternative. Unfortunately, most conventional cow's milk-based formulas lack bioactive components crucial for promoting proper gut bacterial colonization (Miqdady et al., 2020). Studies have shown that exclusively breast-fed infants generally exhibit a healthier microbiome compared to exclusively formula-fed infants (Davis et al., 2017; Houghteling & Walker, 2015). Nevertheless, recent advancements in milk formulas aim to simulate the functionality of breast milk by incorporating various components, including probiotics, prebiotics and different bioactive components, to enhance early microbiota establishment and maturation in newborns.

Probiotics, defined as live microorganisms that confer health benefits when consumed in adequate amounts (Hill et al., 2014), are now commonly added to infant formula. In this study, we selected two probiotic strains, *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, both present in infant gut population and recognized for their beneficial role in infant health (Miqdady et al., 2020; Turpin et al., 2010). However, the lack of long-term effects when supplemented in infant formula suggests that their inclusion as a synbiotic combination (probiotic + prebiotic) could be more effective (Bazanella et al., 2017). Therefore, in this trial, these probiotics were combined with two prebiotics. Prebiotics are defined as substrates selectively utilized by host microorganisms that confer health benefits (Swanson et al., 2020). In this work, we tested two prebiotics naturally present in human breast milk: galacto-oligosaccharides (GOS) and a human milk oligosaccharide (HMOs). Galacto-oligosaccharides (GOS) are non-digestible short-chain oligosaccharide readily fermented by lactobacilli and bifidobacteria (Wang et al., 2022a). They are well-documented to improve intestinal barrier, reduce pathogenic bacteria colonization, significantly increase short-chain fatty acid (SCFA) concentration, and modulate gene expression (Tian et al., 2019; Weng et al., 2019). Human milk oligosaccharides (HMOs) represent the third-largest solid component in human milk (following lactose and lipids), yet they are present in bovine milk at 20 times lower concentrations (Thomson et al., 2018; Vandenplas et al., 2018). Human milk contains over 200 different HMO structures, with 2'-Fucosyllactose (2'FL), 3-Fucosyllactose (3FL), 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL), lacto-N-neotetraose (LNnT), being the most important ones (Zuurveld et al., 2023). These human-specific milk oligosaccharides are intricate, non-digestible carbohydrates that reach the infant colon and have been linked to the growth enhancement of *Bacteroides*, *Lactobacillus* and Bifidobacterium (Marcobal et al., 2010; Thomson et al., 2018). They play a

role in modulating the immune system by inhibiting pro-inflammatory responses, altering intestinal gene expression (Carr et al., 2021), and directly influencing intestinal epithelial cells (Zhang et al., 2021b). According to the definition of synbiotic as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson et al., 2020), we could anticipate that the combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001 with GOS and HMOs might exhibit synbiotic properties when added to the milk formula.

Osteopontin is an acidic phosphorylated glycoprotein expressed in various cell types with multiple functionalities. It is involved in processes such as activation and regulation of the immune system, biomineralization, tissue-transformative processes and interactions with bacteria, among other (Sørensen & Christensen, 2023). Recently, osteopontin have been reported to promote intestinal proliferation and maturation, brain myelination, neurodevelopment and immune development (Jiang & Lönnerdal, 2020). Human milk has the highest concentration of this bioactive protein, being 10 times higher compared to bovine milk (Donovan et al., 2014). Osteopontin is believed to initiate and regulate developmental, immunological and physiological processes in infants, potentially playing a significant role in early-age development.

Despite extensive literature documenting the key roles of these breast milk components in infants, further research is needed to better understand their mode of action and their effects when combined in infant formulas. We hypothesize that the combination of these components could promote a synergic effect, resulting in earlier maturation of intestinal microbiota and improvement in intestinal development and functionality. Therefore, the aim of this study is to evaluate the impact of enhancing an infant milk formula by

adding the described multi-strain synbiotic and/or osteopontin on intestinal health, using a lactating pig model.

4.2. Material and methods

The experiment was carried out at the Experimental Unit of Universitat Autònoma de Barcelona (UAB) and received prior approval (permit no. CEAAH 4928) from the Animal and Human Experimental Ethics Committee (DMAH 10947). The treatment, management, housing and slaughtering conditions adhered to European Union Guidelines (Directive 2010/63/EU, European Commission, 2010). All efforts were made to minimize animal suffering.

4.2.1. Animals, housing and experimental design

A total of 96 male piglets (Large White × Landrace) × Pietrain, aged 5 days (± 0.14), from a high-sanitary-status commercial farm were included in this study. The animals were transported from a commercial farm to UAB's experimental unit.

The study was organized into four experimental periods of 15 days each, to ensure dedicated care of the animals during the initial days. In each period, 24 new piglets were introduced to UAB's facilities. Animals were chosen from 8 litters, selecting males of intermediate weight. Each period, animals were allocated to two rooms, each with four pens (3 animals per pen). The rooms were equipped with automatic heaters and forced ventilation. Each pen had individual heating lights, a dish with a tank for liquid dispensing (Minitainer, Rotecna, Spain), water nipples, and a plastic toy for environmental

enrichment. The experiment was conducted during the autumn season (Sept-Nov).

Upon arrival, animals were distributed among treatments preserving littermates. The trial followed a 2×2 factorial design, encompassing four dietary groups (with/without synbiotic × with/without osteopontin): control formula (CTR), CTR supplemented with probiotics and prebiotics (SYN), CTR supplemented with osteopontin (OPN), and CTR supplemented with the synbiotic and osteopontin (SYN+OPN=CON). Each experimental group had eight replicates across the four experimental periods, with the pen considered as the experimental unit.

4.2.2. Probiotic strains, prebiotics, bioactive peptide and diets

The tested probiotics were *Bifidobacterium longum* subsp. *infantis* CECT 7210, supplied by Laboratorios Ordesa S.L., and *Lactobacillus rhamnosus* HN001 from Danisco USA Inc. Both strains were supplied in lyophilized form, with concentrations of 5×10^{10} and 3×10^{10} colony forming units (CFU) per gram of product, respectively, in a maltodextrin base. Administered in the milk formula, the final dosage was 6.36×10^{8} and 1.09×10^{8} CFU/g, respectively. Before the trial, probiotic viability was confirmed through plate counting immediately after suspension in the milk formula and at various storage times on the farm, indicating good stability for both strains up to 24h.

The experimental prebiotics consisted of a mix of galacto-oligosaccharides (GOS) and human milk oligosacharide (HMO), specifically 2'-Fucosyllactose (2'FL). GOS, in a syrup form, was thoroughly mixed with the milk formula to ensure a homogenous blend with no visual lumps. The final GOS concentration was 4.36 g/L, simulating the present amount in human milk (5-10 g/L) (Kunz et al., 2017), as indicated in previous studies (Alizadeh et al., 2016; Tian et al., 2019). HMO, in powder form, was added for a final dosage

of 0.436 g/L. The tested bioactive peptide, osteopontin, provided in powder form, was added for a final dosage of 0.436 g/L.

The milk formula given to piglets was tailored to their nutritional requirements and manufactured ad hoc by Cargill for this study. The formula excluded acidifiers, any other probiotic strains, or derivatives different from those tested, and it reduced immunoglobulin levels. The formula's main ingredients included powdered lactose, whey powder, soybeans' protein concentrate, porcine plasma meal, wheat hydrolysed gluten, sucrose, wheat, monopotassium phosphate, calcium carbonate, fructo-oligosaccharides and magnesium oxide. The chemical composition details are provided in Table 4.1. Milk was made available continuously, and fresh milk was prepared three times per day (at 7am, 1pm and 7pm). Following milk reconstitution, all additives were incorporated and manually mixed until achieving complete homogeneity. Prior to introducing the probiotic strains, careful verification ensured that the milk temperature remained below 37°C.

Table 4.1. Milk formula composition.

Analytical constituents	(%)
Crude protein	20.9
Crudes oils and fats	10.5
Crude fibre	0.10
Ash	9.50
Calcium (Ca)	0.57
Phosphorus (P)	0.79
Sodium (Na)	0.78
Lysine	1.75
Methionine	0.50

Nutritional additives/kg: 25000 IU vitamin A; 5100 IU vitamin D3; 150 mg vitamin E; 87.0 mg Fe, 130 mg Cu, 50.0 mg Zn, 100 mg Mn, 2.0 mg I, 0.25 mg Se.

4.2.3. Experimental procedure

Each experimental period lasted 15 days, during which animals were introduced to the experimental diets upon arrival. Daily observations were conducted, and any clinical signs such as apathy, diarrhoea, dehydration, or anorexia were recorded. No antibiotic treatment was administered.

Animal performance was monitored, with individual life weight and feed intake recorded daily. Weekly calculations were made for ADG and ADFI. Faecal scores were assessed per pen using the following scale: 1 = solid and dry; 2 = well formed; 3 = very soft or viscous liquid; 4 = watery greyish; 5 = watery yellowish.

For microbiota sequencing, faecal samples were collected on days 3, 9 and 15 through spontaneous defecation or digital stimulation, encompassing all animals (n = 96).

At the end of the trial, two piglets per pen, chosen for higher weight or healthier condition, were euthanized. Euthanasia and subsequent sampling occurred in the morning (between 9:00 and 13:00h). Remaining animals were euthanized at the end of each experimental period. Deep sedation was induced by intramuscular injection of 20 mg/kg ketamine (Ketamidor; Wels, Austria) and 2 mg/kg of xylazine (Xilagesic; Les Franqueses del Vallès, Spain). Blood collection tubes without anticoagulant (Aquisel; Madrid, Spain) and with anticoagulant (Aquisel; Madrid, Spain) were used to collect 10 ml of blood from the cranial cava vein of each animal. After blood sampling, animals were euthanized with an intravenous injection of 200 mg/kg sodium pentobarbital (Euthasol; Le Vet B.V., Oudewater, The Netherlands). Afterwards, animals were bled, the abdomen was immediately opened, and the gastrointestinal tract was excised and transferred to a tray.

Content from the ileum and proximal colon was collected and homogenized, and their pH measured (Crison 52 - 32 electrode, Net Interlab; Barcelona,

Spain). For ammonia determination, ileal and colonic digesta aliquots were store at -20°C in 0.2N H2SO4 as a preservative solution (3 ml of digesta + 3 ml of 0.2 N H2SO4). Another subsample was kept at -20°C for SCFA and lactic acid determination.

Jejunal tissue samples were collected for gene expression study. Approximately 0.5 cm2 tissue from the jejunum was preserved in RNAlater®, initially in the fridge for 24 hours and subsequently at -20°C.

Additionally, jejunal sections of 2 - 3cm length were cut, opened longitudinally, washed thoroughly with sterile PBS, and fixed by immersion in a 4% formaldehyde solution (Panreac; Castellar del Vallès, Spain) for histomorphology.

4.2.4. Analytical procedures

4.2.4.1. Short-chain fatty acids, lactic acid and ammonia analysis

Short-chain fatty acids and lactic acid determinations were conducted on ileal and colonic content using gas-liquid chromatography (GLC). The samples underwent an acid-base treatment followed by ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide (MBTSTFA) plus 1% tertbutyldimethylchlorosilane (TBDMCS) agent, following the method outlined by Richardson et al.'s method (1989) modified by Jensen et al. (1995).

Ammonia concentrations in ileal and colonic content were determined using a gas-sensitive electrode (Hatch Co.; CO, USA) paired with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain). For the determination process, preserved samples were homogenized and centrifuged at 1500 × for 10 min. Subsequently, samples were diluted 1:4 in distilled water based on ammonia concentration. The ammonia released

after the addition of 10M NaOH was measured in the supernatant as a change in voltage (mV) using a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.).

4.2.4.2. Blood analysis

Blood samples were centrifuged (2500 × g, 8 min at 4°C) after 4 hours of refrigeration, and serum samples were stored at -20°C until analysis. Tumor Necrosis Factor-α (TNF-α) concentration in serum samples was analysed using the Quantikine Porcine TNF-α kit (R&D Systems; Minneapolis, United States) with enzyme-linked immunosorbent assay (ELISA).

4.2.4.3. Histological analysis

Histological studies were conducted on jejunum, ileal, and colonic samples. Tissues were dehydrated, embedded in paraffin wax, sectioned at 4 µm, and stained with hematoxylin and eosin. Morphological measurements were made with a light microscope (BHS, Olympus) following the method outlined by Nofrarías et al. (2006). Parameters measured included villus height (VH), crypt depth (CD), the ratio of villus height:crypt depth (VH:CD), intraepithelial lymphocytes (IEL), goblet cells (GC) and mitosis (M). Between 7 and 10 intact villi were measured per animal.

4.2.4.4. Gene expression analysis

Gene expression was quantified by quantitative PCR (qPCR) to assess the expression of 56 genes in jejunal tissue using an Open Array Real-Time PCR Platform (Applied Biosystems, Waltham, MA, USA), following the method described by González-Solé et al. (2020) in the Veterinary Service of

Molecular Genetics at the Veterinary Faculty of the Universitat Autònoma de Barcelona (Spain).

A total of 56 genes involved in multiple physiological functions related to intestinal health were analysed and categorized into groups: 1) genes responsible for maintaining the intestinal barrier function, including OCLN, ZO1, CLDN1, CLDN4, CLDN15, MUC2, MUC13, and TFF3; 2) genes crucial for immune responses, including pattern recognition receptors, cytokines, chemokines, and stress proteins, including TLR2, TLR4, IL1B, IL6, IL8, IL10, IL17A, IL22, IFNG, TNF, TGFB1, CCL20, CXCL2, IFNGR1, HSPB1, HSPA4, REG3G, PPARGC1A, FAXDC2, and GBP1; 3) genes encoding enzymes and hormones involved in the digestive process, such as GPX2, SOD2, ALPI, SI, DAO1, HNMT, APN, IDO1, GCG, CCK, IGF1R, and PYY; 4) genes participating in nutrient transport, including SLC5A1, SLC16A1, SLC7A8, SLC15A1, SLC13A1, SLC11A2, MT1A, SLC30A1, and SLC39A4; and 5) genes associated with stress responses, specifically CRHR1, NR3C1, and HSD11B1. Detailed information regarding genes is provided in Supplementary materials (Table S4.8).

4.2.4.5. Microbiota sequencing

Microbiota studies were conducted on faecal samples obtained on days 3, 9, and 15. The V3-V4 region of the 16S rRNA gene was amplified and sequenced.

For this method, DNA was extracted with the QIAamp Fast DNA Stool Mini Kit (Qiagen; West Sussex, United Kingdom), following manufacturer's protocol. Subsequently, microbiome analysis was performed by sequencing 16S (Illumina MiSeq). For this procedure, a total of 50 ng of DNA was amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). In the first amplification step, primers were designed containing: 1) a universal linker sequence allowing amplicons for

incorporation indexes and sequencing primers by Nextera XT Index kit (ILLUMINA); and 2) 16S rRNA gene universal primers (Klindworth et al., 2013). In the second and last assay amplification indexes were included. 16S based libraries were quantified by fluorimetry using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher). Libraries were pooled prior to sequencing on the NovaSeq platform (Illumina), 300 cycles paired reads configuration. The size and quantity of the pool were assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 20%). Image analysis, base calling and data quality assessment were performed on the NovaSeq instrument (NovaSeq Control Software (NVCS v1.7). Raw sequences, forward (R1) and reverse (R2), were imported into QIIME2 plattform (Bolyen et al., 2019). Cutadapt v3.4 (Martin, 2011) plugin was used to filter specific V3-V4 16S region adapters. R1 and R2 reads were processed using 'denoise-paired' command of DADA2 plugin (Callahan et al., 2016). Low-quality reads were filtered by the function 'filterAndTrim' and were truncated where they started to lose quality (240 for R1, 200 for R2). Error models were generated using 'learnErrors' function, and DADA2 algorithm was applied using 'dada' function. ASVs ('Amplicon Sequence Variants') generated by R1 and R2 reads were merged using 'mergepairs' function. Quimera sequences were removed 'removeChimeraDenovo' function. Taxonomy of resulting ASVs was annotated using blastn v2.2.29+ (Altschul et al., 1990) against 16S specific database from the NCBI (v. August 2021). Assigned taxonomies with an identity percentage lower than 97% were reassigned using NBAYES algorithm (Bokulich et al., 2018) against SILVA v.138 16S database.

4.2.5. Statistical analysis

The effects of experimental treatments on various parameters (excluding microbiology) were analysed using the free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA) with the stats package.(Team, 2014) An ANOVA was conducted (lm function for a two-way ANOVA) to determine the main effects of synbiotic addition, osteopontin addition, or any possible interaction. The following generalized linear model was employed:

$$Y_{ijkl} = m + Synbiotic_i + Oseopontin_j + (Synbiotic \times Osteopontin)_{ij} + Period_k + e_{ijkl}$$

Where Y represents each observation of the studied variable, m is the global mean, $Synbiotic_i$ is the main effect of adding the synbiotic to the diets, $Osteopontin_j$ is the main effect of adding the osteopontin to the diets, Synbiotic × $Osteopontin_{ij}$ is the possible interaction, and $Period_k$ is the main effect of the period. Finally, e_{ijkl} is the experimental error term.

For the analysis of the evolution of ADFI and faecal scores along time, a mixed-effects model (lme function) was used following:

$$Y_{ijk} = m + Diet_i + Time_j + (Diet \times Time)_{ij} + e_{ijk}$$

Where Y is each observation of the studied variable, m is the global mean, $Diet_i$ is the main effect of the experimental diets, $Time_j$ is the main effect of the day, and Diet × $Time_{ij}$ is the possible interaction. Finally, e_{ijk} is the experimental error term.

When the effects of additives were established ($p < 0.05$), the least squares means were separated using the probability function of differences adjusted by Tukey-Kramer.

The analysis of the percentage of casualties was conducted through a frequency analysis using a Fisher test (fisher.test function) with the same statistical package.

The pen was considered the experimental unit for all parameters, and results are expressed as means with their standard errors unless otherwise stated. The α level used to determine statistical significance was $p = 0.05$, with statistical trend levels between $p = 0.05 – p = 0.10$ considered.

In the gene expression study, the Open Array data were prepared using ThermoFisher Cloud 1.0 software (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method for relative quantification was applied, using the geometric mean of 4 reference genes (ACTB, B2M, GAPDH and TBP) and a reference sample with a representative profile from the CTR group as a calibrator. The maximum allowed cycle relative threshold was set to 26, amplification score < 1.240, quantification cycle confidence > 0.80, and maximum allowed SD between duplicates was set to < 0.38. Three samples showing inadequate amplification were removed. All data underwent a logarithmic transformation to approximate the Gaussian distribution. Statistical analysis were performed using free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA). A two-way ANOVA was conducted following the generalized linear model mentioned before, without considering period effect. Subsequent pairwise post hoc comparisons of treatments were executed using Tukey's honest significant difference test (Tukey, 1949). Statistical differences for treatments were considered when p-values were under 0.05 for the ANOVA and Tukey’s analysis.

A principal component analysis (PCA) was performed using samples as cases and gene log10-expressions as variables, following the method described by González-Solé et al. (2020).

Microbiota sequencing data were treated as follows. The relative taxonomic abundances of the samples were displayed with collapsed histograms plotted by 'ggplot2' library in R (v.4.0). Data were normalized using the rarefaction technique from Phyloseq R package (Weiss et al., 2017) to perform alpha diversity analysis. Shannon, Simpson and Richness indexes were calculated using 'vegan' R package (Oksanen & Simpson, 2009). Boxplots were performed by 'ggplot2' library in R (v.4.0), and Wilcox test was used to find significant differences in alpha diversity between groups.

To illustrate taxonomic dissimilarities based on ASVs, Principal Coordinate Analysis (PCoA) across the samples was carried out using the Bray-Curtis distance matrix. The effects of the factors on taxonomy were evaluated with a permutational multivariate analysis of variance (PERMANOVA) using a Bray-Curtis dissimilarity matrix that was previously calculated considering the relative abundances of functional categories in all samples. The Bray-Curtis dissimilarity matrix and PERMANOVA analysis were performed using 'vegan' R package. PCoA plot was constructed using 'ggplot2'. DESeq2 (Love et al., 2014) was used for biomarkers identification between the different diets on each of the days. A taxon was considered differentially abundant if the corrected p-value > 0.1 and if it was present on at least 50% of the samples of one of the compared groups. Heatmaps were constructed using 'ComplexHeatmap' R package (v.2.10.0). MaAslin2 (Mallick et al., 2021) was used to study correlations between microbial abundances and organic compounds, gene expression and histology parameters in day 15 samples. A linear model test was performed for each variable, with the variable as fixed effect. The microbial taxa counts were normalized using DESeq normalization

method, and the normalized counts were log-transformed. Only taxa present in more than 10% of the samples were considered.

4.3. Results

In general terms, the trial proceeded as anticipated. Upon arrival, all animals exhibited good health. However, considering the challenges of rearing early-age piglets without the sows, the mortality rate at the end of the study reached 15.6%, with 5, 1, 3, and 6 casualties for the CTR, SYN, OPN, and CON treatments, respectively. The causes for mortality included: 2 humanitarian euthanasians due to acute meningitis (post-mortem diagnosis), from CON treatment; 1 humanitarian euthanasia due to exudative epidermitis (post-mortem diagnosis), from CON treatment; 10 casualties due to acute diarrhoea and dehydration (6 humanitarian euthanasians, 4 sudden deaths), 3 from CTR, 1 from SYN, 3 from OPN and 3 from CON; and 2 humanitarian euthanasia undiagnosed, from CTR treatment. No significant differences in mortality could be attributed to the experimental treatments (Fisher-test p-value > 0.18).

In total, 8 piglets needed subcutaneous hydration: 3 for CTR (2 unsuccessful); 2 for SYN (1 unsuccessful); 2 for OPN (unsuccessful); 1 for CON (unsuccessful). No significant differences could be attributed to the treatments (Fisher-test p-value = 1).

4.3.1. Performance parameters

Results for LW, average daily feed (milk) intake (ADFI) and ADG are presented in Table 4.2. Regarding the live weight of the animals, there were no differences due to treatments, but there were differences related to the experimental period at day 0 (1940, 2414, 2666, 2563 g for period 1-4

respectively, p = 0.005). This was related to the age at which animals arrived at the farm. In the first and third periods, animals arrived at 4 days of life, whereas in the second and fourth periods, they arrived at 5 days of life.

Regarding milk intake, the animals adapted well to artificial lactation, and all animals learned how to consume milk from the plates after the first 24 hours. Milk intakes could be considered within normal limits, showing a linear increase over time (Figure 4.1.). Milk intakes during the first week exhibited variations related to the period, but not with the treatments or the interaction. In the second week, a higher milk intake was observed in animals supplemented with osteopontin as these animals consistently exhibited a higher milk consumption on days 12, 13, and 14 (Figure 4.1.).

The ADG of the animals also fell within the normal range, although with a high variability between litters (pens). No differences were observed in ADG in relation to the additives or the period.

Ultimately, no significant differences in faecal consistency were noted among treatments over time (mean values of 2.9, 2.6, 2.6, 2.7 for CTR, SYN, OPN, CON respectively, p = 0.739). However, upon examining the percentage of days when animals (pens) exhibited diarrhoea (defined as a score ≥ 4), a notable effect was observed with the synbiotic supplementation. Animals consuming the synbiotic additives experienced a lower percentage of days presenting diarrhoea (Table 4.3).

Table 4.2. Effect of experimental treatments on growth and milk consumption.
Life weight (LW) (g) at the study's onset, day 7, and day 15 (end of study); average daily feed intake (ADFI) (ml/day) and average daily gain (ADG) (g/day) during the first experimental.

Main effects	Osteopontin - Synbiotic -	Osteopontin - Synbiotic +	Osteopontin + Synbiotic -	Osteopontin + Synbiotic +		*p-value*			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Period**
LW (g)									
D0	2376	2436	2390	2383	367.5	0.839	0.885	0.798	**0.006**
D7	3358	3524	3603	3425	325.5	0.692	0.389	0.313	0.193
D15	4628	4756	5028	4794	536.8	0.225	0.120	0.954	0.549
ADFI (ml)									
D0-d7	1075	1030	1122	1080	108.2	0.172	0.161	0.670	0.004
D8-D15	1504	1397	1788	1626	189.5	0.057	**0.002**	0.746	0.604
total	1290	1214	1450	1351	133.0	0.061	**0.005**	0.962	0.369
ADG (g)									
D0-D7	140.4	155.5	173.3	148.9	46.49	0.691	0.389	0.313	0.193
D8-D15	181.4	175.9	203.6	195.6	89.83	0.883	0.549	0.906	0.194
total	160.9	165.7	188.4	172.2	38.35	0.680	0.228	0.452	0.545

Osteopontin +/-: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates p < 0.05 and statistical difference present. RSE, residual standard error.

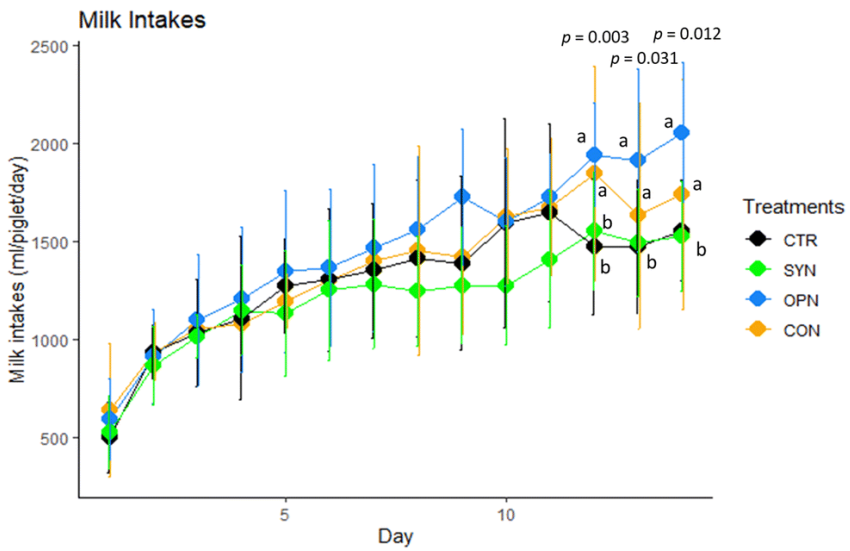


Figure 4.1. Effect of experimental treatments on milk consume with standard error bars.
Milk intakes (ml) per piglet per day during the 15-day trial. **CTR (in black)**: no additives in the diet; **SYN (in green)**: prebiotic + probiotic additives in the diet; **OPN (in blue)**: osteopontin in the diet; **CON (in orange)**: synbiotic + osteopontin in the diet. n = 8.
[a,b] Indicate statistically significant differences between means.

Table 4.3. Effect of experimental treatments on the percentage of days animals were presenting diarrhoea.
Considering diarrhoea as a score ≥ 4.

Main effects	Osteopontin - Synbiotic -	Osteopontin - Synbiotic +	Osteopontin + Synbiotic -	Osteopontin + Synbiotic +		*p-value*			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Period**
% of days presenting diarrhoea	39.98	25.53	46.63	26.65	18.25	**0.039**	0.612	0.716	0.062

Osteopontin +/-: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and statistical difference present. RSE, residual standard error.

4.3.2. Intestinal fermentation

Concerning the fermentation profile in the ileum and colon, 9 piglets exhibited a substantially altered profile (outliers). Analyzing the history of these animals in the sampling book revealed that they had very little digesta content along gastrointestinal tract (almost empty) and/or presented yellowish ileal content (clear signs of diarrhoea). Therefore, these animals were excluded from the NH3 and SCFA data subset. These exclusions were as follows: 3 from the CTR group (periods 1 and 2), 2 from SYN (period 3), 2 from OPN (periods 1 and 3) and 2 from CON (period 1). No animals were excluded from the period 4.

The results of fermentation products in ileal and colonic digesta after excluding these individuals are presented in Table 4.4. Concerning ammonia levels, there was a significant interaction for ileal concentration exhibiting lower values only when all additives were combined in the CON treatment. Significant reductions were observed in colonic ammonia concentration attributed to the synbiotic and the osteopontin supplementation that showed an additive effect in the CON treatment (no significant interaction).

Lactic fermentation predominated in the ileum, with no significant differences noted due to treatments, neither in SCFA. In the colon, however,

significant changes were evident concerning diets. The addition of the synbiotic trended to increase total SCFA and acetate and significantly increased propionate, butirate and valerate. Osteopontin supplementation significantly increased total SCFA and acetate concentrations, showing a trend for butyrate. No significant interactions were found showing additive effects for SCFA, acetate and butyrate.

Regarding molar ratio, a significant interaction was found for acetate that only was increased when both additives were added separately.

Lactic acid in the colon was only detected in some animals, with 63.5% below the minimum level of detection (1.69 mM/kg).

Table 4.4. Effect of experimental treatments on ileal and colonic fermentation.

Main effects	**Osteopontin - Synbiotic -**	**Synbiotic +**	**Osteopontin + Synbiotic -**	**Synbiotic +**		***p-value***			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Period**
Ileum digesta (mM/kg FM)									
Total SCFA	4.02	4.57	4.66	5.16	2.485	0.746	0.340	0.802	**0.039**
Acetate	3.91	4.49	4.60	5.08	2.413	0.725	0.308	0.766	**0.037**
Lactate	20.6	22.3	19.4	20.2	7.449	0.148	0.954	0.495	0.498
mmol NH_3/kg MF	2.99^{xy}	3.64^{x}	3.53^{xy}	2.69^{y}	0.91	0.676	0.453	**0.011**	**<0.001**
Colonic digesta (mM/kg FM)									
Total SCFA	69.0	83.9	89.3	105.4	31.04	0.053	**0.026**	0.834	**0.006**
Acetate	44.5	54.4	58.0	66.5	20.19	0.067	**0.032**	0.958	**0.003**
Propionate	14.8	19.6	18.6	22.3	6.00	**0.005**	0.190	0.548	0.558
Butirate	6.90	8.87	8.54	12.17	4.53	**0.019**	0.059	0.511	0.056
Valerate	1.50	2.40	2.00	2.54	1.00	**0.040**	0.110	0.903	0.214
Total BCFA	1.50	1.86	2.11	1.88	0.92	0.701	0.292	0.271	**0.041**
Lactate[1]	13.02	3.70	10.93	6.04	21.33	-	-	-	-
Molar ratio of SCFA (%)									
Acetate	63.71	68.03	66.58	63.13	8.50	0.211	0.685	**0.035**	**0.022**
Propionate	21.56	20.35	19.68	21.83	5.51	0.153	0.277	0.715	0.369
Butirate	10.76	10.27	9.29	10.92	2.56	0.350	0.245	0.406	**0.007**
Valerate	1.88	2.57	2.26	2.36	1.00	0.768	0.329	0.339	0.346
BCFA	2.24	2.05	2.19	1.76	1.00	0.285	0.490	0.574	0.081
mmol NH_3/kg MF	274.8	78.3	147.2	37.25	161.9	**0.004**	**0.033**	0.119	**0.003**

SCFA, Short-chain fatty acids; BCFA, branched-chain fatty acids. **Osteopontin +/-**: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and

statistical difference present. [x,y] Indicate a statistical trend between means. RSE, residual standard error. [1] It is not possible to provide the statistics since in many animals the values were not detectable. Number of detectable animals were 6, 5, 3 and 5 for CTR, SYN, OPN and CON respectively.

4.3.3. Intestinal histomorphology and serum TNF-α

Histomorphometry analysis of the jejunum, ileum and colon included parameters such as villus height (VH), crypt depth (CD), ratio between villus height:crypt depth (VH:CD), intraepithelial lymphocytes (IEL) (only in the villus), goblet cells (GC) (only in the villus) and mitosis (only in the crypt) (Table 4.5).

