Universitat Autònoma de Barcelona

Evaluation of the synbiotic strategy as prevention and treatment of swine digestive pathologies

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“Evaluation of the synbiotic strategy as prevention and treatment of swine digestive pathologies”

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Certifiquen:

Que la memòria titulada **"Evaluation of the synbiotic strategy as prevention and treatment of swine digestive pathologies",** presentada per **Agustina Rodríguez** amb la finalitat d'optar al grau de Doctor en Veterinària amb menció internacional, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació per que sigui jutjada per la comissió corresponent.

Susana María Martín Orúe Lorena Castillejos Velázquez

I perquè consti als efectes oportuns, signen la present a Bellaterra, 29 de Octubre de 2019.

La present memòria de tesi ha sigut realitzada gràcies a la concessió de la beca predoctoral FPU 2014 pel Ministerio de Educación i al finançament proporcionat pel Centro para el Desarrollo Tecnológico Industrial (CDTI) del Ministerio de Economía y Competitividad conjuntament amb Ordesa S.L. provinent del projecte SMARTFOODS amb referència IDI-20141206.

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SUMMARY

The main objective of this Thesis was to evaluate the efficacy of different synbiotic compounds to improve health and performance of pigs after weaning, as well as their capacity to fight digestive pathogens, like *Salmonella* or enterotoxigenic *Escherichia coli* (ETEC) F4. Furthermore, it was aimed to establish the distinct actions that may be originated by the probiotic or prebiotic alone, or their jointly administration.

To accomplish this objective four experimental trials were performed. In trial 1 and trial 2, the effects of a synbiotic composed by *Bifidobacterium longum* subsp. *infantis* CECT 7210 and oligofructose-enriched inulin in pigs challenged or not with *Salmonella* Typhimurium or ETEC F4, respectively, were evaluated. Trial 3 assessed the efficacy of a multistrain probiotic composed by the former strain and *Lactobacillus rhamnosus* HN001, mixed or not with oligofructose-enriched inulin against *Salmonella* Typhimurium. Lastly, trial 4 evaluated the response of the same multistrain probiotic, once again, administered or not together with galacto-oligosaccharides in weaned piglets experimentally challenged with ETEC F4.

A similar protocol was used in all the trials. Briefly, 72 (trial 1) or 96 (trials 2, 3 and 4) weanlings coming from commercial farms were transported to the experimental facilities of the UAB. In trials 1 and 2, animals were distributed in 24 or 32 pens, respectively, following a 2 x 2 factorial design; treated or not with the synbiotic and challenged or not with the pathogen. In trials 3 and 4, a completely randomized design composed by five treatment groups was used: one non-challenged (CTR+) and four challenged (with *Salmonella* Typhimurium in trial 3 and ETEC F4 in trial 4): same diet (CTR-), or supplemented with the multistrain probiotic (PRO), prebiotic (PRE) or their combination (SYN).

After an adaptation period of one week, animals were orally challenged with the corresponding pathogen and one animal per pen was euthanized at day 4 and 8 (in all trials, except for trial 2: days 3 and 7) post-inoculation (PI). For all experiments, main variables assessed were animal performance, clinical signs, pathogen excretion, fermentation profile, immune response and intestinal morphology.

Summary

The synbiotic combination consisting of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and oligofructose-enriched inulin reduced the numbers of ileal attached enterobacteria at day 7 PI (trial 2) and enhanced the presence of intraepithelial lymphocytes (IEL) in ileum of healthy weaned pigs. When facing a challenge, the synbiotic mixture could not counteract pathogen loads in animals challenged with ETEC F4 (trial 2); however, it tended to accelerate *Salmonella* clearance when it was tested against this pathogen (trial 1) as a 25% of the animals receiving the synbiotic turned negative on day 8 PI to excretion, in comparation to a 0% of the control group (P = 0.076). Interestingly, in both trials (trial 1 and trial 2), the synbiotic treatment had a significant impact on the colonic fermentation but with differential effects depending on if animals had been or not challenged.

In trial 3, the combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* HN001 and oligofructose-enriched inulin did not show to have any significant impact neither on growth performance nor faecal consistency of pigs one week after weaning. After the oral challenge, the multistrain probiotic was able to promote a faster faecal clearance of *Salmonella* (65% negative animals in comparation to 0% of CTR- on day 7 PI; P = 0.028) and an improved recovery of intestinal damage, as reflected in a higher villi height/crypt depth ratio at day 8 PI. Regarding the oligofructose-enriched inulin treatment, this prebiotic was associated to an increased number of ileal intraepithelial lymphocytes (IEL) at day 8 PI. The synbiotic combination did not reflect any synergistic activity against the pathogen, losing, in addition, most of the previous mentioned effects.

The synbiotic evaluated in trial 4, composed by *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* and galacto-oligosaccharides, decreased the number of faecal enterobacteria and coliforms one week after weaning compared to PRE or PRO. Nevertheless, 8 days after the ETEC F4 challenge, animals receiving the SYN treatment showed the greatest loads of enterotoxigenic *E. coli* F4 in colonic digesta. In addition, an enhanced pro-inflammatory status was suggested by the increased serum concentrations of TNF-α (day 4 PI) and Pig-MAP (day 8 PI). This combination was not able, therefore, to demonstrate any synergistic benefits against the pathogen, losing some of the

effects found for the probiotic blend or the prebiotic when administered alone, like the reduced growth impairment after the challenge or the lower levels of Pig-MAP found on day 4 PI.

The results exposed in this Thesis highlight that the combination of probiotics and prebiotics not necessarily result in an additive or synergistic effect. Their impact on the intestinal microbiota and the response of the individual probably depend on the challenges that the animals need to face. More studies are needed to understand the complex interactions produced in the gastrointestinal tract and the involved mechanisms.

RESUMEN

El principal objetivo de esta Tesis fue evaluar la eficacia de diferentes compuestos simbióticos para mejorar la salud y productividad del ganado porcino después del destete, así como también su capacidad para enfrentar patógenos digestivos, como *Salmonella* o *Escherichia coli* enterotoxigénica (ETEC) F4. Se quisieron establecer, además, las distintas acciones que pueden ser originadas por el probiótico o el prebiótico de forma separada, así como de su administración conjunta.

Para cumplir este objetivo se llevaron a cabo cuatro pruebas experimentales. En las pruebas 1 y 2, se evaluaron los efectos de un simbiótico formado por *Bifidobacterium longum* subsp. *infantis* CECT 7210 e inulina enriquecida con oligofructosa en cerdos desafiados o no con *Salmonella* Typhimurium o ETEC F4, respectivamente. La prueba 3 valoró la eficacia de un probiótico multicepa compuesto por la cepa anterior junto con *Lactobacillus rhamnosus* HN001, mezclados o no con inulina enriquecida con oligofructosa frente a *Salmonella* Typhimurium. Finalmente, la prueba 4 evaluó la respuesta del mismo probiótico multicepa nuevamente, administrado o no conjuntamente con galacto-oligosacáridos en lechones destetados experimentalmente desafiados con ETEC F4.

Se utilizó un protocolo similar en todas las pruebas. Resumidamente, 72 (prueba 1) o 96 (pruebas 2, 3 y 4) lechones destetados provenientes de granjas comerciales fueron transportados a las instalaciones experimentales de la UAB. En las pruebas 1 y 2, los animales se distribuyeron en 24 y 32 corrales, respectivamente, siguiendo un diseño factorial 2 x 2; tratados o no con el simbiótico y desafiados o no con el patógeno. En las pruebas 3 y 4, se usó un diseño totalmente aleatorizado constante de cinco grupos de tratamiento: uno no desafiado (CTR+) y cuatro desafiados (con *Salmonella* Typhimurium en la prueba 3 y ETEC F4, en la 4): la misma dieta (CTR-), o suplementada con el probiótico multicepa (PRO), el prebiótico (PRE) o la combinación de ambos (SYN).

Después de un periodo de adaptación de una semana, los animales fueron desafiados oralmente con el patógeno correspondiente y se eutanasió un lechón por corral los días 4 y 8 (en todas las pruebas, exceptuando la prueba 3: días 3 y 7) posinfección (PI). Las principales variables evaluadas en todas las

pruebas fueron productividad, signos clínicos, excreción de patógeno, perfil de fermentación, respuesta inmunitaria y morfología intestinal.

La combinación simbiótica formada por *Bifidobacterium longum* subsp. *infantis* CECT 7210 e inulina enriquecida con oligofructosa redujo el número de enterobacterias enganchadas en íleon a día 7 PI (prueba 2) y aumentó la presencia de linfocitos intraepiteliales (LIE) en íleon de lechones destetados sanos. Frente a un desafío, la mezcla simbiótica no pudo contrarrestar la carga de patógeno en animales desafiados con ETEC F4 (prueba 2); sin embargo, tendió a acelerar la eliminación de *Salmonella* cuando se testó frente a este patógeno (trial 1), volviéndose negativos a la excreción el día 8 PI un 25% de los animales que recibieron el simbiótico en comparación al 0% del grupo control ($P = 0.076$). Curiosamente, en ambas pruebas (pruebas 1 y 2), el tratamiento simbiótico tuvo un impacto significativo en la fermentación colónica, pero con efectos diferentes en función de si los animales habían sido o no desafiados.

En la prueba 3, la combinación de *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* HN001 e inulina enriquecida con oligofructosa no mostró ningún impacto remarcable en la productividad o consistencia fecal de los cerdos una semana después del destete. Posteriormente al desafío oral, el probiótico multicepa promovió una eliminación fecal acelerada de *Salmonella* (65% animales negativos en comparación con 0% de CTR- a día 7 PI; $P = 0.028$) y mejoró la recuperación del daño intestinal, hecho reflejado en la ratio entre altura de vellosidades y profundidad de criptas a día 8 PI. Respecto al tratamiento con inulina enriquecida con oligofructosa, este prebiótico trajo asociado con un mayor número de linfocitos intraepiteliales a día 8 PI. La combinación simbiótica de los dos compuestos no mostró ninguna actividad sinérgica frente al patógeno, perdiendo, además, la mayoría de los efectos previamente mencionados.

El simbiótico evaluado en la prueba 4, formado por *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* HN001 y galacto-oligosacáridos, redujo el número de enterobacterias y coliformes fecales una semana después del destete de los animales, en comparación a PRE y PRO. No

obstante, 8 días tras el desafío con ETEC F4, los animales que recibieron el tratamiento SYN mostraron la mayor carga de ETEC F4 de todos los grupos desafiados en contenido de colon. Asimismo, las concentraciones elevadas de TNF-α (día 4 PI) y Pig-MAP (día 8 PI) sugirieron la presencia de un estado proinflamatorio sobreestimulado. Por lo tanto, esta combinación no fue capaz de demostrar ningún beneficio sinérgico frente al patógeno, perdiendo algunos de los efectos observados para el probiótico o el prebiótico cuando son administrados independientemente, como la mejora en el deterioro de la productividad de los animales después del desafío o los niveles menores de Pig-MAP observados a día 4 PI.

Los resultados expuestos en esta Tesis señalan que la combinación de probióticos y prebióticos no necesariamente desemboca en un efecto aditivo o sinérgico. Su impacto en la microbiota intestinal y la respuesta por parte del individuo dependen, probablemente, de los desafíos a los que los animales deben enfrentarse. Son necesarios más estudios para entender las complejas interacciones que se producen en el tracto gastrointestinal y los mecanismos implicados en ellas.

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Abbreviations

ADFI	Average daily feed intake	IFN-γ	Interferon gamma
ADG	Average daily gain	IgA	Immunoglobulin A
AIDA	Adhesins involved in diffuse adherence	IgG	Immunoglobulin G
Anti-SBRC	Anti sheep blood red cell antibodies	IgM	Immunoglobulin M
ANOVA	Analysis of variance	IL	Interleukin
BUN	Blood urea nitrogen	IMO	Isomalto-oligosaccharides
BW	Body weight	LDH	Lactate dehydrogenase
BWG	Body weight gain	LDL	Low density lipoprotein
C:P ratio	Cholesterol/phospholipids ratio	MDA	Malondialdehyde
CF	Colonization Factor	MOS	Mannano-oligosacharides
CFU	Colony forming units	MRS	Man Rogosa Sharpe
COS	Chitosan oligosaccharide	MUC4	Mucin4 gene
CNS	Central nervous system	NF-κB	Nuclear factor-κB
CRP	C reactive protein	NO	Nitric oxide
CTR	Control	PBS	Phosphate buffered saline
DM	Dry matter	PCR	Polymerase chain reaction
E:L ratio	Enterobacteria/lactobacilli ratio	Pig-MAP	Pig Major Acute Phase Protein
ENS	Enteric nervous system	PRE	Prebiotic
EFSA	European Food Safety	PRO	Probiotic
EPEC	Enterotoxigenic *Escherichia coli*	PUN	Plasma urea nitrogen
ETEC	Enteropatgogenic *Escherichia coli*	PWD	Post weaning diarrhoea
EU	European Union	QPS	Qualified Presumption of Safety
FAO	Food and Agriculture Organization	RFO	Raffinose
FCR	Feed conversion ratio	SCFA	Short chain fatty acids
FDA	Food and Drug Administration	SOD	Superoxide dismutase
FI	Feed intake	TAG	Triacyclglycerols
FM	Fresh matter	TJ	Tight junctions
FOS	Fructo-oligosaccharides	TNF-α	Tumoral Necrosis Factor α
G:F	Gain feed ratio	TRL	Toll-like receptor
GABA	Gamma-amonibutyric acid	UAB	Universitat Autoònoma de Barcelona
GC	Goblet cells	V:C ratio	Villous height/ crypt depth ratio
GI	Gastrointestinal	WBC	White blood cells
H:L ratio	Heterophil:lymphocyte ratio	XLT4	Xylose-Lactose-Tergitol-4
Hp	Haptoglobin	XOG	xylo-oligosaccharide
IEL	Intraephitelial lymphocytes	WHO	World Health Organization

Chapter 1. General introduction

The increasing incomes of society, growing human populations and other sociocultural factors have contributed to the rising demand for meat and other animal products (Sanchez-Sabate & Sabaté, 2019). Data from the FAO shows that pork meat is, nowadays, the most produced and consumed in the world, together with poultry, and the tendency in the last years is towards a continuous growth. In the European Union it occupies the same position, with big difference over poultry production and consumption, being Germany and, secondly, Spain, the main producers and exporters of this product (European Commission, 2019). Because the higher demand is not associated to the availability of greater areas for animal farming, the most adequate way to satisfy the consumers' requests is through intensive farming (Ilea, 2009). Keeping animals in such overcrowding conditions leads to an increased risk of diseases apparitions, with the consequent economic repercussions due to morbidity, mortality and increased reposition rates, among others (Amadori & Zanotti, 2016). This danger is aggravated in animals that are going through immunosuppression and stress periods, thing that occurs during the weaning of pigs, which are commonly separated from the sows at 21-28 days of life (Moeser et al., 2017). This process comprehends one of the most stressful events in life for a pig, in which they are forced to make an abrupt change from liquid milk to solid grain-based feed and be exposed to a new environment with animals coming from different sows and different bacterial loads (Campbell et al., 2013). Furthermore, piglets lose the source of antibodies, which is sow's milk, in a period in which they are not completely immunocompetent (Bailey et al., 2005). Thus, this delicate situation leads frequently to the onset of diarrheic episodes that are caused by opportunistic pathogens, like enterotoxigenic *Escherichia coli* (ETEC) or, less commonly, *Salmonella* (Zimmerman et al., 2012; Rhouma et al., 2017). In order to alleviate or avoid these situations, antibiotics, like colistin, have been used as prophylactic or metaphylactic measures in first instance in swine farming. However, increasing concern about antimicrobial resistance has led to new legislations in the European Union in order to promote a more rational use of antimicrobials substances. Consequently, the European Parliament approved in 2018 a new law project, to be implemented in 2022, with the objective of banning the

use of antimicrobials in prophylaxis and, in addition, all of which are reserved for human use in any circumstances.

Given this scenario, a change in control and treatment of post-weaning associated gastrointestinal pathologies is required. A wide variety of alternatives have been tested in farming animals, being probiotics and prebiotics certainly promising (Thacker et al., 2013).

Firstly, probiotics have been proved capable of interfering with pathogenic agents like ETEC or *Salmonella* by reducing their faecal shedding and/or intestinal colonization (Daudelin et al., 2011; Ahmed et al., 2014; Barba-Vidal et al., 2017) and, parallelly, these bacteria have also shown immunomodulatory actions on the host animal (Zhou et al., 2015; Upadhaya et al., 2017), which is also beneficial against infectious diseases. In the same vein, prebiotics have similar effects on pathogens and immune system increasing numbers of pig's endogenous beneficial bacteria, like lactobacilli and bifidobacteria (Smiricky-Tjardes et al., 2003; Tzortzis et al., 2005; Nadiq et al., 2015).

During the last years the administration of probiotics and prebiotics as a single mixture, known as "synbiotic" or "symbiotic", has gained interest due to the potential enhanced profits than it can cause in the host by two mechanisms: a summation of the effects of each component or, on the other hand, a potentiation of probiotic's action when a selective fermentable source is added (Kolida & Gibson, 2011).

It will be presented in this PhD dissertation the characteristics that probiotics and prebiotics must accomplish in order to be considered candidates for their inclusion in a synbiotic/symbiotic compound, as well as the steps needed to be followed during its design and development. It can also be found a literature compilation evaluating the positive (or negative) outcomes that diverse authors have reported during the last two decades for synbiotics/symbiotics in monogastric farming animals, such as laying hens, broiler chickens and swine.

Chapter 2. Literature review

2.1 WEANING-ASSOCIATED DISEASES

2.1.1 REPERCUSSIONS OF WEANING IN GUT HEALTH

Weaning is known to be one of the most distressing events occurring in the life of a pig, especially in the actual intensive farming conditions. Whereas in a natural environment this process will take place gradually starting at 8 weeks of age, in commercial farms piglets are separated from their mothers between 14 to 30 days of life, being 21 or 28 days the most common (Coffey et al., 2000; Moeser et al., 2017). From this moment onwards, there are several factors that cause a stress response from the animal and potentially affect their body systems development and function. Besides being isolated from the sows, weaned piglets face a transportation, an abrupt change from liquid milk to solid diet, a mix with pigs belonging to different litters (which leads to hierarchy confrontations), an adaptation to a new environment and a risk of contact with pathogens (Campbell et al., 2013). All these challenges, inopportunely, happen in a period of life in which structures of the organism are developing, gastrointestinal system included among them. Moese and collaborators (2017) describe that there is a critical phase during postnatal period key for the GI development that lasts from birth of the piglets until their 12-14 weeks of age. This stage is characterized by a restructuration of the GI structures, which includes a development of the intestinal epithelium, intestinal immune system and the enteric nervous system (ENS). The epithelium plays a defence physical role by the presence of tight junctions (TJ) and Goblet cells, which are responsible of the secretion of a mucous layer and, furthermore, microvilli are relevant in the digestion process by implication of brush-border enzymes (lactase, maltase, sucrase, aminopeptidases) (Marion et al., 2005). Secondly, all lymphoid tissues present in the GI system compose the largest immune organ in the body and its correct maturation is of high importance, as a balance is required between a hyper or hypo reactivity to the antigens present in the gut lumen (Bailey et al., 2005). The third component, the ENS, consists of two plexuses integrated by interconnected neurons and glia located in the muscular and submucosa layers of the

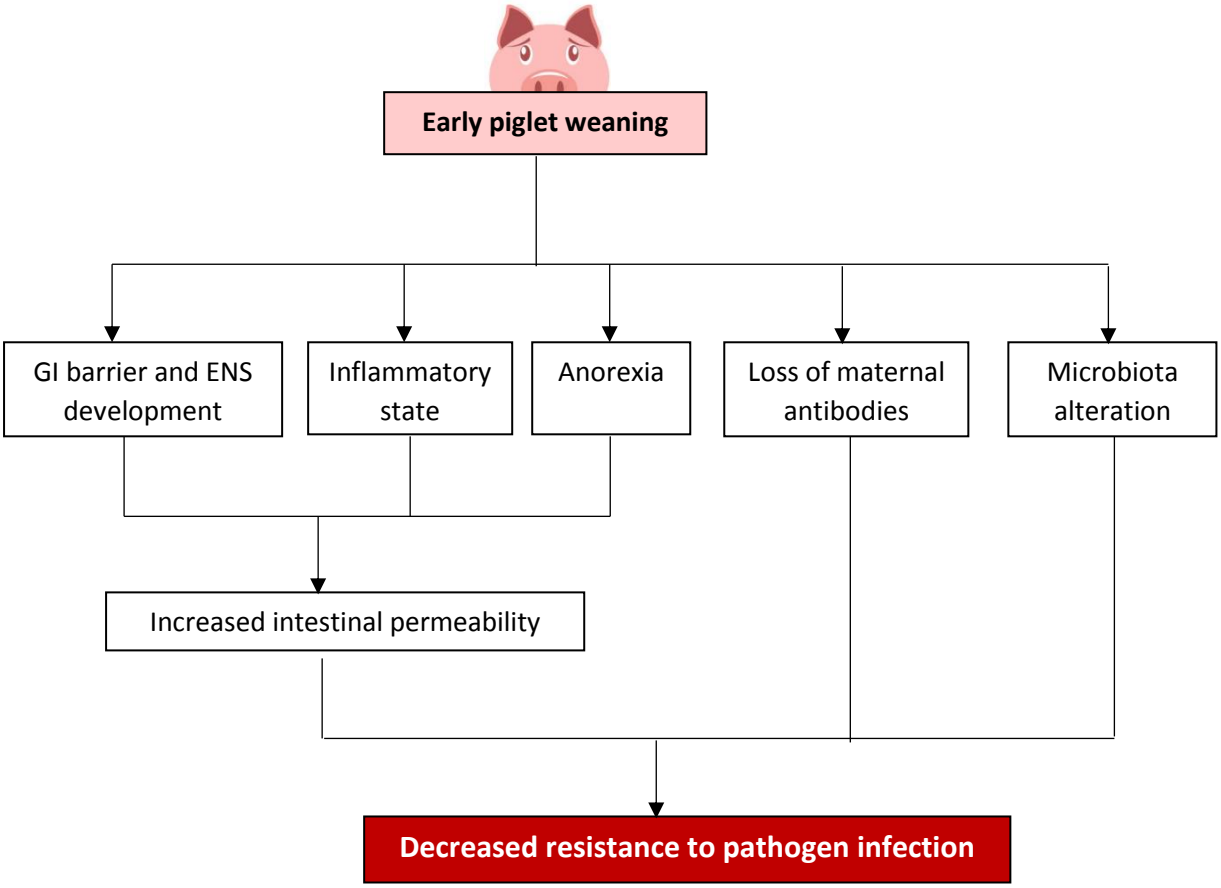
intestine which have an important role in motor, secretory and immune functions (Dhawan et al., 2012; Yoo et al., 2017; Walsh & Zemper., 2019; Waxenbaum & Varacallo, 2019). The activation of the hypothalamic pituitary adrenal axis due to a prompt weaning provokes an elevation of stress mediated relators, which are responsible of an alteration of the natural evolution and dysfunction of the GI barrier system (Moeser et al., 2007).

In addition, this process stimulates the setting of an inflammatory state on the animals which, at the same time, can result harmful to the GI health. Pié et al. (2004) associated the weaning of 28 days-old piglets to an upregulation of expression of genes encoding for the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. Ten years later, in 2014, Bomba and colleagues (2014) reaffirmed an evident higher expression of genes related to antimicrobial and inflammatory response. Moreover, mast cells are activated during weaning (Moeser et al., 2007; Pohl et al., 2007). This activation is induced by corticotropin releasing factor (present in every stress response) and produces the release TNF-α and proteases by the mastocytes, which are responsible of an increase of intestinal paracellular permeability (Overman et al., 2012). This permeability, moreover, is affected by another phenomenon: the post-weaning anorexia. A decrease in feed intake during the first period after weaning is highly common, and, as a consequence of the low energy levels, can impair the intestine condition (McCracken et al., 1999; Spreeuwenberg et al., 2001). Weaning, thus, has been associated to a shortening of enterocyte villi height and deeper crypts (Campbell et al., 2013; Bomba et al., 2004) and to increased permeability of the gut barrier (Overman et al. 2012; Cao et al., 2018).

Furthermore, there is a loss of the protection by the sow's antibodies present in the milk in a moment that the immune system of the piglet is not completely competent (Bailey et al., 2005) that, added to all effects previously mentioned, makes animals susceptible to disease. Another risk factor that should be considered is the alteration of the microbiota in weaned animals (Li et al., 2018). Microbiota has an important paper in the defence of organism, as it is capable of modifying the animal's immune

system (Belkaid & Hand, 2014). Hence, a change in its composition can result detrimental, favouring pathogen colonization.

Figure 2.1. Diagram of factors leading to decreased pathogen infection after early weaning.



2.1.2 POST WEANING DIARRHOEA (PWD) - COLIBACILLOSIS

The process known as post-weaning diarrhoea (PWD) affects pigs during the first weeks after weaning and it is characterized by apparition of diarrhoea episodes that can lead to dehydration and sudden death. Surviving animals, on the other hand, present worsened performance with growth retardation (Fairbrother et al., 2005; Reis et al., 2010).

The aetiology includes a wide range of infectious agents and can be multifactorial: bacteria, such *Escherichia coli* and some species of the genera *Clostridium, Lawsonia and Brachyspira,* and viruses

(rotavirus, coronavirus, etc) (Martella et al., 2007; Vondruskova et al., 2010; Hanke et al., 2017). Among all of them, the most common causative agent is the enterotoxigenic pathotype of *Escherichia coli* (ETEC) (Luppi et al., 2016). ETEC produces two types of virulence factors: in first place, fimbrial adhesins (known as colonization factor (CF) antigens), that permit the binding to the host intestinal epithelium and its colonization (although adhesion can be produced by non-fimbrial adhesins (AIDA: adhesins involved in diffuse adherence)); and enterotoxins, which are responsible of the epithelial damage (Nagy & Fekete, 1999; Kopic & Geibel, 2010). The bacteria present in the environment (faeces) enters the animal via oral route and travels through the GI system, until arriving to the intestine. Once there, it attaches to the intestinal epithelium, using CF or AIDA, recognizing specific receptors on the small intestine (Dubreuil et al., 2016). When the attachment is completed successfully, ETEC starts secreting heatstable (ST) and/or heat labile (LT) toxins. ST toxins cause an increasement of cyclic GMP as they mimic the native intestinal hormone guanylin whereas LT toxins are associated to an elevation of cyclic AMP. In both cases, ion absorption/secretion is deregulated because of the phosphorylation of the cystic fibrosis transmembrane regulator, which causes an osmotic diarrhoea apparition. There are two types of ST, STa/STI and STb/STII, and LT toxins, Type I (LT-I) and Type II (LT-II) (Clements et al., 2012).

In pigs, the most common fimbrial types causing PWD are the F4 and F18; but F5, F6 and F7 may also be causative agents. Furthermore, there are three variants of F4: F4ab, F4ac and F4ad (being F4ac the most predominant); and two of F18: F18ab and F18ac associated with PWD (Prieto et al., 2017).

Figure 2.2. Pathogeny of enterotoxigenic Escherichia coli.