In the jejunum sections, a period effect was observed (data not shown). The number of GC showed abnormally high values with the CON treatment in the third period but not in the other periods.

In the ileum, no significant differences were observed (data not shown).

In the colon, the addition of osteopontin significantly affected the number of IEL with lower numbers. An effect of the synbiotic addition was observed regarding mitosis with lower mitosis counts.

No statistically significant effects were detected in the serum levels of TNF-α (data not shown).

Table 4.5. Effect of experimental treatments on jejunal and colonic histomorphology.

Main effects	Osteopontin - Synbiotic -	Osteopontin - Synbiotic +	Osteopontin + Synbiotic -	Osteopontin + Synbiotic +		*p-value*			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Period**
Jejunum									
VH (µm)	236	319	319	291	67.59	0.344	0.344	0.098	0.134
CD (µm)	181	177	185	172	20.98	0.254	0.791	0.458	0.111
VH:CD	1.33	2.00	1.80	1.78	0.503	0.364	0.118	0.419	0.228
IEL (cell nº)	8.53	14.1	12.9	11.0	5.046	0.294	0.655	0.070	0.339
GC (cell nº)	2.20	3.72	2.95	4.34	1.784	0.452	0.920	0.337	**0.010**
M (cell nº)	1.75	1.44	1.42	1.33	0.701	0.338	0.360	0.946	0.604
Colon									
CD (µm)	278	270	269	284	35.06	0.916	0.736	0.497	0.602
IEL (cell nº)	2.72	1.98	1.49	1.32	0.866	0.263	**0.028**	0.688	0.114
GC (cell nº)	26.4	24.3	24.8	28.3	5.336	0.697	0.560	0.168	0.760
M (cell nº)	0.257	0.157	0.219	0.085	0.123	**0.043**	0.326	0.794	0.702

Measured parameters: villous height (VH); crypt depth (CD); ratio villous height:crypt depth (VH:CD); intraepithelial lymphocytes (IEL); goblet cells (GC); mitosis (M). **Osteopontin +/-**: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. n = 8. *Indicates $p < 0.05$ and statistical difference present. RSE, residual standard error.

4.3.4. Gene expression

The genes exhibiting a significant treatment effect are listed in Table 4.6, while comprehensive results for the entire gene set can be found in Supplementary materials (Table S4.7).

Within the category of genes associated with enzyme/hormone functions, a significant interaction was observed between additives on intestinal alkaline phosphatase (ALPI) and sucrase-isomaltase (SI) genes, with higher expression in animals only when the synbiotic was supplemented alone (SYN treatment). Significant difference associated to the synbiotic supplementation was noted in the cholecystokinin gene (CCK) gene, with higher expression observed. Among the group of digestive enzymes, an interaction effect was found for indoleamine 2,3-dioxygenase (IDO1) gene, resulting in similar lower expression levels for all three supplemented diets compared to the CTR diet.

Regarding proglucagon (GCG), an effect was observed with osteopontin supplementation, resulting in higher expression. In the immune response related genes, results indicated a significant interaction for interleukin 1 beta (IL-1β) with all three experimental treatments showing similar lower values compared to the CTR group. Transforming growth beta factor 1 (TGF-β1) expression also exhibited an interaction effect with reduced levels only when the additives were supplemented separately (SYN or OPN diets). The toll-like receptor 4 (TLR4) gene also showed a significant interaction exhibiting lower expression in the SYN treatment compared to the CTR treatment, with intermediate values observed in the OPN and CON treatments. Lastly, the guanylate binding protein 1 (GBP1) presented lower expression when the synbiotic was supplemented. Regarding the expression of nutrient transport function genes, (SLC13A1, SLC15A1, SLC5A1 and SLC7A8), the synbiotic supplementation promoted a higher expression. In terms of barrier function genes, mucin 13 (MUC13) and mucin
2 (MUC2) displayed an interaction effect. Specifically, when the synbiotic was supplemented alone (SYN diet) resulted in higher values for MUC13, while lower values for MUC2. Ocludin (OCLN) was also more expressed with the synbiotic supplementation.

A principal component analysis (PCA) was conducted to assess the degree of correlation among gene expression values across samples belonging to the four different diets, as illustrated in Figure 4.2. The sample identifier numbers are visually represented in the individual factor map (a), with colours corresponding to the assigned diets for each sample. In the variables factor map (b), the diets that exhibit correlations across the gene expression are depicted.

The findings reveal a discernible correlation in the patterns of gene expression within this tissue. In Figure (a), samples are visually grouped by

treatments in 2D space. Figure (b) clearly shows how the experimental diets with additives differ from the CTR in relation to gene expression.

Table 4.6. Effect of experimental treatments on jejunal gene expression.
Values are expressed relative to a reference sample of the control treatment. The average values for the treatments are presented without the logarithmic transformation employed during statistical analysis.

Main effects	**Osteopontin - Synbiotic -**	**Osteopontin - Synbiotic +**	**Osteopontin + Synbiotic -**	**Osteopontin + Synbiotic +**		***p-value***			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Period**
Genes									
ALPI	1.140^{b}	2.204^{a}	1.859^{a}	2.091^{a}	1.588	**0.001**	0.084	**0.027**	EH
CCK	2.07	2.252	1.823	2.655	1.581	**0.025**	0.897	0.257	EH
CXCL2	1.286	0.543	0.586	0.829	2.194	0.066	0.307	0.075	IR
GBP1	1.415	0.781	0.9	0.9	2.289	**0.045**	0.347	0.217	IR
GCG	0.924	0.891	1.13	1.129	1.468	0.883	**0.040**	0.900	EH
GPX2	1.826	1.433	1.868	1.433	2.094	0.091	0.846	0.957	EH
IDO1	1.364^{a}	0.284^{b}	0.458^{ab}	0.466^{ab}	5.198	**0.024**	0.372	**0.035**	EH
IFNG	3.15	1.002	1.151	1.618	4.136	0.082	0.902	0.145	IR
IL1beta	1.276^{a}	0.406^{b}	0.393^{b}	0.560^{b}	2.389	**0.036**	0.053	**0.027**	IR
IL6	2.899	1.103	1.379	1.356	2.304	0.050	0.356	0.103	IR
MUC13	0.815	1.116	0.988	0.871	1.506	0.492	0.605	**0.015**	BF
MUC2	1.299	1.008	1.094	1.291	1.385	0.447	0.877	**0.040**	BF
OCLN	1.202	1.571	1.383	1.441	1.302	**0.041**	0.815	0.212	BF
SI	2.286^{b}	4.729^{a}	2.771^{ab}	3.569^{ab}	1.884	**0.004**	0.840	**0.026**	EH
SLC11A2	1.503	1.307	1.409	1.285	1.281	0.096	0.702	0.874	NT
SLC13A1	0.666^{y}	1.086^{x}	0.934^{xy}	1.113^{x}	2.255	**0.026**	0.072	0.073	NT
SLC15A1	1.632	2.440	1.893	2.171	1.490	**0.010**	0.731	0.166	NT
SLC39A4	1.24	1.753	1.5	1.529	1.448	0.146	0.566	0.072	NT
SLC5A1	1.694	2.791	1.844	2.349	1.668	**0.005**	0.909	0.215	NT
SLC7A8	1.077	2.604	2.375	3.175	3.187	**0.043**	0.296	0.082	NT
TGFbeta1	1.550^{a}	0.904^{b}	0.916^{b}	1.105^{ab}	1.583	0.108	0.163	**0.010**	IR
TLR2	0.817	0.431	0.635	0.651	2.178	0.073	0.911	0.171	IR
TLR4	2.542^{a}	1.297^{b}	1.577^{ab}	1.620^{ab}	1.755	0.068	0.465	**0.017**	IR

Osteopontin +/-: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. n = 8. Gene abbreviations detailed in Supplementary materials (Table S4.8). EH - enzyme/hormone; IR – immune response; BF – barrier function; NT – nutrient transport. *Indicates $p < 0.05$ and statistical difference present. ^{a,b} Indicate statistically significant differences between means. ^{x,y} Indicate a statistical trend between means. RSE, residual standard error.

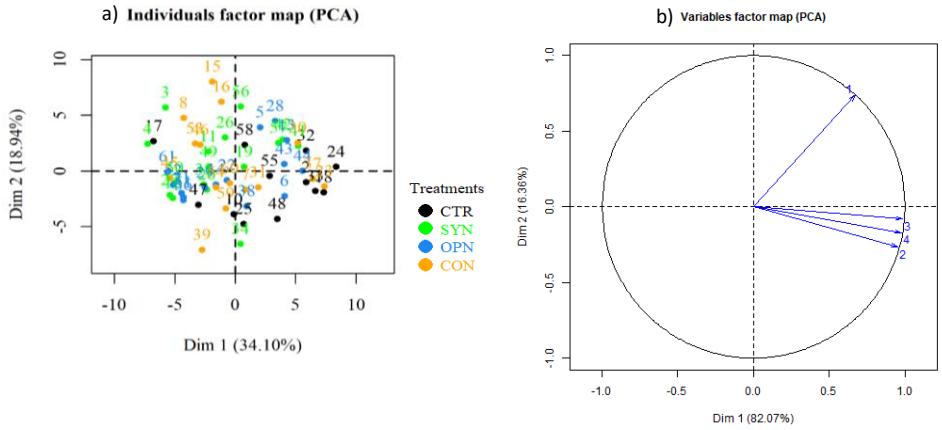


Figure 4.2. Principal component analysis (PCA) for jejunal gene expression data.
a) samples' picture from jejunum (individuals factor map); b) treatment arrow diagram from jejunum (**1 - CTR; 2 - SYN; 3 - OPN; 4 - CON**). **CTR (in black)**: no additives in the diet; **SYN (in green)**: prebiotic + probiotic additives in the diet; **OPN (in blue)**: osteopontin in the diet; **CON (in orange)**: synbiotic + osteopontin in the diet. n = 8.

4.3.5. Analysis of the microbiota by sequencing the 16S rRNA gene

To analyse the impact of experimental diets on the microbiota of piglets, faecal samples were collected on three different days: 3, 9 and 15. The rarefaction curves for all samples reached the plateau phase, indicating comprehensive identification of bacterial species. The predominant phyla in microbial communities were Firmicutes (46.8 ± 16.65%), Bacteroidetes (36.5 ± 17.86%), and Proteobacteria (9.2 ± 10.76%) (Figure 4.3). A total of 69 families were detected, with the most abundant being Bacteroidaceae (6.8 ± 6.28%), Lactobacillaceae (6.2 ± 9.03%), and Oscillospiraceae (4.4 ± 3.67%). A total of 148 genera were identified, with the 30 most abundant ones contributing to 67.6 ± 7.89% of the total abundance. Notably, *Escherichia* (6.2 ± 4.25%), *Bacteroides* (5.8 ± 3.37%), and *Phocaeicola* (5.8 ± 4.54%) exhibited the highest relative frequencies, although with a considerable variability among samples.

Regarding alpha diversity of microbiota species, it remained stable over time, and there were no significant effects of the treatment on Richness, Simpson, or Shannon indexes. However, at the beta diversity level, the PERMANOVA test revealed statistical differences related to Treatment ($R2 = 0.023$ and $p < 0.001$), although most of the variability was explained by Animal and Day (40.7% and 4.5%, respectively).

A treatment effect was observed in the bacterial populations among the different diets. Significant differences at different taxonomic level are presented in the Supplementary Materials (Figures S4.8 - S4.10). In general, synbiotic supplementation exhibited a more pronounced effect with longer administration of the additive (day 15) across various populations. These changes were evident at different taxonomic levels, including phylum, family, and genus. For instance, the phylum Synergistetes, family Synergistaceae, and genus *Cloacibacillus* showed increased abundance due to synbiotic supplementation on day 15. Conversely, the family *Sutterellaceae* and *Helicobacteraceae*, along with their genera *Sutterella* and *Helicobacter*, respectively, were reduced by synbiotic supplementation on day 15. On the contrary, osteopontin supplementation resulted in a more immediate effect on day 3, which diminished as the study progressed. At the family level, there was an increase in *Lactobacillaceae* on day 3, which disappeared by day 15. At the genus level, on day 3, there was an increase in *Oribacterium*, *Mucispirillum*, *Prevotellaceae*, and *Ligilactobacillus* due to osteopontin supplementation. However, by day 15, the genera *Mediterranea* and *Christensenella* exhibited growth promotion, while populations of *Veillonella* and *Faecalicatena* were reduced.

Figure 4.4 illustrates the most notable changes induced by the dietary interventions on specific potentially beneficial or dysbiotic genera according to the literature. As mentioned earlier, these changes gradually consolidated as the study progressed. After 15 days of supplementation, a trend was

observed wherein most of the beneficial genera increased, while a notable decrease was registered in most of the potentially dysbiosis genera. However, evolution along the experimental period was not the same for all bacterial genera. For beneficial genera, supplemented diets led to a temporary decrease on day 9, which reversed by day 15, as evidenced by *Enterococcus* and *Limosillactobacillus*. However, for dysbiotic bacteria the changes had the same trend at day 9 and 15 but consolidating at day 15. Concerning potentially dysbiotic genera at day 15, the combination of synbiotic and osteopontin demonstrated superior control over this group. A significant reduction in *Helicobacter*, *Tyzzerella*, and *Sutterella* may be attributed to the synbiotic component, whereas osteopontin appears to impact *Veillonella*. The CON diet benefits from both effects, owing to an additive synergy. However, some outcomes are not solely attributable to the additive effect but suggest an unexpected interaction. For instance, a notable decrease in *Campylobacter* was observed exclusively with the CON diet.

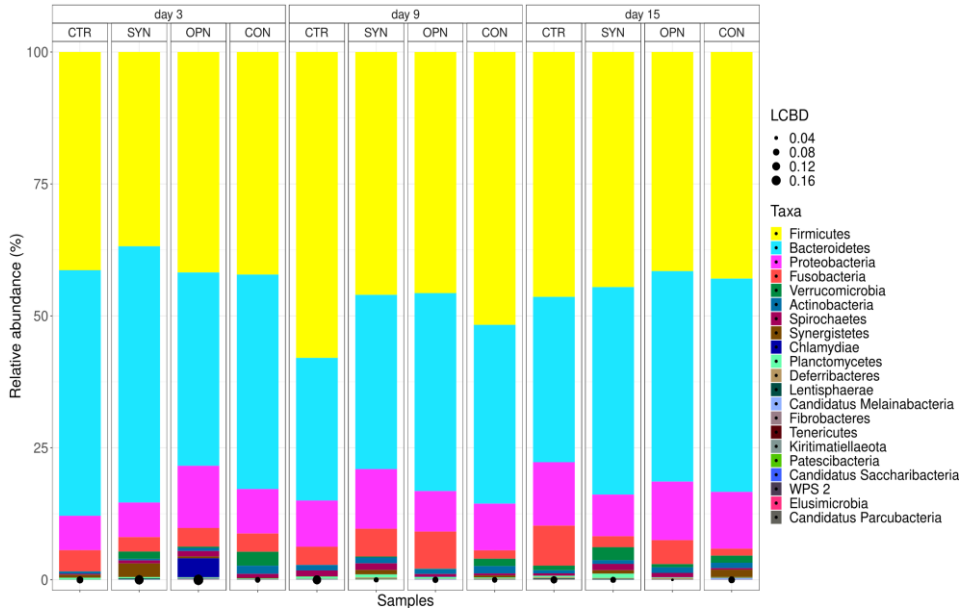


Figure 4.3. Effect of experimental treatments on microbial faecal communities (phylum).
CTR: no additives in the diet; **SYN**: prebiotic + probiotic additives in the diet; **OPN**: osteopontin in the diet; **CON**: synbiotic + osteopontin in the diet. n = 8.

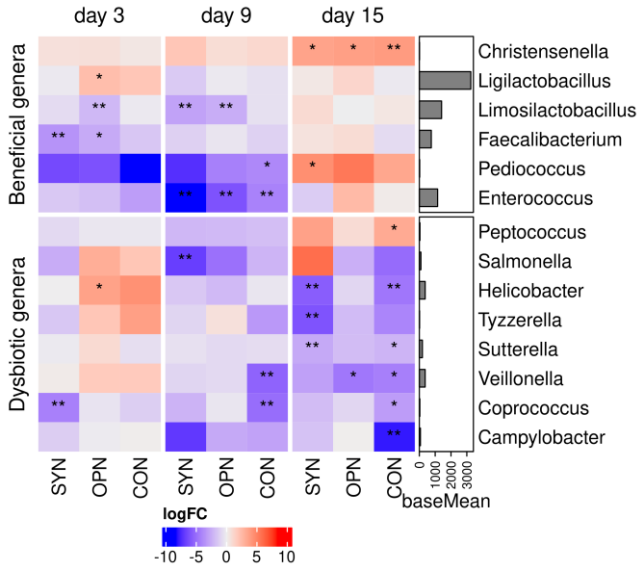


Figure 4.4. Heatmap of the impact of experimental treatments on potentially beneficial and dysbiotic genera observed in faecal samples.
CTR: no additives in the diet; **SYN**: prebiotic + probiotic additives in the diet; **OPN**: osteopontin in the diet; **CON**: synbiotic + osteopontin in the diet. n = 8. *Indicates $p < 0.1$; **indicates $p < 0.05$.

4.4. Discussion

The benefits of additives supplemented in infant formulas, as used in this trial, are well-documented in the literature. Nevertheless, our aim was to elucidate whether the combination of the synbiotic with osteopontin would result in improvements through a synergistic approach.

While there is inconsistent information about the beneficial effects of combining additives of different nature, notable achievements have been documented with the incorporation of probiotics (specifically *Lactobacillus* and *Bifidobacterium*) in conjunction with prebiotics (such as bovine milk oligosaccharides and GOS) into infant milk formulas. These combinations have successfully created an intestinal environment in infants that closely resembles that of breastfed infants, as reported by Meli et al. (2014). Regarding osteopontin supplementation in infant milk formulas, limited research has been conducted, especially in combination with a synbiotic. Nevertheless, important bioactive roles have been attributed to osteopontin (Lönnerdal, 2014) and when supplemented to infant formula, it has promoted shifts in jejunal gene expression (Donovan et al., 2014) and functional systemic benefits (Jiang & Lönnerdal, 2019) approximating a breast-fed condition. Our hypothesis, therefore, is that each additive would endorse benefits to neonates, and the combination of all would be translated into a better response.

In this trial, suckling piglets were employed as an animal model for newborn infants. Attempting to replicate the characteristics of a neonate in an animal model has proven to be intricate. However, the suckling pig has been accepted as a suitable model for human nutritional interventions (Darragh & Moughan, 1995; Litten-Brown et al., 2010; Roura et al., 2016), presenting a very similar digestive system anatomy and morphology compared to human infants (P.J. Moughan, 1992), along with similarities in digestive function and gastrointestinal fermentation profiles (Roura et al., 2016). Nevertheless, it is

important to consider the distinct predominant bacterial groups that these two species host during the early stages of development. In human infants, the intestinal gut is primarily colonized by bifidobacterial, whereas in newborn piglets, the dominant microbial community comprises lactobacilli (P.J. Moughan, 1992). Maternal passive immunity transfer also differs across species. In the case of humans, maternal antibodies are transferred to the fetus through the placenta during gestation, while in piglets, there is no passive immunity transfer. Consequently, the intake of colostrum becomes crucial for them (Goncharova et al., 2017). Despite these unavoidable differences, suckling piglets have already been successfully used as model for humans in nutritional research (Roura et al., 2016).

Supplemented formula in this study proved to be safe. Regarding performance, animals supplemented with osteopontin registered statistical higher feed intake during the second week and overall. This effect, however, was more markedly seen in the first experimental period when animals arrived at an earlier age and lighter weights, suggesting that these additives may have a greater impact in more challenging situations. The weights at the end of the study were numerically higher in all treated groups, particularly in the animals receiving osteopontin ($p = 0.120$), although this difference did not reach statistical significance. The absence of statistical differences in performance could also be attributed to the limitations of the experimental design, which was primarily intended to elucidate possible mechanisms of action rather than assess the impact of diets on performance. Alternative designs, with increased numbers of replicates and extended administration periods, may have resulted in more pronounced differences in growth. Nevertheless, while animal growth remains a reliable indicator of health, other analysed parameters can also offer valuable information in this regard. For instance, the presence or absence of diarrhoea can also be considered a

useful indicator. In this case, the addition of the synbiotic demonstrated a significant impact, reducing its prevalence.

The presence of beneficial microbiota in the hindgut is generally associated with higher concentrations of SCFA (Miqdady et al., 2020; Tian et al., 2019; Zhang et al., 2021b). In our study, the addition of both synbiotic and osteopontin had an effect increasing colonic SCFA concentration. Notably, the highest values were achieved when the additives were combined in the CON diet due to an additive effect. This result could indicate a greater development of colonic microbiota in these animals, promoted by a synergic effect of these additives. In connection with this finding, higher SCFA denotes a healthier gut environment by enhancing intestinal barrier and promoting an anti-inflammatory condition in the area (Goehring et al., 2016; Miqdady et al., 2020; Peng et al., 2009). In accordance with that, colonic IEL counts were found significantly lower when all the additives were combined in CON group. Differences in gene expression related to immune response were seen as well, although they were detected in a different intestinal compartment (jejunum). The expression of the pro-inflammatory cytokine IL-1β was found statistically lower in all three supplemented diets compared to CTR. Similar results were seen in other studies where additives of the same nature helped preserve a non-inflammatory tone of the intestinal mucosa (Ashkar et al., 2000; Meng et al., 2020; Tian et al., 2018, 2019; Weng et al., 2019; Woo et al., 2019). The expression of TGFβ1, IL1beta, GBP1 and TLR4, two inflammation-modulating cytokines (Bent et al., 2018; Vander Ark et al., 2018) and two pathogen recognition receptors (Ghosh et al., 2015; Johns & Galam, 2022), respectively, was also lower in supplemented diets. In all the cases, a downregulation suggests fewer presence of pathogens and a closer approximation to a homeostatic environment (Badia et al., 2012a; Ewaschuk et al., 2011). Continuing with gene expression in the small intestine, the findings suggest that the additives, particularly the synbiotic, influence the

expression of various genes. Genes associated with intestinal maturation and protection against pathogens (ALPI and SI) (Geddes & Philpott, 2008) increased with the synbiotic supplementation alone. The synbiotic supplementation also led to increased expression in nutrient transport genes (SCL13A1, SLC15A1, SLC5A1 and SLC7A8). Both results highlight the beneficial effect of the synbiotic combination. In terms of intestinal barrier function, existing literature suggests that both GOS and osteopontin promote higher expression in this gene group (Tian et al., 2018, 2019; Weng et al., 2019). In our study, synbiotic supplementation led to increased expression of OCLN. However, contrary to what the literature indicates (Woo et al., 2019), the addition of osteopontin did not result in increased expression of OCLN, ZO1, or mucin mRNA. Mucin 2 (MUC2) and mucin 13 (MUC13) showed an upregulation when the synbiotic was supplemented alone (SYN diet). Regarding digestive enzymes and hormones genes, such as proglucagon (GCG), osteopontin exhibited a significant upregulatory effect. This gene encodes for various peptides involved in digestive processes, with the most notable ones being glucagon and glucagon-like peptide-1 (Drucker, 2018; Sandoval & D'Alessio, 2015), which play crucial roles in regulating individual intake and satiety. Interestingly, osteopontin supplementation affects milk consumption in animals, suggesting a potential connection with the upregulation of genes related to digestive function.

This study probably represents one of the first to provide detailed information on the early gut microbial colonization in artificially reared suckling piglets. In general, the three dominant phyla present in faecal samples, regardless of the animals' age, were Firmicutes, Bacteroides and Proteobacteria, consistent with findings from other studies on weaning piglets (Chen et al., 2017; Holman et al., 2017; Pajarillo et al., 2014; Saladrigas-García et al., 2021a, 2022). However, the taxonomic pattern observed in this study during early ages (day 3) was more akin to the pattern

reported in previous studies for days 14 and 21 of age (Saladrigas-García et al., 2022). Fusobacterium phyla is typically identified as the third dominant phylum in preweaning piglets, and its abundance tends to decrease around 2-3 weeks of life. In our trial, Fusobacterium, was consistently detected in low abundances from the beginning (day 3), and the three dominant phyla remained relatively stable throughout all the sampling times. This lack of variation could be attributed to the consistent composition of the artificial formula during the trial, unlike sow milk, which undergoes slight changes in composition as lactation progresses. Furthermore, it is important to note the compositional differences between sow's milk and the milk formula used in this trial, particularly regarding its wheat content, providing a certain amount of starch. Major changes in gut microbiota are typically observed when dry feed is introduced to piglets, characterized by complex carbohydrates from dietary fibre and starch (Li et al., 2018b; Saladrigas-García et al., 2021a). A greater diversity of microbiota species is associated with a more mature gut ecosystem and a healthier environment, contributing to resilience and stability during periods of stress (Konopka, 2009; Naeem et al., 1998). However, in our trial, no significant differences in alpha diversity were observed over time (day 7 vs. day 20 of age) in any of the treatment groups. These findings contrast with those reported by Saladrigas-García et al. (2022), which showed an increase in biodiversity with age. It is possible that the lack of data in the first week of life makes it difficult to discern differences. Added to the previous comment, a milk formula that does not modulate over time may not induce the same changes in gut microbiota. Regarding beta diversity, the PERMANOVA analysis revealed significant differences due to Treatment, Day, Animal, and Period, with Animal being the predominant source of variation. This aligns with previous studies (Saladrigas-García et al., 2022; Thompson et al., 2008), which have noted a high degree of individuality among piglets at 1-2 weeks of age. It is suggested

that the gut community in young piglets is highly dynamic and tends to reach a state of greater stability around the age of 4 weeks. Regarding the influence of milk formula supplementation on microbiota composition, significant changes in family and genera abundances induced by the synbiotic were observed by day 15. In contrast, changes induced by osteopontin were predominantly noticed by day 3, diminishing later in some cases. This observation logically aligns with the distinct functions of the additives. The synbiotic, being a biotic compound, aims to gradually alter the microbiota, requiring time for noticeable effects. Conversely, osteopontin, being a peptide with multifaceted functions, appears capable of effecting more rapid changes. The most noteworthy impact induced by the additives was the improved control of potentially dysbiotic genera at day 15, predominantly observed in the CON diet group. This enhanced control could be attributed to an additive effect of the synbiotic and osteopontin. Among these potentially dysbiotic genera, it is worth noting the significant reduction observed in Campylobacter genera with the CON diet. Most Campylobacter species cannot utilise glucose and therefore rely on free amino and keto acids from the intestinal digesta for growth, making them considered proteolytic bacteria (Linz et al., 2023). The reduced levels of colonic ammonia observed in the supplemented formula, particularly with CON, support a potential shift toward a less proteolytic and more beneficial microbiota due to supplementation. The higher colonic SCFA concentrations found in CON animals reinforces this hypothesis.

Other specific microbial groups were also significantly altered by the experimental treatments; however, drawing conclusions regarding their potential use as diet health-promoting biomarkers is challenging. Identifying which microbiota would best enhance host health remains a complex area of research. Although some progress has been made in recent years, there is still much ground to cover. We now have a better understanding of the gut

colonization process in early-life piglets, which involves shifts from initial populations of LAB, such as genera like *Ligilactobacillus* or *Limosilactobacillus*, to other predominant populations as the animals grow (Saladrigas-García et al., 2021a). In this regards osteopontin supplementation was associated with increases of *Ligilactobacillus* at day 3. However, despite these insights, the variable environmental conditions influenced by individual hosts and their diets, along with the intricate interactions among microorganisms within the gut ecosystem, complicate the identification of specific microbial biomarkers. This makes it a challenging and likely oversimplified task.

To better understand these complex interactions and how gut microbiota impacts intestinal piglet health, we performed correlation analysis between data from SCFA concentration, histomorphology, and gene expression with sequenced microbiota at day 15. The most relevant correlations ($r > 0.5$; adjusted $p < 0.05$) are shown in Figures 4.5-4.7. Some significant correlations between SCFA bacteria producers and SCFA concentration were detected (Figure 4.5). For instance, *Collinsella aerofaciens*, an acetate producer (Flint et al., 2015), was positively correlated with acetate concentration; *Butyricicoccus pullicaecorum*, a butyrate producer (Singh et al., 2022), positively correlated with butyrate; *Megasphaera elsdenii*, a valerate producer (Yoshikawa et al., 2018), was positively correlated with valerate; and *Ligilactobacillus* salivarius, a lactate producer (Guerrero Sanchez et al., 2021), positively correlated with lactate.

In examining the correlation with histomorphology (Figure 4.6), it is noteworthy to consider the following genera due to their notable presence in the samples. The genus *Prevotella*, known for harboring species described as butyrate producers that tend to increase with the introduction of a solid cereal-based diet (Amat et al., 2020), showed a negative correlation with villus height in the jejunum and ileum. *Bacteroides*, a milk oligosaccharides

fermenter genus (Frese et al., 2015), exhibited a negative correlation with crypt depths in jejunum. Lastly, *Phocaeicola* was negatively correlated with intraepithelial lymphocytes in the jejunum. These findings could elucidate how bacteria interact with the host, influencing variable absorption surface, villus regenerating area, or immune modulation. Although not detected in large proportion, the recently reviewed *Akkermansia muciniphila*, a promising probiotic well-known to improve the host metabolism function and immune responses (Effendi et al., 2022), was positively correlated with jejunal and ileal villus height. In our trial, this genus was also associated with increases in CCK (digestive hormone involve in satiety) (Rehfeld, 2021) and PPARGC1α (related to obesity and oxidative stress) (Kobayashi et al., 2021) gene expression. These findings suggest a potential mode of action for this beneficial species. A higher presence of *Akkermansia* could translate into a greater surface area for intestinal absorption and enhanced communication with satiety control mechanisms. It is important to note that these data originate from distinct intestinal compartments. While the microbiota came from faeces, gene expression was obtained from jejunum tissue, and histology from the jejunum, ileum and colon. The potential influence that microbiota may exert on intestinal histology and gene expression is unlikely to be only a direct local effect. Instead, it could result from the complex interaction or cross-talk of the microbiota with the host, where, for example, the metabolites produced could instigate alterations in other regions of the intestine.

Lastly, when gut microbiota was correlated with gene expression (Figure 4.7), positive correlations were found between microbiota families known as disbyotic (*Suturella*, *Tyzzerella*) (Horne et al., 2020) with genes well-known to be linked with a pro-inflammation condition (IDO1, TLR4, TGFβ). These same microbial groups were also found to be significantly reduced by the synbiotic supplementation, suggesting that these reductions could be associated with

the lower incidence of diarrhoea registered. Similarly, microbiota families known to be beneficial (*Christensenella*) (Mazier et al., 2021) and that were increased with all the supplemented formulas, were negatively correlated with pro-inflammatory genes (IL6, TLR4, TGFβ). This discovery proposes a way of exerting effects, where the microbiota directly or indirectly seems to modulate different gene expression, developing an inflammatory habitat or not. Complementary information regarding correlation analysis can be found in Supplementary materials (Figures S4.11-S4.16).