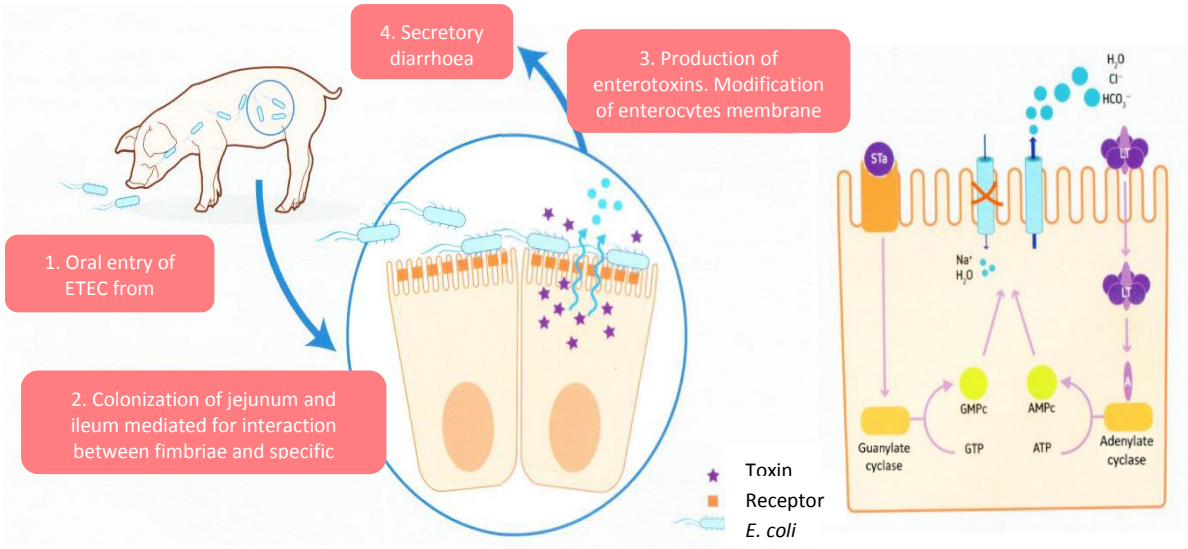


Figure from Prieto et al., 2017

2.1.3 SALMONELLOSIS IN WEANED PIGLETS

Salmonellosis is an infection more common in finisher pigs, although it can occur in weanlings. In young pigs the clinical signs are evident, whereas in fattening pigs, the course is normally asymptomatic (Bonardi, 2017). These signs include fever and watery yellow diarrhoea, initially without blood, that lasts for 3-7 days (Zimmerman et al., 2019). The transmission occurs normally via faecal-oral route, although the bacteria can also enter the host via the respiratory system (Boyen et al., 2008). *Salmonella* first heavily settle in the palatine tonsils of the animal and, when it surpasses the low pH in the stomach, bile salts, lysozyme and defensins, it colonizes to the distal parts of the intestine. Once attached, the invasion of the intestinal epithelium takes place: *Salmonella* enters mature enterocytes and also affects M cells from Peyer's patch developing a macropinocytosis process, creating vacuoles in which the bacteria can replicate and survive. Once inside, an inflammatory response is set, with the release of proinflammatory cytokines (IL-8) and the recruitment of neutrophils and macrophages. Ultimately, *Salmonella* can induce the apoptosis of both, epithelial cells and macrophages (Sansonetti, 2002; Schauser et al., 2005; Boyen et al., 2008; Fàbrega & Vila, 2012). Diarrhea onset is associated to

this damage and to the increased vascular permeability, as water and electrolytes are released to intestinal lumen (Figueroa & Verdugo, 2005).

There are two different species inside the genera *Salmonella: S. enterica* and *S. bongori. S. enterica* includes, at the same time, 6 subspecies. The serotypes included in the subspecies *enterica* have been classified depending on the antigens O, formed by the polysaccharide fraction of the cell wall lipopolysaccharide, and H, constituted by flagellar proteins (Prieto et al., 2017). Among them, serotypes Typhimurium (serovar monophasic), Derby and Infantis are commonly affecting pigs in the European Union (Bonardi, 2017).

This disease acquires significant importance due to its zoonotic condition. Although *Salmonella* control programmed were implemented in 2003 in poultry in the EU, there is nothing similar to achieve *Salmonella* reductions stablished for pigs. Consequently, there was an important reduction of *Salmonella* Enteritiditis human cases reported until 2016 with a contrarily increase of the outbreaks caused by Salmonella Typhimurium, linked to pork derived products (Martínez-Avilés et al., 2019). Nevertheless, there are monitoring systems that are implemented in concordance to the European Commission Directive 2003/99/EC. This control can result difficult as, as mentioned previously, the course of the infection is in most of the cases asymptotic and, hence, sick animals are not easy to be detected. Furthermore, the shedding can course as intermittent, only being detectable in episodes of stress like transport, interfering with a correct monitoring (Bonardi, 2017). *Salmonellosis* causes a worsened performance in swine: losses of individuals, increase in the time needed to reach slaughter weight and non-uniform batches, which leads to economic repercussions (Rodríguez & Suárez, 2014).

2.1.4 PROFILAXIS AND METAPHYLAXIS FOR DIARRHOEA IN WEANGLINGS

On the 25th October of 2018, the European Parliament approved a new legislation banning routinely use of antibiotics as meta- or prophylactics, which is expected to be implemented in 2022. This decision is triggered because of the alarming rise in antimicrobial resistances in human and veterinary

medicine and the high amount of antibiotics destined to food-production animals (7787.1 tons in 2016). Actually, enterotoxigenic *Escherichia coli* has shown to be resistant to multiple antibiotics in several studies blocking this resistance, in some cases, the action of more than one drug. The percentage of resistances to the different antimicrobials varies depending on the geographic situation, but the most affected ones are gentamicin, enrofloxacin, trimethoprim-sulfamethoxazole and some beta-lactams such as amoxicillin or ampicillin (Luppi, 2017). In addition, the presence of resistance to antibiotics by different *Salmonella* serovars in swine is common, being tetracycline, ampicillin, streptomycin, and sulfisoxazole the most affected ones. What is more, multidrug-resistant *Salmonella* has also been identified in swine herds. As a consequence, these resistant bacteria have been isolated in pork products, with the risk that it entails as a zoonotic pathogen (V T Nail et al., 2018). One antibiotic that was frequently used as a feed additive and to prevent and treat gastrointestinal infections, especially those produced by Enterobacteriaceae is colistin. (Kempf et al., 2016). In the past, its use was mainly destined to animals because of its toxicity and the availability of less harmful substances for human use. However, as resistance to them are rapidly growing, colistin is nowadays one of last-resort antibiotic for the treatment of severe infections in humans (Liu et al., 2018). Therefore, as resistance also exists for this antibiotic, its use in production animals should be restricted. In fact, the Committee for Medicinal Products for Veterinary Use recommended in 2016 the suspension of the marketing authorisations for veterinary oral medicinal products containing colistin in combination with other antimicrobial substances (EMA, 2016). Given this situation, alternatives to the classic antimicrobials are needed to be implemented in animal farming.

Some options are summarized in the reviews published by Vondruskova et al. (2010) and Thacker et al. (2013), in which are included the following components, among others:

- Organic acids: Their antimicrobial activity is explained by two mechanisms; pH acidification and penetration through bacterial membrane, causing their destruction.

-Essential oils: Aromatic oily liquids with plant origin that may contain phenolic compounds, terpenes, lectins, aldehydes, polypeptides or polyacetylenes. They have demonstrated antimicrobial properties which is believed to be associated to variations in lipid solubility of bacteria's surface.

- Clay minerals: Net of stratified tetrahedral or octahedral layers formed by molecules of silicon, aluminium and oxygen. These minerals are affective binders and thus, they are capable of immobilizing toxic elements present in the gastrointestinal tract and effective in diarrhoea prevention in piglets.

- Antimicrobial peptides: Includes peptides that are components of the defence mechanisms of the host and have antimicrobial and innate immunity mediator functions.

-Egg yolk antibodies: This strategy involves the use of IgY antibodies produced by laying hens to target enteric pathogens like *E. coli* or *Salmonella*, however, the degree of success may vary.

-Recombinant enzymes: These include proteins with a biological action that permits the hydrolysis of specific chemical bonds, resulting in an enhanced digestion and absorption of certain nutrients. The addition of carbohydrases to the diet is beneficial in a double way: firstly, it allows the animal to take profit of a greater number of carbohydrates and, second, it avoids the use of these substances by bacteria, retarding or inhibiting their growth activity due to competition.

- Probiotics, prebiotics and synbiotics: This category englobes the use of live microorganisms that exert beneficial effects on the host (probiotics), non-digestible substances of the diet that promote the growth and/or activity of probiotics or beneficial microbes present on host's microbiota (prebiotics) or the combination of both, known as synbiotic. The study of this last concept is highly interesting as, a priori, the jointly administration of probiotics and prebiotics can bring excellent outcomes.

Table 2.1. Summary of the alternatives for classic antimicrobials.

Alternatives for classic antimicrobials						
Organic acids	Essential oils	Clay minerals	Antimicrobial peptides	Egg yolk antibodies	Recombinant enzymes	Probiotics, prebiotics & synbiotics
pH acidification, bacterial destruction	Lipid surface solubility variation	Toxic elements inhibition	Bacterial destruction, immune system mediation	Bacterial targeting	Resources competition	Resources & adherence competition, bacterial destruction, toxin inhibition

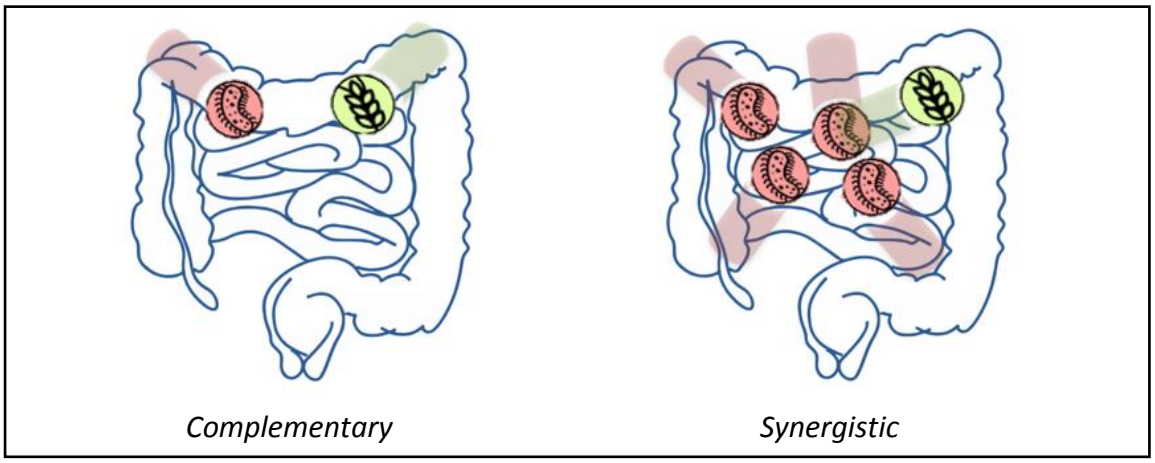
2.2 SYNBIOTICS

The first approach to the definition of the concept "synbiotic" was made by Roberfroid in the year 1998 as "mixture of a probiotic and a prebiotic that beneficially affects the host by improving the survival and the implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria". Although this description was created twenty years ago, the idea has not changed since then. It should be considered, however, that not all combinations between any probiotic and prebiotic will fulfil this principle. The administration of probiotics and prebiotics in a mixture can result in two different outcomes. Thus, their relation can be classified in two categories (Kolida & Gibson, 2011) (***Figure 2.3***):

-Complementary ("symbiotic"): the probiotic is chosen due to the beneficial effects that it exerts on the host, but the prebiotic is chosen independently to increase concentrations of other components of the host microbiota or for its desirable consequences on the host's health.

-Synergistic ("synbiotic"): the probiotic is chosen based on specific beneficial effects on the host, and the prebiotic is chosen to specifically stimulate growth and activity of this probiotic improving its survival and growth in the host.

Figure 2.3. Classification of synbiotic/symbiotic compounds.



The main characteristics that probiotics and prebiotics must accomplish to be considered as potential components of a synbiotic compound will be reviewed in the following sections.

2.2.1 PROBIOTICS

2.2.1.1 Definition

The term "probiotic" was introduced in the '50 decade, being used by Kollath in 1953 and Vegin in 1954, as active substances that are essential for a healthy development of life. Shortly after, in 1974, Parker, modifying the statement made by Lilly and Stillwell in 1965, described probiotics as animal feed supplements containing live bacteria and spores that could help in reducing antibiotic use. In 1989, Fuller considered a probiotic as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". However, current accepted definition for probiotics is "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" which was formulated by FAO (Food and Agriculture Organization) and WHO (World Health Organization) in 2002.

2.2.1.2 Selection criteria

2.2.1.2.1 Functional basic properties

Stress tolerance

Once the selected bacterial strains enter the gastrointestinal system of the host, they must be able to survive to various detrimental conditions that may affect their survival (de Melo Pereira et al., 2018). The first obstacle that they find are the enzymes present in the oral cavity (amylase and lysozyme principally). Lysozyme is responsible of the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall (Manchenko, 1994). Continuedly, probiotics must face low pH and pepsin

from the stomach. Gastric pH can reach values between 1.2 – 2.5 which can be lethal for a wide variety of bacteria. Lactobacilli and bifidobacteria are able to stand these conditions, however, this last genus is less resistant to the gastric lumen conditions (Fontana et al., 2013). Finally, last barrier is constituted by pancreatic enzymes and bile salts acting in the duodenum, having these salts antimicrobial activity due to their detergent properties, which produces the degradation of bacterial membranes (Begley et al., 2006; de Melo Pereira et al., 2018). Several strains of *Bifidobacteria* and *Lactobacillus* have demonstrated their ability to defeat the action of bovine and porcine bile salts (Dunne et al., 2001). Indeed, they have the capacity to deconjugate bile salts, resulting in salts with stronger antimicrobial effect (Begley et al., 2006; Oelschlaeger, 2010).

Host epithelium adhesion ability

Once surpassed the above-mentioned handicaps, probiotic bacteria need to attach to the gastrointestinal (GI) epithelial cells in order to avoid being washed out and to achieve a proper colonization and multiplication. De Melo Pereira and collaborators express that this ability depends on the balance of electrostatic and Van der Waals interactions on the target surface, as well as on bacterial extracellular components, like mucus-binding and other surface proteins, fimbriae or pili (Monteagudo-Mera et al., 2019). They suggest that adhesion is also determined by the autoaggregation capacity, which allows the probiotic to reach high cell density, and hydrophobic properties of the cell surface, responsible of an enhanced interaction between bacteria and host epithelial cells. It should be remarked that adhesion is species-specific, being strains more likely to have a successful colonization on their natural host (Dogi & Perdigón, 2006; Dowarah et al., 2018).

Anti-pathogenic activity

Probiotic strain should be capable of negatively impact pathogenic microorganisms that may potentially cause the apparition of disease in the host. This activity can be carried out through three direct and one indirect mechanisms of action. The first one encompasses the competition of probiotic strains with pathogens for nutrients and adhesion sites. It is obvious that both microorganisms will

compete for common substances that they need for survival and growth but, in addition, some probiotics strains, like *Lactobacillus* species may have an advantage over pathogens as, for example, they don't need iron as a substrate and, furthermore, such as bifidobacteria, are able to bind ferric hydroxide to their surface membrane making it inaccessible to pathogens (Bezkorovainy & Kot, 1998; Elli et al., 2000). In regard of anti-adhesive effects, they are produced by the protective layer originated from the co-aggregation of the probiotic cells, as reported by Kos et al. (2003), for the competition to the same receptor in the host epithelial cells and, lastly, due to increased production of mucus (Oelschlaeger, 2010). For example, studies conducted by Hafez et al. (2012) and Mattar et al. (2002) show that probiotics formed by *Lactobacillus* strains or *Escherichia coli* Nissle 1917 stimulate mucin (especially MUC2) gene expression in different cell culture media.

Secondly, probiotic can produce antimicrobial substances that antagonize pathogens. Hydrogen-peroxide produced by some lactobacilli induces pathogen cell death (Hertzberger et al., 2014). In addition, lactobacilli produce lactic acid that, besides reducing pH in the gut lumen, enters the cytoplasmic membrane of detrimental microorganisms in its associated form, lowering their intracellular pH (Anderson et al., 2015). Probiotics can also promote higher concentrations of short-chain fatty acids (SCFA) as fermentation products (Nagpal et al., 2018) that lower gut lumen pH and, that can lead to SCFA-induced toxicity. This toxicity is due to that organic acids cross the cell membrane in the non-dissociated form and, once inside, it dissociates in protons and SCFA anions. When the pH is low in the exterior of the cell it is due to high amounts of protons, thus, the cell ATPase is not that efficient in pumping the H^+ deriving from SCFA to the outer space (Sun & O'Riordan, 2013). These SCFA also can have an anti-toxin effect, blocking toxin expression and blocking pathogenicity of bacteria that use this mode of action (Oelschlaeger, 2010).

Probiotic strains may also synthesize bacteriocins, which are peptidic toxins that inhibit the growth of other bacteria. Some authors reported the ability of bifidobacteria and lactobacilli to inhibit pathogen growth via production of bacteriocins (bifidin, bifidocin B, biflong) and bacteriocin-like substances

(Yildirim & Johnson, 1998; Touré et al., 2003; Cheikhyoussef et al., 2007; Jeong & Moon, 2015; Mokoena, 2017; Gaspar et al., 2018). Furthermore, *Lactobacillus reuteri*, for example, produces reuterin, which is a broad-spectrum antimicrobial substance, acting against Gram-positive and Gram-negative bacteria, fungi, yeasts, protozoa and virus (Cadieux et al., 2008; Cleusix et al., 2008).

The third direct effect against pathogens relies on the inhibition of bacterial toxin production. As explained above, it can be caused by the production of SCFA, but, in addition, some probiotics produce anti-toxins or proteases than destroy harmful toxins. Several studies are found in the literature proving the capacity *of Saccharomyces boulardii* to block the action of *Clostridium difficile* toxin-A creating proteases and stimulating antibodies against the toxin (Castagliuolo et al., 1996; Rim & Pothoulakis; 2010). Guo et al. (2017) reported that *Lactobacillus fermentum* and *Lactobacillus acidophilus* degrade the α-toxin produced by *Clostridium perfringens*.

Finally, the indirect effect on infectious agents is mediated by an immunomodulation of the host, which will be discussed in the next section.

2.2.1.2.2 Targeted effects on the host

Probiotics have been associated to a wide range of advantageous effects on the host. Most of them are of high significance especially in human medicine. These bacteria are helpful resulting in desirable outcomes in neoplastic diseases, hypercholesterolemia, obesity, diabetes, irritable bowel syndrome (IBS), inflammatory bowel disorder (IBD), lactose intolerance and diarrhoea scenarios (Pandey et al., 2015). Although some repercussions might be of interest in clinical veterinary medicine, are irrelevant in animal production. Nevertheless, probiotic administration is generally associated to immunomodulatory effects and gut-brain axis modification, which can have a relevant impact in animal husbandry.

Immunomodulation

Probiotics modify both, innate and adaptative immunity and, according to Markowiak and Śliżewska (2017), this result in different consequences: induction and maintenance of a tolerant state to environmental antigens and induction and control reactions against pathogens with inhibition of auto-directed and hyper sensible reactions. The immunomodulatory properties are originated by the interaction of the probiotic cell wall components, DNA and metabolites with the host cells.

Innate immune response englobes reactions of the host body to antigens that are not specific. Part of this response is effectuated by pattern recognition receptors (PRRs) that recognize micro-organism-associated molecular patters in order to activate the immune response. Among these PPRs, toll-like receptors (TLR) can be found. Some of these receptors, when bound, activate NFκB signalling, leading to the production of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Llewellyn & Foey, 2017). Probiotics can act at this level by avoiding proinflammatory detrimental states and enhancing the response when a pathogen is present. Castillo et al. (2011) found that *Lactobacillus casei*, for example, upregulated TLR2, TLR4 and TLR9 expression in *Salmonella*-challenged mice, leading to higher concentrations of TNF-α, IFN-γ and IL-10. Nonetheless, when these animals were not infected, this *Lactobacillus* diminished TNF-α, maintaining IFN-γ, IL-6, IL-10. In pigs, *Lactobacillus rhamnosus* was also able to modulate inflammatory response in an ETEC F4+ challenge by avoiding increased expression TLR2, TLR9, NOD1 and TNF-α in high-dose supplemented animals (Li et al., 2012). Another part of innate response lies on phagocytosis performed by macrophages. Several studies supporting stimulation of macrophages by probiotic bacteria are published in the literature. Manzarrino et al. (2012) and Rocha- Ramirez et al. (2017), among others, obtained an enhancement of macrophages activation in human and mice administering different *Lactobacillus* strains, which resulted in a better response of the subjects against infections.

Regarding adaptative immune response, which is antigen-specific, probiotics can also potentiate or depress it (enhancing it in case of presence of strange antigens and avoiding the activation with auto-

antigens) and produce shifts between T-effectors types. Pochard et al. (2002) described a decreased Th2 (humoral) cytokine production in allergic human patients by lactic acid bacteria and, similarly, in animal models, *Bifidobacterium longum* CECT 7210 reduced CD4+ T-cells in hypersensitized animals (Laparra et al., 2012). In contrast, in pigs, bifidobacteria and lactobacilli showed to provoke an increase of T cell response after rotavirus vaccination (Wen et al., 2014; Ishizuka et al., 2016) and, in addition, other probiotics such as *Pediococcus acidilactici* and *Saccharomyces boulardii* induced the same response in ETEC challenged piglets (Lessard et al., 2009).

Gut brain axis modification

As reviewed previously, in the intestine is located the enteric nervous system (ENS), known informally as "second brain". This ENS contains more than 500 million neurons that connect to the central nervous system (CNS) of the host via vagus nerve (Martin et al., 2018; Lyte & Lyte, 2019). Furthermore, the gut epithelium includes chemosensory pathways that are responsible for the detection of neuroactive substances that may be present in the lumen (Breer et al., 2012). Authors like De Vadder et al (2018) and Heiss & Olofson (2019) have reported a modification of ENS and, thus, CNS, due to actions of the microbiota. This makes sense as, expressed by Lyte & Lyte (2019), neurosubstances produced by the host are exactly the same as the ones synthesized by microbes, thus, a bidirectional communication occurs. Firstly, impact of catecholamines produced by the host in a situation of stress has been associated to a promotion of pathogen settlement, as, for example, norepinephrine increases adherence of *E. coli* to intestinal mucosa and shedding of *Salmonella* (Green et al., 2004; Pullinger et al., 2010). Secondly, and in the other direction, a production of neurochemicals by components of the intestinal microbiota has an impact on host's CNS. Among the substances that can be generated by microbes are butyric acid, serotonin, norepinephrine and dopamine and GABA. Low concentrations of GABA are associated to depressive disorders (Luscher et al., 2011). Ko et al. (2013) observed that fermentation of soymilk by lactobacilli was able to produce GABA when soymilk was fermented, alleviating these depression-derived behaviors in rats. Related to this, serotonin (5-HT) is a tryptophan-derived metabolite whose low levels in the CNS are related to apparition of depression

episodes in humans (Sharp & Cowen, 2011). Depression clinical signs have been reported to be ameliorated by administration of probiotics containing lactobacilli or bifidobacteria (Wallace & Milev, 2017). In pigs, while studying tail-biting, Ursinus et al. (2014) stablished a connection between low level of serotonin and animals showing this behaviour, both, biters and their victims. Furthermore, piglets supplemented with tryptophan are able to face weaning with less stress (Liu et al., 2013), thus, production of tryptophan by probiotic bacteria might also help in reducing weaning-derived problems.

2.2.1.2.3 Regulatory affairs

As stated in the Commission Regulation No 429/2008 of 25 April 2008, bacterial strains need to accomplish a series of requirements to be considered as safe and included as authorized feed additives. According to the document they have to be perfectly taxonomic identified and origin, quantitave and qualitative composition have to be determined, as well as purity in order to detect potential microbiological, mycotoxins, heavy metals contamination. These strains have to be deposited in an internationally recognised culture collection and maintained by the culture collection for the authorised life of the additive. Furthermore, a description of all relevant morphological, physiological and molecular characteristics necessary to provide the unique identification of the strain and the means to confirm its genetic stability is necessary.

Probiotic strains must be non-pathogenic and non-toxic, assuring the absence of any toxin or virulence factor. In addition, they shall be genetic stable and lack of antibiotic activity and transferable antibiotic resistance genes (Sanders et al., 2010).

It is important to verify that the microbial strains are safe for the target animals using, if possible, at least a 100-fold overdose in the experimental group and proving that there are no adverse effects for the microbiota of the animal.

Lastly, but not less important, the safety for workers, consumers and environment has to be guaranteed.

Food and Drug Administration (FDA) or European Food Safety Authority (EFSA) can assess the risk of the microorganisms (and other components) using means like “Generally Recognized as Safe (GRAS)” status (FDA) or “Qualified Presumptions of Safety (QPS)“(EFSA) (Shewale et al., 2014). As stated in a review elaborated by Herman et al. (2019), QPS is a concept that was created with the objective to be a harmonic generic pre-assessment for safety meaning that, for example, if a microorganism strain matches the requirements needed to get the QPS status, no other further assessment would be needed. The QPS includes information about taxonomy, safety concerns, use, among others. Nonetheless, there are aspects that are not covered and may be concerning such as hazards related to formulation or processing, to allergenicity or for users and workers, as well as environment impact. Thus, included microbes can be generally considered as safe to be used as feed additives, however, there is still not enough evidence to discard a complete totality of hazards (Barba-Vidal et al., 2019).

2.2.1.2.4 Technological aspects

The last aspect that might affect the decision for a bacterial strain to be selected or not as a probiotic is the technological usability. Probiotic viability is highly affected by external conditions. As reported by Shewale et al. (2014), there are some stability abilities that probiotic need to accomplish:

To survive in the storage without loss of viability.

To grow quickly to maximum concentration in a simple and cheap fermentation medium.

To grow and survive in microaerophilic or aerobic condition.

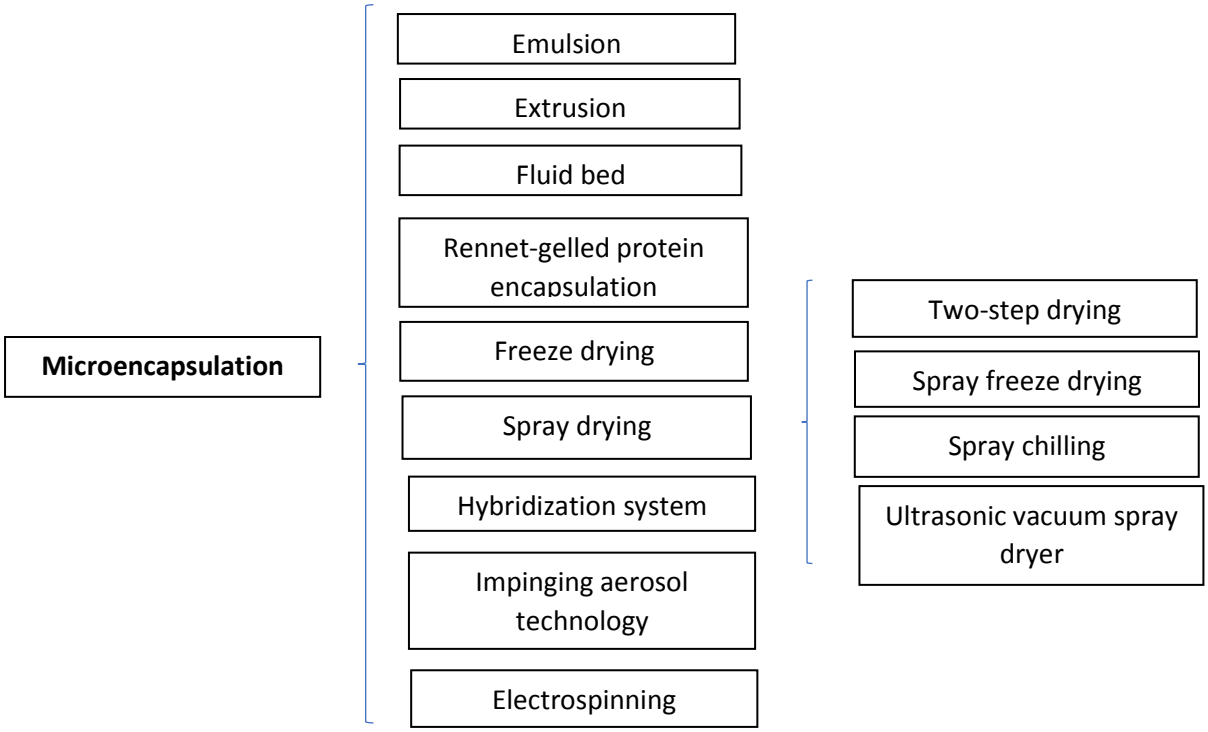
To withstand physical handling without significant loss of viability.

To survive in the food matrices and during the processing.