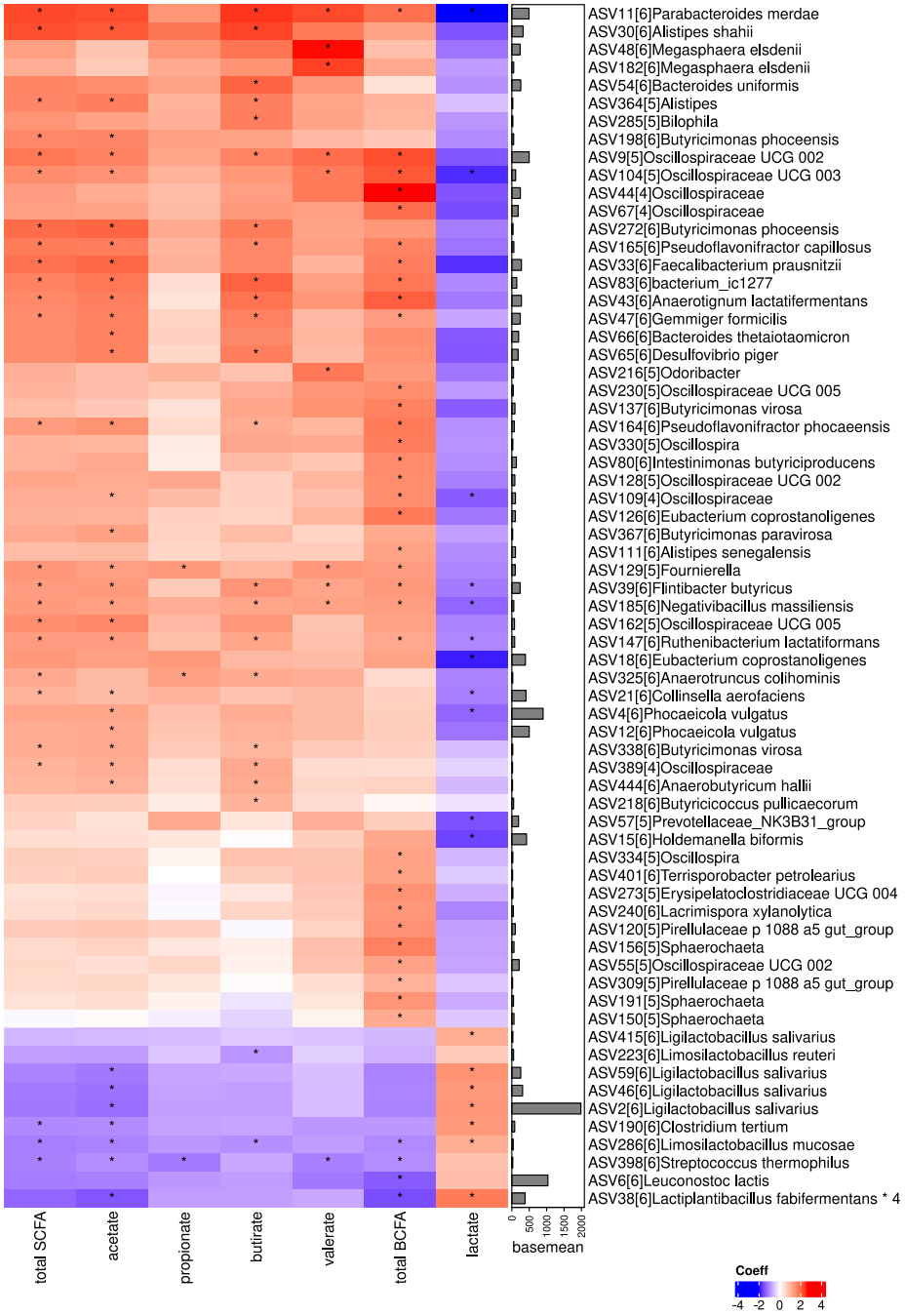


Figure 4.5. Heatmap of correlations between colonic SCFA concentration and ASVs detected on faecal samples from day 15.
*Indicates $p < 0.05$ and statistical difference present.

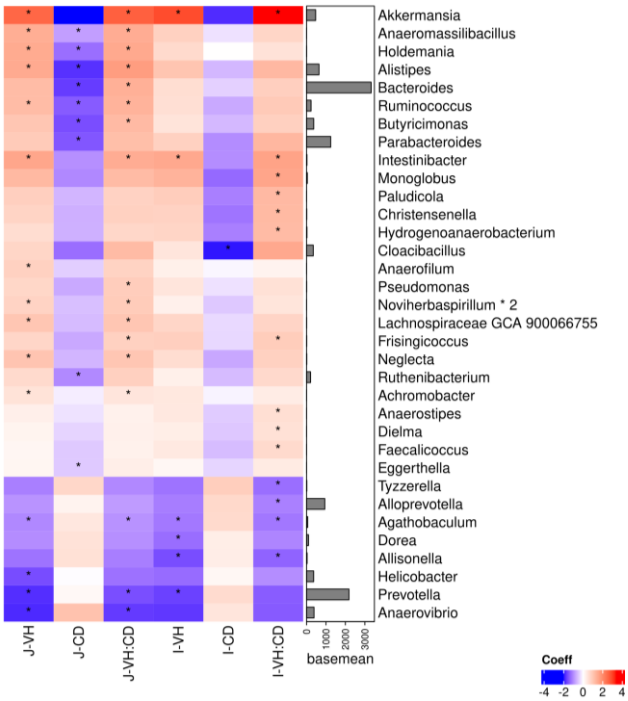


Figure 4.6. Heatmap of correlations between histology (jejunal and ileal) and bacterial genera detected on faecal samples from day 15.
J = jejunal; I = ileal. VH = villus height; CD = crypt depth; VH.CD = ratio villus height:crypt depth. *Indicates $p < 0.05$ and statistical difference present.

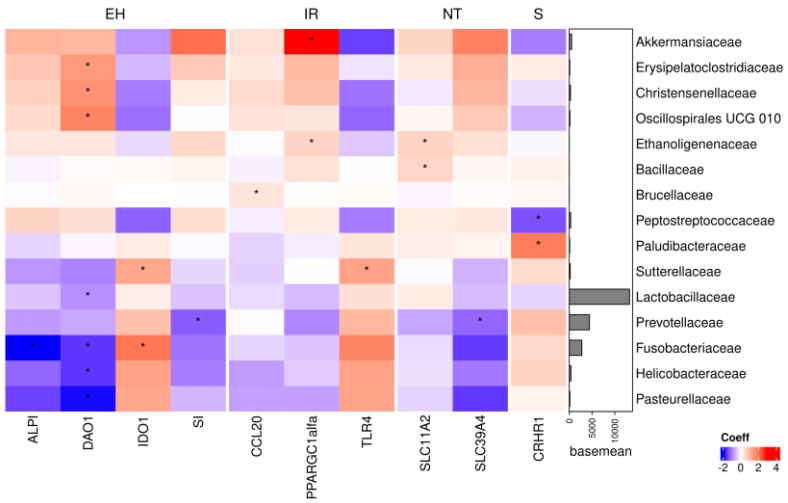


Figure 4.7. Heatmap of correlations between jejunal gene expression and bacterial families detected on faecal samples from day 15.
EH = hormones/enzymes; IR = immune response; NT = nutrient transport; S = stress. *Indicates $p < 0.05$ and statistical difference present.

4.5. Conclusions

In conclusion, the findings suggest that supplementing milk formulas with synbiotic and/or osteopontin during the initial days of life may impact the establishment of intestinal microbiota, digestive maturation, and neonate immune response. Synbiotic additives encourage the growth of beneficial microbiota genera and better control of dysbiotic genera in the lower digestive tract after 15 days of administration. Additionally, these additives led to reduced episodes of diarrhoea in these animals. Furthermore, they significantly influenced jejunal gene expression. Osteopontin supplementation resulted in increased milk formula consumption, alongside some promotion of beneficial microbiota genera and a reduction in dysbiotic genera in the lower digestive tract. However, more substantial results were observed with the combination of the synbiotic and osteopontin. Significant increases in SCFA concentration, reduced ammonia levels, and modulation of jejunal gene expression suggest an intriguing synergic effect of the tested products, potentially aiding in controlling the overgrowth of opportunistic pathogen and enhancing the organism's response to challenging situations. Furthermore, the correlations between gut microbiota and various intestinal parameters (SCFA concentration, histology, and gene expression) shed light on the intricate cross-talk between the microbiota and the host. For example, beneficial microbiota genera exhibited negative correlations with pro-inflammatory gene expressions, while dysbiotic genera showed contrary correlations. These findings imply a mechanism of interaction where the microbiota appears to regulate various parameters, including immune response gene expressions, either directly or indirectly.

4.6. Supplementary materials

Table S4.7. Effect of the experimental treatments on jejunal gene expression.
Values are expressed compared to a reference sample of the control treatment. The average values of the treatments are expressed without the logarithmic transformation used for the statistical analysis.

Main effects	Osteopontin - Synbiotic -	Osteopontin - Synbiotic +	Osteopontin + Synbiotic -	Osteopontin + Synbiotic +		*p-value*			
Diets	**CTR**	**SYN**	**OPN**	**CON**	**RSE**	**Synbiotic**	**Osteopontin**	**Interaction**	**Function**
Genes									
ALPI	1.140[b]	2.204[a]	1.859[a]	2.091[a]	1.588	**0.001**	0.084	**0.027**	EH
ANPEP	1.146	1.159	1.088	1.172	1.354	0.710	0.784	0.811	EH
CCK	2.07	2.252	1.823	2.655	1.581	**0.025**	0.897	0.257	EH
CCL20	0.361	0.661	1.049	0.801	2.737	0.198	0.266	0.866	IR
CLDN1	2.022	1.369	1.532	1.265	2.427	0.150	0.779	0.422	BF
CLDN15	0.918	0.992	0.949	1.066	1.388	0.298	0.418	0.968	BF
CLDN4	1.188	1.179	1.141	1.171	1.848	0.958	0.800	0.844	BF
CRHR1	1.839	1.549	1.423	1.638	2.408	0.360	0.688	0.318	S
CXCL2	1.286	0.543	0.586	0.829	2.194	0.066	0.307	0.075	IR
DAO1	0.913	1.164	1.098	1.132	1.450	0.175	0.342	0.169	EH
FAXDC2	1.465	2.899	2.1	2.525	2.530	0.091	0.662	0.182	IR
GBP1	1.415	0.781	0.9	0.9	2.289	**0.045**	0.347	0.217	IR
GCG	0.924	0.891	1.13	1.129	1.468	0.883	**0.040**	0.900	EH
GPX2	1.826	1.433	1.868	1.433	2.094	0.091	0.846	0.957	EH
HNMT	1.954	1.563	1.844	1.724	1.451	0.312	0.956	0.250	EH
HSD11B1	2.3	1.525	1.675	1.872	1.721	0.383	0.631	0.059	S
HSPA4	1.458	1.25	1.266	1.33	1.456	0.555	0.674	0.207	IR
HSPB1	1.616	1.494	1.476	1.62	1.462	0.865	0.961	0.469	IR
IDO1	1.364[a]	0.284[b]	0.458[ab]	0.466[ab]	5.198	**0.024**	0.372	**0.035**	EH
IFNγ	3.15	1.002	1.151	1.618	4.136	0.082	0.902	0.145	IR
IFNγR1	1.356	1.455	1.406	1.506	1.361	0.373	0.662	0.973	IR
IGF1R	1.382	1.112	1.212	1.343	1.437	0.300	0.687	0.202	EH
IL10	3.011	2.181	2.509	1.927	1.918	0.148	0.654	0.547	IR
IL17A	0.773	0.663	0.615	0.921	2.484	0.885	0.519	0.608	IR
IL1β	1.276[a]	0.406[b]	0.393[b]	0.560[b]	2.389	**0.036**	0.053	**0.027**	IR
IL22	1.076	0.614	0.612	1.047	2.664	0.549	0.462	0.166	IR
IL6	2.899	1.103	1.379	1.356	2.304	0.050	0.356	0.103	IR
IL8	0.874	0.548	0.63	0.724	1.698	0.220	0.633	0.092	IR
MUC13	0.815	1.116	0.988	0.871	1.506	0.492	0.605	**0.015**	BF
MUC2	1.299	1.008	1.094	1.291	1.385	0.447	0.877	**0.040**	BF
NR3C1	1.344	1.423	1.264	1.42	1.266	0.192	0.783	0.729	S
OCLN	1.202	1.571	1.383	1.441	1.302	**0.041**	0.815	0.212	BF
PPARGC1α	0.967	1.058	0.785	0.827	1.550	0.400	0.253	0.664	BF
PYY	0.993	1.166	1.014	1.172	1.526	0.194	0.806	0.945	EH
REG3G	5.741	6.127	6.78	7.876	6.391	0.390	0.168	0.463	IR
SI	2.286[b]	4.729[a]	2.771[ab]	3.569[ab]	1.884	**0.004**	0.840	**0.026**	EH
SLC11A2	1.503	1.307	1.409	1.285	1.281	0.096	0.702	0.874	NT
SLC13A1	0.666[b]	1.086[a]	0.934[ab]	1.113[a]	2.255	**0.026**	0.072	0.073	NT

SLC15A1	1.632[b]	2.440[a]	1.893[ab]	2.171[ab]	1.490	**0.010**	0.731	0.166	NT
SLC16A1	1.398	1.187	1.137	1.204	1.457	0.674	0.252	0.283	NT
SLC30A1	1.498	1.508	1.466	1.636	1.372	0.408	0.737	0.467	NT
SLC39A4	1.24	1.753	1.5	1.529	1.448	0.146	0.566	0.072	NT
SLC5A1	1.694[b]	2.791[a]	1.844[ab]	2.349[ab]	1.668	**0.005**	0.909	0.215	NT
SLC7A8	1.077	2.604	2.375	3.175	3.187	**0.043**	0.296	0.082	NT
SOD2 m	1.321	0.96	1.07	1.058	1.402	0.103	0.423	0.121	EH
TFF3	1.313	1.124	1.368	1.327	1.512	0.836	0.682	0.564	BF
TGFβ1	1.550[a]	0.904[b]	0.916[b]	1.105[ab]	1.583	0.108	0.163	**0.010**	BF
TLR2	0.817	0.431	0.635	0.651	2.178	0.073	0.911	0.171	IR
TLR4	2.542[a]	1.297[b]	1.577[ab]	1.620[ab]	1.755	0.068	0.465	**0.017**	IR
TNFα	1.796	1.04	1.146	1.238	1.884	0.137	0.292	0.166	IR
ZO1	1.323	1.109	1.196	1.239	1.351	0.261	0.823	0.265	BF

Osteopontin +/-: presence/absence of osteopontin ingredient in the diet; **Synbiotic +/-**: presence/absence of synbiotic ingredients in the diet. EH - enzyme/hormone; IR – immune response; BF – barrier function; NT – nutrient transport. n=8. Gene abbreviations detailed in Supplementary materials (Table S4.8). *Indicates $p < 0.05$ and statistical difference present. [a,b] Indicate statistically significant differences between means. SEM, standard error of the mean.

Table S4.8. List of 56 genes (abbreviation, name and function group) used for gene expression analysis using Open Array Real-Time PCR custom designed.

Genes	Name	Function group
ALPI	Intestinal alkaline phosphatase	Enzyme hormone
ANPEP	Alanyl aminopeptidase membrane	Enzyme hormone
CCK	Cholecystokinin	Enzyme hormone
CCL20	Chemokine (C-C motif) ligand 20	Immune response
CLDN1	Claudin-1	Barrier function
CLDN15	Claudin-15	Barrier function
CLDN4	Claudin-4	Barrier function
CRHR1	Corticotropin releasing hormone receptor 1	Stress
CXCL2	Chemokine (C-X-C motif) ligand 2	Immune response
DAO1	Diamine oxidase	Enzyme hormone
FAXDC2	Fatty acid hydrolase domain containing 2	Immune response
GBP1	Guanylate binding protein 1	Immune response
GCG	Glucagon	Enzyme hormone
GPX2	Glutathione peroxidase 2	Enzyme hormone
HNMT	Histamine N-methyltransferase	Enzyme hormone
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	Stress
HSPA4	Heat shock protein 70	Immune response
HSPB1	heat shock protein 27	Immune response
IDO1	Indoleamine 2,3-dioxygenase	Enzyme hormone
IFNγ	Interferon gamma	Immune response
IFNγR1	Interferon gamma receptor 1	Immune response
IGF1R	Insulin-like growth factor 1 receptor	Enzyme hormone
IL10	Interleukin 10	Immune response
IL17A	Interleukin 17 A	Immune response
IL1β	Interleukin 1 beta	Immune response
IL22	Interleukin 22	Immune response

IL6	Interleukin 6	Immune response
IL8	Interleukin 8	Immune response
MUC13	Mucin 13	Barrier function
MUC2	Mucin 2	Barrier function
NR3C1	Glucocorticoid receptor	Stress
OCLN	Ocludin	Barrier function
PPARGC1α	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	Immune response
PYY	Peptide tyrosine tyrosine	Enzyme hormone
REG3G	Regenerating-islet derived protein 3 gamma	Immune response
SI	Sucrase-isomaltase	Enzyme hormone
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2	Nutrient transport
SLC13A1	Solute carrier family 13 (sodium/sulfate symporters) member 1	Nutrient transport
SLC15A1	Solute carrier family 15 (oligopeptide transporter) member 1	Nutrient transport
SLC16A1	Monocarboxylate transporter 1	Nutrient transport
SLC30A1	Solute carrier family 30 (zinc transporter) member 1	Nutrient transport
SLC39A4	Solute carrier family 39 (zinc transporter) member 4	Nutrient transport
SLC5A1	Solute carrier family 5 (sodium/glucose cotransporter) member 1	Nutrient transport
SLC7A8	Solute carrier family 7 (amino acid transporter light chain, L System) member 8	Nutrient transport
SOD2	Superoxide dismutase	Enzyme hormone
TFF3	Trefoil factor 3	Barrier function
TGFβ1	Transforming growth factor beta 1	Immune response
TLR2	Toll-like receptor 2	Immune response
TLR4	Toll-like receptor 4	Immune response
TNFα	Tumor necrosis factor alpha	Immune response
ZO1	Zonula occludens 1	Barrier function

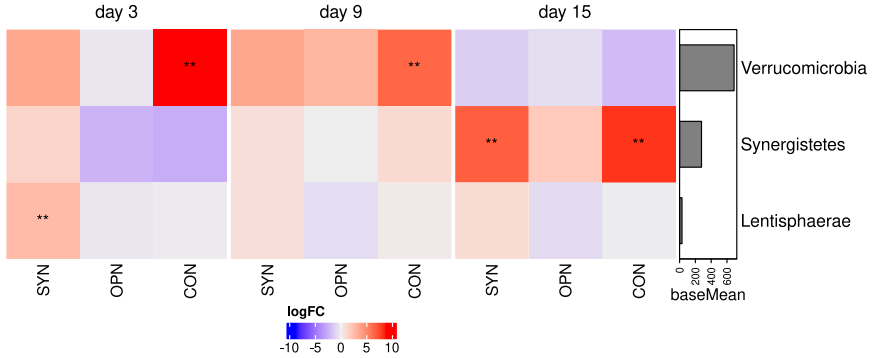


Figure S4.8. Heatmap of the impact of experimental treatments on bacterial phyla in faecal samples.
CTR: no additives in the diet; **SYN**: prebiotic + probiotic additives in the diet; **OPN**: osteopontin in the diet; **CON**: synbiotic + osteopontin in the diet. n = 8. *Indicates p < 0.1; **indicates p < 0.05.

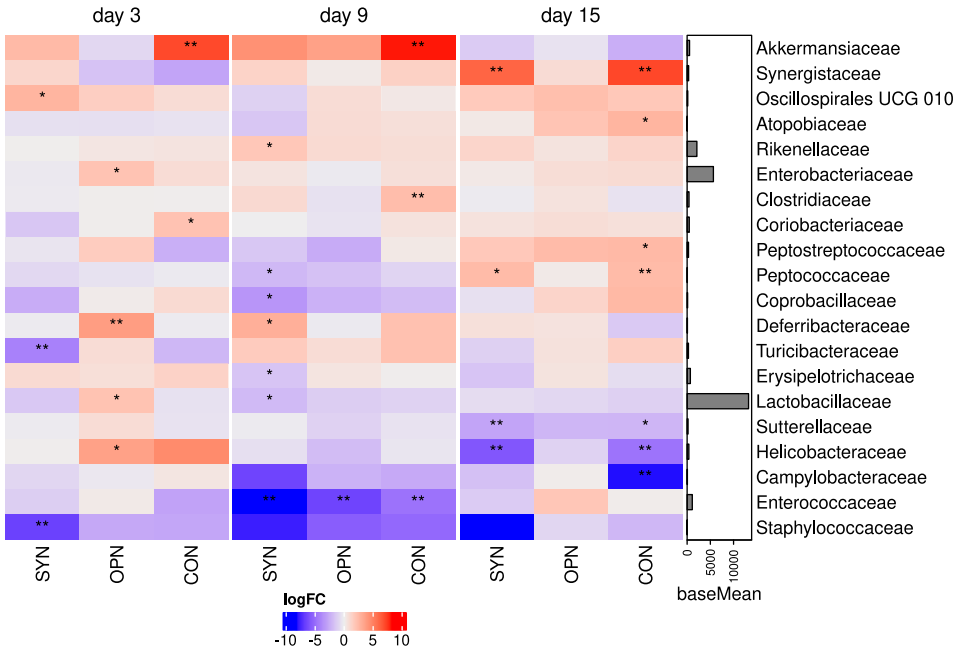


Figure S4.9. Heatmap of the impact of experimental treatments on bacterial families in faecal samples.
CTR: no additives in the diet; **SYN**: prebiotic + probiotic additives in the diet; **OPN**: osteopontin in the diet; **CON**: synbiotic + osteopontin in the diet. n = 8. *Indicates p < 0.1; **indicates p < 0.05.

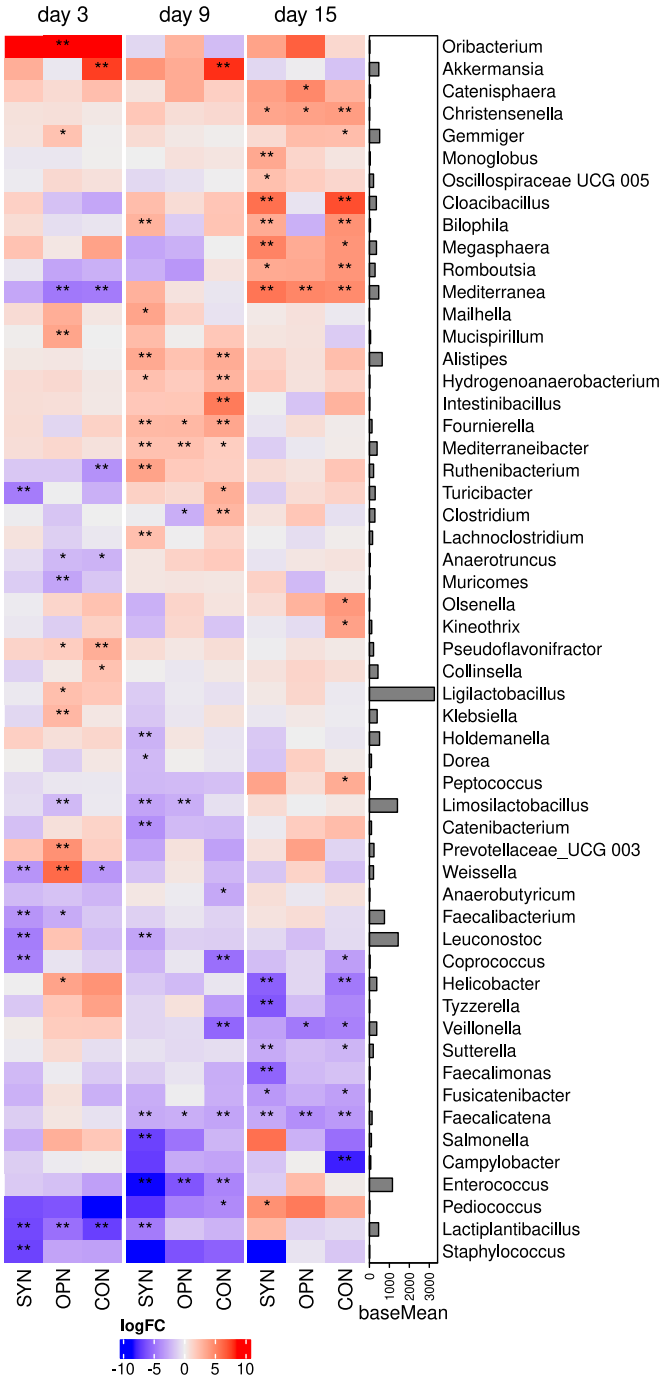


Figure S4.10. Heatmap of the impact of experimental treatments on bacterial genera in faecal samples.
CTR: no additives in the diet; **SYN**: prebiotic + probiotic additives in the diet; **OPN**: osteopontin in the diet; **CON** synbiotic + osteopontin in the diet. n = 8. *Indicates $p < 0.1$; **indicates $p < 0.05$.

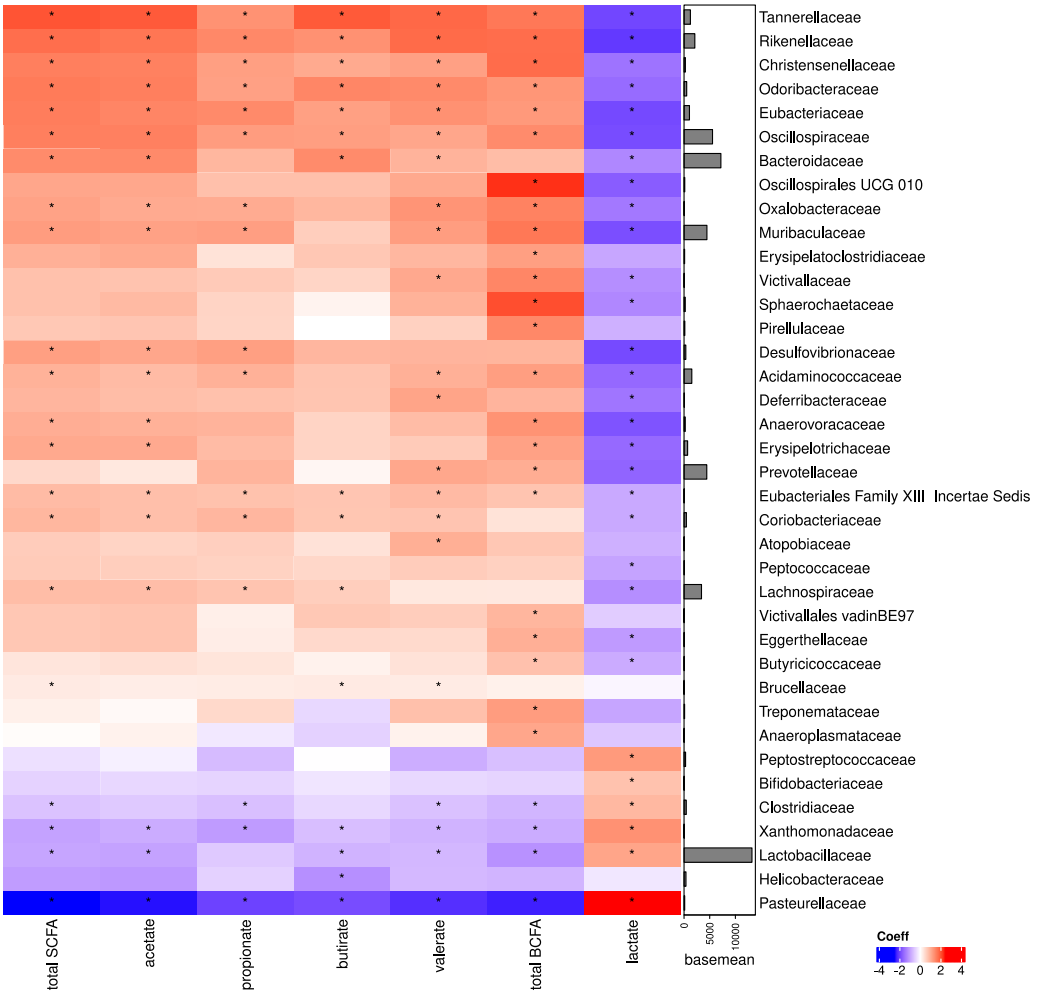


Figure S4.11. Heatmap of correlations between colonic short-chain fatty acids (SCFA) concentration and bacterial families in faecal samples from day 15.
BCFA, Branched-chain fatty acids. *Indicates $p < 0.05$ and statistical difference present.

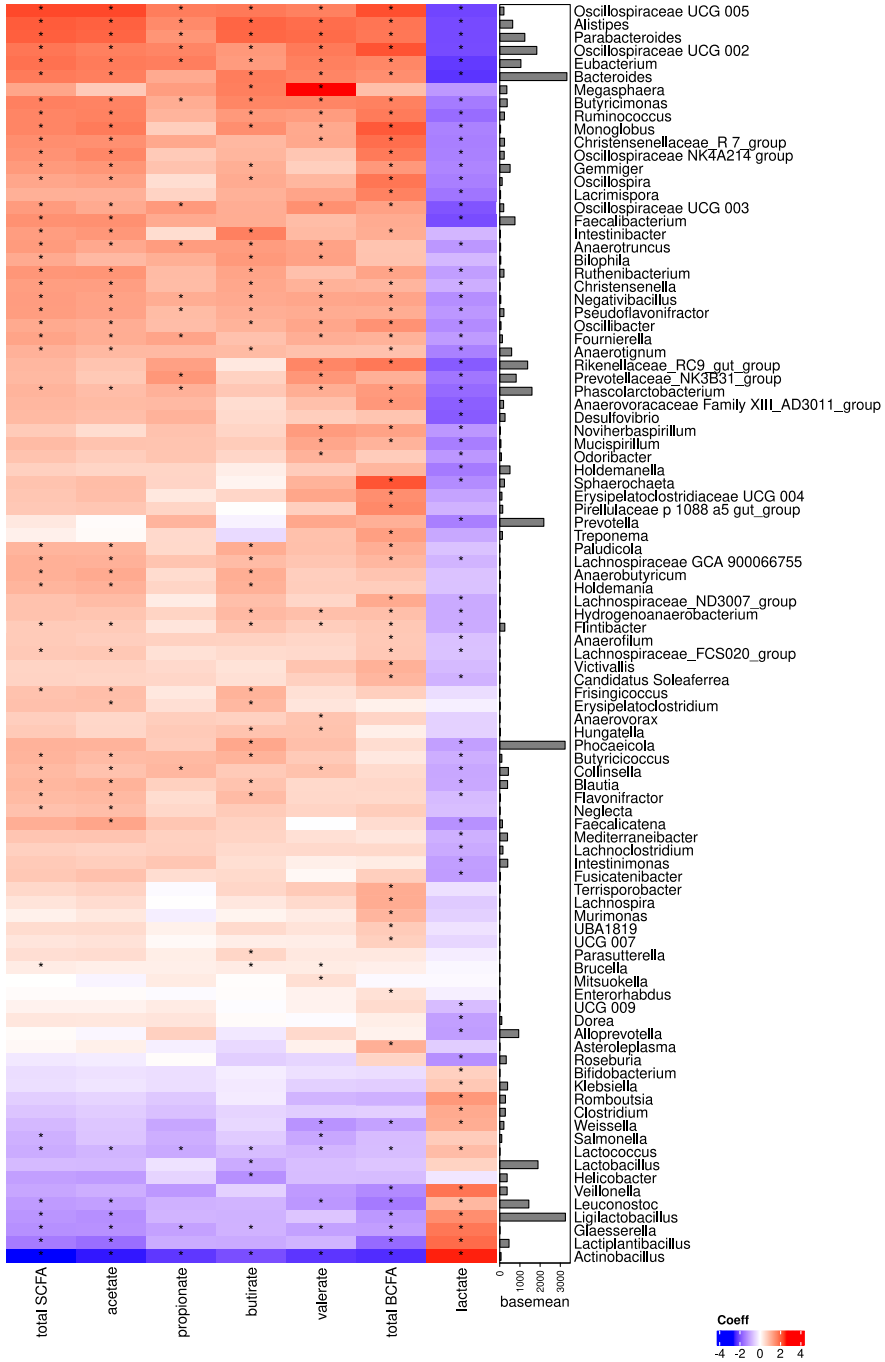


Figure S4.12. Heatmap of correlations between colonic short-chain fatty acids (SCFA) concentration and bacterial genera detected in faecal samples from day 15.
BCFA, Branched-chain fatty acids. *Indicates $p < 0.05$ and statistical difference present.

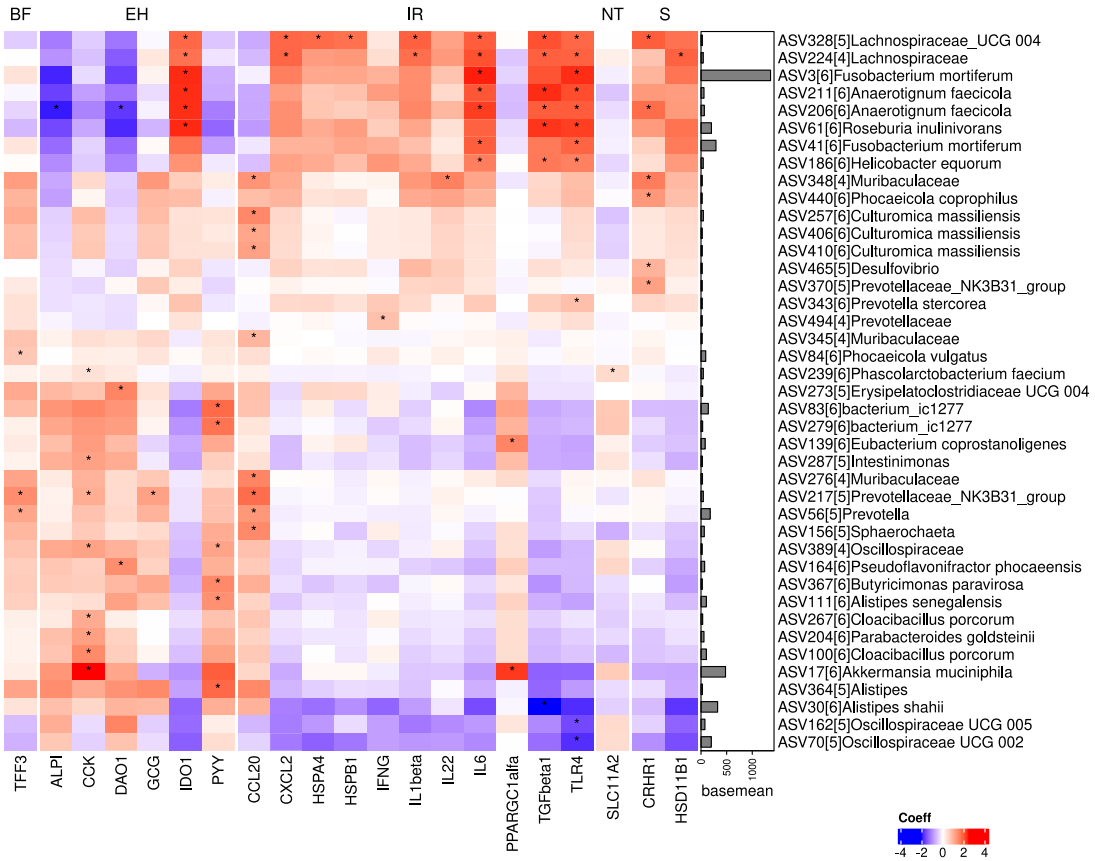


Figure S4.13. Heatmap of correlations between jejunal gene expression and ASVs detected in faecal samples from day 15.
*Indicates $p < 0.05$ and statistical difference present.

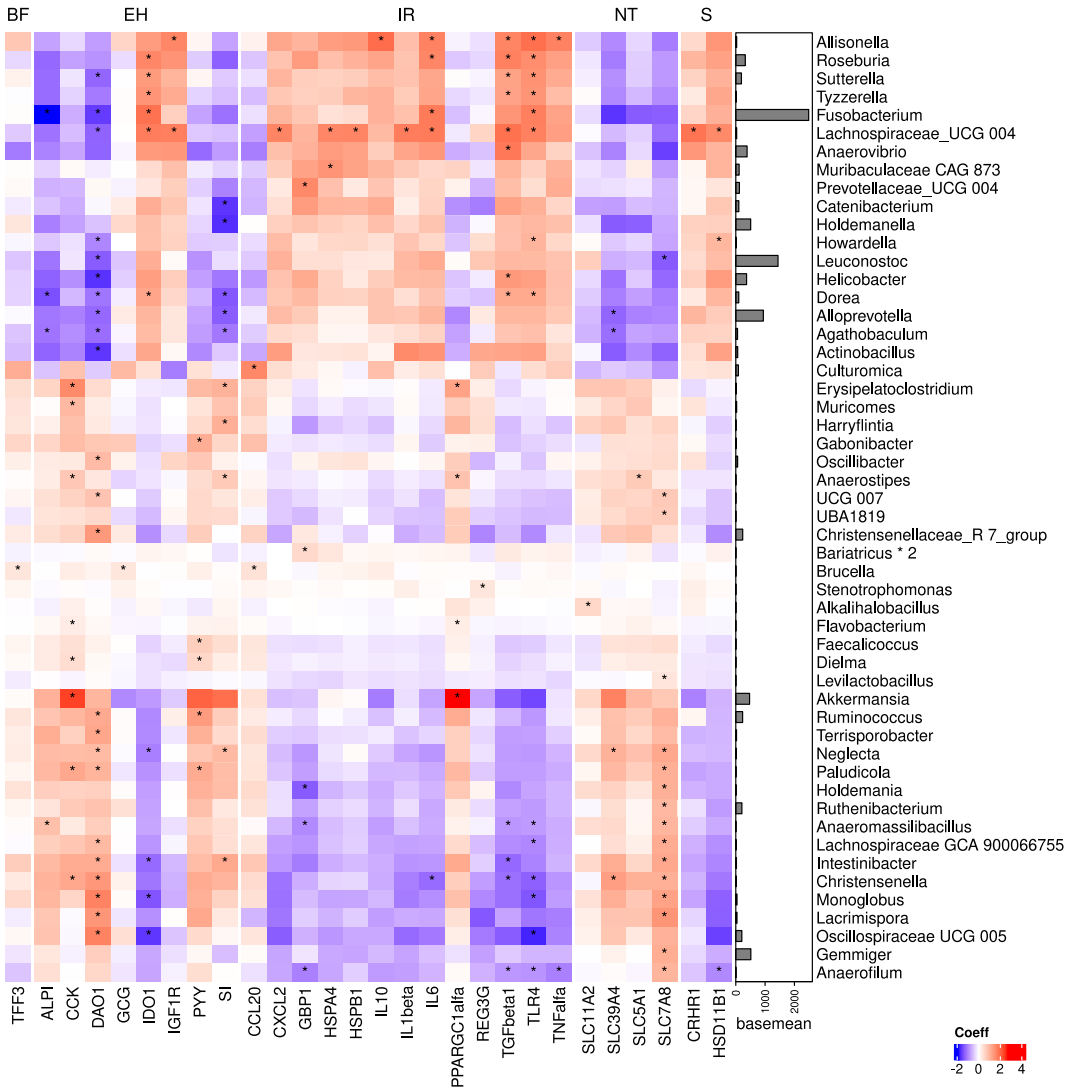


Figure S4.14. Heatmap of correlations between jejunal gene expression and bacterial genera detected in faecal samples from day 15.
*Indicates $p < 0.05$ and statistical difference present.

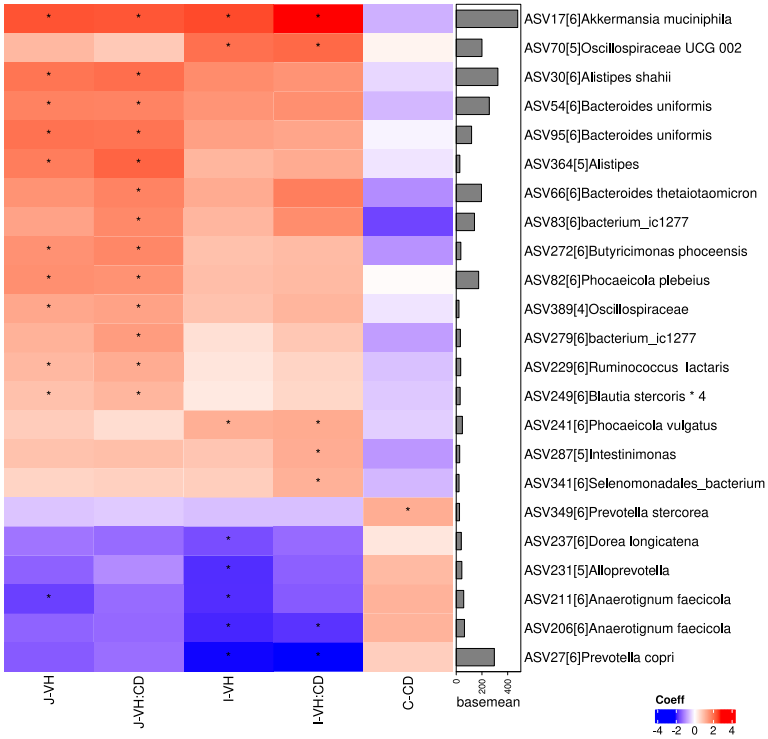


Figure S4.15. Heatmap of correlations between histology (jejunal, ileal and colonic) and ASVs detected in faecal samples from day 15.
J = jejunal, I = ileal, C = colonic. VH = villus height; CD = crypt depth; VH.CD = ratio villus height:crypt depth. *Indicates $p < 0.05$ and statistical difference present.

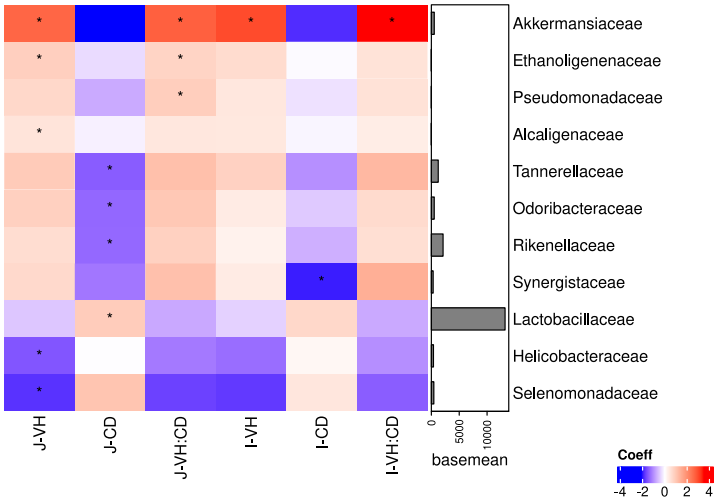


Figure S4.16. Heatmap of correlations between histology (jejunal and ileal) and bacterial families detected in faecal samples from day 15.
J = jejunum, I = ileum. VH = villus height; CD = crypt depth; VH.CD = ratio villus height:crypt depth. *Indicates $p < 0.05$ and statistical difference present.

Chapter 5. Looking for possible benefits of combining short-chain fructo-oligosaccharides (scFOS) with *Saccharomyces cerevisiae* Sc 47 on weaned pigs orally challenged with *Escherichia coli* $F4^+$



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5.1. Introduction

In the modern swine industry, weaning has become the main critical period piglets have to overcome. This stage is characterized by sudden dietary, environmental, and social changes (Lallès et al., 2007) that turn into new stressors for the piglet. Altogether, weaning can lead to a poorer immune response, an impairment of intestinal barrier function, and an unbalanced gut microbiota (Pié et al., 2004). As the intestine plays a primordial role in protecting the animal from harmful microorganisms, post-weaning piglets become more susceptible to opportunistic pathogens (Schokker et al., 2015). ETEC $F4^+$ is one of the pathogens frequently causing post-weaning colibacillosis (Gresse et al., 2017; Luise et al., 2019a) an infection characterized by the presence of watery faeces, poor growth performance, and increased morbidity and mortality in piglets (Campbell et al., 2013; Pan et al., 2017). Until now, antibiotics had been widely used to control these incidences (Cromwell, 2002); however, the increase in the amount of antimicrobial resistant pathogens creates an urgent need to find alternative strategies (Laird et al., 2021; Yang et al., 2015). Furthermore, in some countries, the prophylactic and/or therapeutic use of antibiotics has been banned or highly restricted (Murphy et al., 2017; Muurinen et al., 2021).

Some compounds, such as probiotics and prebiotics, have been gaining prominence in the feed industry as a new dietary strategy due to their potential antimicrobial and growth-promoting effects on animals (Tang et al., 2020; Vohra et al., 2016; Yang et al., 2015). Probiotics are defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts (Hill et al., 2014). Within the most frequently studied microorganisms with this potential, we can find: *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and yeast, such as different species of *Saccharomyces* (Su et al., 2022). Yeasts, from the genus *Saccharomyces*,

have been demonstrated to improve gut integrity, strengthen the immune system, and enhance small intestine development in weaned piglets (Pan et al., 2017; ZHU et al., 2017). In addition, in-feed supplementation of *Saccharomyces* in weaned piglets has been suggested to decrease enterotoxigenic *E. coli* $F4^+$ adhesion to the intestinal mucosa, in addition to this pathogen's jejunal and cecum populations (ZHU et al., 2017). Prebiotics are defined as a substrate that is selectively utilized by host microorganisms to confer a health benefit (Gibson et al., 2017; Gibson & Roberfroid, 1995). Polymers such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) belong to this group of prebiotic ingredients (Strube et al., 2015) and are associated with the best documented benefits (Markowiak & Ślizewska, 2018). They promote the growth and activity of specific favorable bacteria and help in the maintenance of an optimal gut environment (Metzler et al., 2005; Strube et al., 2015). Differences have been reported in the prebiotic activity of FOS depending on the oligomer chain length and composition. Short-chain FOS (scFOS) promote a quicker growth of beneficial bacteria and short-chain fatty acid (SCFA) production compared with long-chain prebiotics, such as oligofructose or inulin (Pompei et al., 2008). In this study, we tested a source of scFOS produced from sucrose presenting a low degree of polymerization (DP 3-5).

Combining probiotics and prebiotics, generally referred as synbiotics, has been proposed to maximize the benefits of both strategies (Gibson & Roberfroid, 1995). According to the recent ISAPP definition for the term, a synbiotic is a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host (Swanson et al., 2020). Benefits can be attributed to the complementary effects of both (complementary synbiotics), as well as the selective growth of the probiotic strain promoted by the prebiotic that is

chosen to specifically stimulate its growth and activity (synergistic synbiotics) (Kolida & Gibson, 2011; Swanson et al., 2020).

Therefore, the main objective of this study was to test the beneficial effects of including scFOS or *S. cerevisiae* Sc47 in the post-weaning diets of piglets orally challenged with *E. coli* $F4^+$ and to evaluate the possible additional benefits of combining both strategies with a synbiotic approach.

5.2. Materials and methods

The study was performed at the Experimental Unit of the Universitat Autònoma de Barcelona (UAB) with prior approval from the institution's Animal and Human Experimental Ethical Committee (permit no. CEAAH 4026; DMAH 10118). The treatment, management, housing, husbandry, and slaughtering conditions conformed to European Union Guidelines (Directive 2010/63/EU).

5.2.1. Animals, housing, and experimental design

The trial was organized as a level 2 high-risk biosecurity procedure, and all involved personnel received appropriate training.

A total of 96 21-day-old male piglets (Large White × Landrace × Pietrain) weighing 5.7 (± 0.20) kg were used. All the animals came from a high-sanitary-status farm in which mothers were not vaccinated against *E. coli*. Piglets were selected from 24 sows previously confirmed to be homozygous for the Mucin 4 (MUC4) gene, a factor related to a greater susceptibility to infections caused by ETEC $F4^+$ (Luise et al., 2019b). Animals were transported to the UAB's facilities and placed in four rooms of eight pens each (32 pens,

with 3 animals per pen). Each pen (3 m^2) was partially slatted and had a feeder and a nipple drinker to provide food and water ad libitum. Each pen was provided with a plastic toy specifically designed for pigs of this age. The weaning rooms were equipped with automatic heating and forced ventilation. The experiment was carried out during the spring season (May–June).

The trial was conceived as a 2 × 2 factorial design (receiving or not probiotics × receiving or not prebiotics) with 4 experimental diets: control (CTR), pigs receiving a pre-starter basal diet; prebiotic (scFOS), pigs receiving short-chain fructo-oligosaccharides; probiotic (YEA), pigs receiving *Saccharomyces cerevisiae* Sc47; and prebiotic + probiotic (SYN), pigs receiving both. Upon arrival, the animals were distributed among rooms and pens according to their initial body weight to ensure a similar average within pens. The four experimental treatments were balanced within rooms (2 pens/treatment in each room). The number of replicates for each experimental diet was eight, considering the pen as the experimental unit. All the groups were orally challenged with the pathogenic ETEC $F4^+$ strain one week after arrival. Animals received the experimental diets during 15 experimental days.

5.2.2. Diets and tested products

The tested prebiotic was short-chain fructo-oligosaccharides (scFOS; Profeed® P95; Beghin-Meiji) obtained from sugar beet sucrose through an enzymatic reaction. It is composed of a terminal glucose molecule (G) linked to fructose molecules (F) by a β1–2 bound, with 37 ± 6 % 1-kestose (GF2), 47 ± 6 % nystose (GF3), and 16 ± 6 % 1F-β-fructofuranosyl nystose (GF4), and therefore presents a low degree of polymerization comprised between 3 and 5 (DP 3-5). It was included at a concentration of 0.5% (5 g/kg) in the scFOS

and SYN diets. The tested probiotic strain was *Saccharomyces cerevisiae* Sc 47 (Actisaf® Sc 47 HR+; Phileo Lesaffre). It was included at a concentration of 0.1% (1 g/kg) in the YEA and SYN diets according to manufacturer's instructions.

The basal diet was formulated and manufactured in a mash form to satisfy the nutrient requirement standards for pigs of this age (National Research Council (NRC), 2012). Details regarding ingredient and chemical composition are shown in Table 5.1. The basal diet was manufactured in 2 batches of 500 kg. The prebiotic was included in one of the mixer batches (batch 2) by adding 2.5 kg over 500 kg of basal diet. All these procedures were performed in a feed mill (Pinsos Molinet SL; Gaià, Barcelona). The probiotic was subsequently included in 150 kg of plain basal diet (batch 1) and 150 kg of basal diet with prebiotic (batch 2) by using a small mixer in the UAB's facilities (Servei de Granges i Camps Experimentals SGiCE) just before starting the trial.

Table 5.1. Ingredients and analysed composition of basal diet.

Ingredients	(g/kg FM)
Maize	205.50
Wheat	180.40
Barley 2 row	170.30
Extruded soybean	150.00
Sweet whey powder (cattle)	112.00
Fishmeal LT	60.00
Soybean meal 47	70.00
Whey powder 50% fat	25.00
Monocalcium phosphate	6.50
Calcium carbonate ($CaCO_3$)	3.80
L-Lysine HCL (78)	4.50
Vit min GPlus (Balsa)*	4.00
Sodium chloride	2.40
DL-Methionine 99	2.60
L-Threonine	2.30
L-Tryptophane	0.70
Analyzed chemical composition	(%)
Dry matter	91.2
Ash	4.98
Crude protein	18.6
Crude fiber	1.86
EE	6.55

FM, fresh matter. LT, low-temperature preparation of fishmeal to preserve amino-acid content. EE, ether extract. *Provided per kilogram of complete diet: 12,000 IU vitamin A; 1,400 IU vitamin D3; 55 UI vitamin E; 2 mg vitamin K3; 3 mg vitamin B1; 7 mg vitamin B2; 7.33 mg vitamin B6; 0.06 mg vitamin B12; 17 mg calcium pantothenate; 45 mg nicotinic acid; 0.2 mg biotin; 1.5 mg folic acid; 60 mg Fe; 90 mg Cu; 95 mg Zn; 40 mg Mn; 0.7 mg I; and 0.3 mg Se.

5.2.3. Bacterial strain

Piglets were orally challenged with an *Escherichia coli* $F4^+$ strain isolated from the faeces of a 14-week-old pig and provided by the Infectious Diseases Laboratory of the UAB (Ref. COLI30/14-3). This strain presented virulence factors F4ab, F4ac, LT, STb, and EAST1. It was negative for factors K99, F6, F18, F41, STa, VT1, VT2, and EAE.

Oral inoculums were prepared after 24 hours of incubation at 37°C in LB medium (Luria broth) with slow agitation (250 rpm) in an orbital incubator. The final concentration of the inoculum was $3x10^9$ CFU/ml, determined by plate counting in Luria agar. Plates were seeded just before inoculating the animals. A total volume of 6 ml was orally administered to each animal by using a disposable syringe without needle, reaching a dose of $1.8x10^{10}$ CFU/animal, to ensure an effective infection.

5.2.4. Experimental procedure

Piglets were transferred from the commercial farm to the experimental unit at 21 days of age. Upon arrival, the animals were weighed, identified with ear tags, and distributed in pens according to their weight.

After an adaptation period (day 8), 6 ml of ETEC $F4^+$ inoculum was orally administered to all the animals. To ensure that the stomach was full at the time of inoculation, facilitating bacterial colonization and standardizing conditions for all the animals, we removed food the same day early in the morning and replaced 15 minutes before inoculation (approximately from 9 am until 2 pm).

From the challenge onward, the animals were examined daily for clinical signs to assess their post-inoculation (PI) status (e.g., dehydration, apathy, and diarrhoea). The faecal samples for microbiological analysis were taken from the heaviest pig in each pen (n = 32) on arrival day (0), and from the same animal before inoculation at day 8 (0 PI), 12 (4 PI), and 16 (8 PI). The rectal temperature was assessed one day before the challenge and on days 1, 2, and 3PI. Individual faecal scores were registered on days 0, 1, 2, 3, 5, and 7PI by tactile faecal stimulation/sampling and/or visual inspection. The faecal score was evaluated using a 5-point scale (1: hard and dry faeces; 2: well-

formed firm faeces; 3: formed faeces; 4: pasty faeces; 5: liquid diarrhoea). Diarrhoea was considered to be indicated by a score ≥ 4.

Performance was monitored throughout the experimental period. The piglets were weighed on days 0, 4, and 8 PI, and the feed consumption was recorded on days 0, 1, 2, 3, 4, 7, and 8 PI. The mortality rate was also noted. No antibiotic treatment was administered to the animals during the experiment.

At day 4 PI (day 12) and 8 PI (day 16), the intermediate- and the high-weight piglets from each pen (from the beginning of the trial) were euthanized, respectively. The animals were sequentially sampled during the morning (between 9:00 and 14:00 hours). The remaining animals were euthanized at the end of the study. For that, animals were first sedated with the synergic effect of 2 mg/kg xylazine (Xilagesic; Les Franqueses del Vallès) plus 20 mg/kg ketamine (Ketamidor; Wels, Austria) IM. Blood samples were taken from the cranial cava vein, and then, the animals were euthanized with an intravenous injection of 200 mg/kg sodium pentobarbital (Euthasol; Le Vet B.V., Oudewater, Netherlands). The abdomen was immediately opened, and the digestive package was excised and transferred to a tray. Different samples were collected for analysis. The digestive contents of the ileum (considered as the distal 1/3 of small intestine) and full colon were emptied and homogenized, and their pH was measured. Subsequently, different aliquots of ileum and colon digesta were preserved for future analysis. For SCFA and lactic acid analysis, the samples were frozen immediately using dry ice and subsequently stored at -20°C until analysis. For the ammonia analysis, 3 ml of colonic contents were collected with 3 ml of 0.2 N H_2SO_4 as a preservative solution. Subsequently, the sample was stored at -20°C. For microbiological analysis, ileum and colon contents were collected in sterile containers, and ileum mucosa scrapings were placed in eppendorfs before being stored at 4°C until analysis. For the quantification of ETEC $F4^+$ by qPCR, colon digesta

aliquots and ileum mucosa scrapings were also collected and immediately frozen using dry ice, and stored at -20°C. For the histological study, 2-3 cm-long sections of the distal ileum were cut, thoroughly washed with sterile PBS, and fixed by immersion in a solution of formaldehyde (4 %).

5.2.5. Analytical procedures

5.2.5.1. Feed analysis

Chemical analysis of the diets, including the dry matter, ash, crude protein, diethyl-ether extract and crude fiber, were carried out according to the standard procedures of the Association of Official Agricultural Chemists (AOAC International, 1995). The gross energy of the diets was also determined on an adiabatic pump calorimeter.

5.2.5.2. Microbiological analysis

Lactobacilli and entero-hemorrhagic *E. coli* (EHEC) were measured by plate counting in selective MRS media (CM0361B MRS Agar OXOID) and selective chromogenic media (413697 CHROMIC- EHEC 6x200 mL Biomerieux), respectively. The inoculated strain was confirmed to thrive on this last medium; however, many other ETEC strains could also have potentially grown. Since no growth was observed in the media for faecal samples collected before the challenge, the plate counts on this medium were ultimately considered as ETEC plate counts.

ETEC $F4^+$ was quantified in colonic digesta and ileum mucosa scrapings by qPCR using SYBR green dye. DNA from the colonic digesta and ileum scraping samples was extracted and purified using the commercial QIAamp Fast DNA

Stool Mini Kit (Qiagen; West Sussex, United Kingdom). The DNA was eluted in 200 ml of Qiagen buffer AE and stored at −20°C until use. Afterwards, a qPCR targeting the gene coding F4 fimbria was performed using the SYBR green dye according to the procedure described by Hermes et al. (2013).

5.2.5.3. Serum analysis

The blood samples were centrifuged (2500 × g, 8 min at 4°C) after 4 hours of refrigeration, and the serum obtained was divided into different aliquots before storage at -20°C.

The concentrations of Tumor Necrosis Factor-α (TNF-α) and major acute-phase protein (PigMAP) in the serum were determined by ELISA (Quantikine Porcine TNF-α kit, R&D Systems; Minneapolis, United States) and a sandwich-type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A.; Segovia, Spain), respectively, following the manufacturer's recommendations.

5.2.5.4. Short-chain fatty acids, lactic acid, and ammonia analysis

The concentrations of short-chain fatty acids (SCFAs) in the colonic digesta and faeces were analyzed by gas chromatography, after which the samples were subjected to an acid–base treatment followed by an ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide (MBTSTFA) plus 1 % tertbutyldimethylchlorosilane (TBDMCS) agent using Richardson et al.'s method (1989) modified by Jensen et al. (1995).

The ammonia concentrations on H_2SO_4-preserved colonic digesta samples were assessed by using a gas-sensitive electrode (Hatch Co.; CO, United

States) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain). For that, the preserved samples were homogenized and centrifuged at 1500 × g for 10 min before the analysis, and diluted with distilled water (1:2 and 1:4 according to the ammonia concentration), and 10M NaOH was added to ensure a final pH > 11. Once the ammonia was released, it was measured in the supernatants as the voltage change (in mV).

5.2.5.5. Histological analysis

Morphological measurements of ileal sections were performed with a light microscope (BHS, Olympus; Barcelona, Spain) following Nofrarías et al.'s method (2006). Measured parameters included: villus height (VH), crypt depth (CD), ratio villus:crypt (VH:CD), intraepithelial lymphocytes (IEL), goblet cells (GC), and mitosis (M). We measured between 7 and 10 intact villi per animal.

5.2.5.6. Mother genotyping for ETEC F4^{+} susceptibility

The Mucin 4 (MUC4) gene is proposed as one of the genetic markers for pig ETEC F4^{+} resistance/susceptibility (Luise et al., 2019a, 2019b). To assure the susceptibility of piglets in this trial, the mothers were genotyped for this gene polymorphism. Hair follicles were collected from 72 mothers to extract the DNA, following the procedure described by Luise et al. (2019b). A restriction fragment length polymorphism analysis (PCR-RFLP) was performed following the method described by Jørgensen et al. (2003). Piglets were selected from 24 sows homozygous for the susceptible gene (MUC4^{GG}).

5.2.6. Statistical analysis

The effects of the experimental diets on all the data were analyzed using the free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, United States) using the stats package (Team, 2014).

The results are expressed as means with their standard errors unless otherwise stated. The microbiological data were previously log transformed.

Most of the data were analyzed by a two-way analysis of variance (lme function) to determine the main effects of prebiotic addition (PRE), probiotic addition (PRO), or any possible interaction (PRE × PRO). Analysis were carried out using a generalized linear model as follows:

$$Y_{ijk} = m + PRE_i + PRO_j + (PRE \times PRO)_{ij} + e_{ijk}$$

where Y is each observation of the studied variable, m is the global mean, PRE_i is the main effect of adding the prebiotic to the diets, PRO_j is the main effect of adding the probiotic to the diets, and PRE × PRO_{ij} is the possible interaction. Finally, e_{ijk} is the experimental error term.

When the effect of the interaction (PRE × PRO) was established ($p = 0.05$), the means were compared using the probability function of differences adjusted by the Tukey–Kramer method.

For an analysis of the evolution of LW, ADG, ADFI, and faecal scores along time, a mixed-effects model (lme function) was used following:

$$Y_{ijk} = m + Diet_i + Time_j + (Diet \times Time)_{ij} + e_{ijk}$$

where Y is each observation of the studied variable, m is the global mean, $Diet_i$ is the main effect of the experimental diets, $Time_j$ is the main effect of the day/period, and Diet × $Time_{ij}$ is the possible interaction. Finally, e_{ijk} is the experimental error term.

The mortality percentage and ETEC $F4^+$ prevalence were analyzed by means of a frequency analysis using a Fisher test (fisher.test function) with the same statistical package.

The score faecal data in Figure 5.1 were analyzed using a non-parametric approach, namely the Kruskal–Wallis test (kruskal.test) (Myles Hollander et al., 1973). When the effect of treatment was established ($p = 0.05$), the means were compared using Dunn's test (dunnTest) with the FSA package (Ogle DH, 2022).

For all the parameters, the pen was considered as the experimental unit. The α level used to determine statistical significance was $p = 0.05$. The statistical trend was considered for $P > 0.05$ and < 0.10.

5.3. Results

In general terms, the trial developed as expected without any remarkable incidents. Upon arrival, all animals showed a good health status, and no presence of ETEC $F4^+$ was detected in faeces before inoculation. After the oral inoculation, the animals showed a mild course of diarrhoea that resolved spontaneously at the end of the study without the need of any pharmacological treatment. During the PI period, three pig deaths were recorded: one from the CTR group at day 2PI and two from the SYN group at days 2PI and 3PI.

One pen from the CTR group was excluded from the study and treatment due to negligible feed intake along the first week. Average daily feed intake in this pen during the first week was 18.6 g/day.

5.3.1. Performance parameters

Average values of LW, ADG, ADFI, and gain to feed ratio (G:F) are presented in Table 5.2.

No significant differences due to treatments were found in LW at the beginning or end of the study. No remarkable differences were found in ADG despite a trend in PRO group presenting lower values during adaptation week. However, ADFI showed differences between treatments after the challenge with a lower feed intake in those animals receiving the probiotic (YEA and SYN treatments). G:F did not show significant differences related to the experimental treatments.

Table 5.2. Effect of experimental treatments on animal performance.
LW (g), ADFI (g/day), ADG (g/day), and G:F for adaptation period (days 1-7), first post-inoculation (PI) period (days 0-4 PI), second PI period (days 4-8 PI).