Probiotics used in swine farming are administered in doses that range from 10^5 to 10^{10} cfu per gram of diet (Liao & Nyachoti 2017). According to Barba-Vidal et al. (2019), to achieve these concentrations, probiotics strains need to be capable of surviving to all detrimental conditions that take place during technological processing and storage, as well as the ones that conform farm environment, such as

high temperatures, humidity, high oxygenation, that can reach toxic levels. In addition, to be successfully considered as probiotics, these bacteria have to be able to be produced at high scale and possess good organoleptic properties to avoid being rejected by the animals at the moment of feeding. Lastly, it should be mentioned that, although viability is critical, it can be improved through different interventions: use of oxygen impermeable containers, two step fermentation, stress adaptation, incorporation of the micronutrients and microencapsulation (Sarkar, 2010). Microencapsulation is highly useful in increasing probiotic survival and it can be carried out through variety of techniques, as summarized in the following figure (Martín et al., 2015).

Figure 2.4. Microencapsulation techniques.



2.2.2 PREBIOTICS

2.2.2.1 Definition

Gibson and Roberfroid described prebiotics in the year 1995 as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health”. This definition has been modified several times until 2007, when experts of FAO and WHO stablished another definition of prebiotics as “non-viable diet ingredients that, once fermented, selectively produce specific changes in composition and/or activity of intestinal microbiota conferring health benefits to the host”. Finally, the last definition of this concept was made by World Gastroenterology Organisation (WGO) in 2011 as following: “substances of the diet (especially non-starch polysaccharides and oligosaccharides) that nourish a selected group of microorganisms favouring the growth of beneficial upon pathogenic bacteria”.

As expressed by Davani-Davari et al. (2019), normally, prebiotics are constituted by carbohydrates, but not only these substances are prebiotics. The list of the most common compounds use as prebiotics are listed below:

- Fructans: In this category are included inulin and fructo-oligosaccharides. They are formed by a molecular chain of fructose monomers united with β(2→1) linkage with a terminal glucose unit. The degree of polymerization is variable, being inulin the longest chain and FOS obtained by enzymatic partial hydrolysis of inulin by the action of β-fructofuranosidades (Grzybowski et al., 2014). Nonetheless, FOS can also be chemically synthetized by the utilization of glycosidase and glycosyl-transferase.
- Galacto-oligosaccharides (GOS): GOS are produced from lactose extension by two mechanisms. The first one consists of the addition of extra galactose units and, the second, of an enzymatic trans-glycosylation. The GOS obtained by this last process are known as Trans-galacto-oligosaccharides

(TOS). TOS have from tri- to pentasaccharide with β (1→6), β (1→3), and β (1→4) linkages. Apart from these, there are other GOS derived from lactulose and sucrose, receiving these last ones the name of raffinose. (Roberfroid, 2007; Davani-Davari et al., 2019).

- Starch and other oligo and polysaccharides: There is a type of starch that is resistant to host's digestion process, resistant starch, that is associated to increased production of butyric acid levels. Furthermore, polydextrose, a glucose-derived glucan is also considered a prebiotic.

- Other oligosaccharides: Other possible promising substances are pectic-oligosaccharide, isomalto-oligosaccharides, lactosucrose, soybean oligosaccharides, xylo-oligosaccharides, mannano-oligosaccharides, etc. (Roberfroid, 2007; Davani-Davari et al., 2019).

- Polyphenols: In this category substances of flavonoids derived from cocoa can be included, as they have been related to increases of lactic acid bacteria (Tzounis et al., 2011). In addition, other polyphenols derived from grapes and blueberries have also demonstrated a prebiotic effect, increasing number of lactobacilli and bifidobacteria (Cueva et al., 2012; Vendrame et al., 2011).

2.2.2.2 Selection criteria

2.2.1.2.1 Functional basic properties

Non-digestibility

Prebiotics must resist the action of gastric acidity, enzymes present in the gastrointestinal tract and absorption in order to reach the distal part of the intestine almost intact (de Vrese & Schrezenmeir, 2008).

Fermentation by intestinal microbiota

Prebiotics should be fermented by bacteria of the gut environment to exert the desired effect. Fermentation can be tested by quantifying prebiotic's disappearance *in-vitro* using chemical,

physicochemical or enzymatic methods, but also *in-vivo* with laboratory and other animals (Wang, 2009).

Selective stimulation of growth and/or activity of beneficial bacteria

The stimulation exerted by prebiotic must affect exclusively beneficial bacteria present in the natural microbiota of the host or beneficial bacteria introduced as probiotics (Folks & Gibson, 1999). Normally, the two suitable genres to be stimulated by prebiotics are lactobacilli and bifidobacteria for the potential advantageous effects that they have on the host's health, as reviewed in the former section.

2.2.1.2.2 Targeted effects on the host

As for probiotics, some results of prebiotic administration are of particular importance in human medicine. For example, prebiotics are useful to counteract dyslipidaemias, as inulin-type fructans are able to cause a reduction of LDL cholesterol and triglycerides in western-diet fed mice models and human patients (Liu et al., 2017; Hiel et al., 2018). In addition, prebiotics have also been associated to cancer prevention, especially useful in colorectal tumours: in rodent models, FOS and galactooligosaccharides (GOS) decreased the aberrant crypt foci formation (Wijnands et al., 2001; Hsu et al., 2004). Furthermore, inulin diminishes immunoreactivity of cyclooxygenase-2, transcription nuclear factor kappa beta and inducible nitric oxide synthase (Hijova et al., 2014). These effects are not of interest in animal husbandry, however, there are some more that can positively affect livestock.

Positive changes in intestinal microbiota

It is well documented the capacity of prebiotics to promote the growth of potential protective and beneficial bacteria and to inhibit potential pathogenic microorganisms. Patterson et al. (2010) proved that inulin of different chain lengths was able to promote the survival and presence of bifidobacterial and lactobacilli in young pigs and, furthermore, they suppressed the viability of undesirable bacteria like *Clostridium spp.* and some enterobacteria. Similar effects were observed by Alizadeh et al. (2016)

regarding lactobacilli and bifidobacteria when they fed pigs with GOS. They also reported a lowering of the pH and an increment of SCFA concentrations, which contributed, in addition, to stabilize the intestinal environment.

Stimulation of mineral absorption

Related to the previous paragraph, the enhanced production of SCFA and lowered pH can have an impact in mineral absorption and, thus, in bone structure. As stated by Scholz-Ahrens et al. (2007), prebiotics have caused an increasement of different mineral and metal absorption, like iron, calcium, magnesium and zinc. By doing this, they improve bone structure as these elements are necessary for a proper collagen and other bone matrix components formation. Other mechanisms mentioned by these authors and Whisner and Castillo (2018) involved on this effect are: enlargement of the absorption surface by promoting proliferation of enterocytes due to bacterial fermentation products (like butyrate), increased expression of calcium-binding and calcium-transport proteins, improvement of gut health (p.e. bifidobacteria and Bacteroides have been related as mediators of Ca absorption), degradation of mineral complexing phytic acid, release of bone-modulating factors such as phytoestrogens from foods, stabilization of intestinal mucus, impact of modulating growth factors and alleviation of systemic inflammation.

This improved mineral absorption would be of special interest in broiler chickens due to the high incidence of tibial dyschondroplasia (TD), which has both, welfare and economic repercussions. However, effects of prebiotic inclusion to counteract this pathology is not clear. Houshmand et al. (2011) reported a better performance of prebiotic-supplemented birds receiving low calcium concentrations in the diet, but the episodes of TD were not reduced. Similarly, Swiatkiewicz and colleagues (2011) obtained comparable results, as inulin and FOS administration to chickens feed low Ca and P diets did not generate modifications in performance or bone quality.

Immunomodulation

Prebiotics can exert an action on immune system direct and/or indirectly. Indirect effects are produced because of their influence on intestinal microbiota. The promotion of beneficial bacteria, as mentioned in the probiotic's section, can deeply influence immune system of the host. Pigs infected with *Tricuris suis* that were treated with inulin suffered an up-regulation of Th2-related immune genes with a suppression of Th1-related pro-inflammatory genes in the colon. In addition, and parallelly, some pro-inflammatory genes were also inhibited (Myhill et al., 2018). These authors suggest that these changes might correspond to changes in microbiota composition, as they observed an increased Bacteroidetes:Firmicutes ratio, being this last phylum associated with increased inflammation. Herfel et al. (2011) experienced a decreased in pro-inflammatory cytokines TNF-α, IL-8, IL-1β and increment of IL-10 in suckling piglets treated with polydextrose that they attributed to the possible pathogen inhibition, and hence less antigenic loads, by the greater presence of lactobacilli and their associated lactic acid.

Besides this, some prebiotic compounds could also act on immune system by themselves. As reported by Shokryazdan et al. (2016), several studies relate the inclusion of different oligosaccharides to changes in gene expression of the host's cells. However, it depends on the degree of polymerization (PD) of the saccharide as might exist a correlation between less PD and enhanced intestinal absorption and further recognition by the gut-associated immune system.

2.2.2.2.3 Regulatory affairs

Similarly to probiotics, prebiotics are considered as feed additives and its incorporation in animals' diets is regulated, once again, in the Commission Regulation No 429/2008. The rules regarding identity, characterisation, conditions of use and safety are identical as those presented in the former probiotic section. Moreover, as prebiotics are not expected to be metabolized or absorbed, metabolic or residue studies are not required and beneficial effects of prebiotics on the specie of destination

must be assessed. Lastly, like probiotics, prebiotic substances can acquire the status of as GRAS (Generally Recognized As Safe), which grants them consideration of being, a priori, secure (Markowiak & Śliżewska, 2018).

2.2.2.2.4 Technological aspects

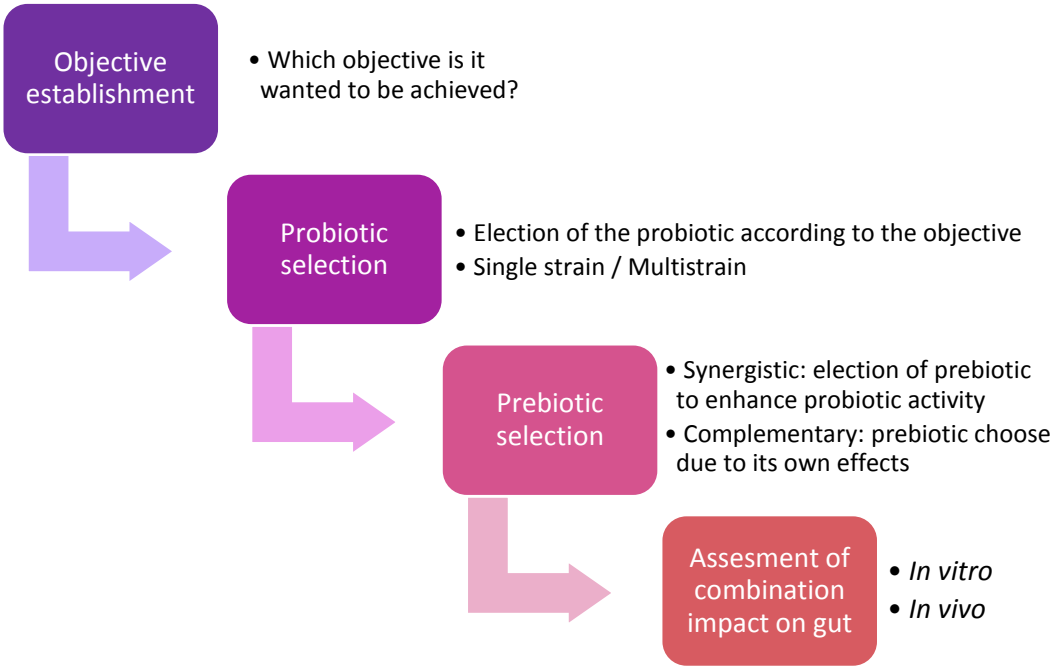
To conserve their functionality, prebiotics need to withstand adverse conditions occurring during their processing, such as low pH, heat and Maillard reactions. If it was not the case, long-chained prebiotic could be degraded to mono- or disaccharides, that would be unavailable for bacterial utilization as they might be digested and absorbed by the host instead. Huebner et al. (2008) tested the stability of fructo-oligosaccharides (FOS) and inulin upon different physico-chemical conditions. They observed that these fibres were not altered by low pH values (being the lowest pH value 4) but if low pH is combined with high temperatures (85°C), inulin and FOS suffered partial hydrolysis to sucrose, glucose and fructose. When analysing them upon Maillard reaction conditions, inulin showed less browning than FOS.

2.2.3 STAGES IN THE DEVELOPMENT OF A SYNBIOTIC

As synbiotics are composed by the mixture of one or more probiotic strains and a prebiotic (or prebiotic mixture), each one of them must accomplish the selection criteria above mentioned for probiotics or prebiotics. It means that, apart from being safe and capable of endure the processing procedure, both components must exert a beneficial effect on the host. Furthermore, in case of a synergistic synbiotic design, the prebiotic must be strictly selected to be fermented by the specific probiotic strain to enhance its growth and activity or, in case of complimentary synbiotics, to increase concentrations of microorganisms already present in the host GI system that could benefit it.

In order to create a new synbiotic compound, there are a series of stages that need to be followed, which are listed in the following section.

Figure 2.5. Diagrams of the steps required for a synbiotic development.



2.2.3.1 Objective establishment

It is fundamental to determine the main objective which is wanted to be achieved by the action of the synbiotic. As reviewed above, probiotic strains have a wide variety of potential beneficial effects on the host's health, which are strain-dependent. In addition, the prebiotic has also to be carefully chosen by its capacity to stimulate probiotic strain activity or growth or by its direct action on the host's microbiota or organism.

2.2.3.2 Selection of the probiotic. Single strain or multistrain

As stated previously, probiotic strain or strains must be chosen carefully in accordance to the changes that are wanted to be introduced in the target host. For example, if the objective is to fight enteric

pathogens, several strains of lactobacilli and bifidobacteria can be considered as potential probiotics, as they have proven their efficacy in numerous studies. *L. rhamnosus* GG, strains of *L. plantarum* and *Bifidobacterium thermophilum* are capable of interfering with *Salmonella* and *Escherichia coli* growth and/or activity (Kobayashi et al; 2002; Tanner et al., 2016; Wang et al., 2017; Mohanty et al., 2019; Splichalova et al., 2019; Song et al., 2019). Furthermore, the production of bacteriocins by some of these bacteria can also be detrimental for pathogen survival (Hegarty et al, 2016). Differently, in case that the goal is to change the animal's behaviour in order to, for example, enhance animal welfare, strains that are able to produce neurotransmitters, like serotonin, should be considered. In this line, *Lactobacillus plantarum* IS-10506 and *Clostridium butyricum* have demonstrated a capacity to increase this molecule concentration in brain (Sun & O'Riordan, 2018; Ranuh et al., 2019). A multistrain probiotic can also be interesting if the objective is to have complementary effects on the host or to potentiate one using two or more strains that may result in a similar outcome.

To evaluate the selected probiotics, different methods can be used. To assess the efficacy against pathogens, *in vitro* assays can be run, like competition tests using microbiological cultures or cell lines (Moreno-Muñoz et al., 2011; Delcaru et al., 2016; Guantario et al., 2017; Song et al., 2019). If successful, they can be followed by *in vivo* testing with different animal models (firstly, rodents) and pathogen challenges to evaluate if the probiotic strains they can ameliorate the damage or diminish the prevalence of disease (Moreno-Muñoz et al., 2011; Wang et al., 2017). *In-vivo* trials can also be desing to verify the impact of probiotics on gut-brain axis or immunity (Papadimitriou et al., 2015; Abildgaard et al., 2017; Sun et al., 2018). Regarding microbiological status of the animals, holoxenic subjects can reflect the idea of how the probiotic strains interfere in their systems with an established microbiota and, when evaluating a probiotic in gnotobiotic (especially axenic) individuals, it can be assured that the produced changes are due to its administration.

Furthermore, the selected probiotic bacteria must also comply with all the characteristics and requirements mentioned previously in this review as for example proving its ability to survive in the GI tract conditions, to adhere to the gut epithelium of the host or being safe.

2.2.3.3 Selection of the prebiotic. Synergistic or complementary

Once chosen the probiotic, the next step for a synbiotic creation depends on if the objective is to obtain a "synergistic" or "complementary" combination. In the first case, the prebiotic is chosen to enhance growth and activity of the specific probiotic in the host. For this, selected bacteria must possess the tools to metabolise the prebiotic and use it as a fermentation source, such enzymes like β-fructofuranosidases or β-galactosidases in the case of using fructo-oligosaccarides or galacto-oligosaccarides as prebiotic (Andersen et al., 2012; Watson et al., 2012). To evaluate the ability of a probiotic to grow on different substrates, *in vitro* tests can be performed. Watson et al. (2012), for example, used a modified Man–Rogosa–Sharpe (MRS) growth medium to test the utilization of several prebiotics by bifidobacteria and lactobacilli. Particularly, this medium does not allow the growth of any of the strains in the absence of a supplemented carbohydrate. Although almost all strains were able to growth in supplementation with glucose or lactose, with lactulose, maltodextrin, polydextrose and FOS the growth rate varied between species of the same genera. For example, *L. plantarum* NCDO326 just survived when glucose was added, whereas others, like strains of *L. reuteri* and *L. rhamnosus* GG could use any of those sources. Regarding bifidobacteria, they resulted less selective, being some strains just restricted by polydextrose. Apart from growth, it should be also evaluated if the prebiotic can increase parallelly the metabolic activity of the probiotic, expecting to find an increase in the production of substances like SCFA, bacteriocins or even neuro-molecules. Gaspar and collaborators (2018) have proved that production of bacteriocins by lactobacilli takes place during the exponential growth phase, thus, an enhanced growth promoted by a prebiotic should be followed by a high production of these substances.

In contrast, when the focus is set in obtaining a "complementary symbiotic", the prebiotic must be selected for its own effects on the host health or microbiota. Once again, changes in microbiota, as commented before, can be assessed *in vitro* or *in vivo* and, for those prebiotics chosen for stimulation of host's mineral metabolism, the most used models are constituted by oestrogen-deficient rats (Whisner and Castillo, 2018).

In addition, it is also of high importance to assure than the selected prebiotic does not promote growth of potentially opportunistic pathogens, particularly if the synbiotic is designed to be prevent or treat digestive infections. Although prebiotics are meant to selectively benefit beneficial bacteria (endogenous or not), pathogen microbes could also take profit from them, which would be counterproductive especially when the objective of the synbiotic is their activity/survival abolishment. Hence, the ability of harmful microorganisms to grow in media containing the selected prebiotic should be also tested *in vitro*. In this regard, Martín-Peláez et al. (2008) measured growth rates of *Salmonella* Typhimurium in cultures supplemented with xylo-oligosaccharides, oligofructose-enriched inulin, genti-oligosaccharydes, fructo-oligosaccharides and lactulose. They observed that the lowest growth rate occurred when MRS culture was combined with lactulose and fructo-oligosaccharides, and, after 24 hours, the lowest optic density belonged to those media containing lactulose or xylo-oligosaccharides. More recently, Jakobsen et al. (2019) evaluated the capacity of purified bovine milk oligosaccharide, galacto-oligosaccharides and pure lactose to stimulate bacteria growth. Interestingly, in this study*, Escherichia coli* and *Clostridium perfringens* showed an increased concentration were cultures contained galacto-oligosaccharides and their combination with lactose. Contrarily, the rest of mixtures and, especially, purified bovine milk oligosaccharide seemed to retard their growth. However, in another work, these two pathogens behaved differently in the presence of xylo-oligosaccharides, being just *E. coli* apparently able to use this carbohydrate source (Chen et al., 2016).

2.2.3.4 Assessment of the combination on the gut environment

The last aspect that should be considered when developing a new synbiotic is to know the impact that it will have when introduced in a complex ecosystem like the gut microbiota. Effects not necessarily will be the addition of those observed for the probiotic o prebiotic when administered separately.

Nowadays there is still no perfect *in vitro* model that fully predict what is going to happen in the gut environment, however, there are available some approximations that can be used (***Figure 2.6***). In this regard, the simplest *in vitro* model to study microbiome is the batch culture, which consists in a closed system in which subtracts are rapidly consumed by the introduced microorganisms that can come from an animal or human faecal material (Pham and Mohajeri, 2018). Batch culture is an ideal method to screen the abilities of the desired compound, knowledge than can be afterwards extended by the determination of the mechanisms of action that are responsible of those actions. A superior model of study is the continuous culture, it allows the addition of new nutrients and waste removal, giving to the system a higher level of complexity, allowing long-term fermentations and a simulation closer to the *in vivo* conditions. Among the continuous fermentation models, the PolyFermS model introduces the possibility of immobilising faecal microbiota, reducing bacterial wash-out and loss of density. (Inness et al., 2011; Wright et al., 2011; Pham and Mohajeri, 2018). With a higher level of complexity, we also find an interesting and intensively used model (with human faecal microbiome) that permits the simulation of the whole gastro intestinal tract that is the SHIME "Simulator of the Human Intestinal Microbial Ecosystem". This system consists of a series of reactors that mimic each one of the gastrointestinal tract parts: stomach, small intestine, ascending colon, transverse colon and descending colon. All parameters are toughly controlled: temperature, pH, retention times, flow rates, volumes, gas mixtures, etc. (Van de Wiele et al., 2015). Among the multiple variables of the SHIME, M-SHIME mimics, in addition, the mucosa layer, allowing the study of the effect of surface-attached and mucin-degradation community (Pham and Mohajeri, 2018). Apart from SHIME, there is another model called TIM-2 (part of TNO models), which was introduced to imitate the colonic tract, and is

able to induce a peristaltic mixing and simulate the uptake of metabolites from the intestinal epithelium (Minekus et al., 1999).

Once studied *in vitro*, consequences of synbiotic administration can be deeper analyzed in *in vivo* animal models, however, it should be reminded that microbiome is specie and individual specific and effects may be different from the target host.

Figure 2.6. In vitro and in vivo models for conducting research on probiotics and prebiotics.

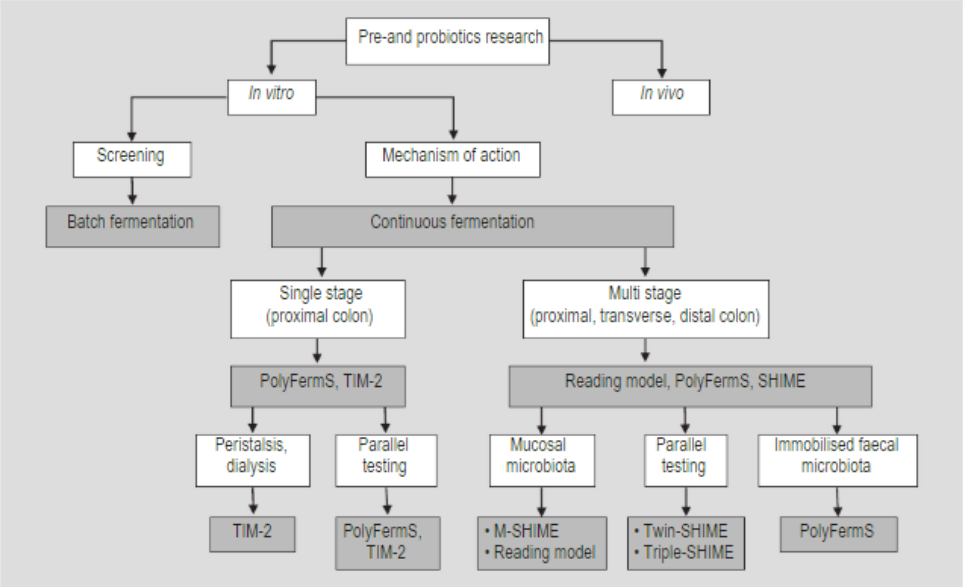


Figure extracted from Pham and Mohajeri, 2018.

2.2.3.5 Effect of synbiotics on complex organisms

Once the potential synbiotic is tested using *in vitro* methods, their potential effects on the target host should be analyzed using *in vivo* animal models. Although several aspects can be assessed *in vitro*, such as effects against pathogenic organisms or general changes in microbiota profile, there are others that are impossible to be studied using exclusively this methodology. As commented earlier in this section, some impacts that synbiotic administration can induce are reflected in changes in behavior or immune parameters. Alterations of behavioral patterns are due to effects of synbiotics on gut-brain axis and, reasonably, this can be only seen when studying them using *in vivo* models. For example,

Barrera-Bugueño et al. (2017) were able to detect improvements of stress-related behaviors in rats treated with *Lactobacillus casei* 54-2-33 and inulin. Regarding immunomodulation properties, effects of synbiotics are normally reflected in inflammatory markers (IL-1, IL-6, TNF- α) (Cazzola et al., 2010) and numbers of immunology-related cells, like natural killers (Ogawa et al., 2006). Thus, it can be deducted that modulation of immune system can be only assessed *in vivo* as it comprehends a certain degree of complexity.

Some synbiotics compounds have been studied in hosts that included farm animals. The most interesting results of their administration to monogastric animals are explained in the following section.

2.3 SYNBIOTICS IN MONOGASTRIC ANIMAL NUTRITION

As introduced previously, the jointly administration of probiotics and prebiotics to animals in order to enhance performance through improvements of their health condition is promising. However, works evaluating synbiotics in farming animals in the published literature are not highly numerous. Subsequently, the aim of this section is to provide a summary of the most outstanding results obtained in the last years due to synbiotic treatment of monogastric animals, including laying hens, broiler chickens and pigs.

2.3.1 POULTRY

2.3.1.1 Laying hens

Although the number is not high, nowadays some studies can be found evaluating the efficacy of synbiotic mixtures administered to laying hens or chickens, most of them run during the last decade. Regarding laying hens, number is even lower, and, in some cases, they just evaluate the changes produced by the synbiotic versus a control group, not including groups of animals treated with the probiotic or prebiotic independently. In two works conducted by Luoma et al. (2017) and Markazi et al. (2018), it was observed that, in *Salmonella*-challenged birds, a synbiotic containing FOS with *L. reuteri*, *B. animalis*, *P. acidilactici* and *E. faecium* administered in the feed or water, reduced the loads of the pathogen and enhanced the concentration of anti-*Salmonella* IgA. Nevertheless, these outcomes attributed to the synbiotic mixture could also have been produced by the probiotic or the prebiotic without the necessity of being administered together. Contrarily, the following experiments did include all four treatments (control, probiotic, prebiotic and synbiotic) and, unexpectedly, in most of the cases, the result of the probiotic and prebiotic mixture did not exert a synergistic effect. Results obtained by Tang et al. (2007) explain that the administration of isomalto-oligosaccharyde (IMO) and

a multispecies probiotic (*L. acidophilus*, *L. casei*, *B. bifidum, S. faecium* and *A. oryzae*) once, again, improved performance and egg weight, but not synergistically, meaning that the results were similar to those produced by the probiotic or IMO by their own. Other modified parameters included a reduction of serum LDL-cholesterol, ALT and AST, as well as a lower heterophil:lymphocyte ratio, which suggests that all treatments help chickens to overcome physiological stress, although administering a synbiotic combination does not increase promoted benefits. Abdelqader et al., in 2013, administrated *Bacillus subtilis* ($2.3 \cdot 10^8$ cfu/kg) with and without inulin at a dose of 0.1% in the feed of eighty Lohmann White hens for twelve weeks. Egg production and egg weight, as well as feed conversion ratio were positively affected by the probiotic, prebiotic and synbiotic, without any synergy. However, although all treatments improved egg quality, synbiotic treated hens had the densest and more marketable ones. Furthermore, exclusively synbiotic administration produced an increase in duodenal villus height and crypt depth and was capable of modifying the hen's microbiota by stimulating the presence of lactobacilli and bifidobacteria in detriment of clostridiobacteria and coliforms. Lastly, a study conducted by Pineda-Quiroga et al. (2017) showed the negative impact of a synbiotic formed by *Pediococcus acidilactici* and whey powder in floor-housed hens. Animal's weight gain was reduced by the synbiotic and it did not improve the egg production, despite that the probiotic and prebiotic could do it separately. When they analysed more exhaustively the microbiota (Pineda-Quiroga et al., 2019), they obtained a diminished west Pielou's evenness and Shannon diversity in the synbiotic diet, indicative of less richness of the caecal microbiome, and a decreased *Bacteroidetes*:*Firmicutes* ratio, which can be considered as undesirable due to the negative impact on poultry performance.

2.3.1.2 Broilers

The number of studies found in the literature with synbiotic combinations in broilers is greater than the one available and presented previously for laying hens. In this regard, ***Table 2.2*** shows a selection of works found in the literature with different outcomes.

It is important to emphasise that, only 14 of the 34 studies here presented (as explained in the table) evaluated the effects of the inclusion of the prebiotic and probiotic separately, thus, it can be said that most of them only were compared with a control group.

- **Impact on performance**

Compared to a control group, many of the reviewed publications show improvements in performance with the administration of synbiotics combinations. Better outcomes include higher body weight at the end of the trials but also reductions of feed intake for a similar final weight (increased feed conversion ratio) (Awad et al., 2008; Chen et al., 2018; Tayeri et al., 2018). Some authors, however, did not find improvements. Erdoĝan et al. (2010), using exactly the same mixture evaluated by others, consisting of *E. faecium* and FOS, did not observe any benefit on animal's performance compared to the control diet and neither did Vineetha et al. (2017), Bogucka et al. (2018) nor Roth et al. (2019) when they tested different combinations.

Nevertheless, in these previous studies it cannot be assessed if effects are due to the combination mixture of just to one of its components. Some works however can be found in the literature using fully controlled designs also including the prebiotic and probiotic separately (Abdel-Hafeez et al., 2017; Min et al., 2016; Mokiah et al., 2014). Among all cited literature, just the combination of *Bacillus subtilis, Clostridium butyrium* and MOS in chicken fed diets with low calcium had a proper synergistic effect in performance when the two components were administered together (Houshmand et al., 2011).

Table 2.2. Repercussions of different synbiotic combinations on performance, immunology, intestinal histology, microbiota and other parameters in broiler chickens.

Study	Probiotic + Prebiotic		PRO/PRE separately evaluation	Special conditions	Main effects				
					Performance	Immunology	GI histology	Microbiota	Others
Awad et al. 2008 **Awad et al. 2009**	Commercial: *Enterococcus faecium* + FOS (+ extracts from sea algae, cell wall)	Dose: 0.1% grower, 0.05% finisher	NO, commercial product		↑ BW, BWG ↓FCR		↑ V:C ratio ↓crypt depth		↑ H_2O and electrolyte absorption ↑ carcass %
Erdoğan et al. 2010		Dose: 0.1%	NO, commercial product	± other phytobiotic from plant extracts	No effect			↓ coliforms	↑ MDA ↑ NO with phytobiotic
Ghasemi et al. 2010		Dose: 0.05%, 0.1%, 0.15%	NO, commercial product	*Eimeria* challenge	0.1,0.15%: ↑ BW ↓FCR				↓ *Eimeria* damage, oocyst shedding
Hassanpour at al. 2013		Dose: 0.1%, 0.2%	NO, commercial product		↑ BW, FI	↑ Newcastle vaccine antibodies	0.1%: ↑villous height ↑surface area 0.2%: ↓ ileal surface area		
Dibaji et al. 2014		Dose: 0.1% grower, 0.05% finisher +25%, 50% - 25%	NO, commercial product					↑ total bacteria ↓ coliforms +50%: ↑ lactic acid bacteria	
Mousavi et al. 2015		Dose: 0.1% grower, 0.05% finisher	NO, commercial product		+25,50%:↑ BWG				

		+25%, 50% - 25%			-25%: Worse performance				
Talebi et al. 2015		Dose: 0.1% grower, 0.05% finisher	NO, commercial product			↑ various vaccine antibodies			
Tayeri et al. 2018		Dose: 0.015%	NO, commercial product		↑ BW ↓FCR			Loss of PRE effect of ↑ lactic acid bacteria	↓ intestinal wall thickness↑ gizzard weight and duodenal length
Ateya et al. 2019		Dose: 0.1%	NO, commercial product	*E. coli O78 challenge*	No effect	↓ TLR4, IFN-γ, IL-10, IL-6		↓ *E. coli* shedding	
Mohammed et al. 2018	Commercial: *Lactobacillus reuteri, Enterococcus faecium, Bifidobacterium animalis, Pediococcus acidilactici* + FOS	Dose: 0.1% and 0.05%	NO, commercial product	Heat stress	Normal & heat conditions: ↑ BW, BWG, FI ↓FCR				Behaviour: ↑ time sitting, standing, walking ↓ wing spread, panting
Roth et al. 2019		Dose: 0.001% (1 kg/ton)	NO, commercial product	± organic acids APEC *E. coli* X-7122 challenge	No effects			↓ *E. coli* shedding	
Houshmand et al. 2011	*Bacillus subtilis, Clostridium butyrium*	MOS 0.2%	YES	Low Ca diet	**↑ BW, FI ↓FCR**				↑ tibial weight, length and ashes
Chen et al. 2018	*Bacillus subtilis, Clostridium butyrium*	XOG 0.015%	YES		↑ BW ↓FCR	↑ IgA	↑ V:C ratio	No effect	↑ thymus weight
Min et al. 2016	*Bacillus subtilis*	XOG 0.015% MOS 0.1%	YES		↑ BW ↓FCR		↑villous height V:C ratio		↑ SOD, lysozyme ↓ MDA (d.42)
Abdel-Hafeez et al. 2017	*Bacillus subtilis, Bacillus licheniformis*	MOS 0.2% starter 0.1% grower 0.05% finisher	YES		↑ BW ↓FCR				↓ abdominal fat ↑ liver, gizzard and

									proventriculus, heart, small intestine and ceca relative weight
Mookiah et al. 2014	*Lactobacillus reuteri, Lactobacillus gallinarum, Lactobacillus brevis, Lactobacillus salivarius* strains	IMO 0.5 and 1%	YES		↑ BW ↓FCR			↑ bifidobacteria, lactobacilli	**<u>↑ SCFA, propionate, butyrate ↑non-VFA, acid lactic</u>**
Ghasemi et al. 2014	*Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium bifidum, Enterococcus faecium*	MOS 0.1%	YES		↑ BWG ↓FCR	↑ immune responses ↑ anti-SBRC			No effect on carcass %, organs or H:L ratio ↓ LDL
Saiyed et al. 2015	*Lactobacillus acidophilus, Lactobacillus casei, Pediococcus acidilactici, Bacillus subtilis, Saccharomyces boulardii*	MOS 0.05% and 0.025%	YES		↑ BWG				↓ abdominal fat No effect on organs
Sarangi et al. 2016	*Lactobacillus bulgaricus, Lactobacillus plantarum, Streptococcus faecium, Bifidobacterium bifidus,*	MOS 0.05%	YES		↑ BW ↑FCR (d.14)				No effect on carcass %, organs

	Saccharomyces cerevisiae								
Salehimanesh et al. 2016	*Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium thermophilum, Enterococcus faecium*	MOS 0.09%	YES		No effect	↑ anti-SBRC (PRE also ↑ IgM)	No effect	No effect	
Vineetha et al. 2017	*Lactobacillus acidophilus*[(1)] or *Lactobacillus plantarum* LGFCP4[(2)]	MOS 0.1%	NO		No effect		Doudenum: ↑villous height, crypt depth	↑ lactobacilli (↑↑ [(2)]) [(2)] ↓ coliforms, *Salmonella*	[(2)] ↑ relative weight of immune organs
Cheng et al. 2017	*Clostridium butyricum, Bacillus licheniformis, Bacillus subtilis*	XOG 0.025%	NO		↑ BW ↑FCR				↓ abdominal fat ↑ breast yield ↓ MDA
Bogucka et al. 2018	*Lactococcus lactis* B/00039, *Carnobacterium divergens* KKP 2012p, *Lactobacillus casei* B/00080, *Lactobacillus plantarum* B/00081, *Saccharomyces cerevisiae* KKP 2059p	RFO 0.8%	NO		No effect				No effect on fat, breast yield ↑ muscular capillaries ↓ fibre splitting
Salah et al. 2019	*Bacillus subtilis, Saccharomyces cerivisiae,*	MOS 0.1%	NO		↓FI, FCR				↑ carcass % ↑ serum proteins

	Streptococcus faecium								↓ LDL, triglycerides ↓ n-6:n-3 fatty acid ratio
Baffoni et al. 2011	Microencapsulated *Bifidobacterium longum* subsp. *longum* PCB133	FOS 0.5% GOS 3%	NO	*Campylobacter jejuni* M1 challenge	No effect			GOS: ↑ bifidobacteria FOS: ↑ lactobacilli GOS, FOS: ↓ *C. jejuni* shedding	
Baffoni et al. 2017	Microencapsulated *Bifidobacterium longum* subsp. *longum* PCB133	XOG 0.087%	NO	*Campylobacter jejuni* M1 challenge				↑ bifidobacteria ↓ richness and diversity ↓ *C. jejuni* shedding	
Al-Baadani et al. 2016	*Bacillus subtilis*	MOS 0.6%	YES	*Clostridium perfringens* challenge			Jejunum: ↑villous height ↑surface area Ileum: ↓villous height ↑villi width		Better control of necrotic enteritis
Poorbagui et al. 2016	*Lactobacillus acidophilus* (LA-5) ± encapsulation	Inulin 0.1%	YES	H_9N_2 Influenza virus challenge					Meat: ↑ water holding capacity ↑ protein Encapsulated: ↑ fat
	Lactobacilli: plantarum, acidophilus,	MOS 0.5% *(+ Aspergillus oryzae, Candida pintolopesii)*	YES	Heat stress					↓ oxidant and antioxidant compounds ↑ [Zn, Cu]

Sohail et al. 2011, 2012, 2013, 2015	*bulgaricus, rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus, Enterococcus faecium*	MOS 0.5%	YES		Loss of PRE effect of ↑ BW ↓FCR		↑villous width, crypt depth		
		MOS 0.5% *(+ Aspergillus oryzae, Candida pintolopesii)*	YES		↑ BW Loss of PRO effect of ↓FCR			No effect	↑ spleen, bursa of Fabricius, intestine and caeca weight
		MOS 0.5%	YES					↑ richness numerically, ↓ less *lactobacillus* than PRO and CTR	
Yan et al. 2019	*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis Lactobacillus reuteri*	FOS 0.1% or 0.05%	NO	Heat stress	↑ BW (0.05<0.1%)				↓ gait score (0.05<0.01%) 0.1%: ↑ mineralization of tibia, femur and humerus

Synergistic effects are underlined and highlighted in bold letters.

Abbreviations-

Anti-SBRC: anti sheep blood red cell antibodies
BW: body weight
BWG: body weight gain
CTR: control
FCR: feed conversion ratio
FI: feed intake
FOS: fructo-oligosaccharides
H:L ratio: heterophil: lymphocyte ratio
IL-6: interleukin-6
IL-10: interleukin-10
IFN- γ: interferon gamma
IMO: isomalto-oligosaccharides
LDL: low density lipoprotein
MDA: malondialdehyde
MOS: mannano-oligosacharides
NO: nitric oxide
PRE: prebiotic
PRO: probiotic
RFO: raffinose
SCFA: short chain fatty acids
SOD: superoxide dismutase
V:C ratio: villus height/crypt depth ratio
XOG: xylo-oligosaccharides

- **Immunology**

In all the studies of the table in which immune components of the animals were analysed, the synbiotic treatment caused an immune modulation or potentiation. Hassanpour et al. (2013) and Talebi et al. (2015) experienced an enhancement of vaccination response as they observed an increment of antibody titters against diverse pathogens while treating chicken with *Enterococcus faecium* and FOS in comparison with a control group. Furthermore, in studies that include a probiotic and a prebiotic treated groups separately, Ghasemi et al. (2014) reported increased primary and secondary immune responses with the synbiotic but in a non-synergistic way. Similarly, Salehimanesh et al. (2016) found non-synergistic increased concentration of sheep blood red cell antibodies, which are commonly used to test the action on the humoral immune response, using different synbiotic combinations. Nonetheless, these last authors describe an increment of serum IgM concentration due to the administration of the prebiotic alone (mannano-oligosaccharides in this case), effect that was lost when MOS are mixed with the probiotic.

- **Intestinal histological structure**

Regarding changes in histological architecture of the intestinal epithelium, in general, synbiotics mixtures have been associated to enlargement of ileal villi height, simultaneously with a higher ratio between it and crypt depth, and greater absorptive area structure when compared to a control group, which are suggestive of a healthier gut. Al- Baadani et al. (2016) when evaluating also each component separately, observed that, the synbiotic formed by Bacillus subtilis and MOS, and not each component alone, enlarged jejunal villi height and, in consequence, its surface area. However, the beneficial action could be dose dependent as, for example Hassanpour et al. (2013) lost an observed increment of the surface area when they incremented the synbiotic dose from 0.1 to 0.2%.

- **Microbiota**

Although there are works in which the microbiota is not altered by the synbiotic (Chen et al., 2018; Salehimanesh et al., 2016; Sohail et al., 2013), normally its administration has an impact on it. When

compared to non-treated broilers, the most common changes are decreases in potential harmful bacteria, like, coliforms, and increases of bifidobacteria and lactobacilli (Erdoĝan et al., 2010; Mookiah et al., 2014). However, as mentioned before, effects can depend on the combinations used and the doses in which they are tested. In this regard Dibaji et al. (2014) obtained an increment of lactic acid bacteria only when the dose used was 50% higher than the recommended for the commercial synbiotic, in counterpart, Vineetha et al. (2017), found an unexpected higher concentration of lactobacilli in animals treated with L. plantarum instead than with L. acidophilus (+ MOS).

As stated above, synbiotics mixtures have been proved to reduce loads of certain pathogens, such as pathogenic *E. coli*, *C. jejuni* and *Eimeria* spp. (Ateya et al., 2019; Ghasemi et al. 2010; Baffoni et al., 2011) compared to animals receiving a non-supplemented diet.

Despite the clear beneficial changes promoted by synbiotics when compared to a non-supplemented diet, most of the times no synergistic impacts on intestinal microbes were observed in the studies that included probiotic, prebiotic and synbiotic groups (Mookiah et al., 2014; Salehimanesh et al., 2016). What is more, negative effects have been described. For example, by combining them, the action of the probiotic or prebiotic can be lost. Several authors report a decrease in the richness of the microbial population (Baffoni et al., 2017 and Tayeri et al., 2018) that could explain the loss of beneficial bacteria described by Sohail et al. (2015) with lower number of *lactobacillus* in animals receiving the synbiotic compared to the probiotic alone.

- **Other effects**

One of the parameters that synbiotics can affect is meat quality. Synbiotics have been shown to improve it by reducing fat content (Cheng et al., 2017; Salah et al., 2019) compared to a control group, but it must be noted that the effects were not different from the originated by the probiotic or prebiotic inclusion (Abdel-Hafeez et al. 2017; Poorbaghi et al., 2016).

Lastly, and in the line of the effects commented in the previous section for probiotics, synbiotics could possibly influence the gut-brain axis. In this regard *Lactobacillus reuteri*, *Enterococcus faecium*,

Bifidobacterium animalis, *Pediococcus acidilactici* mixed with fructo-oligosaccharides has been shown to modify chicken's behaviour towards a welfare-compatible conducts in situations of heat stress (Mohammed et al., 2018). Nonetheless, once again, it cannot be discarded that this improvement may be caused by exclusively the probiotic or prebiotic as these treatments were not include in the experimental design.

2.3.2 SWINE

There are scarce works evaluating the effects of synbiotics administration in different life stages in pigs but those found are summarized in ***Table 2.3***. In this case, almost half of the reviewed experiments (12 of 25) integrated full controlled 2 x 2 design including also the probiotic and prebiotic alone to check if synergy was originated with their mixture. However, is it important to remark that, again, no synergistic effect was observed in the majority of the researches (except for the conducted by Chae et al. (2016) and Krause et al. and Mair et al. in 2010), meaning that the synbiotic results can be obtained simply by the inclusion of the probiotic or prebiotic individually.

- **Performance**

Productive performance of pigs is an important factor that has to be considered because of its economic impact. Among analysed studies, there are some of them that did not find any repercussion in productive parameters, especially in older animals, like growing pigs and sows when compared with a control diet (Barnes et al., 2012; Cheng et al., 2018; Lei et al., 2018). Nonetheless, when effects are present, they produce most of the times shift towards desirable outcomes. In weaned young piglets, the tested combinations commonly result in an increased body weight gain with a parallel better feed conversation ratio, meaning that animals need to consume less amount of feed to gain the same weight than pigs that did not receive any treatment. Wang et al. (2018) proved this effect comparing a combination of microencapsulated *Lactobacillus plantarum* with fructo-oligosaccharides against non-supplemented animals.

Similar results were obtained by Guerra-Ordaz et al. (2013) when they tested the same species of lactobacillus with or without lactulose at a dose of 1%, although they attributed these effects to the prebiotic inclusion. These last authors hypothesize that the result could be due to an enhanced gut health and, consequently, a better adaptation to weaning conditions, but they also speculated that the sweet flavour of lactulose (that also FOS have) could have been associated to a higher voluntary feed intake. The way of administration of the synbiotic could also play a role as suggested by Wang et al. (2018) that imputed the increased animal performance to the use encapsulation to increases probiotic survival. However, as they did not include groups of animals treated exclusively with the probiotic without being encapsulated and separated from the prebiotic, this hypothesis cannot be confirmed. Lastly, in situations of oral challenges, the synbiotic treatment have also been proved to be effective (but not synergistic) in some cases (Guerra-Ordaz et al., 2014; Krause et al., 2010) and not in others, even using the same probiotic strain (Nadiq et al., 2015; Aluko et al., 2017).

- **Fermentation**

Fermentation products are a reflex of changes of the bacterial profile or their activity in the gut, thus, their analysis is worthy when determining impact of synbiotic compounds as seems plausible that they will alter fermentative activities being reflected on short-chain fatty acid concentration and profile. In fact, the use of synbiotic is generally associated to changes in fermentation products in piglets but, not so consistently in older animals. Combinations of *L. plantarum* + inulin or/and maltodextrin prebiotic (Nemcová et al., 2007); *E. coli* UM-2/UM-7 + starch (Krause et al., 2010) and multispecies probiotics + inulin/FOS (Grela et al., 2016) have been demonstrated to increase the concentration of acetate when compared to control groups and these first two synbiotics also increase propionate, butyrate and, synergistically, valerate. Guerra-Ordaz et al. (2014) also reported an increase of butyrate with a decrease of BCFA when the combination of *Lactobacillus plantarum* + 1% lactulose was tested in ETEC K88 challenged animals. However, the impact of this synbiotic was not the same when it was given to not challenged animals (Guerra-Ordaz et al., 2013). In this case the synbiotic combination lacked the capacity of the probiotic and prebiotic to decrease acetate and increase butyrate.

Table 2.3. Repercussions of diverse synbiotic combinations on health status, performance, fermentation, intestinal histology, microbiota and other parameters in swine.

Study	Probiotic (Pro) + Prebiotic(Pre)		PRO/PRE separately evaluation	Special conditions	Main effects				
					Health & performance	Fermentation	GI histology	Microbiota	Others
NEONATAL PIGLETS									
Barnes et al. 2012	*Lactobacillus rhamnosus* GG	FOS (10g/L)	YES	Jejunoileal resection 20%:80% enteral: parenteral nutrition	No effect		↑villous height ↓ apoptosis		↑ spleen weight ↑ jejunal and ileal mass (PRE also mucosal mass) ↑mucosal [DNA] ↑ electr. glutamine transport Loss of PRE capacity to ↑ cell diff.
Nemcová et al. 2007	*Lactobacillus plantarum*	Oral maltodextrin [(1)], inulin&FOS[(2)] or both[(3)] 1.2 g/day each	Only PRO	ETEC K88 challenge *No CTR group*		(2),(3): ↑ acetate and lactic acid		(1),(3): ↓ ETEC K88 jejunum (2), (3): ↓ ETEC K88 colon No effect on lactobacilli	
WEANED PIGLETS									
Wang et al. 2018	Microencapsulated *Lactobacillus plantarum* ACCC 11016	FOS 0.15%	NO		↑BWG, FI ↓ diarrhoea		↑ V:C ratio	↑ acid lactic bacteria	Serum: ↑ albumin, ↓ BUN No effect on MDA

								Trend to ↓ *E. coli*	↑IgA, IgG
Andrejčáková et al. 2016	*Lactobacillus plantarum* Biocenol^{TM} LP96 (CCM 7512) and *Lactobacillus fermentum* Biocenol^{TM} LF99 (CCM 7514) -in cheese-	Flaxseed 10% (Fiber + lipid source)	NO		↑ faecal consistency		Jejunum: ↓ apoptotic enterocytes, ↓ inflammatory infiltrate		↓ LDH in heart, liver and skeletal muscle ↑ WBC and haemoglobin ↑ non-specific immunity
Chae et al. 2016	*Enterococcus faecium* NCIMB 11181	Lactulose 0.5%	YES					↑ richness and diversity **↑ lactobacilli** ↓ enterobacteria RE)	
Sattler et al. 2015	*Enterococcus faecium, Lactobacilus. salivarius, Lactobacilus reuteri, Bifidobacterium thermophilum*	Inulin 0.4%	YES					↑ richness caecum (PRE: ↑↑ colon, caecum) ↑ bifidobacteria ↓ enterobacteria Loss of PRE capacity to ↑clostridia and PRO to ↑ enterococci	
Guerra-Ordaz et al. 2013	*Lactobacillus plantarum* -sprayed-	Lactulose 1%	YES		↑ BWG, FI ↓FCR	↓ BCFA Loss of PRE capacity to ↓pH and PRE and PRO capacity to ↓ acetate and ↑ butyrate		No effect on richness, coliforms, clostridia, lactic acid bacteria ↓ E:L ratio ↓ peak area for *E. coli*	↓ BUN No effect on Pig-MAP

Modesto et al. 2011	*Bifidobacterium animalis* subsp. *lactis* Ra 18 [(1)] or *Bifidobacterium choerinum* Su 891 [(2)]	FOS 4%	YES		(1): ↑ BWG			(1): Trend to ↑ bifidobacteria	
Mair et al. 2010	*Enterococcus faecium, Lactobacillus salivarius, Lactobacillus reuteri, Bifidobacterium thermophilum*	Inulin 0.4%	YES		↓ BWG week 2 **↑ BWG week 4**	**↓ pH ileum** ↑ acid lactic colon Loss of Pro capacity to ↑SCFA and acetate		Colon: ↑ lactobacilli ↑ enterococci Numerically ↑ coliforms, ↑ enterobacteria than PRE and PRO	
Piva et al. 2005	*Lactobacillus brevis* P6 4/9 and *Lactobacillus salivarius* 1B 4/11	Lactitol 0.3%	NO		↓FCR			No effect on lactobacilli or coliforms	↓ PUN
Aluko et al. 2017	Microencapsulated *Enterococcus faecalis* CG1.0007	COS 0.04%	YES	ETEC K88 challenge	No effect Loss of PRE trend to ↓ diarrhoea		↑ V:C ratio		No effect on BUN
Guerra-Ordaz et al. 2014	*Lactobacillus plantarum* -sprayed-	Lactulose 1%	YES	ETEC K88 challenge	↑ BWG Loss of PRO trend to ↓ diarrhoea	↓ NH_3 ↑ butyrate Tended to ↑ SCFA and ↓ BCFA (sum of PRO + PRE effects)	↑ V:C ratio ↑ GC ↓ IEL	↑ lactobacilli Loss of PRO trend to ↓ enterobacteria	↓TNF-α and Pig-MAP
Krause et al. 2010	*Escherichia coli* UM-2 and UM-7	Potato starch 14%	YES	ETEC K88 challenge	After challenge: **↑ BWG, FI**	Colon: ↑SCFA, acetate, propionate, butyrate and **valerate** Ileum: **↑ acetate**		↓ *E. coli* **↓ clostridia** ↑ unclassified clostridials **↑ ileum, colon richness** **↑ diversity**	
Naqid et al. 2015	*Lactobacillus plantarum* B2984	Lactulose 1%	YES	*Salmonella* Typhimurium challenge	No effect				↓ *Salmonella* shedding ↓ IgM, IgG than PRO

									Loss of PRE and PRO capacity to ↑ IgA
Dimitrescu et al. 2014	*Rhodotorula rubra*	Inulin 0.3%	NO	Mycotoxin ZEA					Protective effect
GROWING/FINISHING PIGS & SOWS									
Cheng et al. 2018	*Clostridium butyricum, Bacillus licheniformis and Bacillus subtilis*	XOG 0.01& + yeast wall	NO		No effect				↑ SOD l. dorsi ↓ MDA gluteus ↓ drip & cooking loss; Pb
Lei et al. 2018	*Clostridium butyricum, Bacillus subtillus, Rhodopseudomonas capsulata*	FOS 0.1%	NO		No effect			No effect	No effect on digestibility
Czyżewska-Dors et al. 2018	*Lactobacillus: L. reuteri ŁOCK 1092 L. plantarum ŁOCK 0860 L. pentosus ŁOCK 1094 Saccharomyces cerevisiae ŁOCK 0118* (1) + *Lactobacillus rhamnosus ŁOCK 1087* (2) + *Lactobacillus paracasei ŁOCK 1091* (3)	Inulin 0.05%	NO						(2)(3): ↑IgM, IgA, (3) ↑ IgG No effect on WBC, granulocytes, lymphocytes, IL-8, IL-10, TNF-α, Pig-MAP, CRP or Hp
Grela et al. 2016	*Lactococcus lactis, Carnobacterium divergens, Lactobacillus casei, Lactobacillus plantarum, Saccharomyces cerevisiae*	Inulin 2%	YES			↑ acetate, propionate, valerate, butyrate	↑ µm muscularis externa No effect on villi or crypts	↓ enterobacteria	
Modesto et al. 2011	Microencapsulated *Bifidobacterium animalis* subsp. *lactis* Ra 18	FOS 4%	NO		↑ BW, ↓FCR			↑ number of lactobacilli & bifidobacterial ↓ *E. coli*	

Weiss at al. 2013	*Pediococcus acidilactici* MA18/5M	FOS 2%	Only PRO + Xylanase	Cannulated		No effect on pH, NH_3, lactic acid		↓ enterobacteria ↓ E:L ratio	
Böhmer et al. 2005	*Enterococcus faecium* DSM 10663	Inulin 2%	Only PRE	Ileo-rectal anastomosis (IRA) (50% pigs)		No effect of treatment, only procedure		↑ bifidobacteria (intact pigs) ↑ enterococci (IRA pigs, Pre intact pigs)	
Liong et al. 2007	*Lactobacillus acidophilus* ATCC 4962	FOS 1.25 + Mannitol 1.56 + Inulin 2.20 (g/pig*day)	NO	Hypercho -lesterolemic pigs	No effect				↓ cholesterol, LDL, TAG ↓ C:P ratio ↓ lipids on erytrocytes
OTHER									
Tanner et al. 2015	*Bifidobacterium thermophilum* RBL67	FOS 2%	NO	Adult Göttingen minipigs		No effect on SCFA		↑ bifidobacteria ↑ relative abundance of *Lactobacillaceae* and *Spirochaetaceae*	

Synergistic effects are underlined and highlighted in bold letters.

Abbreviations-

BCFA: branched-chain fatty acids
BUN: blood urea nitrogen
BW: body weight
BWG: body weight gain
COS: chitosan oligosaccharide
C:P ratio: cholesterol/phospholipids ratio
CRP: C reactive protein
CTR: control
E:L ratio: enterobacteria/lactobacilli ratio
ETEC: enterotoxigenic E. coli
FCR: feed conversion ratio
FI: feed intake
FOS: fructo-oligosaccharides
GC: Goblet cells
Hp: haptoglobin
IEL: intraepithelial lymphocytes
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IL-6: Interleukin-6
IL-8: Interleukin-8
LDH: lactate dehydrogenase
LDL: low density lipoprotein
MDA: malondialdehyde
Pig-MAP: Pig Major Acute Phase Protein
PRE: prebiotic
PRO: probiotic
PUN: plasma urea nitrogen
SCFA: short chain fatty acids
SOD: superoxide dismutase
TAG: triacyclglycerols
TNF-α:Tumoral Necrosis Factor α
V:C ratio: villus height/ crypt depth ratio
WBC: white blood cells
XOG: xylo-oligosaccharide

Similarly, Mair et al. (2010) observed a loss of the capacity of a multispecies probiotic to increase SCFA and acetate when it was mixed with 0.4% inulin.