Main effects	**PRO-**		**PRO+**			***p-value***		
	PRE-	**PRE+**	**PRE-**	**PRE+**	**RSE**	**PRE**	**PRO**	**Interaction**
Diets	**CTR**	**scFOS**	**YEA**	**SYN**				
LW (g)								
D0	5659	5662	5643	5657	116.7	0.842	0.802	0.896
D15	7905	7671	7613	7795	720.5	0.921	0.752	0.438
ADFI (g)								
adapt.	308	347	310	300	80.54	0.430	0.294	0.674
0-4 PI	257	214	211	178	60.38	0.182	**0.038**	0.591
4-8 PI	416	386	347	314	70.19	0.439	**0.005**	0.740
ADG (g)								
adapt.	79.8	83.6	62.2	63.1	29.63	0.830	0.091	0.896
0-4 PI	108.2	58.9	48.1	43.0	95.89	0.649	0.164	0.342
4-8 PI	340.7	311.7	351.7	359.1	143.80	0.831	0.889	0.445
G:F								
adapt.	0.294	0.230	0.202	0.226	0.1028	0.621	0.247	0.281
0-8 PI	0.354	0.297	0.330	0.405	0.1291	0.856	0.391	0.183
total	0.233	0.205	0.222	0.259	0.0646	0.847	0.382	0.197

PRO+/-: presence/absence of probiotic ingredient in diet. **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. RSE, residual standard error.

5.3.2. Clinical signs

Faecal scores were measured from the inoculation day until one week later (Figure 5.1). At day 0 PI, the mean faecal score for the different treatments was around 3-3.5. Once inoculated, the animals' faecal score worsened, with increased scores up to day 3 PI and decreasing afterwards. At day 3 PI, the SYN treatment showed significantly lower (better) scores than the CTR group, while scFOS and YEA presented intermediate values.

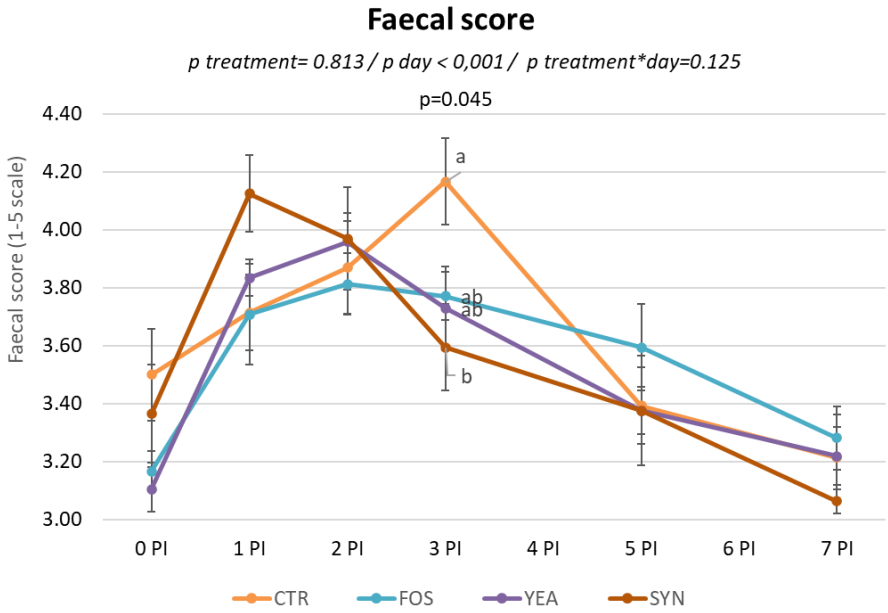


Figure 5.1. Evolution of mean faecal score in different experimental groups during post-inoculation period.
CTR (in orange): animals receiving pre-starter basal diet; **scFOS (in blue)**: animals receiving basal diet with short-chain fructo-oligosaccharides supplementation; **YEA (in purple)**: animals receiving basal diet with *Saccharomyces cerevisiae* Sc 47 supplementation; **SYN (in red)**: animals receiving both supplementations (scFOS + YEA). n = 7 for CTR group, n = 8 for rest of groups. [a,b] Indicate statistically significant differences between groups.

Table 5.3 shows the changes induced by the treatments in the incidence of diarrhoea determined as the percentage of animals per pen with faecal scores ≥4. After one week of adaptation and just before the challenge (day 0 PI), there was a significant PRE × PRO interaction (p = 0.001), resulting in CTR

and SYN groups showing higher diarrhoea prevalence. Treatments including only the probiotic or prebiotic showed the lowest levels of diarrhoea. One day after the challenge (day 1 PI), animals receiving the probiotic showed significantly higher diarrhoea incidence, regardless of whether or not they were receiving the prebiotic (66.3 vs. 35.3 %, respectively, p = 0.006). However, at day 3 PI, animals receiving the prebiotic registered a lower diarrhoea rate (34.1 vs. 55.2 %, p = 0.035), and there was also a trend for a lower incidence with the probiotic (36.3 vs. 52.9 %, p = 0.083). SYN treatment benefits from an additive effect (no interaction), resulting in the lowest numerical values.

Table 5.3. Effect of experimental treatments on percentage of animals with diarrhoea per pen. Considering diarrhoea as a score ≥4.

Main effects	PRO-		PRO+			*p-value*		
	PRE-	PRE+	PRE-	PRE+	RSE	PRE	PRO	Interaction
Diets	**CTR**	**scFOS**	**YEA**	**SYN**				
% diarrhoea/pen								
0PI	37.7[a]	8.3[b]	8.3[b]	37.1[a]	20.81	0.969	0.969	**0.001**
1PI	37.7	33.1	57.9	74.6	28.54	0.559	**0.006**	0.308
2PI	57.0	53.8	70.6	49.6	34.08	0.332	0.702	0.476
3PI	71.0	37.1	41.4	31.1	27.54	**0.035**	0.083	0.244
5PI	9.4	26.9	16.5	12.4	20.87	0.463	0.727	0.206
7PI	14.1	12.4	8.25	0	16.91	0.418	0.145	0.599

PRO+/-: presence/absence of probiotic ingredient in diet. **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. [a,b] indicate statistically significant differences between groups. PI, post-inoculation; RSE, residual standard error.

The rectal temperature of each animal was recorded at 0, 24, 48, and 72 hours PI. Rectal temperature showed normal values throughout the study, with no fever detected (> 40.5°C). No significant differences due to treatments were registered after the oral challenge. However, on day 0PI (before inoculation), supplementing the prebiotic was associated with

significantly lower temperatures (38.8 vs. 39.1, p = 0.032), which was a similar trend to the probiotic (38.8 vs. 39.1, p = 0.082).

5.3.3. Microbiological analysis

Faecal plate counts of ETEC on selective chromogenic media (Table 5.4) showed no growth before the inoculation. However, after the challenge (day 4 PI), plate counts reached high values. At day 4 PI, animals receiving the prebiotic showed higher faecal counts regardless of whether or not they were receiving the probiotic (9.20 vs. 7.76 CFU/g, respectively, p = 0.048), and no effect was found related to probiotic addition. However, at day 8 PI, there was a significant interaction, and all supplemented diets showed lower counts compared with CTR, despite no additive effect for the combined treatments (SYN) being found.

Contrary to faeces, plate counts of mucosa scrapings at day 4 PI showed that animals receiving the prebiotic exhibited lower numbers of ETEC (5.54 vs. 7.34 CFU/g, p = 0.002), with no impact for probiotic supplementation. At day 8 PI, and similarly to faeces, there were no significantly lower values for the three supplemented diets compared with the CTR diet. Ileum digesta samples also showed similar reductions for the three supplemented diets at day 8 PI, with the combination of the prebiotic and probiotic exhibiting an additive decrease in ETEC (SYN diet); therefore, the interaction was not significant. For colon digesta, significant decreases were also found related to the prebiotic or probiotic supplementation at both 4 PI and 8 PI but without additive effects.

In relation to faecal plate counts of lactobacilli in selective MRS medium (Table 5.4), no differences due to treatments were seen before the inoculation (day 0 PI) or on day 4 PI. However, samples collected on day 8 PI

showed higher counts of lactic bacteria with the supplemented diets; however, no additive effect was found for the SYN combination (significant interaction). Plate counts of mucosa scrapes on day 8 PI showed the clear impact of supplementing the probiotic on lactobacilli counts, with a trend for higher values when supplemented alone (interaction $p = 0.083$). Counts of ileal and colonic digesta did not show significant differences due to treatments in any of the sampling days.

When the microbiological results were also expressed as the log ratio of lactobacilli/ETEC (Table 5.4), in most cases, there was a clear significant effect of supplementing the prebiotic or the probiotic, namely higher ratios; however, there were no additional effects when combining both ingredients.

Table 5.4. Plate counts (log CFU/g) of ETEC (chromogenic media), lactobacilli (selective MRS medium), and ratio of lactobacilli/ETEC (as difference of logarithms) in ileal mucosa scrapings, ileum and colonic digesta, and faecal samples.
Values are given for day 4 and 8 post-inoculation, and for the initial of the trial and day 0 PI for faecal lactobacilli counts.

Main effects		**PRO-**		**PRO+**			***p-value***		
		PRE-	**PRE+**	**PRE-**	**PRE+**	**RSE**	**PRE**	**PRO**	**Interaction**
Diets		**CTR**	**scFOS**	**YEA**	**SYN**				
ETEC	**Day**								
Ileal mucosa	4 PI	7.96	5.61	6.80	5.46	1.505	**0.002**	0.235	0.359
	8 PI	5.43^{a}	4.14^{b}	4.22^{b}	4.50^{b}	0.454	**0.004**	**0.014**	**<0.001**
Ileal digesta	4 PI	7.79	5.45	5.48	6.24	1.932	0.267	0.285	**0.034**
	8 PI	5.42	4.27	4.41	3.98	0.616	**0.001**	**0.007**	0.119
Colonic digesta	4 PI	8.45^{a}	6.52^{b}	6.41^{b}	7.05^{ab}	1.010	**0.086**	**0.047**	**0.002**
	8 PI	7.22^{a}	5.66^{b}	5.29^{b}	4.80^{b}	0.657	**<0.001**	**<0.001**	**0.032**
Faeces	4 PI	7.75	9.91	7.77	8.49	1.939	**0.048**	0.329	0.310
	8 PI	6.28^{a}	4.88^{b}	4.70^{b}	5.01^{b}	0.791	**0.064**	**0.017**	**0.006**
Lactobacilli									
Ileal mucosa	4 PI	6.12	6.72	7.14	6.30	0.837	0.694	0.334	**0.023**
	8 PI	5.23	5.77	7.33	6.46	1.084	0.666	**0.001**	**0.083**
Ileal digesta	4 PI	8.52	8.75	8.19	8.58	0.618	0.176	0.279	0.731
	8 PI	8.89	9.07	8.98	8.94	0.201	0.346	0.804	0.151
Colonic digesta	4 PI	8.84	8.90	8.66	9.10	0.420	0.110	0.929	0.211
	8 PI	8.88	8.88	8.84	8.84	0.231	0.964	0.624	0.975
Faeces	0	8.66	8.35	8.81	8.78	0.695	0.517	0.255	0.586
	0 PI	8.78	9.00	8.97	8.70	1.217	0.956	0.898	0.576
	4 PI	9.65	9.87	8.60	9.86	1.381	0.147	0.291	0.306
	8 PI	6.82^{b}	9.05^{a}	10.06^{a}	10.04^{a}	1.031	**0.006**	**<0.001**	**0.005**
Ratio lactobacilli:ETEC									
Ileal mucosa	4 PI	-1.84^{b}	1.11^{a}	0.341^{a}	0.833^{a}	1.5960	**0.006**	0.108	**0.042**
	8 PI	-0.199^{b}	1.63^{a}	3.11^{a}	1.96^{a}	1.0670	0.381	**<0.001**	**0.001**
Ileal digesta	4 PI	0.728	3.30	2.72	2.34	1.7870	0.100	0.433	**0.030**
	8 PI	3.47	4.79	4.57	4.97	0.6529	**0.001**	**0.012**	0.059
Colonic digesta	4 PI	0.395^{b}	2.38^{a}	2.25^{a}	2.06^{a}	1.1560	**0.040**	0.075	**0.014**
	8 PI	1.66^{b}	3.23^{a}	3.55^{a}	4.04^{a}	0.6835	**<0.001**	**<0.001**	**0.038**
Faeces	4 PI	1.90	-0.038	0.822	1.36	2.4650	0.436	0.860	0.173
	8 PI	0.539^{b}	4.18^{a}	5.36^{a}	5.02^{a}	1.3280	**0.002**	**<0.001**	**<0.001**

PRO+/-: presence/absence of probiotic ingredient in diet; **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. ^{a,b} Indicate statistically significant differences between groups. PI, post-inoculation; RSE, residual standard error.

5.3.4. ETEC F4$^+$ qPCR quantification

The results of qPCR quantification showed that not all the animals obtain quantifiable amounts of F4 genes after the challenge. Regarding colonic digesta, eight animals showed non-quantifiable values (five at day 4 PI and three at day 8 PI), while in ileal mucosa scrapings, nine animals showed non-quantifiable values at day 8 PI. No differences between treatments were found in the number of animals with non-quantifiable ETEC F4$^+$.

Table 5.5 shows ETEC F4$^+$ numbers in ileal mucosa and colonic digesta samples (log F4 genes copies/g FM) only considering quantifiable animals. As expected, the numbers decreased from day 4 to 8 PI in both types of samples; however, no statistical differences were found between treatments, except a trend for the interaction in ileal mucosa at day 8 PI, where higher values for all the supplemented diets were observed.

Table 5.5. Numbers of F4 genes in ileal mucosa and colon digesta according to qPCR (in quantifiable animals).
Values were measured at day 4 and 8 post-inoculation.

Main effects		**PRO-**		**PRO+**		***p-value***			
		PRE-	**PRE+**	**PRE-**	**PRE+**	**RSE**	**PRE**	**PRO**	**Interaction**
Diets	**Day**	**CTR**	**scFOS**	**YEA**	**SYN**				
Log F4 gene copies/g FM									
Ileal mucosa	4 PI	4.36	3.88	4.87	4.43	1.182	0.423	0.456	0.961
	8 PI	2.65	3.26	3.50	3.17	0.322	0.518	0.191	0.051
Colon digesta	4 PI	4.93	4.22	4.42	3.94	1.476	0.231	0.396	0.720
	8 PI	3.43	3.79	3.37	3.29	0.443	0.510	0.191	0.299

PRO+/-: presence/absence of probiotic ingredient in diet; **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. PI, post-inoculation; RSE, residual standard error.

5.3.5. Microbial fermentative activity

Regarding pH values and ammonia concentration in colonic digesta samples, no significant differences were found related to the experimental treatments (data not shown).

Table 5.6 shows SCFA concentrations and their molar proportions, measured in colonic digesta at day 4 PI and 8 PI and in faecal samples at day 0 PI (day 8) and 8 PI (day 16). Colonic digesta samples showed lower SCFA concentrations at day 8 PI with the combination of the pre- and probiotic (SYN diet) when compared with other supplemented diets (p interaction = 0.096). This treatment was also associated with the highest molar proportions of BCFA (branched-chain fatty acids) (p interaction = 0.026). In faecal samples, SCFA concentrations also showed lower values at day 8 PI when both ingredients were combined (p interaction = 0.023), but in this case without significant changes in the molar proportion of BCFA. Statistical trends were found in the SYN group for molar proportions of propionate at day 0 PI (p interaction = 0.057), which presented higher values compared with other treatments, and for molar proportions of valerate at day 0 PI (p interaction = 0.085), which presented lower values than other groups.

Table 5.6. Effect of experimental treatments on colonic and faecal fermentation.
Total short-chain fatty acids (SCFA) (mmol/kg of FM), as well as molar ratio of their components are included. Values are given for days 0, 4 and 8 post-inoculation.

Main effects		PRO-		PRO+			*p-value*		
		PRE-	PRE+	PRE-	PRE+	RSE	PRE	PRO	Interaction
Diets	**Day**	**CTR**	**scFOS**	**YEA**	**SYN**				
Colonic digesta (mmol/kg FM)									
Total SCFA	4 PI	80.9	65.4	76.7	84.5	26.3	0.691	0.445	0.238
	8 PI	105.9	112.5	118.8	100.7	19.9	0.428	0.933	0.096
Molar ratio of SCFA (%)									
Acetate	4 PI	59.6	60.7	61.6	58.3	6.085	0.617	0.918	0.337
	8 PI	58.1	57.4	55.5	58.5	3.969	0.441	0.606	0.205
Propionate	4 PI	27.0	23.6	24.3	27.7	5.795	0.986	0.746	0.119
	8 PI	25.4	25.4	28.4	26.6	3.898	0.531	0.149	0.538
Butyrate	4 PI	9.33	10.61	10.16	9.12	2.849	0.910	0.755	0.276
	8 PI	12.3	13.1	12.3	9.56	2.925	0.357	0.102	0.107
Valerate	4 PI	1.89	2.16	1.75	1.97	0.626	0.287	0.479	0.905
	8 PI	2.18	2.09	2.19	2.36	0.641	0.875	0.570	0.587
BCFA	4 PI	2.16	2.93	2.24	2.91	1.211	0.116	0.949	0.910
	8 PI	1.98^{xy}	1.69^{xy}	1.22^{y}	2.66^{x}	1.019	0.125	0.773	**0.026**
Faeces (mmol/kg FM)									
Total SCFA	0 PI	82.2	89.4	79.1	73.8	23.04	0.919	0.298	0.487
	8 PI	77.4	93.2	88.0	68.2	20.6	0.791	0.340	**0.023**
Molar ratio of SCFA (%)									
Acetate	0 PI	57.2	54.3	60.0	54.5	5.60	0.064	0.501	0.547
	8 PI	56.7	56.7	58.4	58.6	3.60	0.601	0.438	0.523
Propionate	0 PI	22.8	21.3	19.8	23.8	3.53	0.366	0.858	0.057
	8 PI	22.2	21.7	22.3	22.6	2.23	0.973	0.523	0.624
Butyrate	0 PI	12.4	15.5	11.3	12.3	3.36	0.119	0.102	0.407
	8 PI	11.7	13.6	11.4	10.9	2.32	0.398	0.085	0.174
Valerate	0 PI	2.93	3.28	3.20	2.44	0.79	0.514	0.388	0.085
	8 PI	2.70	2.89	2.96	2.64	1.02	0.834	0.984	0.522
BCFA	0 PI	4.76	5.55	5.77	6.53	1.36	0.146	0.065	0.972
	8 PI	4.91	4.82	4.69	4.93	1.18	0.862	0.888	0.698

FM: fresh matter. BCFA: branched-chain fatty acids. **PRO+/-**: presence/absence of probiotic ingredient in diet; **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for the rest of groups. ^{x,y} indicate a statistical trend between groups. PI, post-inoculation; RSE, residual standard error.

5.3.6. Immune response

The animals' inflammatory response was assessed by measuring serological levels of TNF-α (pro-inflammatory cytokine) and Pig-MAP (main acute phase

protein in the pig) at day 0 PI (before inoculation), 4 PI, and 8 PI. The average values are detailed in Table 5.7.

In general terms, no significant effects were reported related to the experimental treatments except for a trend for a reduction in TNF-α levels with the scFOS diet detected after the week of adaptation and just before the challenge (p interaction = 0.063).

Table 5.7. Serological levels of TNF-α and Pig-MAP for different experimental treatments. Values are given for day 0, 4, and 8 post-inoculation.

Main effects		**PRO-**		**PRO+**		***p-value***			
		PRE-	**PRE+**	**PRE-**	**PRE+**	**RSE**	**PRE**	**PRO**	**Interaction**
Diets	**Day**	**CTR**	**scFOS**	**YEA**	**SYN**				
TNFα (pg/mL)	0 PI	124.1	94.5	102.5	109.8	26.40	0.251	0.742	0.063
	4 PI	78.7	101.8	100.1	151.9	64.50	0.118	0.135	0.543
	8 PI	80.4	85.6	83.0	79.0	18.58	0.932	0.770	0.497
PigMap (g/L)	0 PI	1.334	0.948	1.112	1.035	0.570	0.269	0.743	0.457
	4 PI	1.291	1.809	1.620	1.248	1.385	0.886	0.817	0.380
	8 PI	1.653	0.774	0.876	0.823	0.929	0.174	0.286	0.228

PRO+/-: presence/absence of probiotic ingredient in diet; **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. PI, post-inoculation; RSE, residual standard error.

5.3.7. Ileal histomorphometry

Table 5.8 shows the average value for the different histomorphological parameters determined in ileal sections. No significant effects attributed to the experimental treatments were detected except for a significant increase in the number of intraepithelial lymphocytes (IEL) at day 8 PI when animals received the probiotic regardless of whether or not they received the prebiotic (17.17 vs. 13.97 cell nº, respectively, p = 0.046; 6.36 vs. 5.14 cell nº/100 µm, respectively, p = 0.009), as well as a significant increase in the

number of mitosis (M) at day 8 PI with probiotic supplementation (1.15 vs. 0.69 cell nº, p = 0.008; 0.61 vs. 0.36 cell nº/100 µm, p = 0.01).

Table 5.8. Effect of experimental treatments on ileal histomorphology.

Main effects		PRO-		PRO+		*p-value*			
		PRE-	PRE+	PRE-	PRE+	RSE	PRE	PRO	Interaction
Diets	Day	CTR	scFOS	YEA	SYN				
VH (µm)	4 PI	265	257	249	243	45.3	0.656	0.358	0.956
	8 PI	298	253	257	284	50.1	0.617	0.760	0.057
CD (µm)	4 PI	192	202	179	181	30.7	0.579	0.140	0.699
	8 PI	191	198	185	193	25.4	0.402	0.536	0.992
VH:CD	4 PI	1.71	1.29	1.43	1.36	0.325	0.442	0.695	0.824
	8 PI	1.59	1.29	1.42	1.50	0.332	0.355	0.851	0.124
IEL (cell nº)	4 PI	11.6	11.0	10.4	8.41	3.170	0.263	0.107	0.571
	8 PI	14.7	13.4	16.1	18.2	4.195	0.793	**0.046**	0.265
GC (cell nº)	4 PI	9.30	8.15	11.4	12.9	5.736	0.952	0.109	0.541
	8 PI	13.2	8.02	12.6	9.48	4.561	0.096	0.605	0.169
M (cell nº)	4 PI	1.04	1.26	0.766	1.19	0.446	0.056	0.291	0.517
	8 PI	0.517	0.835	1.08	1.21	0.460	0.180	**0.008**	0.585
IEL (cell nº/100 µm)	4 PI	4.35	4.30	4.09	3.51	1.001	0.386	0.160	0.464
	8 PI	4.85	5.40	6.19	6.54	1.225	0.312	**0.009**	0.822
GC (cell nº/100 µm)	4 PI	3.55	3.19	4.30	5.14	1.912	0.727	0.060	0.389
	8 PI	4.45	3.11	3.90	3.46	1.605	0.136	0.866	0.443
M (cell nº/100 µm)	4 PI	0.551	0.648	0.426	0.664	0.254	0.079	0.543	0.450
	8 PI	0.290	0.424	0.595	0.634	0.258	0.360	**0.010**	0.612

Measured parameters: villous height (VH); crypt depth (CD); ratio villous height:crypt depth (VH:CD); intraepithelial lymphocytes (IEL); goblet cells (GC); mitosis (M). **PRO+/-**: presence/absence of probiotic ingredient in diet; **PRE+/-**: presence/absence of prebiotic ingredient in diet. n = 7 for CTR group, n = 8 for rest of groups. PI, post-inoculation; RSE, residual standard error.

5.4. Discussion

This trial aimed to expand our knowledge in the search for non-antibiotic alternatives to strengthen piglets in one of the most critical periods of their lives. To do so, we assessed the effectiveness of dietary supplementation based on a prebiotic or probiotic, as well as the beneficial impact of adding

both as a potential synbiotic in ETEC F4$^+$-challenged piglets (Barba-Vidal et al., 2018, 2019; Luise et al., 2019a).

Ensuring an effective infection is a crucial step in challenge models. In this trial, different parameters confirmed the successful infection of the animals. Clinical signs of moderate diarrhoea, with a peak just after the inoculation, together with the qPCR detection of ETEC F4$^+$ in mucosa scrapings and ileal and colonic digesta, proved the effectiveness of the inoculation. Similar moderate clinical response was observed in our previous studies using a similar model (Barba-Vidal et al., 2017a; Gustavo Hermes et al., 2013a; López-Colom et al., 2020; Rodríguez-Sorrento et al., 2021, 2022). Despite qPCR failing to evidence favourable effects for the experimental diets in post-weaning colibacillosis, ETEC plate counts showed the considerable impact of all supplemented diets, with reductions in all intestinal sampling sites. Apparent discrepancies between both methods could be the result of differences in sensitivity and targets. The qPCR detection of F4 fimbria is a very specific methodology that can detect any *E. coli* strain bearing this gene. In this work, no F4 fimbria were qPCR detected before the inoculation; therefore, we could assume that qPCR values after the challenge corresponded to the inoculated strain. However, it cannot be fully excluded that another indigenous strain could have taken the opportunity to grow. Furthermore, the registered levels were very close to the method's minimum level of detection (approximately 2.5 gene copies/g FM), and several animals presented not-quantifiable levels. Being so close to the minimum level of detection probably precludes from detecting differences with this method.

The ETEC plate count numbers were higher than those obtained by qPCR, even considering the different units used (CFU vs. gen numbers), suggesting that other ETEC strains had also been quantified. By inoculating the animals with the ETEC F4$^+$, we were able to induce colonization and infection of the

gut with a presumable associated dysbiosis. Disrupting the ecological equilibrium of the gut could have favoured the proliferation of other opportunistic pathogens sharing the same ecological niche with the inoculated *E. coli*. From this point of view, the ETEC plate count results not only reflect the impact of diets on the specific inoculated ETEC $F4^+$ but also the growth of other indigenous ETEC strains.

Fructo-oligosaccharides (FOS) are attractive prebiotic fibers, which are suggested to improve weaned pigs' intestinal morphology and growth performance, even after exposure to ETEC $F4^+$. Short-chain fructo-oligosaccharides (scFOS), such as those tested in this study, have a lower degree of polymerization (between 3 and 5) when compared with other FOS or inulin (≥ 9 units). Short-chain FOS are known to be fermented more effectively and quickly than other sources due to this lower degree of polymerization in their structure. Some *in vitro* studies demonstrated that scFOS are more effective in stimulating SCFA production (Hernot et al., 2009) and inducing an effective prebiotic effect than longer chains, such as oligofructose and inulin (Saulnier et al., 2008; Scott et al., 2014). Previous studies demonstrated that this source of scFOS is selectively fermented by gut microbiota, mainly promoting the growth of *Lactobacillus*, *Bifidobacterium*, and *Prevotella* at the expense of *E. coli* growth, and scFOS are also able to increase the concentration of SCFA (Bourgot et al., 2014, 2019; le Bourgot et al., 2017; Xu et al., 2005, 2002; Zhao et al., 2019). Moreover, this type of scFOS has been associated with changes in the immune response and with the enhancement of intestinal architecture in neonatal and weaned piglets (Ayuso et al., 2020; le Bourgot et al., 2016, 2017; Xu et al., 2005, 2002). Other favourable outcomes reported with dietary scFOS supplementation in piglets include the modulation of inflammatory responses by downregulating some cytokines, such as TNF-α and IL-6 in the small Intestine (Tsukahara et al., 2003; Zhao et al., 2019). Until

now, less was known about the effect of scFOS in pigs challenged with ETEC $F4^+$.

In this trial, a reduction in diarrhoea prevalence was seen in animals supplemented with scFOS. Reductions were detected at the end of first week, when scFOS was supplemented alone, and also shortly after the challenge (at day 3PI). One hypothesis that would explain reductions in diarrhoea with scFOS would be the specific promotion of LAB, particularly *Lactobacillus* and *Bifidobacterium*, which have the ability to exclude opportunistic pathogens, such as *E. coli* (Kim et al., 2022; Shim et al., 2007). Therefore, our results showed a promoting effect of scFOS on lactobacilli in faeces at day 8 PI, with reductions in ETEC plate counts in ileal mucosa and in ileum and colon digesta. As a result, the ratio of lactobacilli/ETEC was also lower in ileal and colonic digesta and in faeces at day 8 PI, as well as in ileal mucosa and colon digesta at day 4 PI, proving the beneficial competition between bacteria populations exerted by scFOS supplementation. However, despite reductions in diarrhoea with the prebiotic, we were not able to detect significant improvements on animal growth performance. Probably because of the limited number of replicates, the high individual variability observed in these challenge models, as well as the short period of scFOS supplementation, precluded us from finding statistical differences in performance indexes.

Supplementation with scFOS has been commonly associated with increases in SCFA (Xu et al., 2005; Zhao et al., 2019). In this trial, we also found an increase in the SCFA concentration in faeces and the colon (a trend) at day 8 PI, but only when scFOS was administered alone and not combined with the probiotic. These results would suggest that combining scFOS and *Saccharomyces cerevisiae* Sc 47 led to a decrease in the amount of fermentable carbohydrates (including scFOS) arriving to the hindgut. This

possibility is also suggested by the higher molar proportions of BCFA registered in the colon at day 8 PI. This reduction in the arrival of fermentable carbohydrates could be due to an increased fermentative activity at the end of the small intestine, as fermentation at the ileal level has been reported to be relevant in pigs (Hoogeveen et al., 2020). A cross-feeding phenomenon between *S. cerevisiae* and LAB, in ileum in the presence of scFOS, could be behind such an effect. Actually, Ponomarova et al. (2017) described a similar yeast–LAB cross-feeding community in the presence of lactose, in which *Saccharomyces cerevisiae* enabled the survival of *Lactobacillus plantarum* and *Lactococcus lactis* by secreting a pool of metabolites, especially amino acids, and LAB provided yeast with free glucose from lactose in a commensal association.

These effects, seen in microbial populations and fermentation activity with scFOS, were not associated with changes in ileal architecture, as had been previously reported in challenged piglets, in which the villus height and the ratio V/C were increased after FOS supplementation (Liu et al., 2020b). No effects were seen in the immunity indexes measured in this study (TNF-α, PigMap nor IEL), except a trend in TNA-α, which displayed a decrease at day 0 PI when supplemented alone, in agreement with Zhao et al. (2019). However, other authors have reported differences in cytokine and IgA secretion (le Bourgot et al., 2017; Zhao et al., 2019). Altogether, our results suggest that scFOS reduced diarrhoea, mainly through a preventive activity based on the growth promotion of beneficial bacteria that had competitively excluded potential opportunistic pathogens, such as *E. coli.*

Regarding the probiotic used in this trial, *Saccharamoyces cerevisiae* Sc 47*,* it has been previously documented to generate beneficial effects when tested in piglets challenged with ETEC $F4^+$. Its favourable effects include reductions in diarrhoea scores, ETEC $F4^+$ attachment to intestinal mucosa, and faecal

ETEC F4$^+$ shedding, and improvements to the intestinal architecture (Barba-Vidal et al., 2018; Che et al., 2017; Kiarie et al., 2011; Stuyven et al., 2009; Trckova et al., 2014). Supporting the literature, at day 3 PI, the probiotic-supplemented animals tended to present lower diarrhoea prevalence ($p = 0.083$), which was similar to the reductions observed with scFOS. Lower diarrhoea was also registered at the end of the first week when the probiotic was supplemented alone. However, in contrast with scFOS, animals receiving *S. cerevisiae* Sc 47 exhibited a considerable increase in diarrhoea prevalence just one day after the pathogen inoculation (1 PI). This could suggest the higher intestinal susceptibility of these animals to be colonized. This idea is in consonance with the reduction in ADFI registered with probiotic supplementation after the challenge, which was also seen in this and other trials (Czech et al., 2018). Nevertheless, despite this hypothesized higher susceptibility, the administration of the probiotic could have enhanced the piglets' response to the pathogen, which finally results in a good control of the infection. Considerably lower ETEC counts were found in the ileum, mucosa, colon, and faeces of these animals, with significant increases observed in the lactobacilli/ETEC ratios. These results clearly emphasize an antagonistic effect exercised by the probiotic against ETEC F4$^+$. *In vitro*, this antagonism has been explained by the nutrient competition between yeasts and bacteria, the modification of the physico-chemical conditions of the digestive environment, or by the yeast production of inhibitory substances, such as proteases or ethanol (Etienne-Mesmin et al., 2011). Other *in vivo* trials also supported the beneficial effect of *Saccharomyces cerevisiae*, showing an increase in the lactobacilli population in intestinal contents (Che et al., 2017). Supporting this hypothesis, we also found increased levels of lactobacilli in faeces and ileal mucosa at day 8 PI.