- **Intestinal histological structure**

Synbiotic compounds have been proven by different authors to exert a good action on intestinal epithelium. For example, an increase of the ratio between villous height and crypt depth of the enterocytes with a lower number of apoptotic cells was described for synbiotic treated animals compared to non-treated, facts related by the authors to an enhanced gut health (Andrejčáková et al., 2016; Wang et al., 2018). Others, doing comparisons between probiotic, prebiotic and synbiotic treatments, like Barnes et al. (2012) and Aluko et al. (2017), also observed similar outcomes but they could not evidence improvements compared to the prebiotic or the probiotic alone. In addition, and, also without synergy, the combination of *L. plantarum* and lactulose was able to increase the number of goblet cells under an experimental ETEC challenge (Guerra-Ordaz et al. 2014), obtaining, hypothetically, an improved mucus protective layer as these cells synthetize and secret high-molecular-weight glycoproteins that form mucins (Specian & Oliver, 1991).

- **Microbiota**

As it can be thought that the inclusion of live microorganisms and specific fermentation substrates in a form of a synbiotic mixture can modify endogenous microbiota structure of an individual, its determination might result of a high interest considering the relevant role of intestinal microbiota in the animal homeostasis. Several works report changes in intestinal microbiota promoted by synbiotic mixtures when compared to a control group, generally the mixture inclusion produced an increment of beneficial bacteria like lactobacilli and bifidobacteria with a decrease of potential pathogenic bacteria, like enterobacteria and coliforms (Modesto et al., 2011; Weiss et al., 2013). In addition, in studies that evaluated the synbiotic also against a probiotic and prebiotic fed animal groups, with a 2 x 2 design, further outcomes could be seen. Krause et al. (2010) also observed reductions of *E. coli* and synergistically, of clostridia. However, results do not turn out always beneficial as, sometimes, the

combination of a probiotic with a prebiotic did not have any effect, or even resulted in a worse outcome. For example, Mair et al. (2010) found a higher presence of enterobacteria in pigs that received a multispecies probiotic + 0.4% inulin rather that the ones that only were treated with the probiotic or prebiotic and some analogous pattern was described by Slatter et al. (2015) when testing the same mixture. In spite of these last authors, Krause et al. (2010) and Chae et al. (2016) described an increase of microbial richness and diversity when the synbiotic was administered to weaned piglets, being only synergistic in the last work that tested *Escherichia coli* UM-2 and UM-7 + 14% starch.

- **Other effects**

Lastly, synbiotics can produce a variety of other actions that are included in the table. They have been related to reductions in plasma/blood urea nitrogen. This depletion is attributed by some authors to a lower presence of nitrogen in the gut associated to an enhanced synthesis of microbial proteins (Piva et el., 2005; Guerra-Ordaz et al., 2013; Wang et al., 2018). Moreover, and being an important characteristic for selection of probiotic or prebiotics, potentiation and modulation of the immune system has been also attributed to synbiotics, as treated animals have shown higher amounts of immunoglobulins and lower concentration of pro-inflammatory cytokines (Andrejčáková et al., 2016; Czyżewska-Dors et al., 2018; Guerra-Ordaz et al., 2014; Wang et al., 2018). To end up with, synbiotic administration can, as in broiler chickens, modify meat quality. The combination of C*. butyricum, B. licheniformis, B. subtilis* + XOG improved it by reducing drip and cooking losses (Cheng et al., 2018). They hypothesize that it may be due to the observed increment of the antioxidant enzyme SOD that causes a reduction of lipid peroxidation, responsible of the overproduction of free radicals, increasing water reservation among myofibrils. Nevertheless, once again, all these effects were not potentiated by the combination of the compounds.

To sum up, and bearing in mind all the information above presented, it can be affirmed that the administration of synbiotic mixtures in poultry and pigs can potentially lead to a multitude of beneficial effects, enhancing productive performance, improving intestinal structure, potentiating immune

system or balancing the microbiota towards a beneficial profile. Nevertheless, in the vast majority of fully controlled studies no synergistic action could be demonstrated, as benefits were similar to those found for prebiotics or probiotics by their own. Even in some cases, their combination is associated to a loss of the probiotic or prebiotic benefits. This failure getting synergistic effects could be due to an empiric design of the mixture, disregarding some of the criteria above exposed before, and also to the use of unappropriated doses. Despite this, it is also possible to find some works evidencing additional benefits of combining appropriately probiotics and prebiotics, opening expectations for the design of new more rational synbiotic strategies. From this point of view, it appears worth it to go deeper in the research of the synbiotic concept addressed to improve animal health and particularly reinforce homeostasis and natural defenses of young animals to prevent diseases like diarrheic disorders in piglets.

Chapter 3. Objectives and experimental design

In the latest years, our research group has carried out a variety of experimental trials with piglets in order to evaluate the efficacy of different probiotics to fight enteric pathogens after weaning. In this regard, the INCOMES project, financed by IMPRONTA–CDTI 2011 (Ref. IPT-20111008), through research agreements with Laboratorios Ordesa S.L., tested the effectivity of several probiotic strains, like *B. longum* subsp. i*nfantis* CECT 7210 and B. *animalis* subsp. *lactis* BPL6, against *Salmonella* Typhimurium or enterotoxigenic *Escherichia coli* F4 oral challenges. On the light of the promising results obtained, this project had continuity in the SMARTFOOD project, financed by CIEN - CDTI 2014 (Ref. IDI-20141206), in which this Thesis was framed on. The project follows the goal of improving the action of probiotics by their combination with prebiotics, looking forward to obtaining a synbiotic action.

Therefore, the main hypothesis of the present PhD dissertation is that:

1. The synbiotic strategy may be effective in order to improve animal health and resistance to digestive pathogens in the early life.

Consequently, the main objectives of the PhD dissertation are focused on:

1. To evaluate *in vivo*, through the utilization of a piglet model, the usefulness of new synbiotic combinations to enhance piglet adaptation to weaning.
2. To assess their effectiveness against *Salmonella* or enterotoxigenic *Escherichia coli* (ETEC) F4, by the use of an experimental oral challenge.

With the purpose of achieving these objectives, four experimental trials were performed, which are included from Chapters 4 to 6 of this dissertation:

1. Chapter 4: Includes two trials that were carried out to evaluate a synbiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and oligofructose-enriched inulin in weaned piglets challenged or not with *Salmonella* Typhimurium (trial 1) or ETEC F4 (trial 2). In these trials, the main variables determined were animal performance, clinical signs, pathogen quantification, fermentation profile, immune response and intestinal histomorphology.

2. Chapter 5: Includes a trial (trial 3) that evaluates the effects of two combined probiotic strains of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, administered or not with oligofructose-enriched inulin, in weaned piglets challenged with *Salmonella* Typhimurium. The same parameters as in Chapter 4 were determined.
3. Chapter 6: Incorporates a trial (trial 4) that assesses the effects of two combined probiotic strains of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, administered or not with galacto-oligosaccharides, in weaned piglets challenged with ETEC F4. The same parameters as in Chapter 4 were determined. Additionally, in this trial the impact of MUC-4 gene polymorphism was analyzed.

Chapter 4. Assessment of the effects of the synbiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and oligofructose-enriched inulin against digestive bacterial infections in a piglet model

4.1 INTRODUCTION

Acute enteritis is a pathology that consists of a loss of faecal consistency and/or increased stool frequency, with a duration of less than seven days. In the vast majority of cases it is precipitated by an infectious agent, this being a frequent cause of global childhood mortality and morbidity, especially in the developing world (Thapar & Sanderson, 2004). The list of organisms that can provoke acute infectious enteritis includes viruses, bacteria and parasites. As many as 70% of cases are generated by a virus (principally rotavirus), while a not-negligible 20% are caused by a bacterial infection (Koletzko & Osterrieder, 2009). Among these bacteria, *Salmonella* and *Escherichia coli* can often be isolated, with a total of 18,729 (15,320 *Salmonella*; 3,409 *E. coli*) cases reported to the National Outbreak Reporting System (NORS) in the United States of America (USA) between 2015 and 2017 (CDC, 2018). These infections commonly have their origin in food contamination, although the infection can also be produced via contact with infected animals (Conrad et al., 2017). New-born and young children are particularly susceptible to infections because their immune systems are not mature and they are not fully immunocompetent. An important form of protection is provided by the mother through passive IgG transplacental transfer as well as in the milk (Simon et al., 2015); moreover, it has been demonstrated that breastfeeding diminishes the incidence and severity of infectious diarrhoea (Farthing et al., 2013; Hartman et al., 2019). Probiotics are live beneficial microorganisms that when administered in infant formulas can help reduce the number of episodes and duration of diarrhoea associated with acute infections (Szajewska et al., 2001). Nonetheless, this effect is strain-dependent and different outcomes have been reported in the literature (Skórka et al., 2017). *Bifidobacterium longum* subsp. *infantis* CECT 7210 is a bifidobacteria isolated from infant faeces that when given as a supplement to healthy children, has been associated with a reduction in diarrhoea events (Escribano et al., 2018). Moreover, in pathogen-challenged animal models, it diminishes pathogen shedding and modulates immune response (Moreno-Muñoz et al., 2011; Barba-Vidal et al., 2017). Prebiotics such as inulin or its derivatives can potentially benefit the survival and multiplication of bifidobacteria (Vandeputte et al., 2017) and combat enteric pathogens (Tran et al., 2018). Therefore, it is

hypothesized that the synbiotic administration of an advantageous *Bifidobacterium* strain with these prebiotics will result in an improved outcome regarding digestive bacterial illness. The aim of this work is to determine the efficacy of a synbiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and inulin enriched with oligofructose against *Salmonella* Typhimurium and enterotoxigenic *Escherichia coli* F4, using weaned piglets as an animal model.

4.2 MATERIALS AND METHODS

Two different experiments were executed to evaluate the efficacy of the synbiotic combination against an oral challenge with either *Salmonella enterica* serovar Typhimurium (*Salmonella* trial) or enterotoxigenic *E. coli* F4 (ETEC F4 trial). Both experiments were performed at the Experimental Unit of the Universitat Autònoma de Barcelona (UAB) and received prior approval (Permit No. CEEAH: 4026 DMAH: 10118) from the Animal and Human Experimental Ethical Committee of this institution and its competent authorities. The treatment, management, housing, husbandry and slaughtering conditions conformed 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

These trials were carried out as biosafety Level 2 procedures and all personnel involved received appropriate training. A total of 168 male piglets were distributed between the two trials as follows: 72 [Landrace x Large White] x Pietrain of 24 (± 4) days of age weighing 7.70 (± 0.15) kg for the *Salmonella* trial and 96 [Landrace x Large White] x Pietrain piglets of 21 (± 4) days of age weighing 4.98 (± 0.07) kg for the ETEC F4 trial. All animals came from high-sanitary-status farms and mothers that were serologically negative to *Salmonella* in the *Salmonella* trial or were not vaccinated against *E. coli* in the ETEC F4 trial.

The piglets were transported to the experimental unit located in the UAB, comprising three boxes (*Salmonella* trial) and four boxes (ETEC F4 trial) of eight pens each (24 and 32 pens, respectively, with three animals per pen). Each $2m^2$ pen was separated by a solid fence that prevented any contact between animals of different pens. Each pen had a feeder and water nipple to provide feed and water *ad libitum*. All weaning rooms were equipped with an automatic heater and forced ventilation and each pen had an individual heating light.

At arrival, the animals were distributed according their initial body weight (BW) in order to ensure a homogeneous average body weight between treatment groups. Trials consisted of a factorial design 2 x 2 that included two treatments (control vs. synbiotic) and challenged or not with the pathogen (yes vs. no), resulting in a total of four experimental groups: control non-inoculated animals, NC; non-inoculated animals receiving synbiotic treatment, NS; control inoculated animals, IC; inoculated animals receiving synbiotic treatment, IS. In the case of the first trial, the design was unbalanced, as piglets in two of the three rooms were challenged while the third room remained non-challenged. In contrast, for the second trial we utilized four rooms and therefore had a balanced design. In each room, synbiotic treatment was distributed within four pens on one side of the room, with the four control pens on the other side of the room separated by a corridor to prevent contact between animals. Each experimental group had eight replicates, except for the non-challenged groups in the *Salmonella* trial, which had four replicates instead.

4.2.2 Probiotic strain, prebiotic mixture and diets

In both trials the probiotic tested was *Bifidobacterium longum* subsp. *infantis* CECT 7210 strain, supplied by Ordesa S.L. in a lyophilized form and containing 5×10^{10} colony-forming units [cfu] per gram of product in a maltodextrin carrier. The estimated dosage during the procedures was the same for both trials (1×10^9 cfu per piglet and day). In the *Salmonella* trial, the animals received the probiotic orally each morning using disposable syringes without a needle. To this end, the lyophilized bacteria

were re-suspended in 2 mL of phosphate-buffered saline (PBS) no more than one hour prior to administration. The control groups were administered the same amount of sterile PBS as a placebo. In the ETEC F4 trial, the piglets received the probiotic mixed in their feed: on each day, the lyophilized probiotic was thoroughly mixed manually with fresh feed, with the dose adjusted considering the average feed intake (1 gram of lyophilized probiotic per 1000 grams of feed).

The stability of the probiotic into the feed and feeders had previously been assessed in a viability test to ensure an accurate dosage of the product per day.

The prebiotic consisted of a mixture of oligofructose (FOS) and inulin (Orafti® Synergy1, Beneo; Mannheim, Germany) that was administered through the feed (5%) in both trials.

Pre-starter diets were formulated in concordance with the nutrient requirement standards for pigs (NRC, 2012) and given in a mash form. In the ETEC F4 trial, potential amino acid dilution in the synbiotic diet due to the incorporation of the prebiotic was compensated by the addition of synthetic amino acids: 0.5 g L-valine, 0.9 g L-lysine HCL, 1.2 g DL-methionine, 0.5 g L-threonine and 0.2 g L-tryptophan per kg of feed. Details of the ingredient and chemical composition are given in ***Table 1***

Table 4.1. Ingredient and nutritional composition of the diets.

	***Salmonella* TRIAL**		**ETEC F4 TRIAL**	
Ingredients (g/kg FM)	**Control**	**Synbiotic**	**Control**	**Synbiotic**
Maize	280.8	266.8	207.4	196.4
Wheat	170.0	161.5	180.0	170.1
Barley 2 row	150.0	142.5	170.0	161.0
Extruded soybean	122.4	116.3	149.1	141.2
Sweet whey powder (cattle)	100.0	95.0	100.0	94.7
Fish meal	50.0	47.5	60.0	56.2
Soy bean meal 44	50.0	47.5	80.0	75.8
Whey powder 50% fat	30.3	28.8	25.0	23.7
Mono-calcium phosphate	21.3	20.2	6.8	6.5
Calcium carbonate ($CaCO_3$)	8.2	7.8	3.9	3.7
L-Lysine HCL	4.5	4.3	4.5	5.0
Vit-Min Premix*	4.0	3.8	4.0	3.7
Sodium chloride (marine salt)	3.0	2.9	2.5	2.3
DL-Methionine 99	2.4	2.3	2.6	3.6
L-Threonine	2.3	2.2	2.3	2.6
L-Tryptophan	0.9	0.9	0.6	0.7
L-Valine	1.0	1.0	1.5	1.9
Prebiotic	0	50	0	50
Analyzed composition (g/kg FM)	**Control**	**Synbiotic**	**Control**	**Synbiotic**
Dry matter	903.4	902.9	909.6	912.7
Ashes	68.5	61.6	52.9	50.4
Crude fat	57.7	53.8	60.2	56.1
Crude protein	17.4	161.4	204.7	180.7
Neutral detergent fibre	89.8	119.2	92.2	83.0
Acid detergent fibre	29.4	27.3	30.3	29.4

***** *Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se*

4.2.3 Salmonella and ETEC strains

In the first trial, the bacterial strain used for the oral challenge was a *Salmonella enterica* serovar Typhimurium var. monophasic (*formula: 4,5,12:i:-, resistance profile: ACSSuT-Ge, Fagotype: U302*) that had been isolated from a salmonellosis outbreak of fattening pigs in Spain, provided by the Infectious Diseases Laboratory (Ref. 301/99) of the UAB. Preparation of the oral inoculum consisted of 24-hour incubation at 37°C in buffered peptone water (BPW) (Oxoid; Hampshire, United Kingdom) and diluted (1:10) with sterile PBS (Sigma-Aldrich; Madrid, Spain). The final concentration of the inoculum was 1 x 10^9 cfu/mL. Inoculum concentrations were determined prior to the inoculation by McFarland standards and were doubly plated in Tryptic Soy Agar (TSA) (Liofilche; Italy) on the same day in order that they could be checked by manual plate counting.

In the second trial, the bacterial strain of enterotoxigenic *E. coli* F4 used was isolated from the faeces of 14-week-old pigs and provided by the Infectious Diseases Laboratory (Ref. 30/14) of the UAB. This strain presented the following virulence factors: F4ab, F4ac, LT, STb and EAST1 and was negative for K99, F6, F18, F41, STa, VT1, VT2 y EAE. The oral inoculum was prepared via 12-hour overnight incubation at 37˚C in Brain Heart Infusion broth (Oxoid; Hampshire, England) with slow agitation (250 rpm) in an orbital incubator. The culture was directly given to the animals with a final concentration of 1 x 10^9 cfu/mL. Inoculum concentrations were also determined before the inoculation by McFarland standards and were plated in Luria Agar (LA) (made in-house: tryptase, yeast extract, NaCl, agar, Oxoid; Hampshire, UK) the same day for manual plate counting.

4.2.4 Experimental procedure

Both experiments lasted 15 days. After an adaptation period of seven days in the *Salmonella* trial and eight days in the ETEC F4 trial, the animals were challenged orally with the pathogen. One animal from

each pen was euthanized on days 4 and 8 post-inoculation (PI) in the *Salmonella* trial and on days 3 and 7 PI in the ETEC F4 trial.

After a week of adaptation, the inoculum containing the pathogenic bacteria culture was given to the challenged groups orally: in the first trial this was one 2 mL dose (2 x 10^9 cfu) of *Salmonella* Typhimurium, whereas in the second trial it was one 6 mL dose (6 x 10^9 cfu) of ETEC F4. The same amount of sterile broth was administered to the non-challenged piglets. In order to ensure that the animals' stomachs were full at the time of the oral challenge, the pigs were starved for a period of 12 hours and feed was reintroduced 30 minutes before inoculation.

From the challenge onwards, the animals' clinical signs were checked daily to evaluate their post-inoculation status (i.e. dehydration, anorexia, apathy, general behaviour and faecal score), always by the same person. Faecal score was measured using a scale whereby 1 = solid and cloddy, 2 =soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Rectal temperature was assessed using a digital thermometer (Accuvet, Sanchung City, Taiwan) on days 1, 2 and 3 PI in the *Salmonella* trial and days 1 and 2 PI in the ETEC F4 trial.

The animals' performance was also monitored: individual body weight was registered on arrival and on days 0, 4 and 8 PI (0, 3 and 7 PI in the ETEC F4 trial) and feed intake was determined on days 0, 4 and 8 PI in the *Salmonella* trial, whereas in the ETEC F4 trial feed intake was registered daily, concurring with the regular feed replacement aimed at maintaining probiotic viability. The average daily gain (ADG), average daily feed intake (ADFI) and the gain:feed ratio (G:F) were calculated by pen. The mortality rate was also registered and no antibiotic treatment was given to the animals in any of the experiments.

For microbiological analysis, faecal samples were collected aseptically after spontaneous defecation or by digital stimulation at arrival on the day of the inoculation (0 PI): in the *Salmonella* trial this was from the animal with the highest initial BW in each pen (N = 24), whereas in the ETEC F4 trial faecal samples were obtained from the animal with the medium BW in each pen (N = 32). Furthermore (and

just for the *Salmonella* trial), additional faecal samples were taken on days 1, 3 and 7 PI from the same animal.

On days 4 and 8 PI (3 and 7 PI in the ETEC F4 trial), one pig per pen was euthanized. On day 4 PI, the animal selected was the one with the medium initial BW, while on day 8 PI it was the heaviest piglet in each pen. The animals were euthanized and sequentially sampled during the morning of each day (between 8:00 and 13:00 hours). Before injecting the euthanasia drug, 10 mL sample blood was taken from each animal via venepuncture of the cranial cava vein using 10 mL blood collection tubes without anticoagulant (Aquisel; Madrid, Spain). Immediately after blood sampling, pigs were intravenously administered a lethal dose injection of sodium pentobarbital (140 mg/kg BW; Euthasol, Le Vet B.V.; Oudewater, Netherlands). Once dead, the animals were bled, the abdomen opened and the gastrointestinal tract extracted.

A faecal sample from the rectum was used for traditional microbiology in the ETEC F4 trial, whereas a caecal sample was obtained for microbiology in the *Salmonella* trial. They were kept on ice and analyzed within four hours.

In both experiments, the digesta of the ileum and the proximal colon were collected and homogenized prior to pH determination with a pH meter calibrated on each day of use (Crison 52–32 electrode, Net Interlab; Barcelona, Spain) and the digesta score was registered on a scale as follows: 1 = liquid; 2 = liquid with some formed material; 3 = thick; 4 = semi-solid. Subsamples of the ileal and colonic digesta were preserved for different analyses. One aliquot of colonic content was kept at −80°C for ETEC F4 (ETEC F4 trial) and probiotic quantification by qPCR. A set of ileal and colonic digesta samples were conserved frozen at -20°C in H_2SO_4 solution (3 mL of content plus 3 mL of 0.2 N H_2SO_4) for ammonia (NH_3) determination and an additional set (~10 g) was also frozen (−20°C) for future analysis of short-chain fatty acids (SCFA) and lactic acid.

In the ETEC F4 trial, to determine the number of enterobacteria, coliforms and ETEC F4 attached to the intestinal mucosa, 5 cm sections of distal ileum were collected from each animal, washed

thoroughly with sterile PBS, opened longitudinally and scraped with a microscopy glass slide to obtain the mucosa scraping.

For the histological study, 1 cm sections from the ileum were removed, opened longitudinally, thoroughly and carefully washed with 4% formaldehyde solution (Panreac; Castellar del Vallès, Spain) and fixed by immersion in the same solution.

Blood samples were centrifuged (3,000 × g for 15 min at 4°C) after clothing and the serum obtained was stored at -20°C.

4.2.5 Analytical procedures

Chemical analyses of the diets – including dry matter (DM), ash, crude protein and diethyl ether extract – were performed according to Association of Official Agricultural Chemists standard procedures (AOAC International, 1995). Neutral detergent fibre and acid-detergent fibre were determined according to the method of Van Soest et al. (1991).

For the microbiological analysis of *Salmonella*, samples were transferred to buffered peptone water solution in a concentration of 1:10. The quantitative analysis was performed by seeding serial dilutions of the samples 10^{-2}, 10^{-4} and 10^{-6} in Xylose-Lactose-Tergitol-4 plates (XLT-4) (Merck; Madrid, Spain). For the qualitative analysis, samples were incubated in BPW (37°C, 24h), transferring 100 µl of the culture to 10 mL of Rappaport-Vassiliadis for a second incubation (42°C, 48h) to finally seed them in XLT4 plaques to observe H_2S positive colonies.

For the enterobacteria and coliform counts, samples were serially diluted in Lactate Ringer Solution (Sigma-Aldrich; Madrid, Spain) and proper dilutions seeded in MacConkey agar (Oxoid; Madrid, Spain) and eosin methylene blue agar (Scharlab; Barcelona, Spain). Plaques were incubated for 24h at 37°C and colonies were manually counted.

The presence of ETEC F4 in the colonic digesta and ileal scrapings was determined by real-time PCR. To extract the DNA from these samples, the commercial QIAmp DNA stool minikit (Qiagen; West Sussex, United Kingdom) was utilized. Afterwards, several aliquots of DNA eluted in Qiagen buffer AE (total volume; 200 µl) were stored frozen at -80°C. A qPCR targeting the gene coding the F4 fimbria of ETEC F4 using the SYBR green dye was performed according to the procedure described by Hermes et al. (2013). To express the results, the animals were distributed across five levels according to the number of gene copies per gram of fresh matter that they showed when qPCR was performed. Ranges were defined as follows: negative = under 4 logarithmic units of gene copies per gram of fresh matter; low = 4 – 5.5 logarithmic units of gene copies per gram of fresh matter; medium = 5.5 – 7 logarithmic units of gene copies per gram of fresh matter; high = 7 – 8.5 logarithmic units of gene copies per gram of fresh matter; and very high = more than 8.5 logarithmic units of gene copies per gram of fresh matter.

Short-chain fatty acids and lactic acid analyses were performed using gas chromatography, after the samples had undergone acid-base treatment followed by ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide (MBTSTFA) plus 1% tert-butyldimethylchlorosilane (TBDMCS) agent, using the method of Richardson et al. (1989) that was subsequently modified by Jensen et al. (1995).

Ammonia concentrations were assessed using a gas-sensitive electrode (Hatch Co.; Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain), following a procedure described by Hermes et al. (2009) that was adapted from Diebold et al. (2004). Samples were diluted (1:2) in 0.16 M NaOH and, after homogenization, were centrifuged at 1500 x g for 10 minutes. Once the ammonia was released, it was measured in the supernatants as a change in voltage in mV.

Serum concentrations of Tumor Necrosis Factor-α (TNF-α) were determined by Quantikine Porcine TNF-α kits (R&D Systems; Minneapolis, USA) and pig major acute-phase protein (Pig-MAP)

concentration was determined by a sandwich-type enzyme-linked immunosorbent assay (ELISA) (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A.; Segovia, Spain) according to the manufacturer's instructions. In the *Salmonella* trial, antibodies against *Salmonella* were also assessed using an ELISA *Salmonella* Herdcheck (Idexx; Hoofddorp, Netherlands), establishing the cut-off for positivity in optic density ≥ 40%.

For histological study, tissue samples were dehydrated and embedded in paraffin wax, sectioned at a thickness of 4-µm and stained with haematoxylin and eosin. The measurements of 10 different villus-crypt complexes per sample and the counting of intraepithelial lymphocytes (IEL), goblet cells (GC) and the number of mitosis of each were performed with a light microscope (BHS, Olympus; Barcelona Spain), using as a guideline the procedure described in Nofrarías et al. (2006).

4.2.6 Statistical analysis

The results from both trials are expressed here as means with their standard errors, unless otherwise stated (microbiological counts were transformed [log] for analysis). A two-way analysis of variance (ANOVA) was used to examine the effect of the experimental challenge and synbiotic treatment as well as the interaction between the two (only included when significant). All analyses were performed using a generalized linear model (GLM) or mixed-effects model of R software and by Fisher's exact tests when analyzing frequencies. Version 3.3.1 of R statistical analysis software was used (R Development Core Team; New Jersey, USA). When treatment effects were established, the comparison of means was adjusted by the Tukey-Kramer test. The experimental unit of analysis considered was the pen. The α-level used for the determination of significance for all analyses was $P = 0.05$. The statistical trend was also considered for $P < 0.10$.

4.3 RESULTS

Both experiments proceeded as expected, without any remarkable incidence.

The oral challenge with the pathogenic bacteria induced moderate clinical signs in the animals that were slightly more severe after the *Salmonella* challenge (*Salmonella* trial). In these trials, humane euthanasia of two pigs was indicated (1 IS; 1 NIS). Furthermore, two spontaneous casualties were registered in the *Salmonella* trial (1 IC; 1 IS) and the ETEC F4 trial (1 NIS; 1 IC).

4.3.1 Performance parameters

Changes in average daily gain (ADG), average daily feed intake (ADFI) and gain:feed ratio (G:F) with the experimental treatments are shown in ***Table 4.2***.

The challenge with the pathogen caused a decrease in ADFI and ADG in the *Salmonella* trial (P = 0.010 and P = 0.024, respectively). The effects of the ETEC F4 challenge were milder, with only a numerical trend seen for a lower ADFI in the post-inoculation period (333.65 vs. 367.95 g for challenged and non-challenged groups, respectively, P = 0.152).

No significant changes in ADFI nor ADG were registered that might be associated with the synbiotic treatment regardless of the trial, aside from a numerical difference (P = 0.130) in the ETEC F4 trial for reduced ADG during the post-inoculation phase. Moreover, in the *Salmonella* trial a reduction in the G:F ratio was seen after the adaptation period (P = 0.020).

Table 4.2. Effects of experimental treatments on feed intake and weight gain.

	Treatment				*RSD*	*P-value*	
	IC	**IS**	**NIC**	**NIS**		***Challenge***	***Treatment***
<u>*Salmonella* trial</u>							
BW (kg)							
Initial	7.70	7.74	7.70	7.66	*0.156*	*0.540*	*0.878*
Final	9.23	9.71	10.52	10.26	*1.311*	*0.120*	*0.662*
ADFI (g)							
pre	181.1	189.1	192.1	214.5	*43.66*	*0.347*	*0.480*
post	288.2	287.4	398.7	403.2	*93.08*	*0.010**	*0.979*
ADG (g)							
pre	121.6	89.4	126.1	111.1	*42.85*	*0.146*	*0.488*
post	85.2	109.2	249.9	165.9	*104.90*	*0.024**	*0.781*
G:F							
pre	0.67	0.44	0.66	0.52	*0.197*	*0.704*	*0.020**
post	0.26	0.33	0.60	0.36	*0.268*	*0.125*	*0.737*
<u>ETEC F4 trial</u>							
BW (kg)							
Initial	4.99	5.02	4.97	4.96	*0.790*	*0.107*	*0.723*
Final	7.46	6.94	7.20	7.14	*0.775*	*0.915*	*0.290*
ADFI (g)							
pre	82.8	77.5	98.2	79.2	*17.03*	*0.506*	*0.215*
post	359.3	308.0	370.5	365.4	*65.95*	*0.152*	*0.237*
ADG (g)							
pre	55.5	57.3	69.1	48.7	*32.30*	*0.830*	*0.421*
post	283.2	219.2	250.5	235.2	*72.01*	*0.745*	*0.130*
G:F							
pre	0.61	0.73	0.76	0.53	*0.349*	*0.853*	*0.674*
post	0.79	0.70	0.68	0.63	*0.152*	*0.102*	*0.207*

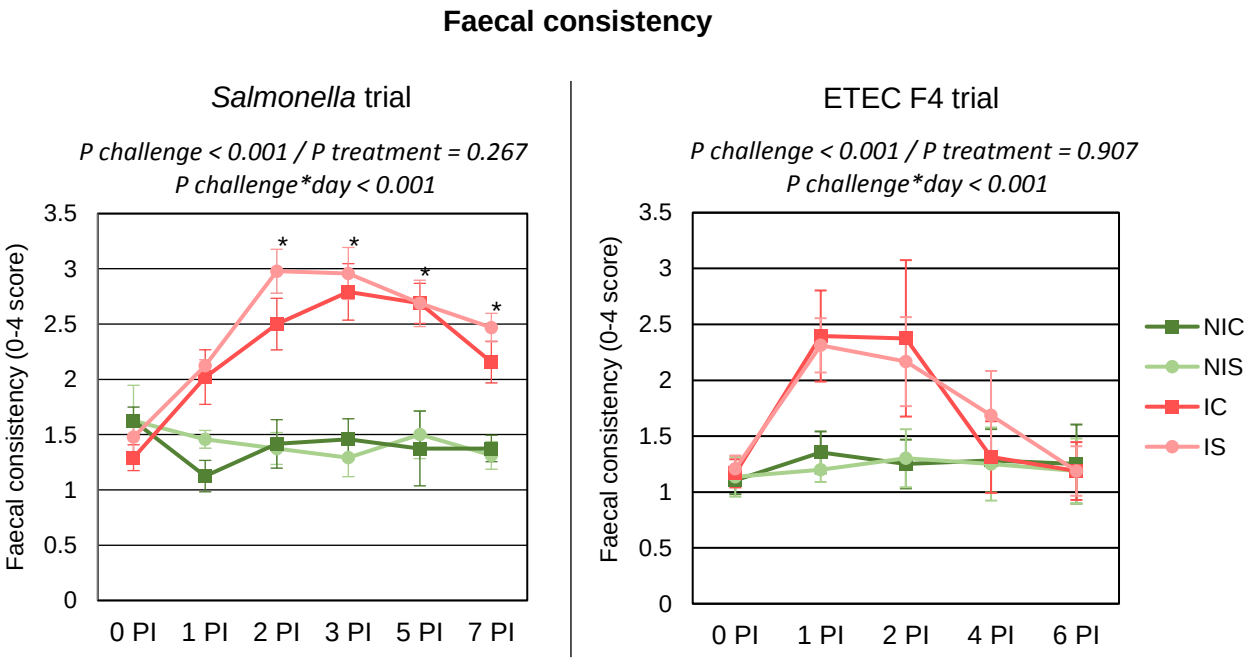
Body weight (BW) (kg), average daily feed intake (ADFI) (g/day), average daily gain (ADG) (g/day) and feed efficiency (gain:feed ratio, G:F) for the pre-inoculation period (pre: days 1 – 8) and post-inoculation period (post: days 8 – 15). IC – Inoculated animals receiving placebo; IS – Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all groups except for non-challenged animals in Salmonella trial, N = 4. No interaction effects between challenge and treatment were found.

4.3.2 Clinical signs

On day 1 PI, an increment of 1°C of rectal temperature was caused by the *Salmonella* challenge (38.9 ± 0.13°C vs. 39.8 ± 0.18°C, P < 0.001), whereas the ETEC F4 challenge did not modify the piglets' temperatures (39.1 ± 0.07°C vs. 39.1 ± 0.17°C, P = 0.974). No significant differences were found related to the synbiotic administration.

Figure 4.1 shows the evolution in faecal consistency after the oral challenge for each trial. In both trials, the challenge was able to significantly impair faecal consistency (P < 0.001) with an increase in the incidence of diarrhoea. However, the progression of the faecal score over time differed between the trials: whereas with the *Salmonella* challenge the faecal inconsistency was registered up to the end of the trial, in the ETEC F4 trial the faecal consistency returned to normal within three to four days. No significant differences were found related to the synbiotic treatment.

Figure 4.1. Evolution in average faecal scores for the different experimental groups in the post-inoculation period.



IC – Inoculated animals receiving placebo; IS- Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N=8 for all groups except for non-challenged animals in Salmonella trial, N = 4. Bars correspond to standard error.

Regarding the consistency of the ileal and colonic digesta, the oral challenge did not instigate significant changes in any of these parameters, although some effects were observed regarding the colon digesta's consistency following the administration of the synbiotic. In the *Salmonella* trial, the synbiotic treatment was associated with improved consistency at day 8 PI (3.44 vs. 2.62; P = 0.010); moreover, in the ETEC F4 trial it was related to a trend towards interaction (P = 0.061) with an improvement with supplementation to the challenged animals (3.62 vs. 3.00), but a looser consistency in the non-challenged group (3.87 vs. 3.62).

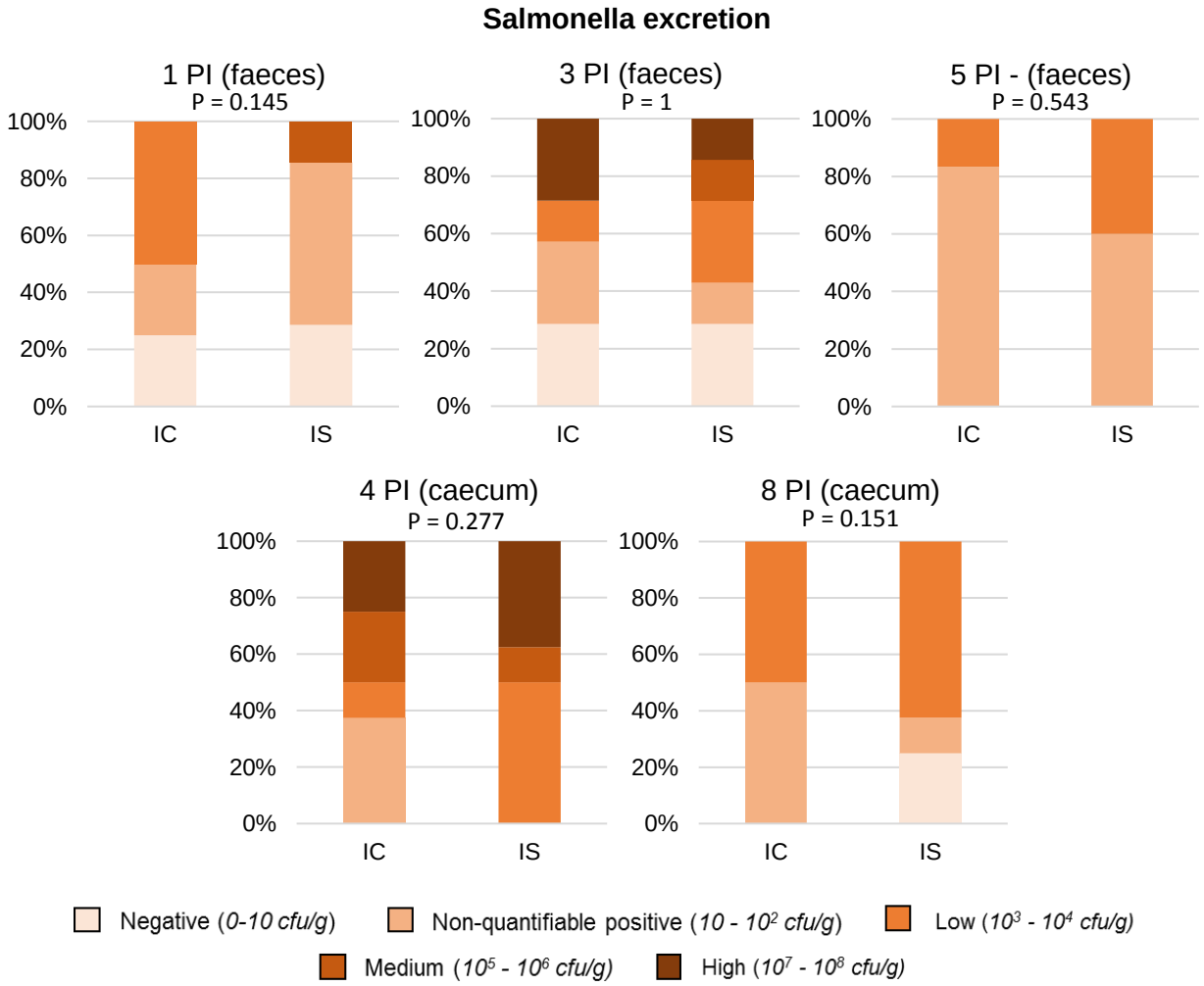
4.3.3 Microbiological analysis

In the *Salmonella* trial, the serological analysis revealed that all of the animals remained seronegative throughout the study, confirming that they had not been exposed to the pathogen prior to the oral challenge. Regarding the presence of the pathogen in the faeces and intestinal digesta, ***Figure 4.2*** shows the evolution of *Salmonella* plate counts along sampling days in the challenged animals. In general terms, the non-challenged piglets remained negative during the study, with the exception of three piglets that recorded positive in at least one sample, albeit always at low-to-uncountable levels ($< 10^2$ cfu/g).

All challenged animals were positive in at least one faecal sample and all presented *Salmonella* in the caecal content.

Regarding synbiotic administration, only a trend on day 1 PI was found, with the faeces revealing that the animals that received the synbiotic presented lower shedding compared to the control group (P = 0.145). Moreover, regarding the caecal digesta, 25% of piglets treated with the synbiotic turned negative to *Salmonella* excretion (vs. 0% control) on day 8 PI (P positive/negative excretion = 0.076).

Figure 4.2. Percentage of animals included in the different faecal and caecal excretion levels of Salmonella. All samples were obtained from the heaviest animal in each pen, except for the caecal sample on day 4 PI, which was obtained from the anima of medium weight.



IC – inoculated animals receiving placebo; IS – Inoculated animals receiving the synbiotic; N = 8 for IC and IS. P-values were obtained using Fisher's Exact Test on R software.

Regarding the ETEC F4 trial, no differences between groups were registered in the plate counts of faecal enterobacteria or coliforms on the day of the piglets' arrival. Furthermore, no changes caused by the synbiotic treatment were observed before the oral challenge, although differences could be noted related to the box (9.79, 10.69, 8.12, 8.44 log cfu/g FM for IC, IS, NIC and NIS, respectively; $P < 0.001$). ***Table 4.3*** shows the impact of the experimental treatments on the enterobacteria and coliform

plate counts post-inoculation. The oral challenge promoted an increase in enterobacteria and coliforms either in the faeces ($P < 0.013$) or the ileal scrapings ($P < 0.013$) on day 3 PI. The effects produced by the challenge were no longer detected at day 7 PI. Moreover, no significant effects were found related to the synbiotic supplementation in any of the parameters analyzed. However, a trend towards interaction in enterobacteria ($P = 0.057$) and coliform ($P = 0.104$) counts in the ileal scrapings could be identified at day 7 PI. On this day, non-challenged animals that received the synbiotic presented lower counts than the control, whereas challenged piglets exhibited the opposite effect.

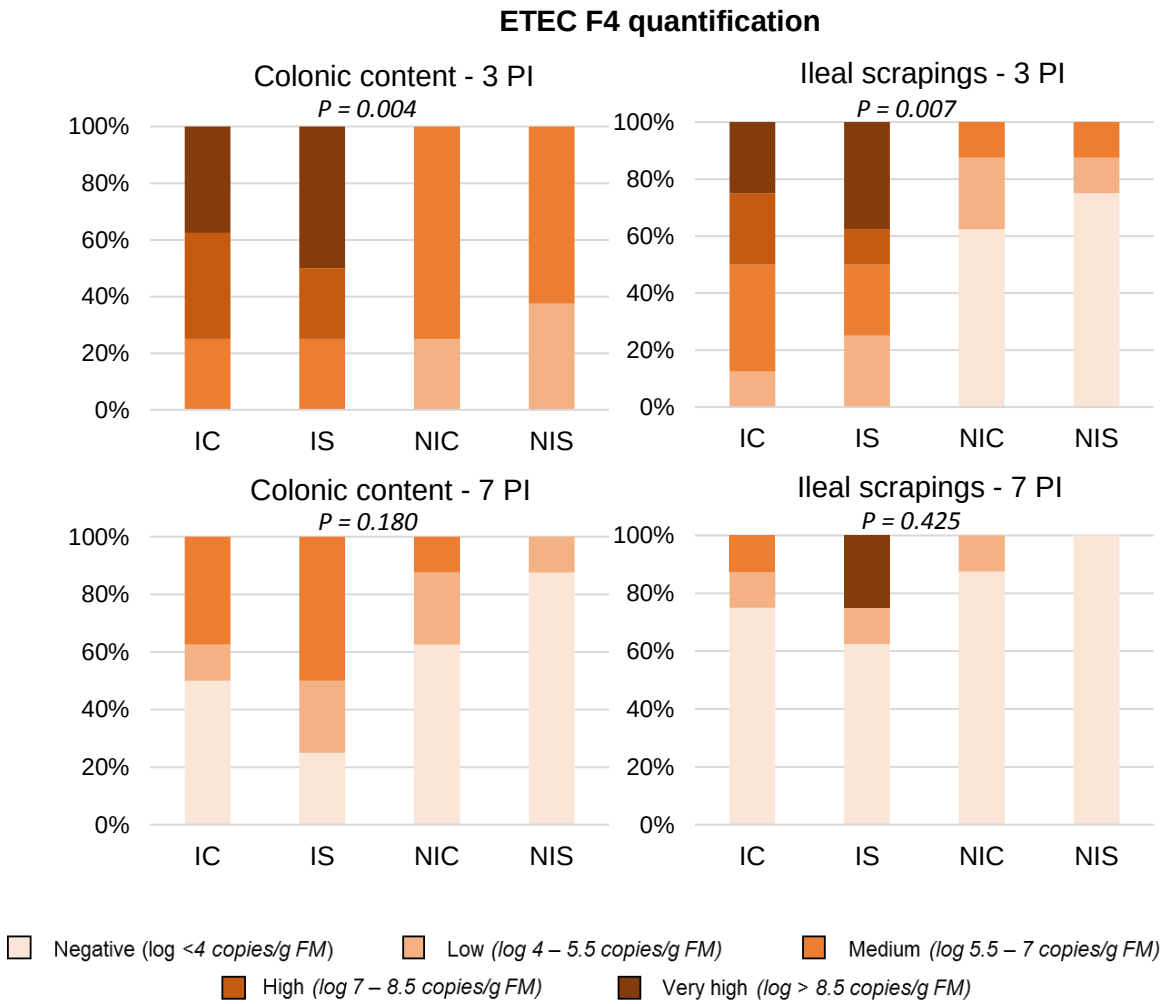
Table 4.3. Effects of experimental treatments on enterobacteria and coliform counts in faecal samples and ileal scrapings.

	Treatment				***RSD***	***P-value***		
	IC	**IS**	**NIC**	**NIS**		***Challenge***	***Treatment***	***Interaction***
Enterobacteria (log cfu/g FM)								
Faeces								
Day 3 PI	10.44	10.51	9.15	8.83	1.564	*0.012**	*0.828*	*0.725*
Day 7 PI	8.52	8.07	8.15	8.62	2.581	*0.925*	*0.993*	*0.615*
Ileal scrapings								
Day 3 PI	8.56	8.37	6.57	7.06	*1.564*	0.012*	*0.828*	*0.726*
Day 7 PI	7.08^{y}	8.53^{xy}	9.25^{x}	7.98^{xy}	*1.942*	0.247	*0.892*	*0.057*
Total coliforms (log cfu/g FM)								
Faeces								
Day 3 PI	9.60	10.36	8.66	8.26	1.630	*0.013**	*0.753*	*0.323*
Day 8 PI	7.90	7.51	8.10	8.55	2.446	*0.480*	*0.974*	*0.632*
Ileal scrapings								
Day 3 PI	8.30	8.07	6.25	6.95	*1.630*	*0.013**	*0.753*	*0.323*
Day 7 PI	6.70	8.55	8.21	7.86	*1.850*	*0.532*	*0.263*	*0.104*

IC – Inoculated animals receiving placebo; IS – Inoculated animals receiving the synbiotic; NIC- Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all experimental groups. P-values were obtained by ANOVA using the generalized linear procedure in R software. Letters x, y and z express differences considered for P < 0.07.

The results corresponding to qPCR targeting the coding gen of the F4 fimbria of *E. coli* F4 are summarized in ***Figure 4.3.*** Given that the pathogen could not be quantified in all animals, the data were analyzed as frequencies. The figure shows the distribution of the percentage of animals within each of the five defined ranges based on the number of copies/g fresh matter found in the analysis. The effect of the oral challenge was clearly evidenced on day 3 PI through a significant increase in the percentage of animals showing large or very large numbers of copies in colonic content (P = 0.004) or ileal scrapings (P = 0.007). No significant differences were found related to the synbiotic administration.

Figure 4.3. Percentage of animals in each presence level of ETEC F4 on days 3 and 7 post-inoculation. Different animals were sampled on day 3 PI (medium weight) and 7 PI (greatest weight).



IC – inoculated animals receiving placebo; IS- Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all experimental groups. P-values were obtained using Fisher's Exact Test on R software.

4.3.4 Intestinal fermentation

Tables 4.4 and 4.5 show all the changes induced by the different experimental treatments in the ileal and colonic fermentation products.

As displayed, the challenge with *Salmonella* stimulated a significant increase in ammonia levels in the colon (34.77 vs. 14.48 mmol/L; P = 0.001) on day 8 PI. It did not influence the total amount of short chain fatty acids (SCFA) or lactic acid, but challenged animals presented a higher molar percentage of valeric acid in the colon on day 4 PI (4.61 vs. 3.02%; P = 0.044) and also tended to have more branched-chain fatty acids on day 8 PI (1.09 vs. 0.53%; P = 0.088). Although the administration of the synbiotic did not modify the ammonia, lactic or SCFA concentrations, it promoted a higher molar percentage of valeric acid in the colon on day 8 PI (6.28 vs. 2.49%; P = 0.014) and provoked two interactions in the molar percentages of acetic (P = 0.012) and propionic acids (P = 0.002). Whereas in non-challenged piglets administration of the synbiotic mixture reduced the molar percentage of acetic acid and increased that of propionic acid, in the challenged animals the effect was the opposite.

In the ETEC F4 trial, the effects of the challenge in terms of fermentative activity were more apparent. In the colon, the challenge was responsible for a drop in pH on day 3 PI (6.11 vs. 6.38, P = 0.035) and an increase in the total amount of SCFA on day 7 PI (129.2 vs. 111.3 mmol/kg; P = 0.026). The molar proportion of propionic was also increased on day 3 PI (24.10 vs. 21.10%; P = 0.016), especially in the synbiotic group (P interaction = 0.022).

Related to the effects of the synbiotic mixture on the fermentation parameters, in the ileum an interaction was found on day 4 PI regarding the concentration of acetic acid and lactic acid as the main products of fermentation. Acetic acid showed the greatest concentration in the non-challenged animals not receiving the synbiotic (P interaction = 0.033), whereas lactic acid presented the highest values in the non-challenged and non-supplemented animals (P interaction = 0.004).

Table 4.4. Effects of experimental treatments on ileal and colonic fermentation in the Salmonella trial. This table includes values corresponding to pH, ammonia concentration (NH_3) (mmol/g of FM), lactic acid (mmol/kg of FM), total short-chain fatty acids (SCFA) (mmol/kg of FM) and molar ratio of these SFCA.

	PI Day	Treatment				RSD	P-value		
		IC	IS	NIC	NIS		Challenge	Treatment	Interaction
Salmonella trial									
ILEUM									
Lactic acid *(mmol/kg)*	4	16.56	17.09	7.07	9.52	*20.010*	*0.341*	*0.911*	*0.913*
	8	22.38	6.01	7.37	3.10	*14.260*	*0.173*	*0.064*	*0.351*
Acetic acid *(mmol/kg)*	4	4.43	2.40	3.34	2.06	*2.301*	*0.479*	*0.077*	*0.711*
	8	2.95	2.97	2.37	2.40	*2.441*	*0.599*	*0.984*	*0.999*
COLON									
pH	4	6.00	5.89	6.20	6.03	*0.547*	*0.475*	*0.559*	*0.900*
	8	6.03	6.21	6.02	5.67	*0.335*	*0.070*	*0.986*	*0.819*
NH_3 *(mmol/L)*	4	7.99	6.66	11.56	7.44	*3.877*	*0.210*	*0.169*	*0.414*
	8	30.75	38.80	15.39	13.57	*12.620*	*0.001**	*0.366*	*0.377*
Lactic acid *(mmol/kg)*	4	6.20	1.80	0.24	1.11	*6.984*	*0.571*	*0.437*	*0.628*
	8	5.76	5.72	23.46	0.24	*14.340*	*0.376*	*0.272*	*0.155*
SCFA *(mmol/kg)*	4	93.1	89.6	107.9	97.1	*40.90*	*0.537*	*0.725*	*0.839*
	8	129.3	97.4	96.9	105.6	*45.16*	*0.543*	*0.332*	*0.313*
SCFA molar ratio (%)									
Acetic	4	53.8	54.3	57.8	54.8	*9.51*	*0.589*	*0.873*	*0.673*
	8	55.5^{ab}	61.3^{ab}	64.4^{a}	45.3^{b}	*10.50*	*0.437*	*0.571*	*0.012**
Propionic	4	25.2	24.5	26.2	25.5	*6.03*	*0.695*	*0.777*	*0.998*
	8	28.5^{b}	22.1^{c}	24.5^{c}	36.4^{a}	*6.22*	*0.069*	*0.891*	*0.002**
Butyric	4	13.1	13.1	11.9	14.0	*4.79*	*0.944*	*0.706*	*0.619*
	8	11.7	10.8	8.8	9.6	*4.22*	*0.268*	*0.865*	*0.650*
Valeric	4	4.19	5.04	2.16	3.89	*1.714*	*0.044**	*0.117*	*0.557*
	8	3.33^{b}	4.36^{b}	1.66^{b}	8.21^{a}	*2.607*	*0.345*	*0.014**	*0.023**
BCFA	4	1.74	1.81	1.43	1.28	*1.388*	*0.491*	*0.997*	*0.856*
	8	0.82	1.36	0.65	0.42	*0.335*	*0.088*	*0.343*	*0.223*

BCFA = branched-chain fatty acids.

IC – Inoculated animals receiving placebo; IS- Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all groups except for non-challenged animals, N = 4. P-values were obtained by ANOVA using the generalized linear procedure in R software.

Table 4.5. Effects of experimental treatments on ileal and colonic fermentation in ETEC F4 trial. This table includes values corresponding to pH, ammonia concentration (NH_3) (mmol/L of FM), lactic acid (mmol/kg of FM), total short-chain fatty acids (SCFA) (mmol/kg of FM) and molar ratio of these SFCA.

	PI Day	Treatment				RSD	P-value		
		IC	IS	NIC	NIS		Challenge	Treatment	Interaction
ETEC F4 trial									
ILEUM									
Lactic acid *(mmol/kg)*	3	7.55	10.58	54.33	10.12	*15.880*	*0.013**	*0.116*	*0.004**
	7	14.42	18.06	15.96	14.52	*15.330*	*0.854*	*0.840*	*0.643*
Acetic acid *(mmol/kg)*	3	2.45	2.31	2.13	5.57	*1.727*	*0.034**	*0.092*	*0.033**
	7	3.93	3.85	3.86	6.20	*3.704*	*0.390*	*0.395*	*0.362*
COLON									
pH	3	6.14	6.09	6.26	6.38	*0.383*	*0.035**	*0.548*	*0.096*
	7	6.05	6.08	6.21	6.15	*0.332*	*0.363*	*0.894*	*0.699*
NH_3 *(mmol/L)*	3	9.56	10.14	5.8	4.82	*7.008*	*0.097*	*0.906*	*0.767*
	7	2.17	2.04	2.44	1.22	*1.288*	*0.532*	*0.150*	*0.253*
Lactic acid *(mmol/kg)*	3	0.65	3.10	1.65	2.64	*2.858*	*0.940*	*0.140*	*0.575*
	7	0.44	0.72	0.25	0.69	*0.397*	*0.667*	*0.106*	*0.704*
SCFA *(mmol/kg)*	3	99.8	99.3	81.8	103.4	*40.60*	*0.716*	*0.517*	*0.489*
	7	132.6	126.1	115.2	107.2	*21.94*	*0.026**	*0.359*	*0.921*
SCFA molar ratio (%)									
Acetic	3	60.4	54.4	60.9	57.7	*4.53*	*0.261*	*0.012**	*0.444*
	7	61.0	55.5	58.1	56.3	*4.45*	*0.515*	*0.029**	*0.241*
Propionic	3	22.6^{ab}	25.6^{a}	22.8^{ab}	19.4^{b}	*3.37*	*0.016**	*0.982*	*0.022**
	7	22.6	23.7	25.0	24.4	*3.44*	*0.217*	*0.846*	*0.485*
Butyric	3	11.9	13.5	11.8	15.5	*3.78*	*0.483*	*0.093*	*0.464*
	7	13.1	15.2	12.4	13.1	*2.67*	*0.154*	*0.140*	*0.459*
Valeric	3	2.82	4.07	2.05	4.62	*1.741*	*0.959*	*0.010**	*0.336*
	7	1.88	3.74	2.52	4.40	*1.328*	*0.174*	*<0.001**	*0.981*
BCFA	3	1.74	1.32	1.37	2.08	*0.893*	*0.469*	*0.729*	*0.117*
	7	1.31	1.69	1.92	1.64	*0.779*	*0.325*	*0.862*	*0.243*

BCFA= branched-chain fatty acids.

IC – Inoculated animals receiving placebo; IS- Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all groups except. P-values were obtained by ANOVA using the generalized linear procedure in R software.