Other evidence supporting the hypothesis of yeast improving the animal response against the pathogen comes from ileal histomorphometry. In

accordance with Bontempo et al. (2006) and Trevisi et al. (2017), increases in mitosis counts were found in the ileum sections of the supplemented animals. Enterocytes are constantly being replaced in crypts, and the rate of replacement should match the rate of loss. During weaning, intestinal mucosa can become thinner due to different insults requiring more proliferating cells (Bontempo et al., 2006). Therefore, our results would suggest that yeast-supplemented piglets had a better capacity to restore the intestinal mucosa architecture by increasing cell replacement; however, despite this, increased mitotic activity without changes in the villus/crypt ratio could also be regarded as an increased pathogen invasiveness resulting from the probiotic. Moreover, an increased number of intraepithelial lymphocytes was also seen in pigs receiving the yeast. Weaning stress has been associated with decreases in these cells and an increased apoptosis, and Bontempo et al. (2006) and Zhaxi et al. (2020) reported that a greater number of lymphocytes in the intestine could represent an enhanced intestinal mucosa immunity. Taking this into account, the moderate increase observed in the IEL with the probiotic could be regarded as a positive effect.

The improved response of the piglets to the pathogen challenge with the probiotic could therefore be due to different mechanisms. On the one hand, supplementing *S. cerevisiae* Sc 47 could have caused ecological changes in the gut microbiota, as previously described by other authors (Kiros et al., 2019) which was suggested by the changes observed in lactobacilli population. Particularly relevant is the increase observed in the number of lactobacilli in ileal mucosa, which could have resulted in an improved mucosa immunity in agreement with the observed increases in IEL. We also hypothesize that a boosted immune response could have been mediated by a directed effect of the probiotic because it has been demonstrated to display multiple immuno-modulatory effects at the molecular level in intestinal porcine epithelial cells (Zanello et al., 2011a). In short, the benefits of

supplementing *S. cerevisiae* Sc 47 for the ETEC $F4^+$ challenge seems to be mediated not so much by a preventive effect, such as scFOS, but by an improved response mediated directly or indirectly by the supplemented probiotic.

In this study, we assessed supplementing animals with a combination of scFOS and *S. cerevisiae* Sc 47 as a potential effective synbiotic for post-weaning colibacillosis (Kim et al., 2019). The results showed that at day 3 PI, the greatest reduction in diarrhoeal clinical signs (faecal scores) was found with the SYN combination, reaching considerably lower values compared with CTR, supporting their effectiveness to fight *E. coli*. However, before the challenge, the combination of both additives did not result in any improvement compared with CTR, whereas the single supplements were shown to reduce the number of diarrheic pigs. These results would support the benefits of using this synbiotic combination in challenging scenarios, such as farms with a high incidence of PWD; however, the benefits may be minimal in farms with high hygienic standards.

In summary, the way this synbiotic would help to fight post-weaning colibacillosis would rely on combining the different mechanisms of action discussed above for each of their components. Combining the preventive benefits from scFOS and a boosted animal response promoted by *S. cerevisiae* Sc 47 would explain why the SYN treatment was associated with the lowest ETEC counts and the highest ratio of lactobacilli/ETEC in the colon at day 8 PI. Moreover, as discussed above, the combination of scFOS and *S. cerevisiae* Sc 47 could have also led to yeast–LAB cross-feeding phenomena, which synergistically promoted the growth of lactobacilli at the end of the small intestine, favouring the exclusion of *E. coli*.

5.5. Conclusions

Our results evidence that supplementing scFOS or *Saccharomyces cerevisiae* in the pre-starter diets is efficacious to fight post-weaning colibacillosis, showing considerable decreases in intestinal ETEC numbers. Their synbiotic administration would benefit from complementary modes of action, including preventive effects based on beneficial changes in the intestinal ecosystem and improvements in the animal response. Our results also suggest the possible synergistic effects of this synbiotic on foregut fermentation. Combining both strategies could be particularly beneficial when animals have to face an elevated risk of post-weaning colibacillosis on the farm.

Chapter 6. Evaluation of a new in feed additive during an enterotoxigenic *Escherichia coli* $F4^+$ oral challenge in weanling piglets, and in farm conditions in transition pigs



6.1. Introduction

In the current swine production system, weaning poses significant challenges for animals. Various stress factors including an abrupt change from milk to solid feed, separation from the mother, animal regrouping with new social hierarchies, and exposure to pathogens, can disrupt the animals' health balance (Lallès et al., 2007). PWD, often caused by ETEC strains F4 and F18, frequently occurs during this period, resulting in poor animal performance, high mortality rate and economic losses (Luppi et al., 2016; Rhouma et al., 2017).

Antibiotics were commonly used to mitigate the negative effects of PWD. However, due to increasing concerns regarding antimicrobial resistance, the use of antibiotics has been restricted in the European Union (EU), among other places. Following these measures, the practice of supplementing feed with therapeutic levels of ZnO during the first two weeks of weaning saw a rise (Canibe et al., 2022). Zinc (Zn) is an essential trace element in animal nutrition, playing a role in multiple physiological functions (Hill & Shannon, 2019; Sloup & Langrova, 2017). Incorporating ZnO into the diet of weaning piglets had demonstrated a reduction in the incidence of diarrhoea and an increase in growth. Although the underlying mechanisms are not fully understood, these beneficial effects may be attributed to antioxidant properties, interference with bacterial fimbriae adhesion, modulation of intestinal microbiota resulting in increased diversity of enterobacteria, and significant improvements in nutrient absorption and intestinal morphology in the animals (Bonetti et al., 2021; Canibe et al., 2022; Maier et al., 2017). Nevertheless, the use of high levels of ZnO has shown adverse effects on both human health and the environment, contributing to the emergence of antimicrobial resistance in bacteria and significant accumulation of heavy metals in the soil (Canibe et al., 2022). Consequently, the European

Medicines Agency (EMA) decided to remove the high dietary supplementation of ZnO by June 2022 (*Zinc Oxide | European Medicines Agency*, n.d.). Thus, in the current context, there is a need for new and effective alternatives to improve the health of piglets during weaning. Several non-antibiotic options are available, including live oral vaccines, bacteriophage cocktails, essential oils, organic acids, prebiotics, and probiotics (Gresse et al., 2017). Although some alternatives look promising, new options are being sought. Antibody technology has emerged as a new field of research. With the discovery of camelid antibodies, devoid of light chains, and later, of nanobodies, camelid heavy-chain variable domain with unique amino acid sequence (V_HH), a new field was opened for exploration. Nanobodies, or bivalent V_HH constructs, are comparable to monoclonal antibodies in terms of selectivity, specificity, and antigen-binding affinity. However, they distinguish themselves through their diminutive size, being the smallest naturally occurring (15 kDa), as well as stability, robustness, and high solubility. Furthermore, they appear suitable for oral administration, as it has demonstrated persistence in the digestive tract and maintained its activity (Fiil et al., 2022). For these peculiarities, these constructs emerge as a promising strategy for both prevention and treatment of infections (Wilken & McPherson, 2018).

Porcine ETEC strains are characterized by presenting a collection of specific fimbriae adhesins and enterotoxins (Gonzales-Siles & Sjöling, 2016). These proteins play a vital role in pathogen's virulence, facilitating bacterial attachment to epithelial cells in the gastrointestinal tract, and leading to inflammation and fluid loss. Previous studies have reported the effectiveness of bivalent V_HH constructs binding these virulence factors and reducing their pathogenicity (Harmsen et al., 2005; Virdi et al., 2013, 2019). These findings were observed *in vitro*, demonstrating a selective binding of the product to ETEC $F4^+$; and *in vivo*, showing a decreased number of ETEC $F4^+$ infected

piglets upon administration (Fiil et al., 2022). Nonetheless, in the latter study, the addition of V_HH did not promote any discernible impact on the productive parameters of the animals, necessitating further research to elucidate this phenomenon.

The present work hypothesizes that bivalent V_HH constructs, designed to specifically target F4 fimbriae, and LT and Stx2e toxin, can disrupt bacterial adhesion to enterocytes and block the toxin activity, thereby reducing their pathogenicity and facilitating their elimination. As a result, the outcomes would include a reduction in observed diarrhoea and noticeable improvements in intestinal health indicators and productive performance among treated pigs. Therefore, the objective of the present study was to assess the impact of bivalent V_HH constructs on intestinal health in two distinct scenarios: firstly, on weaning piglets orally challenged with ETEC $F4^+$ under controlled experimental conditions, and secondly, in nursery piglets reared under commercial conditions.

6.2. Materials and methods

The study included two different trials:

Trial 1 was conducted with ETEC $F4^+$ orally challenged piglets in the Experimental Unit (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona (UAB), with prior approval of UAB's Animal and Human Experimental Ethics Committee (permit nº. CEAAH 4026) and from the Departament de Medi Ambient i Habitatge of the Generalitat de Catalunya (DMAH 10118).

Trial 2 was conducted in a commercial pig farm in Catalunya (Spain), with prior approval of UAB's Animal and Human Experimental Ethics Committee

(permit no. CEAAH 3817) and from the Departament de Medi Ambient i Habitatge of the Generalitat de Catalunya (DMAH 10215).

Treatment, management, housing, and husbandry conditions were conformed to the European Union Guidelines (Directive 2010/63/EU) in both trials.

6.2.1. Experimental products

The products subjected to experimental testing were directly supplied by Bactolife® (Copenhagen, Denmark). They consist of bivalent V_HH constructs, protein-based feed additives designed to selectively bind virulence factors associated with the pathogen. In Trial 1, the bivalent V_HH construct was specifically tailored to target the LT toxin and F4 fimbriae, key virulence factors found in the ETEC $F4^+$ inoculated strain. In Trial 2, the design was expanded to encompass the binding of F4 fimbriae, LT and Stx2e toxins, addressing a broader spectrum of virulence factors commonly found in *E. coli* strains under typical commercial farm conditions.

Trial 1

6.2.2. Animals, housing and experimental design

The study was carried out as a Biosafety Level 2 procedure, with adequate training of personnel involved.

A total of 96 male piglets (Landrace × Large White) were weaned at 21 days of age from a high health status farm and from non-vaccinated mothers against *E. coli*. Piglets were selected from sows that were homozygous for the Mucin 4 (MUC4) gene, a factor related to greater susceptibility to infections

caused by ETEC $F4^+$ (Jørgensen et al., 2003). The genotyping of the sows was carried out in accordance with the guidelines described by Jørgensen et al. (2003). Piglets were transported to the facilities of the UAB and housed in four rooms with eight pens each (32 pens, three animals per pen). Each pen ($3m^2$), slatted, had a feeder and a nipple drinker to provide feed and water ad libitum. The weaning rooms were equipped with automatic heating and forced ventilation. Regarding environmental enrichment, each pen was provided with a plastic toy, according to piglets' age. The experiment was carried out during the spring season (March-April) with an average temperature of 24°C (± 5°C).

At arrival, animals were distributed among rooms and pens according to their initial body weight to ensure a similar average within pens. The experimental design was a randomized block design, blocked by room. The three treatments were randomly allotted to the 32 pens, for a total of 11 pens for treatment CTR, 10 pens for treatment ZnO and 11 pens for treatment V_HH. The pen was considered as the experimental unit. All the animals were orally challenged with a pathogenic ETEC $F4^+$ strain, one week after arrival. The trial had a duration of 15 days.

6.2.3. Experimental diets and treatments

The three experimental treatments consisted of a control diet (CTR); the same control diet supplemented with 3,100 ppm ZnO (ZnO) (Apsamix Zinc 1000 mg/g, Pintaluba, Reus, Spain) as positive control; or supplemented with the bivalent constructs (V_HH) against LT toxin and F4 fimbriae (granulate supplemented at 1.05 kg/T LT and 0.67 kg/T F4). Animals from the V_HH treatment also received and oral solution of the product providing 15 mg per pig, the day 0 (before the challenge) and 1 PI.

Basal diet was formulated to satisfy the nutrient requirement standards for pigs of this age (National Research Council (NRC), 2012) and manufactured in a mash form. Ingredient and chemical composition are shown in Table 6.1. Basal diet was manufactured in 3 batches of 500kg (1.5 tonnes in total) for the CTR, ZnO and V_HH treatments each. One composite sample (200 g) of each diet was collected and stored for further analysis.

Table 6.1. Ingredients and analysed composition of basal diet.

Ingredients	(g/kg FM)
Maize	205.50
Wheat	180.40
Barley 2 row	170.30
Extruded soybean	150.00
Sweet whey powder (cattle)	112.00
Fishmeal LT	60.00
Soybean meal 47% CP	70.00
Whey powder 50% pork lard	25.00
Monocalcium phosphate	6.50
Calcium carbonate (CaCO3)	3.80
L-Lysine HCL (78)	4.50
Vit-Min Premix*	4.00
Salt	2.40
DL-Methionine 99	2.60
L-Threonine	2.30
L-Tryptophane	0.70
Analyzed Chemical Composition	(%)
Dry matter	91.04
Ash	5.22
Crude protein	19.28
Crude fiber	3.35
Ether Extract (fat)	6.70

FM, fresh matter. LT, low-temperature preparation of fishmeal to preserve amino-acid content. *Provided per kilogram of complete diet: 12,000 IU vitamin A; 1400 IU vitamin D3; 55 UI vitamin E; 2 mg vitamin K3; 3 mg vitamin B1; 7 mg vitamin B2; 7.33 mg vitamin B6; 0.06 mg vitamin B12; 17 mg calcium pantothenate; 45 mg nicotinic acid; 0.2 mg biotin; 1.5 mg folic acid; 60 mg Fe; 90 mg Cu; 95 mg Zn; 40 mg Mn; 0.7 mg I; and 0.3 mg Se.

6.2.4. Bacterial strain

Animals were orally challenged with enterotoxigenic *Escherichia coli* $F4^+$ strain isolated from 14-week-old pig's faeces and provided by the Infectious Diseases Laboratory of the UAB (Ref. COLI30/14-3). This strain had the following virulence factors: F4ab, F4ac, LT, STb and EAST1; and it was negative for the factors: K99, F6, F18, F41, STa, VT1, VT2 and EAE.

Oral inoculums were prepared after 24 h of incubation at 37°C in LB medium (Luria Broth) with slow agitation (250 rpm) in an orbital incubator. Final inoculum concentration was 7.7×10^8 CFU/mL, determined by plate counting in Luria agar. Plates were seeded just after inoculating the piglets.

6.2.5. Experimental procedure

At arrival, animals were weighed, identified with ear tags and distributed in pens according to their weight. Faecal samples were obtained from the largest animal in each pen (n = 32) for microbiological analysis. Feed consumption per pen was recorded during the adaptation week. After one week of adaptation (day 8), animals were orally challenged with the pathogen. A total volume of 6 mL of the strain culture was orally administered to each animal using a disposable syringe without needle, reaching a dose of 4.62×10^9 CFU/animal. To ensure that the piglets had a full stomach at the time of inoculation, standardize conditions and facilitating bacterial colonization, food was removed the same day early in the morning and replaced 15 minutes before inoculation.

Animals from the V_HH group (32 in total) also received an oral dose of V_HH at days 0 PI (before ETEC $F4^+$ inoculation) and at day 1 PI, to ensure proper product intake. A total of 1ml of liquid, providing 15 mg of the V_HH product,

was administrated orally to each piglet, each day. The rest of piglets were also dosed with saline solution (10 mM Tris-HCl + 10 mM NaCl at pH 6.5) as placebo.

After the oral ETEC $F4^+$ challenge, animals were examined daily for clinical signs to assess their PI status (e.g. dehydration, apathy, and diarrhoea). For microbiological analysis, faecal samples were taken after spontaneous defecation or by digital stimulation, from the heaviest pig from each pen on arrival day (0), and the same pig at day 5 of the adaptation week, before inoculation at day 8 (0 PI) and at days 12 (4 PI) and 16 (8 PI). Rectal temperatures were assessed one day before the challenge and on days 1, 2 and 3 PI. Individual faecal scores were registered the day before the inoculation and at days 0, 1, 2, 3, 5 and 7 PI by tactile faecal stimulation/sampling and/or visual inspection. A 5-point scale was used (1: hard and dry faeces; 2: well-formed firm faeces; 3: formed faeces; 4: pasty faeces; 5: liquid diarrhoea). Performance was monitored along the experimental period: piglets were weighed on days 0, 4 and 8 PI and feed consumption was recorded on days 0, 1, 2, 3, 4, 7 and 8 PI. The mortality rate was also noted. No antibiotic treatment was administered to animals during the experiment.

At day 12 (4 PI) and 16 (8 PI), the intermediate and the heaviest piglet (at the beginning of the study) from each pen were euthanized respectively. Animals were sequentially sampled during the morning (between 9:00 and 14:00 hours). The remaining animals were euthanized at the end of the study. For that, animals were first sedated with the synergic effect of xylazine (2 mg/kg) plus ketamine (20 mg/kg) IM. Blood samples were taken from the cranial cava vein and then animals were euthanized with an intravenous injection of sodium pentobarbital (200 mg/kg). The abdomen was immediately opened, the digestive package separated from the body and transferred to a tray.

Different samples were collected for analysis. The digestive content of ileum and colon was emptied and different aliquots of colon digesta were preserved for future analysis. For microbiological analysis, ileum and colon content samples were collected in sterile containers and ileal mucosa scrapings in eppendorfs, being stored at 4°C until analysis. For the quantification of ETEC $F4^+$ by qPCR, colon digesta samples and ileal mucosa scrapings were also collected and immediately frozen on dry ice and subsequently stored at -20°C. For the histological study, 2-3 cm long sections of the proximal ileum, jejunum and proximal colon were cut, thoroughly washed with sterile PBS and fixed by immersion in a solution of formaldehyde (4 %).

6.2.6. Analytical procedures

6.2.6.1. Feed analysis

Chemical analysis of the diets including dry matter (DM), ash, crude protein and fiber, and diethyl ether extract were performed according to the Association of Official Agricultural Chemists standard procedures (AOAC 2007).

6.2.6.2. Microbiological analysis

Lactobacilli were measured by plate counting in selective MRS media (CM0361B MRS Agar OXOID) and entero-hemorrhagic *E. coli* (EHEC) in selective chromogenic media (413697 CHROMIC- EHEC 6x200mL Biomerieux). The inoculated strain was confirmed to thrive on this last medium, however numerous other ETEC strains could also had potentially grow. Considering that no growth was found in this media for faecal samples

collected before the challenge, the plate counts on this media were finally considered as ETEC plate counts. Results are expressed as log CFU/g of fresh matter (FM).

ETEC $F4^+$ was quantified in ileum scraping and colonic digesta by qPCR using SYBR green dye and following (Gustavo Hermes et al., 2013b). Briefly DNA was extracted and purified using the commercial QIAamp Fast DNA Stool Mini Kit (Qiagen; West Sussex, United Kingdom) and DNA eluted in 200 mL of Qiagen buffer AE and stored at −20°C until use. For ETEC $F4^+$ real-time PCR quantification, the selected target gene was that coding the F4 fimbria of ETEC $F4^+$. Results are expressed as log F4 gene copies/g FM.

6.2.6.3. Serum analysis

Blood samples were centrifuged (2500 × g, 8 min at 4°C) and the serum obtained divided into different aliquots before storage at -20°C. Serum concentrations of tumour necrosis factor-α (TNF-α) were determined by enzyme-linked immunosorbent assay (ELISA) using the Quantikine Porcine TNF-α kit (R&D Systems; Minneapolis, MN, USA). Serum concentration of pig major acute-phase protein (Pig-MAP) was determined using a sandwich-type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A.; Segovia, Spain), following the manufacturer's recommendations.

6.2.6.4. Histological analysis

Histomorphometry of ileal sections was performed with a light microscope (BHS, Olympus; Barcelona, Spain) following Nofrarías et al. (2006) methodology. Measurements of 7-10 different villus-crypt complexes per samples were performed including: villus height (VH), crypt depths (CD), ratio villus:crypt (VH:CD), intraepithelial lymphocytes (IEL), goblet cells (GC), and mitosis (M).

6.2.6.5. Mother genotyping for ETEC $F4^+$ susceptibility

To ensure the susceptibility of piglets in the trial, mothers were genotyped for Mucin 4 (MUC4) gene polymorphism, as it is proposed as one of the genetic markers for pig ETEC $F4^+$ resistance/susceptibility (Luise et al., 2019a, 2019b). To obtain DNA, hair follicles from 75 mothers were collected and genetic material extracted, following the procedure described by (Luise et al., 2019b). A restriction fragment length polymorphism analysis (PCR-RFLP) was performed following the method described by Jørgensen et al. (2003). Piglets were selected from 33 sows homozygous for the susceptible gene ($MUC4^{GG}$).

6.2.7. Statistical analysis

Data were analyzed using the free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA) and the stats package (Team, 2014). Results are expressed as means with their standard errors unless otherwise stated. Microbiological data were previously log transformed.

The effects of the experimental treatment on performance, microbial counts, and all slaughter measurements were analyzed by a two-way analysis of variance (lm function for a two-way ANOVA) following:

$$Y_{ijk} = \mu + \ Treat_i + Room_j + (Treat \times Room)_{ij} + e_{ijk}$$

Where Y is each observation of the studied variable, μ is the global mean, $Treat_i$ is the main effect of the experimental diet, $Room_j$ is the main effect of the room, and (Treat × $Room_{ik}$) is the interaction. Finally, e_{ijk} is the unexplained random error. Regarding the $Room_k$ and $(Treat \times Room)_{ik}$, they were finally excluded from the model as they did not prove to have any consistent significant effect.

To analyze the evolution of faecal score data along time, a mixed-effects model (lme function) was used following:

$$Y_{ijk} = \mu + \ Treat_i + Time_j + (Treat \times Time)_{ij} + e_{ijk}$$

Where Y is each observation of the studied variable, μ is the global mean, $Treat_i$ is the main effect of the experimental diets, $Time_j$ is the main effect of the day post-inoculation, and Treat × $Time_{ij}$ is the possible interaction. Finally, e_{ijk} is the unexplained random error.

When the effects of treatments were established ($p = 0.05$), the least squares means were separated using the probability function of differences adjusted by Tukey-Kramer.

Mortality percentage was carried out by means of a frequency analysis using a Fisher test (fisher.test function).

To analyze the faecal score data, the non-parametric approach of Kruskal-Wallis test (kruskal.test) was used (Myles Hollander et al., 1973). When the effect of treatment was established ($p = 0.05$), the means were compared using Dunn's test (dunnTest) with FSA package (Ogle DH, 2022).

The pen was considered as the experimental unit for all parameters. The α level used to determine statistical significance was p = 0.05, and for statistical trend were considered levels between p = 0.05 - p = 0.10.

Trial 2

6.2.8. Animals, housing and experimental design

A total of 528 commercial crossing piglets ([Large White × Yorkshire] × Pietrain), male and female were used. The animals were obtained from a commercial sow farm (S1) positive for ETEC $F4^+$ (previously tested), at the day of weaning (23 ± 1.52 days) and moved to the associated commercial nursery unit with truck transport. The animals were distributed in 48 pens by sex (24 pens of males and 24 pens of females) and a balanced initial live weight (11 piglets/pen). Each pen had a commercial non-lidded hopper and a nipple drinker to ensure feed and water ad libitum. Pens were randomly allotted to the three experimental treatments (16 replicates for each treatment).

6.2.9. Experimental diets and treatments

The three experimental treatments consisted of a control diet (CTR) in which animals were offered standard pre-starter and starter diets; the same control diet except that the pre-starter diet was supplemented with 3,100 ppm ZnO (ZnO) (Apsamix Zinc 1000mg/g, Pintaluba, Reus, Spain) as positive control; or the same control pre-starter and starter diets supplemented with the bivalent constructs (V_HH) against LT and Stx2e toxins, and F4 fimbriae (granulate supplemented at 0.88 kg/T Stx2e, 0.67 kg/T LT and 1.05 kg/T F4). The pre-starter and starter diets were fed ad libitum for 14 consecutive days (P1) and 28 consecutive days (P2), respectively.

Basal diets were formulated to meet the requirements for pigs of this age (National Research Council (NRC), 2012). Ingredient and chemical composition are shown in Table 6.2. Basal diets were split into 3 different batches of 1500 kg, for each experimental diet. Experimental products were added to basal diets during the feed preparation process. Diets were produced in pellet form either for P1 (1.8mm) and P2 (3.2 mm). One composite sample (200 g) of each diet was collected and stored for further analysis.

Table 6.2. Ingredients and analysed composition of basal pre-starter (PS) and starter (STR) diets.

Ingredients (g/kg FM)	**PS**	**STR**
Maize	350.00	528.20
Broken rice	120.00	50.00
Sweet whey powder (cattle)	90.00	-
Wheat	63.70	60.00
Soy protein concentrate	49.80	18.70
Extruded soybeans	48.20	117.30
Acid whey powder (cattle)	44.00	-
Lactose	35.00	-
Hydrolysate intestinal mucose 62% CP	34.70	-
Barley	30.00	30.00
Sucrose	30.00	-
Soybean meal 47% CP	30.00	120.00
Wheat bran	20.00	20.00
L-Lysine 50	12.80	9.80
L-Arginine	5.50	2.70
Mono calcium phosphate	5.30	10.30
VevoVitall*	5.00	5.00
Vit-Min Premix**	5.00	5.00
Lard	5.00	-
DL-Methionine 9 %	3.90	2.80
L-Threonine	3.80	2.80
L-Isoleucine 70%	2.60	0.70
L-Valine	2.60	1.80
L-Tryptophane	1.70	1.00
Salt	1.10	9.50

L-Histidine	0.20	-
Limestone	0.00	4.30
Ronozyme (phytase)	0.10	0.10
Analyzed Chemical Composition (%)	**PS**	**ST**
Dry Matter	89.5	88.1
Ash	4.35	4.99
Crude protein	19.5	18.6
Crude fibre	2.19	3.15
Ether Extract (fat)	4.84	5.14

*Vevovitall® (DSM, Shangai, China).
**Supplied the following per kg of diet: 7,000 IU of vitamin A (acetate); 500 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 45 mg of vitamin E; 1 mg of vitamin K3; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 1.75 mg of vitamin B6; 0.03 mg of vitamin B12; 8.5 mg of D-pantothenic acid; 22.5 mg of niacin; 0.1 mg of biotin; 0.75 mg of folacin; 20 mg of Fe (chelate of amino acids); 2.5 mg of Cu (sulphate); 7.5 mg of Cu (chelate of glycine); 0.05 mg of Co (sulphate); 40 mg of Zn (oxide); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (oxide); 7.5 of Mn (chelate of glycine); 0.35 mg of I, 0.5 of Se (organic); 0.1 mg of Se (sodium).

6.2.10. Experimental procedure

At arrival to nursery unit, animals were weighted, identified with an electronic ear tag and distributed in pens according to their weight and sex. Weight was recorded at weaning (day 0), day 14 and 42 post-weaning. Feed consumption per pen was recorded throughout the study (day 0, 14 and 42 post-weaning). The health status of the piglets was periodically evaluated, and any abnormal signs or medication administered was recorded. Pen faecal scores were registered every two days throughout the trial by visual inspection of the faeces found in the wet corner. A 4-point scale was used (0: well-formed firm faeces + clean piglets; 1: soft faeces + clean piglets; 2: pasty faeces + dirty piglets; 3: liquid diarrhoea + dirty and wet piglets). Faecal consistence was visually evaluated by the same experienced person that was blinded to treatments.

Faecal samples were collected at day 0, 7 and 14, for F4ab, F4ac, F18, LT and Stx2e virulence factor detection by PCR, and dry matter analysis, from a pig close to the average live weight of the pen, from each pen. Faecal samples for virulence detection were obtained through rectal stimulation using hyssops with Amies medium (Deltalab, Spain).

6.2.11. Analytical procedures

6.2.11.1. Feed analysis

Proximal analysis of feed was performed following the same methods than in Trial 1.

6.2.11.2. Characterization of *E. coli* isolates.

Genomic DNA of *E. coli* isolates were extracted by the boiling method, as described previously by Al-Gallas et al. (2007). PCR multiplex was used to detect toxin genes (LT, Stx2e) and adhesin genes (F4, F18) as previously described by Toledo et al. (2012). Four different multiplex PCRs for each toxin and adhesin genes were carried out using Apex Taq DNA polymerase (Genesee Scientific). Primers used were as described by Vidal et al. (2020).

6.2.12. Statistical analysis

Productive parameters (ADFI, ADG and feed conversion ratio (FCR)), were analyzed considering the different experimental treatments under study as main factor with ANOVA using the GLIMMIX procedure of the statistical package SAS® (SAS Institute Inc., Cary, NC), following:

$Y_{ij} = \mu + Treat_i + e_{ij}$

Where Y is each observation of the studied variable, μ is the global mean, $Treat_i$ is the main effect of the experimental diets and e_{ij} is the unexplained random error.

To evaluate the treatment effect on LW and ADG of the animals, an analysis based on quantiles was used. The pen was considered the experimental unit, and the 10th, 20th, and 30th quantiles, denoted as Q_{10}, Q_{20} and Q_{30}, of LW and ADG were calculated for each treatment group (CTR, ZnO, and V_HH). These quantiles indicate the weights below which 10%, 20%, or 30% of the animals fall, with the remaining animals above these values. Therefore, a significantly higher Q_{10} value in a treated group compared to a control group would suggest that the 10% of smaller animals in the treated group have shifted to higher weights than those in the control group. To determine if the differences in quantiles are significant, a quantile test (Johnson et al., 1987) was applied separately for each pair in CTR, ZnO, and V_HH. This nonparametric method, implemented in R (EnvStats library in R 4.1.1, (Millard, 2013)), does not require standardization.

Chi-square analysis on data mortality and culled animals was performed by considering exact Fisher's test using free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA). Fisher's test analysis was performed on virulence factor genes detection (positive vs negative) using the same software version of RStudio.

The pen faecal scores were analyzed as an average value evolution (from the measures taken every two days) for each period P1 (0 to 14 d post-weaning) and P2 (15 to 35 d post-weaning) by fitting an ordinary regression model with quadratic adjust, using the free R statistical analysis software version x64 4.0.3 (R Development Core Team; Franklin Lakes, NJ, USA).

The pen was considered as the experimental unit for all parameters. The α level used to determine statistical significance was p = 0.05, and for statistical trend were considered levels between p = 0.05 - p = 0.10.

6.3. Results

Trial 1

The trial run as expected without any noticeable incidence. All animals showed a good health status at their arrival. No presence of ETEC $F4^+$ was detected in faeces before inoculation (day 0, 5 and 8). Once the inoculation was performed, animals showed mild clinical signs of diarrhoea that spontaneously resolved at the end of the study without needing any therapeutical treatment. A total of one death was recorded throughout the study, from CTR group. This casualty occurred at day 4 PI after a sudden acute curse of diarrhoea (faecal score of 5 in scale of 1-5).

Three pens were excluded from data analysis due to negligible feed intake throughout the study. There was one pen excluded from each diet. Average daily feed intake for these pens along the first week were 2.89, 10.06 and 6.95 g/day, from CTR, ZnO and V_HH respectively.

6.3.1. Performance parameters

Average values of LW, ADFI and ADG for each experimental treatment are presented in Table 6.3.

No significant differences between treatments were found in the LW of animals at the beginning of the study neither at the end of it. In relation to ADG, no differences were found due to treatments during the adaptation period. However, after the challenge, significant higher values (p = 0.018) were described for ZnO and V_HH treatments, compared to CTR. Regarding ADFI, there were no significant differences due to treatments during the adaptation period and neither on PI period.

Table 6.3. Effect of experimental treatments on animal performance.
Live weight (LW) (g), average daily gain (ADG) (g/day), average daily feed intake (ADFI) (g/day) for adaptation period (days 1-7), first post-inoculation period (days 0-4 PI), post-inoculation period (days 4-8 PI).

	CTR	ZnO	V_HH	RSE	*p-value* Treatment
LW (g)					
D0	5908	5891	5865	153.2	0.820
D15	6925	7346	7351	849.7	0.455
ADG (g)					
adapt.	29.35	0.624	40.75	54.60	0.279
0-4 PI	27.58[b]	112.4[a]	105.8[a]	68.03	**0.018**
4-8 PI	175.3	250.3	194.2	155.1	0.561
ADFI (g)					
adapt.	66.14	56.18	80.47	30.06	0.226
0-4 PI	157.4	166.2	180.6	51.11	0.598
4-8 PI	228.0	280.8	277.7	99.77	0.434

CTR: animals fed a basal diet; **ZnO**: fed a basal diet supplemented with 3,100 ppm of ZnO; **V_HH**: fed a basal diet supplemented with V_HH constructs together an oral dose of V_HH constructs before and one day after the challenge. For details see material and methods section. n = 10 for CTR and V_HH group and n = 9 for ZnO group. PI, post-inoculation; RSE, residual standard error.
[a,b] Indicate statistically significant differences between groups.

6.3.2. Faecal score

Faecal consistencies were measured from the day before inoculation until one week later. At day 0 PI, mean faecal score for the different treatments was around 3. Once inoculated, faecal score of the animals worsened with increased scores up to day 5 PI, decreasing afterwards. Significant differences related to treatments were seen one day before the challenge, and at day 2 PI, showing the CTR group higher scores than treated diets, reaching statistical significance with ZnO. Faecal score evolution along time can be seen in Figure 6.1.

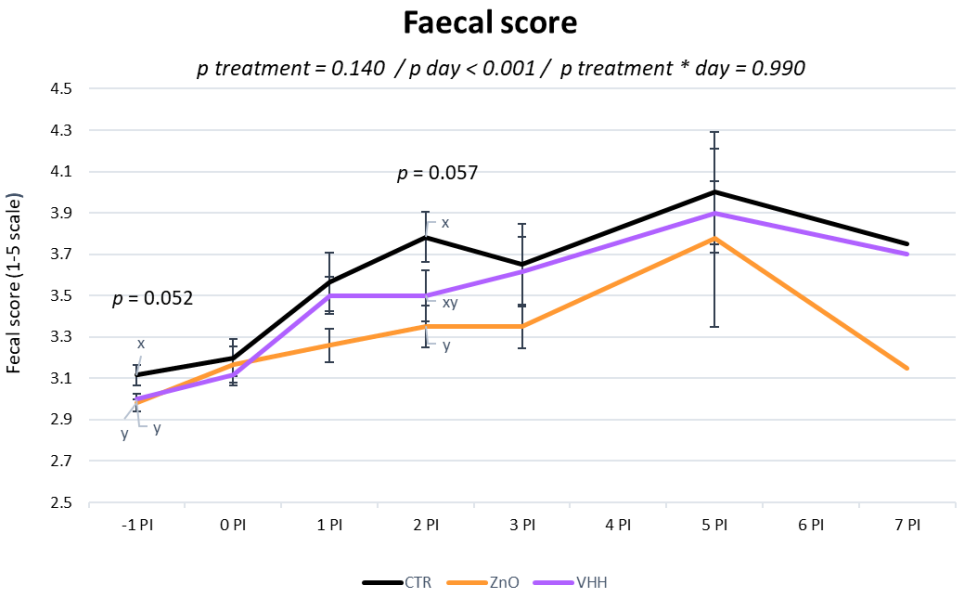


Figure 6.1. Effect of experimental treatments on evolution of the mean faecal score during the post-inoculation period, with standard error bars.
1: hard and dry faeces; 2: well-formed firm faeces; 3: formed faeces; 4: pasty faeces; 5: liquid diarrhoea. **CTR (in black)**: animals fed a basal diet; **ZnO (in orange)**: fed a basal diet supplemented with 3,100 ppm of ZnO; **V_HH (in purple)**: fed a basal diet supplemented with V_HH constructs together an oral dose of V_HH constructs before and one day after the challenge. For details see material and methods section. n = 10 for CTR and V_HH group and n = 9 for ZnO group. ^{x,y} Indicate a statistical trend between groups.

Rectal temperature showed normal values and no-fever was detected (> 40.5°C). No significant differences due to treatments were registered after the oral challenge (data not shown). However, on day 0 PI (previously to the inoculation), ZnO was associated to a trend to lower temperatures comparing to V_HH group (39.0, 38.7 and 39.1, respectively, p = 0.061).

6.3.3. Microbiological analysis

At day 0, 5 and just before the inoculation, there was no growth of ETEC $F4^+$ in faecal samples. However, after the challenge (day 4 PI), plate counts reached higher values suggesting the effective colonization of the gut by the pathogenic bacteria (Table 6.4). At day 4 PI, no significant differences due to treatments were found in ETEC plate counts, neither in ileal scraping, ileum, colon content, nor faecal samples. However, at day 8 PI, animals receiving V_HH showed lower faecal counts in faeces than CTR group. This significant difference was only seen in faecal samples. In relation to plate counts of lactobacilli in selective MRS medium, no differences due to treatments were seen before the inoculation. However, at day 4 and 8 PI, animals receiving ZnO registered lower lactobacilli counts in ileum content and ileal mucosa respectively, when compared to CTR group. Animals receiving V_HH registered intermedium values in ileum content. When microbiological results were expressed as lactobacilli/ETEC ratio, differences due to treatments reached an statistical trend (p = 0.08) in faeces at day 8 PI, showing V_HH the highest values and CTR treatment the lowest.

Table 6.4. Effect of experimental treatments on plate counts (log CFU/g) of ETEC (chromogenic media), lactobacilli (selective MRS medium), and ratio of lactobacilli/ETEC (as difference of logarithms). Ileal mucosa, ileal and colonic content, and faecal samples on day 0, 5, and 0, 4 and 8 post-inoculation.

						p-value
	Day	**CTR**	**ZnO**	**V_HH**	**RSE**	**Treatment**
ETEC						
Ileal mucosa	4 PI	4.28	3.60	4.45	2.696	0.771
	8 PI	4.18	3.48	4.08	1.661	0.620
Ileal content	4 PI	4.58	3.34	4.76	2.901	0.524
	8 PI	4.64	5.30	6.55	2.691	0.288
Colonic content	4 PI	7.21	7.61	6.66	0.999	0.135
	8 PI	5.47	7.13	6.52	2.017	0.212
Faeces	0 PI		No growth			
	4 PI	6.76	7.11	6.56	1.021	0.508
	8 PI	7.21^{a}	6.42^{ab}	5.77^{b}	1.220	**0.046**
Lactobacilli						
Ileal mucosa	4 PI	7.37	7.05	7.35	1.417	0.862
	8 PI	7.86^{a}	6.19^{b}	7.75^{a}	1.178	**0.008**
Ileal content	4 PI	8.54^{a}	7.99^{b}	8.44^{ab}	0.407	**0.015**
	8 PI	9.43	9.56	9.75	0.377	0.191
Colonic content	4 PI	8.61	8.60	8.48	0.304	0.584
	8 PI	8.95	8.62	8.80	0.366	0.169
Faeces	0	7.18	7.09	7.22	0.391	0.750
	5	8.64	8.45	8.51	0.368	0.525
	0 PI	8.49	8.43	9.00	0.592	0.081
	4 PI	8.28	8.02	8.12	0.780	0.764
	8 PI	8.51	8.22	8.39	0.322	0.154
Ratio Lactobacilli:ETEC						
Ileal mucosa	4 PI	1.20	0.95	1.13	0.721	0.738
	8 PI	1.55	1.87	1.70	0.640	0.542
Ileal content	4 PI	1.21	0.79	1.20	0.722	0.363
	8 PI	1.08	1.50	1.59	0.677	0.218
Colonic content	4 PI	1.20	1.14	1.33	0.200	0.145
	8 PI	1.08	1.24	1.40	0.414	0.237
Faeces	4 PI	1.24	1.14	1.28	0.207	0.323
	8 PI	1.20^{y}	1.34^{xy}	1.52^{x}	0.290	0.071

CTR: inoculated animals receiving diet without additives; **ZnO**: inoculated animals receiving ZnO; **V_HH**: inoculated animals receiving V_HH constructs. n = 10 for CTR and V_HH group and n = 9 for ZnO group. ^{a,b} Indicate statistically significant differences between groups. ^{x,y} Indicate a statistical trend between groups. PI, post-inoculation; RSE, residual standard error.

6.3.4. Enterotoxigenic *Escherichia coli* $F4^+$ qPCR quantification

Table 6.5 shows the number of F4 genes quantified in colonic digesta and ileal mucosa by qPCR. In ileal mucosa, 12 samples showed non-quantifiable values, interpreted as 0 log F4 gene copies/g FM. However, in the colonic digesta, all the animals showed quantifiable amounts of F4 genes. No differences were seen between experimental treatments.

Table 6.5. Effect of experimental treatments on quantification of F4 genes in ileal mucosa and colon digesta by qPCR.
Values measured at day 4 and 8 post-inoculation.

						p-value
lLog F4 gene copies/g FM	**Day**	**CTR**	**ZnO**	**V_HH**	**RSE**	**Treatment**
Ileal mucosa	4 PI	3.32	2.95	2.86	1.988	0.863
	8 PI	2.25	3.03	3.05	1.299	0.303
Colon digesta	4 PI	5.44	5.25	5.44	1.351	0.943
	8 PI	3.86	3.92	3.87	0.470	0.943

CTR: inoculated animals receiving diet without additives; **ZnO**: inoculated animals receiving ZnO; **V_HH**: inoculated animals receiving the V_HH constructs. n = 10 for CTR and V_HH group and n = 9 for ZnO group. PI, post-inoculation; RSE, residual standard error.

6.3.5. Immune response

Inflammatory response of the animals was assessed by measuring serological levels of TNFα (pro-inflammatory cytokine) and PigMap (main acute phase protein in the pig) at day 0 PI (before inoculation), 4 PI and 8 PI. No differences were seen between treatments at any day (data not shown).

6.3.6. Ileal histomorphometry

Table 6.6 shows the average value for the different histomorphological parameters determined in ileal sections. No significant effects on intestinal architecture attributed to the experimental treatments were detected except a statistical trend for increase in the ratio VH:CD at day 8 PI when animals received the ZnO supplementation comparing to CTR group (1.52 vs. 1.29, respectively, p = 0.028). It was found higher number of intraepithelial lymphocytes (IEL) at day 4 PI when animals received the V_HH compared to ZnO supplementation (9.46 vs. 5.80 cell number, p = 0.037).

Table 6.6. Effect of experimental treatments on histomorphology parameters in ileal section.

						p-value
Ileum	**Day**	**CTR**	**ZnO**	**V_HH**	**RSE**	**Treatment**
VH (µm)	4 PI	248	240	259	43.93	0.644
	8 PI	273	314	286	41.35	0.105
CD (µm)	4 PI	195	169	188	31.21	0.198
	8 PI	216	209	213	32.48	0.895
VH:CD	4 PI	1.28	1.44	1.43	0.331	0.444
	8 PI	1.29^{y}	1.52^{x}	1.35^{xy}	0.207	0.053
IEL (cell nº)	4 PI	8.06^{ab}	5.80^{b}	9.46^{a}	3.030	**0.045**
	8 PI	10.82	12.83	10.82	3.431	0.360
GC (cell nº)	4 PI	6.40	7.92	6.57	3.322	0.553
	8 PI	5.92	6.59	5.80	3.283	0.854
M (cell nº)	4 PI	0.59	0.78	0.66	0.414	0.615
	8 PI	0.40	0.53	0.32	0.293	0.338

CTR: inoculated animals receiving diet without additives; **ZnO**: inoculated animals receiving ZnO; **V_HH**: inoculated animals receiving the V_HH. n = 10 for CTR and V_HH group and n = 9 for ZnO group. ^{a,b} Indicate statistically significant differences between groups. ^{x,y} Indicate a statistical trend between groups. PI, post-inoculation; RSE, residual standard error. Measured parameters: villous height (VH); crypt depth (CD); ratio villous height:crypt depth (VH:CD); intraepithelial lymphocytes (IEL); goblet cells (GC); mitosis (M).

Trial 2

The trial was carried out as expected without any remarkable incident. A total of 30 casualties were recorded throughout the study. The number of casualties per treatment for the pre-starter period (day 0-14 post-weaning) was 1, 0 and 1 for CTR, ZnO and V_HH, respectively, without differences between treatments (p = 0.605; X-squared = 1.004). For the starter period (day 15-42 post-weaning), casualties per treatment were 9, 6 and 13 for CTR, ZnO and V_HH, being differences neither significant (p = 0.247; X-squared = 2.791).

No piglets were culled in the pre-starter period. In the starter period, 5, 6 and 5 animals were culled from CTR, ZnO and V_HH groups respectively, without showing statistical differences (p = 0.937; X-squared = 0.129). When culled piglets and casualties were pooled together the number of animals were 15, 12 and 19 for CTR, ZnO and V_HH respectively, without statistical differences between groups (p = 0.4143; X-squared = 1.762).

6.3.7. Performance parameters

Table 6.7 shows performance parameters for the pre-starter, starter period, and the entire trial. No significant differences between treatments were found in the LW of animals at the beginning nor at day 14 of the study. However, at the end of the trial (day 42), V_HH animals registered higher LW compared to CTR, showing ZnO intermediate values (p = 0.011).

In relation to ADG and ADFI, no differences were seen between treatments in the pre-starter period. Nevertheless, along the starter period, a trend for higher ADG values was observed when comparing V_HH to CTR, showing ZnO intermediate values (p = 0.056). When the entire experimental period was

considered, these differences reached the statistical significance (p = 0.036). No significant effect of the treatments was found in the FCR.

Table 6.7. Effect of experimental treatments on animal performance.
Live weight (LW) (kg), average daily gain (ADG) (g/day), average daily feed intake (ADFI) (g/day) and feed conversion ratio (FCR) for the pre-starter (day 0 to 14 post-weaning), starter (day 14 to 42 post-weaning) and for the entire trial (day 0 to 42 post-weaning).

					p-value
	CTR	**ZnO**	**V_HH**	**SEM**	**Treatment**
LW (kg)					
0d	5.164	5.087	5.116	0.158	0.941
14d	7.197	7.247	7.397	0.220	0.801
42d	14.630^{b}	15.771^{ab}	16.818^{a}	0.490	**0.011**
ADG (g)					
0-14d	144.5	154.3	163.0	7.16	0.200
14-42d	263.8^{y}	297.9^{xy}	317.4^{x}	17.85	0.056
total	224.0^{b}	250.0^{ab}	265.9^{a}	11.18	**0.036**
ADFI (g)					
0-14d	222.8	222.2	230.7	8.98	0.759
14-42d	551.2	529.4	596.5	37.35	0.439
total	441.8	427.0	474.5	25.49	0.410
FCR (g)					
0-14d	1.596	1.463	1.429	0.0730	0.243
14-42d	2.211	1.906	1.948	0.1915	0.481
total	2.252	1.792	1.826	0.1463	0.401

CTR: basal diet; **ZnO**: basal diet + ZnO (only in the pre-starter period); **V_HH**: basal diet + V_HH (2.2 kg/T or 1 kg/T). SEM, standard error of the mean.
^{a,b} Indicate statistically significant differences between groups. ^{x,y} Indicate a statistical trend between groups.

Experimental treatments had an impact on the growth evolution of the population. Live weight on day 42 post-weaning showed significant differences in quantiles 10, 20 and 30 among treatments (Table 6.8). V_HH and ZnO showed statistical higher LW compared to CTR. Figure 6.2a illustrates a reduction in the left tail of the population weights, remarking greater values in the V_HH and ZnO groups. Additionally, V_HH and ZnO animals demonstrated

higher ADG compared to CTR in quantiles 10, 20 and 30 (Table 6.9). Graphically (Figure 6.2b), enhanced ADG (from day 0 to 42 post-weaning) can be observed for V_HH and ZnO treatments compared to CTR, attributed to a reduction in the left tail of the population.

Table 6.8. Effect of experimental treatments on quantile change (Q_{10}, Q_{20} and Q_{30}) according to the individual live weight at the end of the trial (42 days post-weaning).

Treatment	Q_{10}	*p-value* Q_{10}	Q_{20}	*p-value* Q_{20}	Q_{30}	*p-value* Q_{30}
CTR	10.64	**0.002**	12.04	**0.002**	13.06	**0.011**
ZnO	12.63		13.43		14.50	
CTR	10.64	**0.003**	12.04	**0.003**	13.06	**< 0.001**
VHH	12.61		14.23		14.85	

CTR: basal diet; **ZnO**: basal diet + ZnO (only in the pre-starter period); **V_HH**: basal diet + V_HH (2.2kg/T or 1kg/T).

Table 6.9. Effect of experimental treatments on quantile change (Q_{10}, Q_{20} and Q_{30}) according to the individual average daily gain for the entire trial (0-42 days post-weaning).

Treatment	Q_{10}	*p-value* Q_{10}	Q_{20}	*p-value* Q_{20}	Q_{30}	*p-value* Q_{30}
CTR	129.8	**< 0.001**	175.7	**0.003**	195.3	**< 0.001**
ZnO	180.7		206.0		221.1	
CTR	129.8	**< 0.001**	175.7	**< 0.001**	195.3	**0**
VHH	182.5		226.9		243.2	

CTR: basal diet; **ZnO**: basal diet + ZnO (only in the pre-starter period); **V_HH**: basal diet + V_HH (2.2kg/T or 1kg/T).

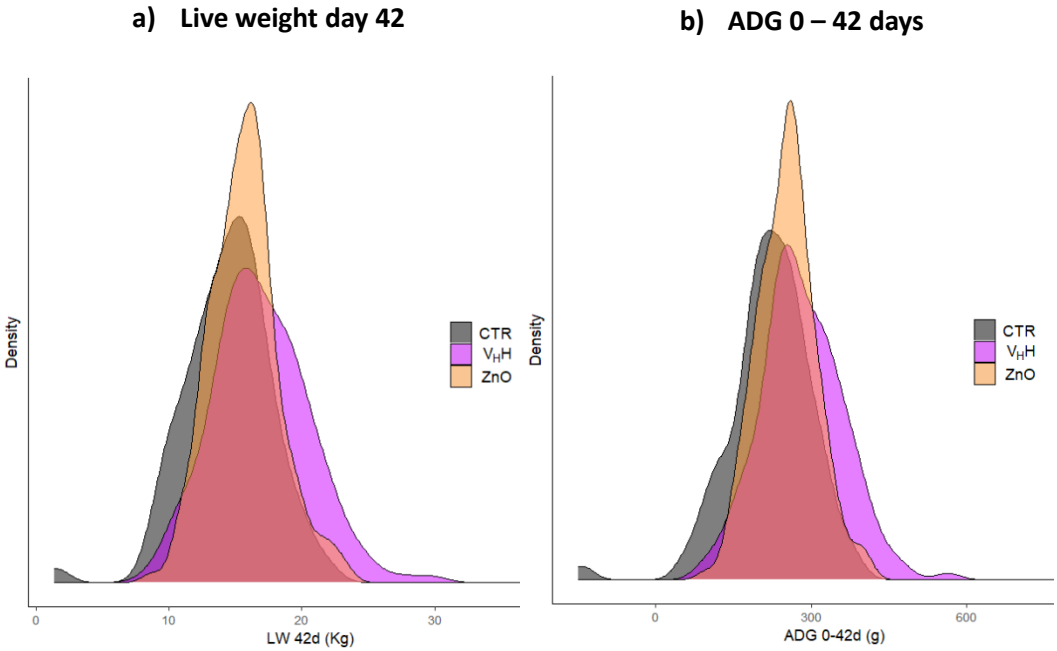


Figure 6.2. Effect of experimental treatments on live weight distribution at the end of P2 (day 42 post-weaning) (a), and ADG distribution for the entire experimental period (0 - 42 days) (b). **CTR (in black)**: basal diet; **V_HH (in purple)**: basal diet + V_HH (2.2kg/T or 1kg/T); **ZnO (in orange)**: basal diet + 3,100 ppm ZnO (only in the pre-starter period).

6.3.8. Faecal score and faecal dry matter

Faecal consistencies were measured every two days along the trial and analysed as an average value evolution. In the pre-starter period, lower average faecal scores were observed for the animals fed V_HH (-0.166; p = 0.005) and ZnO (-0.22; p = 0.0001) compared to CTR. In starter period, lower faecal score was observed only for the animals fed ZnO (-1.69X10-1; p = 0.0172) compared to CTR.

Regarding dry matter content, no differences were seen between treatments (77.2%, 78.9% and 78,2% respectively for CTR, ZnO and V_HH; $p > 0.10$). However, a clear weaning effect was observed with a reduction of dry matter

content (88.7% > 79.5% > 66.1% at weaning day (day 0), day 7 and 14 post-weaning, respectively; $p < 0.001$). No interaction was observed ($p > 0.10$) between treatments, indicating that the linear reduction of the dry matter content after weaning was independent of the treatment used.

6.3.9. Prevalence of virulence genes

Virulence factors (F4ab, F4ac, F18, LT and Stx2e) of ETEC were detected on faeces along P1 by PCR. When the positive values per toxin were cumulated along the entire period (day 0 to 14 post-weaning) no differences were observed for F4ac, F4ab, Stx2e (Vt2) and LT among treatments. However, higher F18 prevalence was observed for the animals fed ZnO diet than those fed CTR or V_HH ($p < 0.05$) (Table 6.10).

Table 6.10. Effect of experimental treatments on positive piglets for each virulence factor. Pre-starter period (cumulated values from days 0, 7 and 14 post-weaning).

Virulence factors	CTR	ZnO	V_HH	fisher test
F4ab	13/48	9/48	10/48	0.674
F4ac	10/48	10/48	11/48	1
Vt2	1/48	1/48	1/48	1
LT	0/48	0/48	0/48	1
F18	0/48	5/48	0/48	**0.011**

CTR: basal diet; **ZnO**: basal diet + ZnO (only in the pre-starter period); **V_HH**: basal diet + V_HH (2.2kg/T or 1kg/T).

Figure 6.3 illustrated the progression of the presence of F4 (F4ab and F4ac), the main virulence factor detected, during pre-starter period. Remarkably, the V_HH group did not show any peak of positive animals one week after weaning, unlike the CTR and ZnO groups. On day 7, 75% of animals in the CTR

group were presenting F4 virulence factors, whereas in the V_HH group, it was 44%, fisher test p = 0.149.

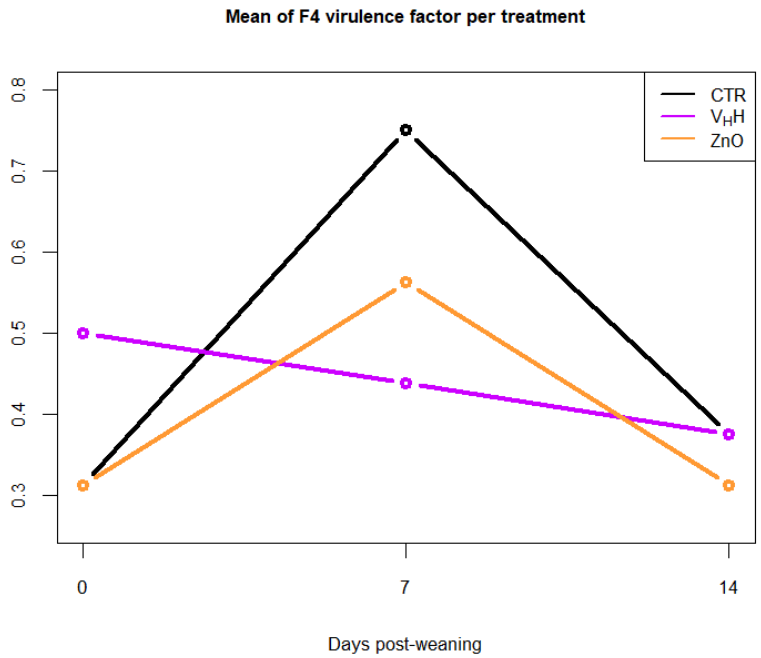


Figure 6.3. Effect of experimental treatments on the prevalence of positive piglets to F4 virulence factors.
F4ab and F4ac included along the pre-starter period (days 0, 7 and 14 post-weaning) (n = 16/treatment × day).
CTR (in black): basal diet; **ZnO (in orange)**: basal diet + ZnO (only in the pre-starter period); **V_HH (in purple)**: basal diet + V_HH (2.2kg/T or 1kg/T).

6.4. Discussion

In the pursuit of innovative strategies to mitigate the widespread issue of PWD, the current study aims to evaluate a novel technology targeting ETEC $F4^+$ infection. The bivalent V_HH constructs tested were specifically designed to bind not only F4 fimbriae, but LT and Stx2e toxins (this last in trial 2). The mechanism of action of these constructs involves binding to F4 fimbriae, disrupting bacterial attachment, promoting bacterial aggregation, and

ultimately facilitating their expulsion. Additionally, binding to LT and Stx2e blocks their toxicity, thereby reducing the virulence of the pathogen.

Two trials were designed to evaluate this new technology. Trial 1 was conceived as a validation of the V_HH constructs against the specific pathogen at experimental level, where factors can be more effectively controlled and modes of action better described (TRL 4 level (*Technology Readiness Level in EU Funding*, n.d.). To achieve this, an oral inoculation of the pathogen was conducted and confirmed by an increase in faecal ETEC CFU and the presence of moderate clinical signs, consistent with previous studies conducted by our group using the same methodology (López-Colom et al., 2019; Rodríguez-Sorrento et al., 2022). In a second stage of the research (trial 2) we aimed to evaluate this technology in a relevant environment (TRL 5-6 level), where pathogens are naturally encountered. To this end, a farm was selected based on previous confirmation of ETEC presence in the herd through the detection of virulence factors in faecal samples.

Regarding animal growth and performance, the additive consistently showed a positive effect in both trials. Piglets receiving the V_HH exhibited significantly higher growth, leading to increased live weights at the end of both studies. This enhanced growth does not appear to be attributed to increased feed intake (not significant) but rather to an improved performance among smaller piglets. Perhaps, due to a decrease in clinical or subclinical pathologies, these smaller individuals may allocate their energy towards growth rather than immune system activation. In fact, the additive reduced the proportion of underweight animals, aligning their weights more closely with the average of the ZnO group and distinguishing them from the CTR group, thereby promoting greater homogeneity. Variability at first ages has been evidenced to be one of the main variables explaining the variability at the end of fattening period in the pig industry (López-Vergé et al., 2018).

Therefore, increasing homogeneity of nursery piglets could be seen as an effective way to reduce the negative consequences of variability on depreciation of carcasses (Douglas et al., 2014) and underuse of barn facilities.

The prevalence of ETEC infection and related clinical signs observed throughout the trials provided crucial insights into the additive's effectiveness. In trial 1, the V_HH additive led to lower faecal scores, despite this difference was not statistically significant. Consistently in trial 2, animals receiving V_HH exhibited a significant reduction in faecal score during the pre-starter period, coinciding with the most critical time for piglets when it used to be reported a higher prevalence of diarrhoea (Tang et al., 2022).

The reduction in clinical signs could be aligned with a reduction in the ETEC $F4^+$ colonization. In trial 1, it was detected a significant reduction of ETEC plate counts on faecal samples at day 8 PI in animals of V_HH group. However, we were not able to evidence reductions in the pathogen by qPCR detection. Discrepancies between qPCR and plate counts for ETEC detection have been also reported in previous work (Ferreres-Serafini et al., 2023). Differences in sensitivity and targets between methods could explain lack of consistency. In trial 2, V_HH group showed a numerical decrease in the prevalence of F4 virulence factors (F4ab and F4ac) on faeces, suggesting a reduction of ETEC $F4^+$ relevance in those animals. These results suggest that the binding of V_HH constructs to the F4 fimbria, essential for effective gut colonization, has led to a negative selection against strains bearing F4, thereby reducing their ability to thrive. Conversely, constructs targeting LT and Stx2e toxins did not induce such negative selection against strains bearing these virulence factors, as they would not interfere with their potential to colonize the gut. However, they did counteract their detrimental effects on the animals. In the second trial, bivalent constructs also targeted a toxin involved in edema disease in

swine, caused by a different strain of *E. coli* (Tăbăran & Tabaran, 2019), thus providing coverage for a wider spectrum of pathogens. Specifically, we did not observe reductions in the prevalence of these toxins in faecal samples, but we did observe significantly higher growth rates in those animals.

ZnO also demonstrated similar positive effects on growth rates compared to the CTR group; however, its mode of action differed from V_HH. The beneficial impact of ZnO did not appear to be associated with any specific reduction in the pathogen, considering that ETEC levels were not reduced in trial 1, nor was the faecal prevalence of F4 virulence factors in trial 2. The positive impact of supplementing ZnO may have been mainly due to an improvement in the animal's response of infection, considering the various functions of Zn as a nutrient (Kambe et al., 2008). The potential antimicrobial effect described for pharmacological doses of ZnO (Bonetti et al., 2021) could have led to indiscriminate changes in the intestinal microbiota. In fact, in this work we observed unexpected increases in the faecal prevalence of F18 virulence factor (trial 2), as well as lower lactobacilli plate counts in the ileal lumen and mucosa at day 4 and 8 PI, respectively. This reduction of lactobacilli is consistent with findings from other studies (Hansen et al., 2022b; Højberg et al., 2005; Montoya et al., 2021). We could hypothesize that the potential broad effect of ZnO on microbiota could therefore negatively affect the microbiota balance, impairing beneficial bacterial populations resulting in undesirable side effects. Beneficial populations that might be affected include species from the genera *Lactobacillus* and *Bifidobacterium*. In this respect, Yoo et al. (2021), investigating the *in vitro* effect of ZnO nanoparticles on different bacterial populations, found that ZnO led to slight decreases in the counts of these bacteria and alterations on their morphology (Yoo et al., 2021). It is important to note that members of the genus *Lactobacillus* are among the most important probiotic bacteria. These beneficial microorganisms help maintain gastrointestinal barrier integrity,

compete against opportunistic pathogens, and participate in the maturation of the immune system in early life, among other functions (Rastogi & Singh, 2022). Interestingly, the higher number of ileal IEL found in the V_HH group compared to CTR group could also be attributed to the higher ileal lactobacilli populations observed with this treatment. A higher number of ileal IEL could be considered positive, as these cells play a crucial role in repairing and safeguarding the integrity of the intestinal epithelium, while also acting as early response effectors against mucosal pathogens (Olivares-Villagómez & Van Kaer, 2018).