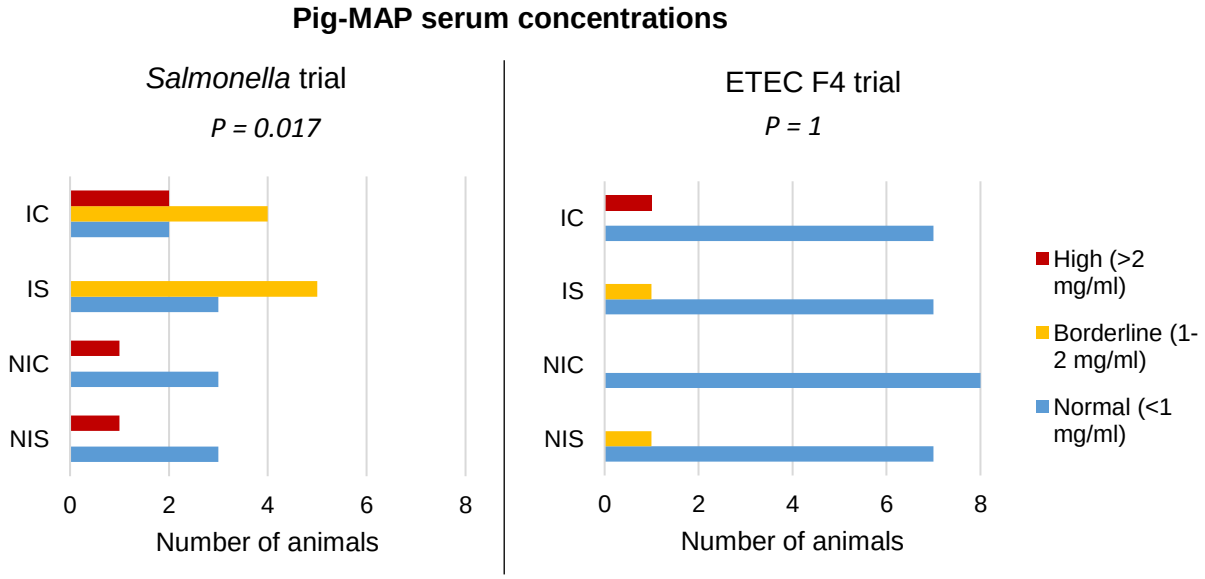
At the colonic level, no effects were found for the pH, total concentration of SCFA or lactic acid, but changes did occur in the profile of fermentation. The percentage of acetic acid fell on both days (56.05 vs. 60.65% for day 3 PI, P = 0.012, and 55.9 vs. 59.55% for day 7 PI, P = 0.029), in line with an increase in valeric acid (4.34 vs. 2.43% for day 3 PI, P = 0.010 and 4.07 vs. 2.20% for day 7 PI, P < 0.001) and a trend towards a higher butyric acid molar percentage (14.52 vs. 11.88% for day 3 PI, P = 0.093 and 14.22 vs. 12.78% for day 7 PI, P = 0.140).

4.3.5 Immune response

No significant differences related to the synbiotic treatment were found in the serum levels of TNF-α or Pig-MAP. However, changes were noted associated with the pathogen inoculation. Regarding TNF-α, animals challenged with ETEC F4 presented higher concentrations than non-challenged pigs on day 7 PI (58.9, 57.2, 48.1, 43.1 pg/mL for IC, IS, NIC and NIS, respectively; P = 0.010) and a similar pattern was found after the *Salmonella* challenge, although in this case the differences did not reach statistical significance (125, 135, 124, 101 pg/mL for IC, IS, NIC and NIS, respectively, day 8 PI; P = 0.132).

When analyzing the Pig-MAP, the values did not adjust to a normal distribution and therefore the results were analyzed as frequencies using Fisher's exact test. Three range levels were defined: high (> 2 mg/mL); borderline (1–2 mg/mL) and normal (< 1 mg/mL) according to Piñeiro et al. (2009). The results analyzed in this way are shown in ***Figure 4.4***. Only the *Salmonella* trial was able to promote an increase in the number of animals with borderline-high levels of Pig-MAP at day 8 PI (P = 0.017), with no significant change induced by the ETEC F4 challenge.

Figure 4.4. Effect of experimental treatments in serum levels of acute-phase protein Pig-MAP in piglets on day 8 (Salmonella trial) and 7 (ETEC F4 trial) following a pathogen oral challenge. Figure represents value frequencies between a normal (0.3-1 mg/mL) and abnormal (>2 mg/mL) range.



IC – Inoculated animals receiving placebo; IS- Inoculated animals receiving the synbiotic; NIC – Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all experimental groups except for non-challenged animals in Salmonella trial, N = 4. P-values were obtained using Fisher's Exact Test on R software.

4.3.6 Intestinal histological structure

The effects of the experimental treatments on ileal histomorphology are summarized in ***Table 4.6***. The *Salmonella* challenge promoted high levels of shorter villous height on day 4 PI ($P < 0.001$) and deeper crypts on days 4 and 8 PI ($P = 0.013$ and $P = 0.042$, respectively).

After the ETEC F4 challenge, no significant differences were found apart from a greater number of mitosis on day 7 PI ($P = 0.014$).Regarding impact of synbiotic administration on ileal histomorphology, different outcomes were found depending on the trial. In the ETEC F4 trial, villous height was greater on day 3 PI in the animals receiving the synbiotic, but only in the non-challenged group (P interaction = 0.032). However, the opposite numerical effect was observed on the second sampling day in both trials (P interaction < 0.156). Crypt depth showed an increasing trend from synbiotic administration

on day 4 PI in the *Salmonella* trial (P = 0.078), but, contrarily, a decreasing one on day 3 in the ETEC F4 trial (P = 0.081).

Table 4.6. Effects of treatments on ileal histomorphological parameters on days 4 and 8 (Salmonella trial) and 3 and 7 (ETEC F4 trial) post-inoculation.

	PI Day	Treatment				RSD	P-value		
		IC	IS	NIC	NIS		Challenge	Treatment	Interaction
Salmonella trial									
Villous height (μm)	**4**	128	149	230	228	*50.4*	*<0.001**	*0.532*	*0.604*
	8	215	202	227	270	*44.7*	*0.061*	*0.716*	*0.150*
Crypt depth (μm)	**4**	290	314	240	277	*24.4*	*0.013**	*0.076*	*0.665*
	8	327	327	282	296	*40.1*	*0.042**	*0.769*	*0.705*
IEL (Cell no./100 μm)	**4**	1.22	1.28	1.19	0.69	*0.636*	*0.276*	*0.626*	*0.320*
	8	1.01	1.41	1.14	1.53	*0.443*	*0.510*	*0.039**	*0.994*
Mitosis (Cell no./100 μm)	**4**	0.90	0.93	0.60	0.85	*0.136*	*0.171*	*0.598*	*0.310*
	8	0.84	0.59	0.63	0.60	*0.408*	*0.142*	*0.008**	*0.094*
ETEC F4 trial									
Villi height (μm)	**3**	256^{ab}	267^{ab}	287^{b}	216^{a}	*50.8*	*0.584*	*0.106*	*0.032**
	7	285	261	261	294	*55.1*	*0.830*	*0.825*	*0.156*
Crypt depth (μm)	**3**	244	231	249	201	*47.8*	*0.475*	*0.081*	*0.308*
	7	223	219	217	245	*24.8*	*0.264*	*0.178*	*0.068*
IEL (Cell no./100 μm)	**3**	0.57	0.45	0.43	0.62	*0.252*	*0.871*	*0.705*	*0.086*
	7	0.49	0.40	0.45	0.43	*0.131*	*0.885*	*0.243*	*0.452*
Mitosis (Cell no./100 μm)	**3**	0.29	0.33	0.27	0.40	*0.084*	*0.546*	*0.006**	*0.173*
	7	0.43	0.38	0.33	0.32	*0.091*	*0.014**	*0.332*	*0.483*

IEL = villous intraepithelial lymphocytes/100 μm; mitosis = number of mitosis in crypts/100 μm. IC – Inoculated animals receiving placebo; IS – Inoculated animals receiving the synbiotic; NIC- Non-inoculated animals receiving placebo; NIS – Non-inoculated animals receiving the synbiotic. N = 8 for all groups except for non-challenged animals in Salmonella trial, N = 4. P-values were obtained by ANOVA using the generalized linear procedure in R software. Letters a and b express differences considered for P < 0.05.

IEL were enhanced by the synbiotic at day 8 PI in the *Salmonella* trial (P = 0.039) and the same trend was observed on day 3 PI in the ETEC F4 trial, although only in the non-challenged animals (P interaction = 0.086). Mitosis were reduced by the synbiotic at day 8 PI in the *Salmonella* trial (P = 0.008), especially in the challenged animals (P interaction = 0.094). In contrast, in the ETEC F4 trial mitosis increased thanks to the synbiotic at day 3 PI (P = 0.006).

4.4 DISCUSSION

The aim of this work was to assess the potential of the combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and a mixture of inulin and FOS as a synbiotic strategy to fight two common gastrointestinal pathogens: *Salmonella and* ETEC F4.

In our previous research (Barba-Vidal et al., 2017), the strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 was found to have an effect against *Salmonella* Typhimurium and ETEC F4 colonization in pigs with a stimulation of local immune response by increasing the number of intraepithelial lymphocytes. Furthermore, it is widely acknowledged that fructo-oligosaccharides (FOS) and inulin are selectively fermented by most strains of bifidobacteria (Wang et al., 1993; Kaplan et al., 2000) due to the production of β-fructofuranosidases (Imamura et al. 1994). In fact, FOS and inulin are two of the most studied prebiotics with bifidogenic properties (Meyer & Stasse-Wolthuis, 2009). Considering these facts, we hypothesized that combining *B. infantis* CECT 7210 with FOS and inulin could therefore enhance its beneficial effects against pathogens, contributing to improved gut health.

With this objective, two different trials were performed to challenge animals with either *Salmonella* or ETEC F4. As is well known, these pathogens exhibit differences in terms of pathogenicity, mediated by distinct virulence factors and mechanisms (Boyen et al., 2008; Clements et al., 2012). Thus, the animals' responses to the challenge and the clinical course differed depending on the pathogen. Whereas after the *Salmonella* challenge growth and feed intake were markedly reduced, with a clear effect on faecal score and an increase in rectal temperature, the challenge with ETEC F4 exhibited a

much milder course of diarrhoea, the differences not being statistically significant for several parameters. Similar effects were observed by Barba-Vidal et al. (2017), viewing the challenge by ETEC F4 as milder than that of *Salmonella* Typhimurium.

The performance of the animals before the oral challenge was not modified by the administration of the synbiotic in terms of feed intake or weight gain, but in terms of gain:feed a significant reduction in feed efficiency was seen in the Salmonella trial (0.48 vs. 0.66 for synbiotic vs. control diet, P = 0.02). We might hypothesize that this decrease in feed efficiency was the result of a dilution of the energy or limiting of amino acids in the SYN diet following the inclusion of 5% of the prebiotic. It may also have owed to changes in the transit time and digestibility of nutrients related to the inclusion of 5% of FOS/inulin (Kelly, 2009; Chen et al., 2017), being the changes that we observed in the consistency of caecal digesta at day 8 PI supported by this hypothesis. Furthermore, the reduction of gain:feed may have been due to modifications in the gut microbiota promoted by the synbiotic with an impact on host energy homeostasis (Rosenbaum et al., 2015; Ley et al., 2016). However, this is mere speculation, as we lack the evidence to support these ideas.

Clinical signs such as diarrhoea incidence were not improved by the administration of the synbiotic compound to the animals, but it is also fair to highlight that no deterioration was observed in any of the trials, verifying the safety of the probiotic strain, as proved by other authors (Moreno-Muñoz et al., 2011; Barba-Vidal et al., 2017) even when it is combined with inulin and fructo-oligosaccharides.

Numerous previous studies have demonstrated that bifidobacteria, inulin and FOS can have a beneficial effect for the host, helping it to maintain a healthy gut environment. Indeed, bifidobacteria may increase the colonic intraluminal concentration of short-chain fatty acids (SCFA) (Servin, 2004), which are responsible for a wide range of effects in the gastrointestinal system. Topping (1996) has described how the reduction in pH associated with an increase in SCFA might help to control the proliferation of pathogenic microorganisms. In addition, inulin and the fructo-oligosaccharides derived can produce the same effect on SCFA concentrations *in vivo* and *in vitro* (Pompei et al., 2008; van der

Beek et al., 2018; Baxter et al., 2019). In our trials, however, the concentration of SCFA in the colonic content was not enhanced by the synbiotic treatment. According to Nyman (Nyman et al., 2002), this outcome should be expected because the concentration of SCFA measured is contingent on the balance between production and absorption, and commonly SCFA produced by fermentation are rapidly absorbed or utilized by the colonic mucosa.

Regarding changes in the fermentation profile, different effects can be attributed to bifidobacteria and inulin or FOS. Several authors have described increases in the molar percentage of butyrate with probiotic bifidobacteria (Rossi et al., 2005; Belenguer et al., 2006), although the main fermentation product of bifidobacteria is acetate. Butyrogenic effects owe to a stimulation of acetate-depending, butyrate-producing colon bacteria by cross-feeding interactions that in parallel are required by some other bacteria that can convert lactate into butyrate, albeit only when acetate is present (De Vuyst et al., 2011; Moens et al., 2016; Moens et al., 2017). Higher amounts of acetate and butyrate have been shown to have favourable effects on the colonic structure; for example, acetate promotes colonic epithelial proliferation and butyrate is responsible for the maintenance of mucosal integrity, reparation and colonocyte proliferation, given that it is the preferred energetic source for these cells (Topping, 1996). Inulin and FOS have been reported as exerting a bifidogenic effect in infants (Meyer & Stasse-Wolthuis, 2009; Paineau et al., 2013) as well as in modulating fermentation products in the gut. Furthermore, various *in vitro* studies have shown how inulin and oligofructose can increase butyrate, propionate (van de Wiele et al., 2007) and acetate (van der Beek et al., 2018) production. Differential effects may be related to the contrasting chemical structure of these compounds as well as to the specific microbial ecosystems in which they are introduced. In this regard, Rossi et al. (2005) have reported differences between inulin and FOS: whereas for inulin the main fermentation product was butyric acid with lower amounts of acetic, lactic and propionic acids, for FOS the main fermentation products were lactic acid and acetic acid, alongside lower amounts of butyric acid and no propionic acid. *In vivo* studies have also shown a variable impact on intestinal fermentation. Scholtens et al. (2006) have demonstrated increases in acetate and decreases in butyrate in the faeces

of humans receiving 25–30 g/d of FOS for a period of two weeks, while Boets et al. (2015) have used stable isotope technology to demonstrate how in humans inulin is mainly fermented into acetate as well as to a lesser extent into butyrate and propionate.

Considering the varied findings of previous works, it was difficult to anticipate what to expect when combining bifidobacteria, inulin and FOS; moreover, the impact of the synbiotic would seem to be contingent on the trial in question. Whereas the synbiotic reduced the molar proportion of acetate in non-challenged animals but increased it in challenged ones, in the ETEC F4 trial acetate was consistently reduced. These lower levels of acetate might be explained by a cross-feeding phenomena, supported in the ETEC F4 trial by the observable increasing trend of butyrate. In the same vein, the increase observed for acetate in the *Salmonella* challenged animals with the synbiotic, could correspond to a more acute dysbiosis that might disturb the normal cross-feeding phenomena within bifidobacteria and colonic bacteria.

Similar kinds of interactions have been described in the literature. For instance, regarding FOS supplements to dogs, Pinna et al. (2018) found increases in the acetate:propionate ratio in low-protein diets but a decrease in high-protein diets.

Another SCFA that is rarely considered in the literature and whose concentration was augmented by the synbiotic treatment in both trials is valeric acid. This fatty acid, which is capable of inhibiting the growth of pathogenic bacteria like *Clostridium difficile* (McDonald et al., 2018), originates in 5-aminovalerate, which is a product of the anaerobic degradation of previously hydrolyzed protein by gut bacteria (Baker et al., 1987). Recent investigations have proved that a strain of *Megasphaera elsdenii* (a major inhabitant of the pig intestine) can utilize lactic acid as a fermentation substrate and convert it into valerate (Yoshikawa et al., 2018). It is feasible that a similar effect occurred in our experiments, as colonic lactic acid was augmented by the synbiotic, albeit only in the ETEC F4 trial.

Together with the inhibitory effects promoted by probiotics on enteropathogens throughout changes in the fermentation products, probiotics have also been shown to fight pathogens via other

mechanisms. In particular, several species of *Bifidobacterium* are deemed capable of enhancing and modulating the immune response (Wagnet et al., 2009; Bermudez-Brito et al., 2013; Presti et al., 2015), of releasing bacteriocins and bacteriocin-like substances (Poltavska et al., 2012; Martinez et al., 2013) and of completing/displacing pathogens from their adhesion sites on the intestinal epithelium (Collado et al., 2005; Candela et al., 2008). The probiotic strain tested – *Bifidobacterium longum* subsp. *infantis* CECT 7210 – has proved to be effective in reducing *Salmonella* loads in piglets in previous works (Barba-Vidal et al., 2017). Nevertheless, in the present trial, combining this probiotic with inulin and FOS was unable to reduce *Salmonella* excretion, suggesting that the combination of this *Bifidobacterium* strain with these prebiotics does not improve its power to fight the pathogen. Regarding its potential to exclude ETEC F4, previous studies have demonstrated this strain's ability to reduce the number of coliforms adhered to ileal mucosa in ETEC F4-challenged animals (Barba-Vidal et al., 2017), but in the present study we were only able to find such an effect in the non-challenged animals. When challenged with the ETEC F4, the synbiotic treatment was associated with a numerical increase in attached coliforms, although not significantly. This interaction (day 7 PI; $P = 0.057$) may be explained by inoculated ETEC F4 potentially profiting from the supplemented inulin and FOS, considering that as stated by Rossi et al. (2005) *Escherichia coli* can use these fermentable sources of carbohydrates as growth substrates, increasing its concentration when it is seeded in faecal cultures supplemented with inulin or FOS. However, despite these effects on coliforms, it is also important to remember that in both the present study and that of Barba-Vidal et al. (2017), no significant effects (nor increases or decreases) were detected in the numbers of ETEC F4 either in the digesta or in the ileal scrapings.

Although this investigation's results do not provide evidence of the ability of the synbiotic to reduce the number of pathogens in the intestine, some insights regarding the potential positive effects can be provided. In the *Salmonella* trial, the reduction of villi height associated with the pathogen challenge (Watson et al., 1995) was not attenuated by the synbiotic mixture, although a decline in the number of mitosis was seen, suggesting that the amount of damaged tissue that needed to be

replaced was reduced. Furthermore, the synbiotic may have also modulated the immune response at the gut level, as an increase in the number of IEL with the synbiotic was found in the *Salmonella* trial (P = 0.039) at day 8 PI as well as in the ETEC F4 trial, albeit only in those animals challenged with the pathogen (P interaction = 0.086). Similarly, with this probiotic strain, Barba-Vidal et al. (2017) have also reported an increase in IEL in the ileum of piglets (whether challenged or not) with *Salmonella* of ETEC F4. A higher presence of IEL might be regarded as beneficial considering that these cells are responsible for the healing and protection of the integrity of the intestinal epithelium as well as acting as early response effectors against mucosal pathogens (Olivares-Villagómez & Van Kaer, 2018). Supporting the immunomodulatory properties of this strain, previous studies with a murine model of rotavirus infection (Moreno-Muñoz et al., 2011) have reported increases in the levels of secretory Immunoglobulin A (IgA) in the faeces. In the light of these results, increases in ileal IEL in the present study with the synbiotic could be attributed to the probiotic strain. However, a possible additional impact of the prebiotic fibres should not be discounted, considering that in the literature several works have reported the ability of inulin and FOS to enhance local immune responses (Shukla et al., 2016; Le Bourgot et al., 2017; Myhill et al., 2018).

To summarize, combining *Bifidobacterium longum* subsp. *infantis* CECT 7210 with inulin and FOS was not able to reduce *Salmonella* or coliforms loads in the gut, as has been previously reported for the single probiotic strain. Nonetheless, this synbiotic combination was able to modify the fermentative activity of the intestine with differential effects depending on the pathogen challenge, most likely disturbing the expected cross-feeding processes between the bifidobacteria and the indigenous butyrogenic colonic bacteria. The combination of this probiotic strain with inulin and FOS was also able to increase the numbers of IEL at the ileal level, suggesting certain immunomodulatory properties. A more in-depth study of the changes produced in the gut ecosystem is necessary in order to develop a greater understanding of the role of this synbiotic combination in a scenario of well-balanced or dysbiotic microbiota.

Chapter 5. Effects of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, combined or not with oligofructose-enriched inulin, on weaned pigs orally challenged with *Salmonella* Typhimurium.

5.1 INTRODUCTION

Salmonellosis is a disease caused by non-typhoidal serotypes of *Salmonella* that, after campylobacteriosis, is the second most common foodborne in the European Union (91,662 human cases reported in 2017; EFSA, 2019). It is characterized by symptoms like an acute onset of fever mainly accompanied by abdominal pain, nausea and diarrhoea. The course of the illness is self-limited and affected individuals recover without treatment, but it can develop to a serious and life-threatening condition in non-immunocompetent patients, like children and elderly people (WHO, 2018).

Different serovars of *Salmonella* isolated from humans have been found to be "high" or "extremely high" resistant to antimicrobials (EFSA 2019). As a consequence, the study of alternatives to these substances that could potentially fight against these pathogenic bacteria, such probiotics and prebiotics, are of a great interest. Different mechanisms could be behind the positive effects against pathogens reported by different probiotics and prebiotics but in general terms we could say that all of them would boost the natural mechanisms of colonization resistance (Lawley and Walker, 2013; Fehervari, 2019).

Probiotics have demonstrated efficacy against a multitude of enteropathogens. Some strains of *Lactobacillus* have been shown to prevent the intestinal damage caused by enterohemorrhagic *Escherichia coli* (Johnson-Henry et al., 2008) and specifically, the HN001 (DR20) strain, was demonstrated to offer protection against *Salmonella* Typhimurium by stimulating the immune response of the host (Gill et al., 2001). In addition, *Bifidobacterium* genus has an important role in the maintenance of gut homeostasis (Tojo et al., 2014) and some strains have also been tested in gastrointestinal pathogen infections. Specifically, *Bifidobacterium longum* subsp. *infantis* CECT 7210 has been proven safe and effective against rotavirus in a murine model (Moreno-Muñoz et al., 2011) and also against enterotoxigenic *Escherichia coli* and *Salmonella* Typhimurium in a piglet model (Barba-Vidal et al., 2017).

Prebiotics have also been demonstrated as a good strategy to fight enteropathogens. Compounds such as inulin and oligofructose have been shown to reduce the adhesion of pathogens to intestinal epithelium by increasing the bifidobacteria and lactobacilli indigenous population able to use these fibres as growth substrates (Bosscher et al., 2006). In the last years different authors have proposed the combined use of probiotics and prebiotics with the aim of increasing selectively the survival and activity of the specific probiotic strain, improving in this way their efficacy. This concept is known as synbiotic (De Vrese & Schrezenmeir, 2008) and has showed promising results against acute diarrhoea in children (Yang et al., 2019).

The aim of this work was to evaluate a multistrain probiotic composed by *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, the prebiotic oligofructose-enriched inulin and their synbiotic combination in weaned piglets orally challenged with *Salmonella* Typhimurium.

5.2 MATERIALS AND METHODS

A trial was carried out at the Experimental Unit of the Universitat Autònoma de Barcelona (UAB) and received prior approval (Permit No. CEAAH1619) from the Animal and Human Experimental Ethical Committee of this Institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to European Union Guidelines *(*Directive 2010/63/EU*, European Commission, 2010)*. All efforts were made to minimize animal suffering.

5.2.1 Animals, housing and experimental design

This trial was carried out as Level 2 High-Risk Biosecurity procedures and involved personnel received appropriate training. A total number of 96 male piglets [Landrace x Large White] x Pietrain of 28 (± 3)

days of age weighting 6.81 (± 0.13) kg were used. All animals came from high-sanitary-status farms whose mothers were serologically negative to *Salmonella.*

Piglets were transported to the experimental unit located in the UAB, which consisted in four boxes of eight pens each (thirty-two pens, three animals per pen). Each pen of $2m^2$ have a feeder and water nipple to provide feed and water *ad libitum*. All weaning rooms were equipped with automatic heater, forced ventilation and each pen had an individual heating light.

At arrival, animals were distributed according their initial body weight (BW) in order to ensure a homogeneous average BW between treatment groups. Trial was conceived as a completely randomized design that included five experimental groups: non-inoculated control (CTR+) and inoculated control (CTR-), probiotic combination (PRO), prebiotic (PRE) and synbiotic (SYN). These last four groups were orally challenged and distributed equally in three of the four rooms, meanwhile one full room was kept for non-inoculated control pigs, being the design hence unbalanced. In the challenged rooms, probiotic and synbiotic treatments were distributed within the four pens along one side of the room, and the control and prebiotic treatments on the other side of the room, separated by a corridor in between. Each experimental group had six replicates except for the non-challenged groups, which had eight replicates.

5.2.2 Probiotic strains, prebiotic mixture and diets

Tested probiotics were *Bifidobacterium longum* subsp. *infantis* CECT 7210, supplied by Ordesa S.L., and *Lactobacillus rhamnosus* HN001 (Danisco USA Inc.) strains. Both were stored in a lyophilized form containing 5×10^{10} and 3×10^{10} colony-forming units [cfu] per gram of product respectively in a maltodextrin base. The lyophilised probiotics were daily mixed into the feed for a final dosage of 5.5×10^7 and 3.3×10^7 cfu/g respectively, being the feed totally replaced every day. The viability of the probiotic along the day in the dry feed was confirmed before the start of the trial.

The experimental oligofructose-enriched inulin (OF) was in powder form and was manually mixed into the feed up to a final concentration of 5% (w/w). OF was mixed previously to the probiotic in the SYN diet.

Table 5.1. Ingredient and nutritional composition of the diets.

Ingredients (g/kg FM)	**Control**	**Synbiotic**
Maize	206.7	196.3
Wheat	179.4	170.4
Barley 2 row	169.4	160.9
Extruded soybean	148.6	141.2
Sweet whey-powder (cattle)	99.6	94.7
Fish meal	59.8	56.8
Soy bean meal 44	79.7	75.7
Whey-powder 50% fat	24.9	23.7
Mono-calcium phosphate	6.8	6.4
Calcium carbonate ($CaCO_3$)	3.9	3.7
L-Lysine HCL	5.3	5.1
Vit-Min Premix*	4.0	3.8
Sodium chloride (marine salt)	2.5	2.4
DL-Methionine 99	3.9	3.7
L-Threonine	2.8	2.7
L-Tryptophan	0.8	0.8
L-Valine	2	1.9
Prebiotic	0	50
Analyzed composition (g/kg FM)	**Control**	**Synbiotic**
Dry matter	916.9	917.4
Ashes	48.2	47.7
Crude fat	61.1	58.8
Crude protein	203.4	193.1
Neutral detergent fibre	80.5	82.0
Acid detergent fibre	25.7	27.1

***** *Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se*

Pre-starter diets were formulated in concordance with the nutrient requirement standards for pigs (NRC, 2012) and given in a mash form. Synthetic amino acids were added to PRE and SYN diets in order to compensate the possible amino acid dilution produced by prebiotic incorporation: 0.5 g L-valine, 0.9 g L-lysine HCL, 1.2 g DL-methionine, 0.5 g L-threonine and 0.2 g L-tryptophan per kg of feed. Details for their ingredient and chemical composition is given in ***Table 5.1.***

5.2.3 *Salmonella* strain

The bacterial strain used for the oral challenge was a *Salmonella* Typhimurium var. Monophasic (*formula: 4,5,12:i:-, resistance profile: ACSSuT-Ge, Fagotype: U302*) isolated from a salmonellosis outbreak of fattening pigs in Spain, which was provided by the Infectious Diseases Laboratory (Ref. 301/99) of the UAB. The preparation of the oral inoculum consisted of 24-hour incubation at 37°C in buffered peptone water (Oxoid; Hampshire, UK) and diluted (1:10) with sterile phosphate buffered saline (PBS) (Sigma-Aldrich; Madrid, Spain). Final concentration of the inoculum was 1 x 10^9 cfu/mL. Inoculum concentrations were determined before the inoculation by McFarland standards and were doubly plated in Tryptic Soy Agar (TSA) (Liofilche; Italy) the same day in order to check them by manual plate counting.

5.2.4 Experimental procedure

The experiment had a duration of fifteen days. After an adaptation period of 7 days animals were orally challenged with the pathogen. One animal of each pen was euthanized on days 4 and 8 post-inoculation (PI).

After a week of adaptation, the inoculum containing the pathogenic bacteria culture was given to the challenged groups via oral route. There were given a total volume of 2 mL to each animal corresponding to a 2 x 10^9 cfu dose of *Salmonella* Typhimurium. The same amount of sterile broth was administered to non-challenged piglets. To ensure that the animals had a full stomach in the moment

of the oral challenge, pigs were starved for a period of 12 hours and feed was reintroduced 30 minutes before the inoculation.