The literature on the use of bivalent constructs in *in vivo* assays against bacterial pathogens remains limited due to their relatively recent introduction. However, these constructs represent one of the fastest-growing categories of biologics for clinical applications (Yong Joon Kim et al., 2023). Considering their potential application in the swine industry, bivalent constructs could be a valuable tool for infection prevention in scenarios where intestinal health balance is compromised, such as during the post-weaning period. These nanobodies could specifically interfere with the virulence factors of opportunistic pathogens, significantly reducing their opportunities to thrive and colonize the gut. This could be particularly relevant in reducing the prevalence of subclinical infections, which are frequently the cause of growth variability along the nursery period. However, in cases of established infections with acute clinical diarrhoea, intestinal dysbiosis, and disruption of the animal's homeostasis, antimicrobial strategies might still be necessary and preferred, as they are essential tools to fight the disease. Nevertheless, even in such scenario, bivalent constructs could serve as a valuable tool to improve the success of antibiotic therapy and prevent recurrences. To date, these constructs have shown promising results in combating existing infections in various *in vivo* assays (Andersen et al., 2016; Lu et al., 2022; Wilken & McPherson, 2018), including those caused

by infectious agents with limited treatment options (Sheoran et al., 2015). Overall, bivalent constructs emerge as a promising alternative to antimicrobials due to their potential efficacy and the lack of promotion of antimicrobial resistance (Sanaei et al., 2020; Seixas et al., 2022).

6.5. Conclusions

Based on the results, incorporating V_HH constructs into the diet during weaning may offer a promising alternative for the swine industry to prevent and treat post-weaning diarrhoea caused by ETEC $F4^+$. The inclusion of this product in the diet consistently demonstrated positive outcomes in both experimental and field trials. It led to increased animal weights, primarily due to enhanced growth in low-weight piglets, potentially reducing clinical or subclinical pathologies. This improved growth could lead to greater homogeneity, reducing the number of underweight pigs. There was also a notable decrease in diarrhoea prevalence during the first post-weaning period, possibly linked to the reduced pathogen levels observed in both trials, identified through plate counts or detection of virulence factors. This reduction in pathogen bacterial population did not negatively impact other beneficial bacterial populations, such as lactobacilli. Thus, the results suggest potential of the tested additive as a strategy to combat ETEC.

Chapter 7. General discussion



General discussion

In the pursuit of alternatives to prevent and alleviate PWD symptoms in weaning piglets, various strategies have been studied over the years. Increasing evidence suggests that microbiota plays a key role in the susceptibility of piglets to pathogens (Dou et al., 2017), making biotic additives worthy of further investigation. Additionally, new technologies with different and promising mechanisms of action could significantly advance PWD control (Yong Joon Kim et al., 2023).

On one hand, this work tested the effects of two different synbiotic combinations administered at different stages of piglet's life, aiming to modulate the animal's microbiota towards a more mature and resilient state capable of better handling the weaning period (Chapter 4 and 5). These studies provide an opportunity to evaluate the potential of microbiota modulators when administered either pre-weaning or post-weaning.

On the other hand, a newly discovered strategy to reduce the pathogenesis of PWD in weaned piglets (nanobodies) was evaluated (Chapter 6). This evaluation was done conducting a very similar experimental model of colibacillosis as such used to evaluate one of the synbiotic strategies (Chapter 5). This allows us to compare the effectivity of such two strategies (a synbiotic aimed to modulate microbiota or nanobodies addressed to fight directly the pathogen) and make a comparison about their effectiveness and modes of action.

In this way the following sections of this discussion will aim first to analyse the differential impact of using biotic strategies early in the lactation period or after weaning, and secondly to compare the effectivity and distinct mechanisms of action of synbiotic strategies or nanobodies. In a final section

the practical applicability of these strategies in the swine industry will be also examined, along with a brief discussion of the piglet as an animal model.

7.1. Synbiotic combination administered at an earlier age results in faster improvement of piglet intestinal health

Two different synbiotic combinations were tested at different early stages of piglets' life: suckling and weaning. One combination consisted of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosaccharides (GOS) and Human Milk Oligosaccharides (HMO), with or without the bioactive peptide, osteopontin, tested in suckling piglets. The other combination included *Saccharomyces cerevisiae* Sc 47 with or without short-chain Fructo-oligosaccharides (scFOS), tested in weaning piglets challenged with ETEC $F4^+$.

The experimental designs differed, introducing certain limitations to the comparison. For instance, the growth rate of the animals could not be compared due to the different ages of the animals. Another differing aspect was the nature of the feed administered, which undoubtedly impacts on fermentative activity. Lactate plays an important role in suckling animals. In our trial, suckling piglets showed higher lactate concentrations in the ileum and colon, while lactate was almost undetected in weaning piglets.

However, the primary effect sought was the modulation of the microbiota towards a more mature and resilient profile, in line with the nature of the additives. Therefore, despite analysing different synbiotic combinations, this comparison aims to discuss the effects promoted on the microbiota depending on the timing of administration.

Changes in the early establishment of the microbiota

The bacterial analysis methodologies differed between the two trials. In suckling animals, 16S rRNA sequencing analysis was performed on faecal samples, while in weaning piglets, microbiological plate counts were done on ileal mucosa, ileal and colon digesta and faecal samples. In the first case, the entire microbiota was screened; in the second case, only ETEC and lactobacilli counts were analysed. Thus, comparison should made carefully.

In both trials, more significant differences were observed between treatments with longer administration times (Figure 7.1). In suckling animals, changes promoted by the synbiotic combination were more consolidated at the end of the trial (day 15). Potentially beneficial genera such as *Christensenella,* increased in relative abundance at day 15 across all three treatments. Similarly, potentially dysbiotic genera such as *Helicobacter*, *Suterella*, *Veillonella*, *Coprococcus* and *Campylobacter* were significantly decreased at day 15 with the combined treatment. It is logical to think that the establishment and colonization of different strains in the digestive tract require time, and greater results are seen with longer administration of the additives.

In weaning animals, the most significant differences were registered at the end of the study (day 8 PI). The animals receiving the three treated diets exhibited lower ETEC counts in the ileal mucosa, colon content and faeces. The lactobacilli:ETEC ratios were also higher at the end of the study for those animals receiving the additives. These results suggest that the additives were able to promote a more beneficial microbiota profile by lowering pathogen counts and maintaining a higher proportion of beneficial microbiota particularly at the end of the trial.

Impact on fermentative activity

Intestinal fermentation serves as a parameter to measure the microbiota profile and its activity, with increased beneficial microbiota translating to higher levels of fermentation products, such as SCFA (Su et al., 2022).

In suckling piglets, there was a clear increase in total SCFA concentrations with the additive combination. Significant higher levels of acetate, propionate, butyrate, and valerate were observed in the CON treatment. These results clearly demonstrate an enhanced effect of combining additives in the CON diet, promoting a beneficial microbiota and increased fermentative activity (Figure 7.1).

In weaned piglets, this additive-promoted effect was not as evident. Although there was a decrease in pathogen count and an increased ratio of lactobacilli: ETEC, these changes were not reflected in fermentative activity. The animals receiving the treatments did not show increased concentrations of SCFA in the gut (Figure 7.1).

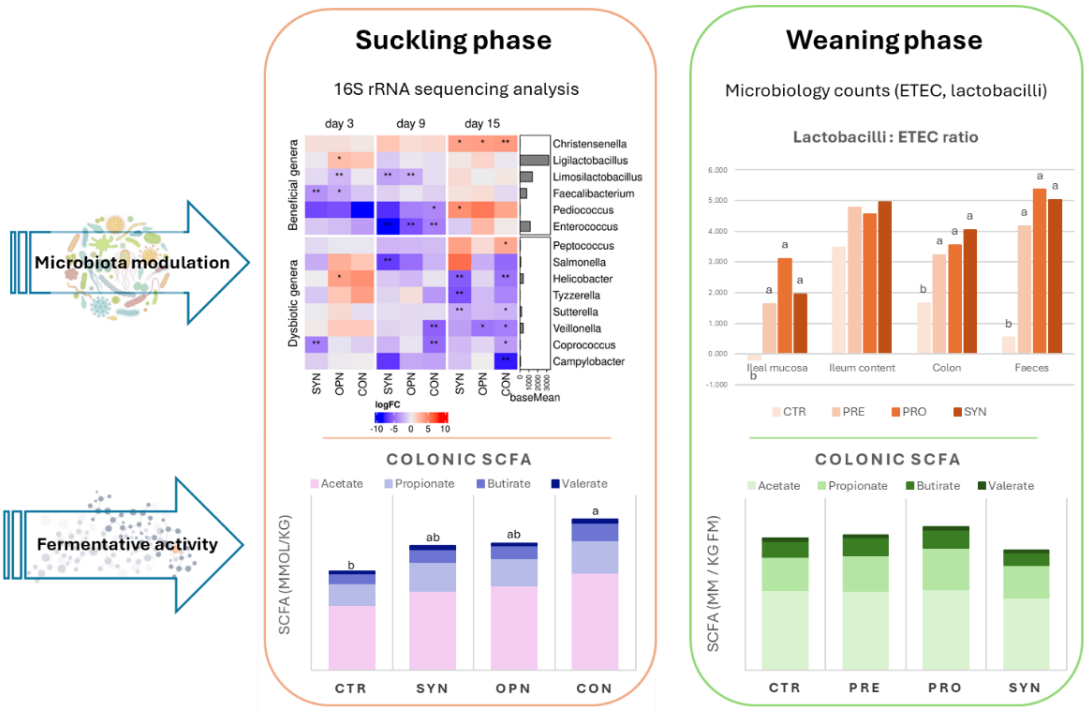


Figure 7.1. Effect of experimental treatments on microbiota modulation and fermentative activity, a comparison between trials.

In light of these results, the comparison of synbiotic additives administered at different stages of piglet's life suggests that earlier intervention yields clearer effects. The most significant and consistent outcome in both trials was the shift in the microbial profile. Given the nature of the additives administered, it is reasonable to hypothesize that the microbiota profile is the first aspect to be influenced. Once stablished, a beneficial microbiota profile could lead to changes in other parameters, such as fermentative activity and intestinal architecture. In suckling piglets, the effects on fermentative activity were evident, and initial changes in intestinal architecture were also observed. In weaning piglets, a more beneficial bacterial profile was noted, even though the microbiota was not sequenced. The effects on the other parameters were more diluted.

7.2. Two different strategies efficiently address the weaning challenge

A synbiotic combination of *Saccharomyces cerevisiae* Sc 47 with or without short-chain Fructo-oligosaccharides (scFOS) was tested in a piglet challenge model with ETEC $F4^+$. Similarly, bivalent V_HH constructs were tested in the same piglet challenge model. Having two tested strategies in a similar challenged piglet model offers a valuable opportunity to compare each approach. Both strategies aim to better manage the challenges associated with weaning, but they employ different mechanisms of action, which are expected to produce differences in various parameters.

Before starting the comparison, several differences should be noted. For instance, the initial live weight of the animals differed between trials. Initially, the animals in the synbiotic combination trial were lighter, with starting weights of 5.66 kg (±0.20) compared to 5.89 kg (±0.16) in the V_HH construct trial. Moreover, the challenge characteristics were slightly different. Although both challenges followed the same methodology, slight variations in the final pathogen dosage were detected: 3.0×10^9 CFU/ml in synbiotic combination trial vs. 7.7×10^8 CFU/ml in V_HH trial. However, it is not only the inoculation dose that impacts the challenge response in the animals. Other factors, such as the health status of the farm of origin, passive immunity transferred by the sow, transport stress, time of year, and temperature, can also affect the animals' response to the challenge.

Considering the objective of both strategies, the main parameters compared will include performance and clinical response, microbiota balance and pathogen control, and intestinal architecture.

Performance and clinical response

As seen in Table 7.1, the synbiotic combination did not alter the growth rate of the animals. Conversely, in the other trial, both ZnO and V_HH constructs registered significantly higher ADG compared to CTR group during the same period. This difference could be attributed to the different mechanisms of action of each strategy.

The synbiotic combination, acting as a microbiota modulator, does not exert an immediate direct effect on the pathogen's strain. Instead, it creates an unfavourable environment for the pathogen growth through the promotion of a beneficial microbiota profile (Shin et al., 2019). Consequently, activation of the immune system is required to combat the infection (Gensollen et al., 2016), which can have a detrimental effect on animal's growth.

In contrast, V_HH supplementation resulted in significantly higher ADG compared to the CTR group. In this study, inoculation of the animals resulted in a pronounced growth reduction, which may more clearly highlight the effect of bivalent V_HH constructs. These products specifically target pathogen virulence factors, including blocking the F4 fimbriae and toxins, thereby giving support to the immune response. Although immune activation occurs, this support may mitigate any adverse effects on the animal's growth.

Table 7.1. Effect of experimental treatments on ADG, a comparison between trials.

	Synbiotic trial				**V_HH constructs trial**		
	PRO-		**PRO+**				
	PRE-	**PRE+**	**PRE-**	**PRE+**			
ADG (g)	**CTR**	**scFOS**	**YEA**	**SYN**	**CTR**	**ZnO**	**V_HH**
0-4 PI	108.2	58.9	48.1	43.0	27.58[b]	112.4[a]	105.8[a]

[a,b] Indicate significant differences between means.

Regarding clinical signs, both trials recorded an increase in diarrhoea incidence after the inoculation, indicating the effectiveness of the challenge. As shown in Figure 7.2, in the biotic trial, all three biotic additives appeared to rapidly alleviate faecal scores after inoculation, around days 2-3 PI, compared to CTR group. Similary, ZnO and V_HH, showed a decrease of faecal scores during the post inoculation period, compared to CTR group.

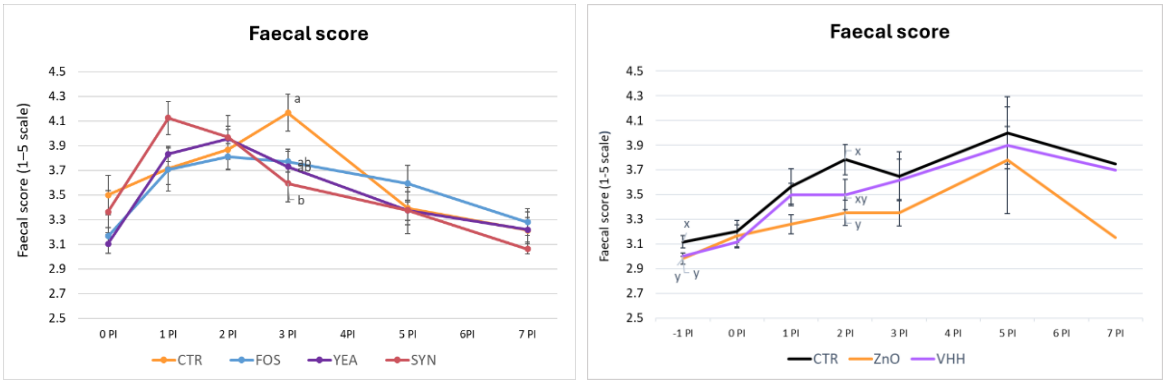


Figure 7.2. Effect of experimental treatments on faecal score, a comparison between trials.

Although there were differences in performance results, both strategies proved effective in decreasing clinical signs.

In the first trial, animals receiving the synbiotic combination likely developed a more diverse, rich and resilient gut ecosystem. With a high presence of beneficial microbiota, proper mucus production, and a functional mechanical barrier, the pathogen would struggle to attach to the mucosa, replicate and exert its pathogenic effects, causing fluid loss. Therefore, a reduction in diarrhoea signs would be observed, compared to CTR group, as depicted in Figure 7.2. However, this beneficial microbiota profile does not prevent immune system activation, which consumes energy and may impair growth.

In the second trial, when the pathogen expressed F4 fimbriae to attach to the epithelium and replicate, the V_HH construct would inhibit this attachment.

Additionally, when the pathogen expressed LT and Stx2e toxins, the other construct would counteract these virulence factors, leading to fewer clinical signs. The presence of these proteins blocking virulence factors would help the immune system response.

Microbiota balance and pathogen control

Microbiological analysis included ETEC and lactobacilli counts in both trials. ETEC counts served to monitor the success of inoculation and as a parameter of treatment efficacy. Lactobacilli counts, on the other hand, served as indicator of health due to the associated benefits of these bacteria.

All three biotic diets showed a decrease in ETEC counts across all intestinal compartments compared to CTR group. The beneficial effect of the additives increased throughout the post-inoculation period, becoming significant in all compartments by day 8 PI (Table 7.2). Considering the objective of the strategy, the reduction of pathogen counts is a key point. Regarding lactobacilli:ETEC ratios, there was a noticeable increase in animals receiving biotic additives. These elevated values stemmed from lower ETEC counts and higher lactobacilli counts (although not significant) compared to the CTR group. These outcomes support the hypothesis of biotic additives as microbiota modulators.

In contrast, bivalent V_HH constructs, exhibited a reduction of ETEC counts in faeces at day 8 PI compared to CTR, but not in other compartments (Table 7.2). Lactobacilli:ETEC ratios did not show significant differences (although a trend was observed), primarily because ETEC counts were not decreased.

The results presented here vary between trials. In one case, pathogen reduction was observed in all the compartments; in the other, only in faeces. Once again, these differences can likely be attributed to the mechanism of

action. Biotic additives create an unfavourable environment for pathogen grow, thereby reducing their counts over time. In contrast, V_HH constructs target virulence factors of the pathogen without affecting the bacteria's viability itself. If the pathogen does not express F4 fimbriae or toxins, it is likely less harmful to the animal but can still proliferate in the gut.

Table 7.2. Effect of experimental treatments on ETEC counts and lactobacilli:ETEC ratio at day 8 PI, a comparison between trials.

	Synbiotic trial				V_HH constructs trial		
	PRO-		PRO+				
	PRE-	PRE+	PRE-	PRE+			
	CTR	scFOS	YEA	SYN	CTR	ZnO	V_HH
ETEC (log CFU/g)							
Ileal mucosa	5.43^{a}	4.14^{b}	4.22^{b}	4.50^{b}	4.18	3.48	4.08
Ileum content	$5.42^{A\dagger}$	$4.27^{A\ddagger}$	$4.41^{B\dagger}$	$3.98^{B\ddagger}$	4.64	5.30	6.55
Colon content	7.22^{a}	5.66^{b}	5.29^{b}	4.80^{b}	5.47	7.13	6.52
Faeces	6.28^{a}	4.88^{b}	4.70^{b}	5.01^{b}	7.21^{a}	6.42^{ab}	5.77^{b}
Lactobacilli:ETEC ratio							
Ileal mucosa	-0.20^{b}	1.63^{a}	3.11^{a}	1.96^{a}	1.08	1.50	1.59
Ileum content	3.47	4.79	4.57	4.97	1.54	1.87	1.70
Colon content	1.66^{b}	3.23^{a}	3.55^{a}	4.04^{a}	1.08	1.24	1.40
Faeces	0.54^{b}	4.18^{a}	5.36^{a}	5.02^{a}	1.20^{y}	1.34^{xy}	1.52^{x}

^{a,b} Indicate significant differences between means. ^{x,y} Indicate statistical trends between means. ^{A,B} Indicate significant differences due to PRO effect. †‡ Indicate significant differences due to PRE effect.

Intestinal architecture

Intestinal architecture serves as an indicator of proper digestive function, which can be influenced by pathogens and also by beneficial microbiota.

While synbiotic additives promote a more beneficial microbiota profile, these effects did not manifest changes to villus architecture, possibly due to the short duration of administration. Similarly, V_HH constructs did not exhibit any

effects on villus architecture; however, in this case microbiota profile was not significantly altered.

Regarding histological cell counts, probiotic supplementation (PRO diet) resulted in higher IEL and higher M numbers in the ileum at day 8 PI, as detailed in Table 7.3. However, interpreting these findings is challenging due to inconsistences in the literature regarding desirable cellular profiles. For instance, GC are associated with mucin production and enhanced intestinal protection (Su et al., 2022), yet their presence has also been linked to decreased efficiency in pigs, as they can interfere with nutrient absorption and represent an energy burden (McCormack et al., 2017).

Table 7.3. Effect of experimental treatments on ileum histology at day 8 PI, a comparison between trials.

	Synbiotic trial				**V_HH constructs trial**		
	PRO-		**PRO+**				
	PRE-	**PRE+**	**PRE-**	**PRE+**			
	CTR	**scFOS**	**YEA**	**SYN**	**CTR**	**ZnO**	**V_HH**
VH:CD	1.59	1.29	1.42	1.50	1.29	1.52	1.35
IEL (cell nº)	14.7^B	13.4^B	16.1^A	18.2^A	10.8	12.8	10.8
GC (cell nº)	13.2	8.02	12.6	9.48	5.92	6.59	5.79
M (cell nº)	0.52^B	0.84^B	1.08^A	1.21^A	0.40	0.53	0.32

^{A,B} Indicate significant differences due to PRO effect.

To sum up, synbiotic additives notably influenced gut microbiota structure, guiding it towards a more resilient profile. This was evidenced by the clear reduction in ETEC counts, leading to better pathogen control and a decrease in clinical signs. However, these additives did not promote higher animal growth rates.

In contrast, V_HH constructs specifically target pathogen virulence factors, reducing pathogenic effects and clinical signs. This targeted approach may

contribute to improved animal growth rates, helping immune system response. However, since V_HH constructs focus on virulence factors rather than directly reducing pathogen counts, their impact on ETEC counts was limited.

Despite their different mechanisms, both strategies efficiently achieved their respective objectives.

7.3. The potential of these strategies in a real farm scenario

In light of the previous comparisons, a discussion regarding the potential of these strategies in real farm conditions is intended.

The mechanisms of action tested here differ significantly, so the desired target must first be defined when choosing a strategy. Biotic strategies should be considered as health promoters and gut microbiota stabilizers, acting to prevent the detrimental effects of PWD, rather than serving as therapeutic treatments (Barba-Vidal et al., 2018). Our study suggests that synbiotic additives enhance intestinal health, starting with improving microbial balance, and subsequently benefiting gut and immune function. On the other hand, the nanobody strategy, though still in development, shows a clear effect by specifically targeting the pathogen without interfering with the gut environment. This makes it more suitable as a treatment therapy, although it could also be useful for prevention.

Certainly, the literature on biotic additives indicates that their effects are not spectacular, especially considering that most trials are conducted under controlled experimental conditions. More diverse results could be expected when testing these additives in a real farm scenario (Barba-Vidal et al., 2018). Regarding nanobodies, there is limited literature supporting their efficacy under experimental conditions and none under field conditions. Our work

provides results from both conditions. In field conditions, nanobodies were shown to enhance growth rates and reduce the number of underweight piglets, thus, reducing the uncertainty of outcomes under these conditions.

The timing of strategy implementation can significantly impact outcomes. This work suggests that early ages present a window of opportunity. It is well-documented that early colonization of the gut microbiota supports the host's health by fostering the development of gut barrier function and maturing the immune system (Cheng et al., 2019). In terms of performance, changes in LW categories are common at young ages and show a low correlation coefficient with slaughter LW. However, intervention capacity diminishes after weaning (Blavi et al., 2021). In this work, early administration of synbiotic additives demonstrated pronounced effects on microbial populations and fermentative activity. Thus, implementing biotic strategies as soon as possible is recommended, despite the practical challenges often encountered on farms. Various administration methods exist for biotics, with in-feed supplementation being the most widely adopted approach for both piglets and sows (Pereira et al., 2022). Other options include solid or liquid suspension in feed (top dressing) (Barba-Vidal et al., 2018), as well as suspension in milk (Gebert et al., 2011) or water (Walsh et al., 2012), although information on these methods remains limited.

The cost implications of these strategies should not be disregarded. The economic burden of PWD is substantial, ranging from 40€ to 314€ per sow depending on disease severity (Luppi, 2017). An economic analysis should be included to determine if a strategy is economically viable. For instance, studies have noted that the total feed cost per kg of weaned piglet was lower when using probiotics (Mazur-Kuśnirek et al., 2023; Patience et al., 2015; Pereira et al., 2022). Each case requires detailed examination, but there are proposed methods to reduce probiotic cost. For example, utilizing probiotics

derived from food industry waste, such as whey fermented with kefir, could be a rentable option (Agregán-Pérez et al., 2021; Chiang et al., 2015). The same should be done for nanobodies; although little is known about production cost, they are reported to be cost-effective (Fiil et al., 2022; Harmsen et al., 2005).

Given the multifactorial nature of susceptibility to PWD, a combination of strategies is recommended. As depicted in Figure 7.3, successfully mitigating the adverse effects of weaning could involve integrating preventive strategies, risk reduction measures, and targeted treatments in a holistic approach. For instance, this approach might entail selecting genetically resistant animals and implementing effective vaccination protocols pre-emptively. Subsequently, administering biotic additives could create a more diverse, resilient and functional intestinal environment in piglets, thereby enhancing protection during challenging periods. Management practices should also be considered, as they directly influence stress levels and overall animal performance (Blavi et al., 2021). Nanobodies could be implemented preventively or therapeutically, specifically targeting pathogen virulence factors. Ultimately, in cases of infection and upon proper diagnosis, therapeutic treatments could be applied.

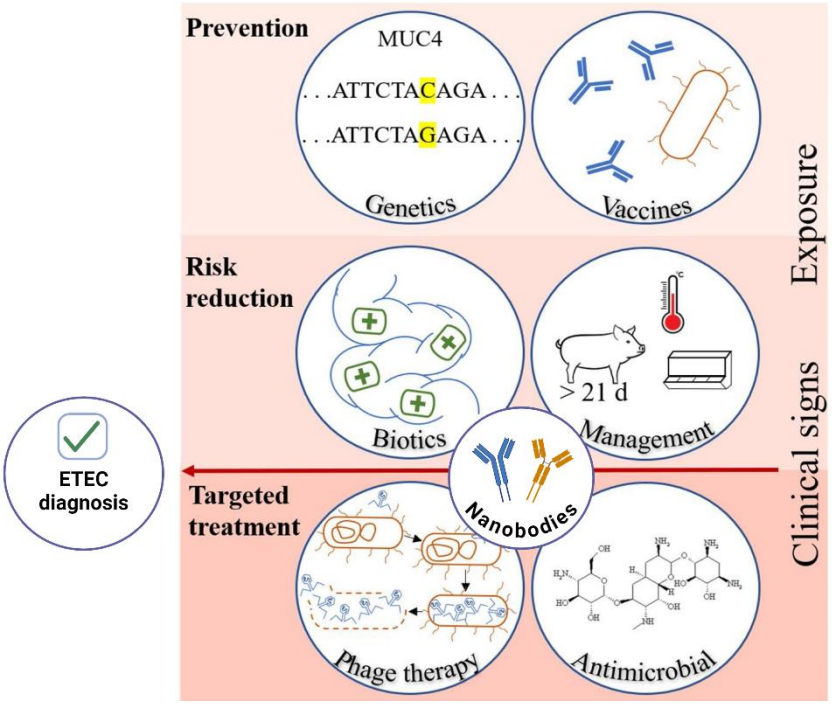


Figure 7.3. An example of a holistic approach for the management of PWD. Adapted from Laird et al. (2021). Created with BioRender.

7.4. Piglet as an animal model

In Chapter 4, the additives tested were intended for inclusion in milk formulas designed for newborn infants, thus using suckling piglets as an animal model. As discussed in the paper, replicating the exact characteristics of human neonates in an animal model poses challenges. However, the use of suckling piglet for such studies is widely accepted and documented (P.J. Moughan, 1992).

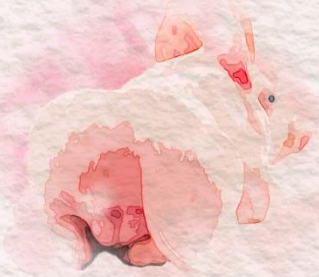
This study offers dual benefits. Firstly, for applications in human infants, preclinical animal studies are necessary to assess the effectiveness of new products or combinations, making suckling piglets a suitable model due their physiological similarities. Secondly, for the objectives of this thesis, valuable insights were gained regarding the administration of additives during early

piglet development, an area less frequently studied. Although, feeding piglets with artificial milk formula is not practical in the swine industry, it provided crucial information about microbial profiles from first week of life until weaning, early jejunal gene expression and overall gut function.

In the past years, the use of pigs in human biomedical research has grown significantly, becoming crucial (Burrin et al., 2020). The neonatal pig has been established as a model for studying various diseases, including fetal infections, fetal obstructions, growth-restrictions, malnutrition, sepsis, weanling diarrhoea and chemotherapy in children. Two notable examples where the preterm piglets have emerged as a preferred model are necrotizing enterocolitis (NEC) and short bowel syndrome (SBS), conditions prevalent among premature and very-low-birth-weight infants. NEC is closely linked to gastrointestinal immaturity, enteral feeding, and bacterial colonization, which are common in premature neonates. Those affected by NEC often require surgical removal of necrotic intestinal sections, leading to SBS characterized by reduced digestive capacity, fluid loss and dependence on parenteral nutrition. Research using preterm pigs has provided valuable insights into these conditions, particularly due to the ability of this animal model to replicate clinically relevant interventions, such as parenteral nutrition, surgical bowel resection, and orogastric tube feeding (Sangild et al., 2013).

Another significant advancement is the gnotobiotic piglet model, which features completely defined bacterial populations. This model has facilitated studies on the effects of human microbiota, using inocula from infants, children and adults. This research promises major developments in understanding host-microbiota interactions, including the microbiota-gut-brain axis (Lunney et al., 2021).

Chapter 8. Conclusions



Conclusions

1. The synbiotic composed of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* NH001, galacto-oligosaccharides (GOS) and Human Milk Oligosaccharides (HMO), combined with osteopontin, administered to suckling piglets for 15 days, can provide a better control of potential dysbiotic genera in faecal microbiota.

2. Such combination of synbiotic and osteopontin also promote a better development of the colonic fermentative activity in suckling piglets with higher concentrations of total SCFA, acetate, propionate, butyrate and valerate.

3. The same combination is also able to modify jejunal gene expression, upregulating genes related to hormonal and enzymatic functions, and nutrient transport functions, while downregulating particular genes related to immune response.

4. Correlation analysis between microbial groups and gene expression revealed significant associations. Dysbiotic microbiota families, such as *Sutterella* and *Tyzzerella*, which were positively correlated with pro-inflammatory genes, are decreased with the synbiotic supplementation. Conversely, beneficial microbiota families like *Christensenella*, which were negatively correlated with pro-inflammatory genes, are increased with the combination of synbiotic and osteopontin supplementation.

5. The synbiotic combination of *Saccharomyces cerevisiae* Sc 47 and short-chain fructo-oligosaccharides (scFOS) administered to weaning piglets orally challenged with ETEC $F4^+$, promote a decrease in ETEC

counts and an increase in the ratio of lactobacilli:ETEC, in all gut compartments at day 8 PI.

6. The novel technology of bivalent V_HH constructs specifically targeting F4 and LT virulence factors, administered to weaning piglets orally challenged with ETEC $F4^+$, promote higher ADG shortly after inoculation (0 - 4 PI) and a trend toward decreased diarrhoea incidence.

7. Bivalent V_HH constructs specifically targeting F4, LT and Stx2e virulence factors, administered in-feed to weaning piglets, can decrease diarrhoea incidence under field conditions, with higher ADG, and increased LW at the end of the nursery period. Moreover, the increased weight of treated animals is primarily due to a growth enhancement of low-weight piglets.

Chapter 9. References



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