From the challenge onwards, animals were checked daily to determine clinical signs and evaluate their post-inoculation status (i.e, dehydration, anorexia, apathy, general behaviour and faecal score), always by the same person. Faecal score was measured using a scale: 1 = solid and cloddy, 2 =soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Rectal temperature was assessed with a digital thermometer (Accuvet, Sanchung City, Taiwan) on days 1 and 3 PI.

In order to monitor animal performance, individual body weight was registered at arrival and on days 0, 4 and 8 PI. Feed intake was monitored daily, associated to the regular feed change to maintain probiotic viability. The average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F) were calculated by pen. Mortality rate was also registered and no antibiotic treatment was given to the animals.

For microbiological analysis, faecal samples were collected aseptically after spontaneous defecation or by digital stimulation at arrival, the day of the inoculation (0 PI) and on days 1, 3 and 7 PI. Faecal samples were always obtained from the same animal corresponding to the largest of each pen at the beginning of the trial (N = 32).

On days 4 and 8 PI one pig per pen was euthanized. On day 4 PI, the selected animal was the one with the medium initial BW, while on day 8 PI, the chosen piglet was the heaviest one of each pen. Animals were euthanized and sequentially sampled during the morning of each day (between 8:00 and 13:00 hours). Prior to injection of euthanasic drug, 10 mL sample blood was taken from each animal via venepuncture of the cranial cava vein using 10 mL blood collection tubes without anticoagulant (Aquisel; Madrid, Spain). Right after blood sampling, pigs were administered a lethal dose injection of sodium pentobarbital intravenously (140 mg/kg BW; Euthasol, Le Vet B.V.; Oudewater, Netherlands). Once dead, animals were bled, abdomen immediately opened and gastrointestinal tract extracted.

A sample from caecal content was obtained for microbiology and kept in ice until analysis within the first 4 hours. Subsequently, digesta of ileum and proximal colon was collected and homogenized prior to pH determination with a pH-meter previously calibrated (Crison 52–32 electrode, Net Interlab; Barcelona, Spain). Subsamples of ileal and colonic digesta were preserved for different analysis. A set of samples were stored frozen at -20°C in H_2SO_4 solution (3 mL of content plus 3 mL of 0.2 N H_2SO_4) for ammonia (NH_3) determination and an additional set (~10 g) for short-chain fatty acids (SCFA) and lactic acid.

For the histological study, 1-cm sections from the ileum were removed, opened longitudinally, washed thoroughly and carefully with 4% formaldehyde solution (Panreac; Castellar del Vallès, Spain) before fixing them by immersion in the same solution.

Blood samples were centrifuged after blood clotting (3,000 × g for 15 min at 4°C) and the serum obtained stored at -20°C.

5.2.5 Analytical procedures

Chemical analyses of the diets, including dry matter (DM), ash, crude protein and diethyl ether extract, were performed according to Association of Official Agricultural Chemists standard procedures (AOAC International, 1995). Neutral detergent fibre and acid-detergent fibre were determined according to the method of Van Soest et al. (1991).

For microbiological analysis of *Salmonella*, samples were transferred to BPW solution in a concentration of 1:10. Quantitative analysis was performed by seeding serial dilutions of the samples 10^{-2}, 10^{-4}, and 10^{-6} in Xylose-Lactose-Tergitol-4 plates (XLT-4) (Merck; Madrid, Spain). For the qualitative analysis, samples were incubated in BPW (37°C, 24h) transferring 100 µL of the culture to 10 mL of Rappaport-Vassiliadis for a second incubation (42°C, 48h) to finally seed them in XLT4 plaques to observe H_2S positive colonies.

Short-chain fatty acids and lactic acid analyses were performed using gas chromatography, after the samples underwent acid-base treatment prior an ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide plus 1% tert-butyldimethylchlorosilane agent, using the method of Richardson et al. (1989), modified by Jensen et al. (1995).

Ammonia concentration was assed using a gas-sensitive electrode (Hatch Co.; Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain) and following the procedure described by Hermes et al. (2009), which was adapted from Diebold et al (2004). Samples were diluted (1:2) in 0.16 M NaOH and, after homogenization, centrifuged at 1500 x g for 10 minutes. Once the ammonia was released, it was measured in the supernatants as a change in voltage in mV.

Serum concentrations of Tumor Necrosis Factor-α (TNF-α) were determined by Quantikine Porcine TNF-α kits (R&D Systems; Minneapolis, USA) and pig major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A.; Segovia, Spain) according to the manufacturer's instructions. Antibodies against *Salmonella* were also assed using an ELISA *Salmonella* Herdcheck (Idexx; Hoofddorp, Netherlands) and stablishing the cut-off for positivity in optic density ≥40%.

For histological study, tissue samples were dehydrated and embedded in paraffin wax, sectioned 4-µm thickness and stained with haematoxylin and eosin. Measurements of 10 different villus-crypt complexes per sample and counting of intraepithelial lymphocytes (IEL), Goblet cells (GC) and number of mitosis of each one were performed with a light microscope (BHS, Olympus; Barcelona Spain) using as guideline the procedure described in Nofrarías et al. (2006).

5.2.6 Statistical analysis

Results are expressed as means with their standard errors unless otherwise stated. Microbiological counts were transformed [log] for analysis. A one-way ANOVA was used to assess the effect of the five experimental treatments. All analysis were performed using a generalized linear model (GLM) o mixed effects model of R software, and by Fisher´s exact tests when analyzing frequencies. Version 3.4.4 of R statistical analysis software was used (R Development Core Team; New Jersey, USA). When treatment effects were established, means comparison was adjusted by Tukey–Kramer test. The considered experimental unit for analysis was the pen. The α-level used for the determination of significance for all the analysis was P = 0.05. The statistical trend was also considered for P < 0.10.

5.3 RESULTS

The course of the experiment went as expected without any remarkable incidence. Feed consumption was within expected values, receiving piglets the calculated daily amount of probiotic and prebiotic.

After the oral challenge with the pathogenic bacteria, animals presented mild to moderate clinical signs of diarrhoea. It was registered only a casualty at day 2 PI (CTR- group) and no humanitarian euthanasia was required.

5.3.1 Performance parameters

Results for BW, average daily feed intake (ADFI) and average daily gain (ADG) are presented in ***Table 5.2***.

Average daily feed intake showed differences between diets from the adaptation period. After the first week animals receiving SYN diet had a lower ingestion compared to CTR+ (P = 0.038) . During the acute phase of the infection (days 0-4 PI), PRE and SYN groups also reached significant decreases

compared to CTR+ (P = 0.049 and P = 0.006, respectively) and during the sub-acute phase (days 4-8 PI) all challenged groups showed a reduced consumption (P < 0.001).

Regarding average daily gain, no significant differences related to diets were found after the first adaptation week. After the challenge, although all the challenged groups showed numerical reductions compared to CTR+, only for the SYN group this reduction was statistically significant (P = 0.001), leading to a lower BW at the end of the experiment (P = 0.004).

Table 5.2. Effects of experimental treatments on body weight (BW) average daily feed intake (ADFI) and average daily weight gain (ADG).

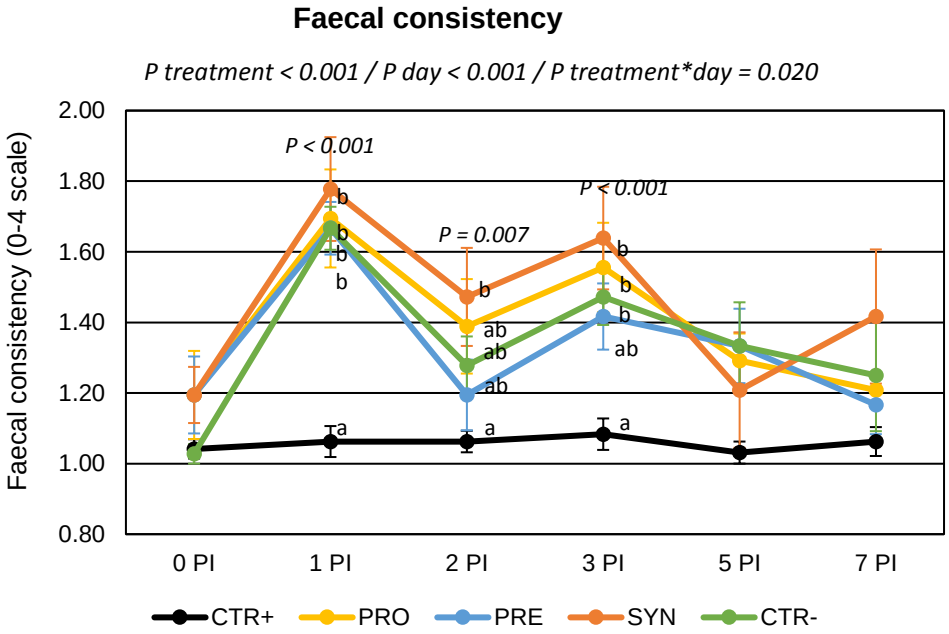
	Treatment					***RSD***	***P-value***
	CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
BW (kg)							
Initial	6.80	6.76	6.78	6.88	6.81	*0.140*	*0.687*
Final	10.45^{a}	9.34^{ab}	8.86^{ab}	8.21^{b}	9.44^{ab}	*1.067*	*0.009*
ADFI (g)							
Adaptation	291.2^{a}	242.2^{ab}	236.4^{ab}	225.2^{b}	249.0^{ab}	*40.16*	*0.040**
0-4 PI	395.9^{a}	316.1^{ab}	287.4^{b}	255.2^{b}	315.8^{ab}	*68.55*	*0.009**
4-8 PI	481.5^{a}	314.9^{b}	299.9^{b}	219.1^{b}	316.1^{b}	*103.10*	*0.001**
ADG (g)							
Adaptation	206.2	172.3	143.0	150.9	146.5	*64.96*	*0.334*
0-4 PI	274.7^{a}	223.7^{ab}	153.9^{ab}	85.5^{b}	222.9^{ab}	*110.60*	*0.042**
4-8 PI	270.0^{a}	156.3^{ab}	136.4^{ab}	38.7^{b}	150.3^{ab}	*97.46*	*0.003**

Adaptation: pre-inoculation week (days 1-8); 0-4 PI: acute post-inoculation period (days 8-11); 4-8 PI: sub-acute post-inoculation period (days 11-15). CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8.

5.3.2 Clinical signs

Evolution of faecal consistency along the post-inoculation period is represented in ***Figure 5.1***. Clinical signs of diarrhoea were mild after the challenge, but faecal scores showed a clear increase in all inoculated groups the day after (P < 0.001, at day 1 PI). On day 2 PI the difference with CTR+ group was only significant for the SYN group (P = 0.006) whereas on day 3 PI all challenged groups except PRE were different from CTR+ (P < 0.001). On days 5 (P = 0.116) and 7 PI (P = 0.140) all inoculated groups showed to recover with no significant differences between treatments.

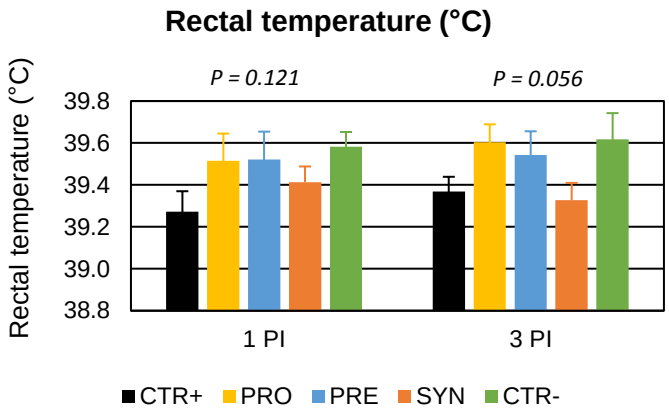
Figure 5.1. Evolution of average faecal scores for the different experimental groups in the post-inoculation period.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. Bars correspond to standard error.

Febrile response (> 40.5°C) could not be registered in the animals after the challenge (***Figure 5.2***) however on day 3 PI a trend for an increased rectal temperature was found in the challenged animals except for those receiving the SYN treatment (P = 0.056).

Figure 5.*2. Effect of experimental treatments on rectal temperature on days 1 and 3 post-inoculation.*



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. Bars correspond to standard error.

5.3.3 Microbiological analysis

Serologic analysis confirmed that none of the animals were exposed to the pathogen previously to their transfer, being all of them seronegative at the end of the experimental trial (data not shown).

In order to analyse data from *Salmonella* faecal plate counts, animals were classified into five different levels high (between 10^7 and 10^8 cfu per gram), medium (between 10^6 and 10^5 cfu per gram), low (between 10^4 and 10^3 cfu per gram), non-quantifiable positive (between 10^2 and 10 cfu per gram) or negative. Expressed in this way, the effect of diets was analysed by Fisher´s Exact Test and no significant differences could be found related to experimental treatments (***Figure 5.3***).

Figure 5.3. Percentage of animals included in the different faecal and caecal excretion levels of Salmonella. All samples were obtained from the same animal of each pen (the highest-weight) except for the caecal sample on day 4 PI, obtained from the medium-weighted animal.

PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained using Fisher's Exact Test on R software.

However, it deserves to be mentioned that on day 7 PI a greater percentage of animals receiving the PRO diet became negative to *Salmonella* compared to CTR- animals reaching this comparison (65% vs 0%) significant P-values (P = 0.028). In addition, when treatments in terms of percentage of animals with non-countable *Salmonella* along the whole experimental period (negative + very low levels) were compared, the only treatment with no animal with countable levels was the PRE treatment, being differences significant when compared to CTR- group (0 % vs. 7%; P = 0.013).

5.3.4 Intestinal fermentation

Values of intestinal pH, ammonia, lactic acid and short chain fatty acid concentrations in ileal and colonic content for the different experimental treatments are presented in ***Table 5.3***.

At ileal level no significant differences were seen on pH nor ammonia concentrations related to the experimental treatments. The concentration of total short chain fatty acids showed a numerical decrease with the challenge that only reached significant levels with the SYN treatment at day 8 PI (P = 0.025). No statistical differences were found in the fermentation profile in terms of molar ratio.

Table 5.3. Effects of experimental treatments on ileal and colonic fermentation. The table include pH values, ammonia concentration (NH_3) (mmol/L), lactic acid (mmol/kg of FM), total short-chain fatty acids (SCFA) (mmol/kg of FM) and molar ratio of these SFCA.

	PI Day	Treatment					*RSD*	*P-value*
		CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
ILEUM								
pH	4	6.66	6.50	6.55	6.62	6.61	*0.253*	*0.812*
	8	6.53	6.63	6.86	6.85	6.88	*0.299*	*0.130*
NH_3 *(mmol/L)*	4	0.59	0.48	0.44	0.54	0.53	*0.206*	*0.715*
	8	0.98	0.99	0.79	1.06	0.70	*0.397*	*0.468*
Lactic acid *(mmol/kg)*	4	9.97	10.48	5.60	3.93	12.26	*10.27*	*0.589*
	8	22.1	22.42	5.38	16.47	5.77	*25.10*	*0.586*

SCFA	4	6.40	7.12	3.11	3.39	6.27	*3.934*	*0.266*
(mmol/kg)	8	8.22^{a}	6.76^{ab}	4.26^{ab}	3.09^{b}	4.81^{ab}	*2.954*	*0.025**
SCFA molar ratio (%)								
Acetic	4	97.3	96.3	94.1	94.7	96.2	*4.06*	*0.623*
	8	96.5	97.3	94.4	85.5	94.9	*8.75*	*0.158*
Propionic	4	3.22	3.36	10.19	6.95	7.20	*3.709*	*0.253*
	8	1.93	1.92	4.55	6.72	4.21	*3.544*	*0.105*
Butyric	4	1.43	2.51	0.83	3.50	2.74	*2.085*	*0.275*
	8	1.52	1.03	1.23	9.26	1.07	*7.032*	*0.286*
COLON								
pH	4	5.82^{a}	5.65^{ab}	5.17^{b}	5.42^{ab}	5.67^{ab}	*0.340*	*0.018**
	8	5.74	5.76	5.85	5.94	5.92	*0.390*	*0.826*
NH_3	4	4.52	8.43	5.31	8.97	9.84	*5.517*	*0.325*
(mmol/L)	8	4.66	8.92	10.22	9.07	11.64	*6.651*	*0.369*
Lactic acid	4	1.16	0.37	1.36	1.41	1.35	*1.173*	*0.695*
(mmol/kg)	8	0.41	0.38	0.00	0.81	2.15	*1.993*	*0.514*
SCFA	4	158	152	138	155	147	*29.7*	*0.756*
(mmol/kg)	8	177	171	165	157	146	*41.4*	*0.706*
SCFA molar ratio (%)								
Acetic	4	59.7^{a}	57.8^{ab}	51.6^{b}	53.9^{ab}	59.4^{a}	*3.83*	*0.002**
	8	55.7	58.8	51.6	58.4	61.6	*6.03*	*0.077*
Propionic	4	25.7	24.1	28.3	26.3	24.4	*3.51*	*0.268*
	8	26.9	23.7	29.7	23.8	23.9	*4.82*	*0.145*
Butyric	4	10.8	13.8	14.4	14.6	11.8	*3.87*	*0.297*
	8	12.1	12.9	11.9	11.5	10.3	*2.86*	*0.398*
Valeric	4	2.25^{a}	2.59^{a}	4.85^{b}	4.11^{ab}	2.79^{ab}	*1.246*	*0.003**
	8	2.68^{ab}	2.77^{ab}	4.81^{c}	4.34^{bc}	2.23^{a}	*1.127*	*0.001**
BCFA	4	1.55	1.70	0.86	1.11	1.59	*0.593*	*0.087*
	8	1.56	1.77	1.95	2.04	1.97	*0.932*	*0.863*

BCFA= branched-chain fatty acids.

CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8.

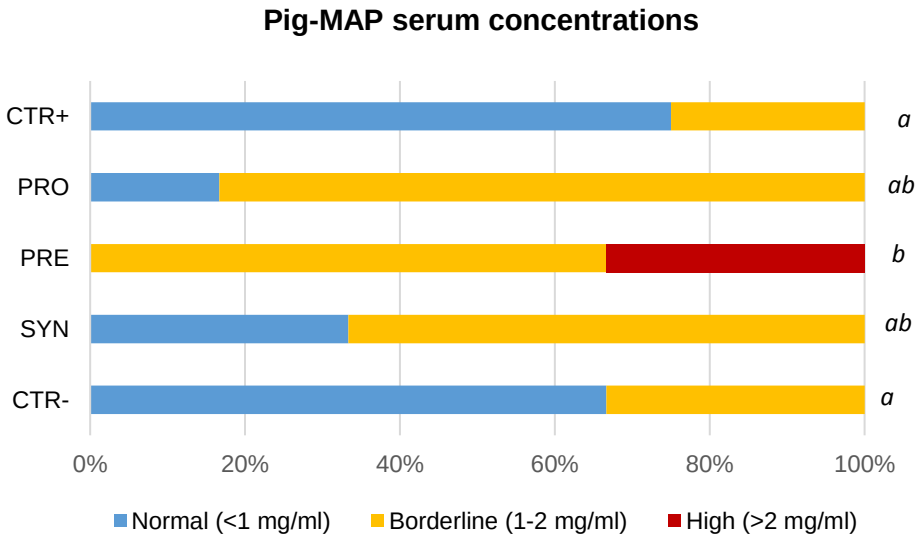
P- values were obtained by an ANOVA using the generalized linear procedure in R software.

With regard to findings in colon, no differences related to the experimental treatments were found in ammonia, lactic acid nor total SCFA concentrations. There were found however at day 4 PI lower pH values for the PRE diet compared to CTR+ ($P = 0.013$). Despite concentration of total SCFA was not modified by the experimental treatments, the molar ratio for the different fermentation products was modified by the treatments. On day 4 PI, PRE group showed a lower percentage of acetic acid than the control groups ($P = 0.004$ and $P = 0.012$ for CTR+ and CTR-, respectively). Moreover the percentage of valeric acid was numerically higher in those animals receiving de prebiotic (PRE and SYN) at day 4 and 8 PI when compared to the rest of the groups, although statistical significances were only found ($P < 0.05$) when compared to CTR+ (PRE at day 4 and 8 PI) and to CTR- (PRE and SYN at day 8 PI). A trend was also found for a lower percentage of BCFA with PRE when compared to PRO at day 4 PI ($P = 0.128$).

5.3.5 Immune response

TNF-α did not respond to the challenge and only a trended to be higher with PRE diet at day 4 PI (106.5, 122.6, 147.8, 140.8 and 111.2 pg/mL for CTR+, PRO, PRE, SYN and CTR- respectively; $P = 0.094$). Pig-MAP concentrations did not adjust to a normal distribution and therefore data were analyzed by frequencies using Fisher's exact test (***Figure 5.4***). In this way statistical differences were found when PRE treatment was compared to both control groups (CTR+ and CTR-) on day 4 PI, showing a higher percentage of animals with levels above those considered normality (>2 mg/mL) (Piñeiro et al., 2009: normal (<1 mg/mL), borderline (1-2 mg/mL) high levels (>2 mg/mL)) ($P = 0.002$ and $P = 0.006$, respectively).

Figure 5.4. Effect of experimental treatments in serum levels of acute-phase protein Pig-MAP in piglets on day 4 after Salmonella oral challenge. Figure represents frequencies for values between a normal (0.3-1 mg/mL) or abnormal (>2 mg/mL) range.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotic; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained using Fisher's Exact Test on R software (Piñeiro et al., 2009: normal (<1 mg/mL), borderline (1-2 mg/mL) high levels (>2 mg/mL)).

5.3.6 Intestinal histological structure

As depicted in ***Table 5.4***, four days after the challenge, the height of ileal villi showed a numerical decrease in all inoculated groups but only for SYN this decreased was significant (P = 0.022). Same pattern was observed for crypt depth that numerically increased with the challenge but only for SYN the change was significant (P < 0.001). Consequently, a significantly reduced villous height/crypt depth ratio was found in all challenged groups (P < 0.001) at day 4 PI, that showed a faster recovery at day 8 PI with PRO diet being the only one not different from CTR+.

Regarding number of mucosal intraepithelial lymphocytes, on day 8 PI animals receiving the PRE diet had higher numbers of these cells when compared to CTR+ and CTR-, showing PRO and SYN

intermediate levels. A similar pattern was seen on day 4 PI, but in this case, not statistically significance was found.

Table 5.4. Effects of treatments on ileal histomorphological parameters on days 4 and 8 post-inoculation.

	PI Day	Treatment					*RSD*	*P-value*
		CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
Villous height (μm)	**4**	282^{a}	229^{ab}	213^{ab}	184^{b}	246^{ab}	*52.5*	*0.033**
	8	259	256	227	227	242	*36.1*	0.320
Crypt depth (μm)	**4**	228^{a}	263^{abc}	249^{ab}	299^{c}	282^{cb}	*26.2*	*<0.001**
	8	245	266	275	267	282	*31.3*	*0.241*
Villous height/crypt depth ratio	**4**	1.24^{a}	0.88^{b}	0.85^{b}	0.64^{b}	0.87^{b}	*0.201*	*<0.001**
	8	1.06^{a}	0.96^{ab}	0.84^{b}	0.84^{b}	0.85^{b}	*0.123*	*0.008**
IEL (Cell no./100 μm)	**4**	0.46	0.68	1.02	0.54	0.57	*0.776*	*0.320*
	8	0.50^{a}	0.67^{ab}	0.98^{b}	0.65^{ab}	0.45^{a}	*0.286*	*0.028**

CTR+ - Non-Inoculated animals receiving placebo; PRO- Inoculated animals receiving the probiotic; PRE- Inoculated animals receiving the prebiotic; SYN- Inoculated animals receiving the synbiotic; CTR- - Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained by an ANOVA using the generalized linear procedure in R software.

5.4 DISCUSSION

One of the main characteristics of probiotics is their ability to antagonize pathogenic bacteria and, thus, improve host health. As summarized by Markowiak and Śliżewska (2017), the capacity to reach this goal is achieved by four different mechanisms such as production of antimicrobial substances (bacteriocins, SCFA, etc.); competition for the adhesion sites in the intestinal epithelium and for nutrients; modulation of the immune system of the host; and blockage of the toxin production by pathogenic bacteria. This experimental trial exhibits that the probiotic compound formed by *Bifidobacterium longum* subsp. *infantis* CECT7210 and *Lactobacillus rhamnosus* HN001 (DR20) exerted a negative impact on *Salmonella* Typhimurium infection as it reduced its faecal shedding seven days

after the challenge. Moreover, it showed to help the animals to recover faster from the intestinal damage produced by the challenge considering the improved villi /crypt ratio registered at day 8 PI with this diet. It is not clear, however, which one of the two strains was the main actor of this action, or if the effect was, precisely, due to the administration of both together. Regarding the strain *Lactobacillus rhamnosus* HN001, previous works have demonstrated effectiveness against *Salmonella* Typhimurium in a murine model by reducing pathogen loads in visceral organs and enhancing the immune system (Gill et al., 2001). This strain has been also considered as responsible of incrementing the blood leucocyte phagocytic activity of mice challenged with *Escherichia coli* O157:H7 (Shu et al., 2002). Other strains, like *Lactobacillus rhamnosus* (GG), have been also shown to reduce presence levels of *Salmonella* Infantis in the jejunum of inoculated pigs mitigating intestinal inflammation response caused by this bacterium (Yang et al., 2017). These outcomes are, therefore, consistent with the improved *Salmonella* clearance found in our study in the PRO group.

Regarding *Bifidobacterium longum* subsp. i*nfantis*, it has been also described to exhibit benefits against infectious agents. Mice pre-treated with the 35624 strain and, in a scenario of a *Salmonella* infection, showed a diminished enterocyte damage and reduced expression of interleukins IL-8 and IL-10 (Sydmons et al., 2012). This down-regulation of pro-inflammatory cytokine was consistent with results reported by O'Mahony et al. (2008) that showed, in mice consuming this probiotic, a decrease in the release of IFN-γ, TNF-α and IL-10 following CD3/CD28 stimulation by a challenge with *Salmonella* Typhimurium or LPS. In addition, other authors also reported with this probiotic more CD4+CD25+ cells in the spleen, associated with pro-inflammatory cytokine inhibition (Maloy et al., 2003; Scully et al., 2013). More specifically, the tested CECT 7210 strain manifested effectiveness against rotavirus infection in mice (Moreno-Muñoz et al., 2011) and, furthermore, it also showed a good outcome against digestive pathogens (*Salmonella* and ETEC) in piglets (Barba-Vidal et al., 2017). In this case, the probiotic reduced pathogen intestinal colonization and modulated the immune response with an increase in the intraepithelial lymphocytes at ileal level. In the present trial, however, we did not find such an effect on IEL numbers with the PRO treatment and neither in TNF-α or Pig-MAP. This suggests

that the combined use of these two strains do not show so much clear immunomodulatory activity as previous studies with the single strains.

Nonetheless, the inclusion of the prebiotic mixture of inulin and oligofructose in the PRE treatment showed changes in immunity parameters lighting up a possible immunomodulatory effect for this additive. PRE diet promoted an increase in the concentration of serum Major Acute-phase Protein (Pig-MAP) and also in the number of ileal IEL. Pig-MAP is a protein that is majorly synthetized in the liver when an acute phase response is occurring, being this an unspecific reaction to tissue damage. We could think that the registered increase in Pig-MAP with the PRE diet was a response to a higher damage induced by the *Salmonella* infection, however, with this treatment, we did not find changes in histomorphological parameters and neither in *Salmonella* loads that actually were shown to be lower compared to CTR-. In this regard, it should be reminded that the expression of Pig-MAP is, in a certain way, conditioned by cytokine IL-6, as its presence induces a higher production (Gonzalez-Ramón et al., 2000). Simultaneously, IL-6 has been shown to be affected by the ingestion of some prebiotic fibres, such inulin and oligofructose. It is described that β2→1-fructans can induce an NF-κB/AP-1 activation (Vogt et al., 2013) which can provoke an up-regulation of the IL-6 gene expression (Liebermann & Baltimore 1990) although results found in the bibliography at this respect are sometimes contradictory. In this regard Shukla et al. (2016) reported an increment of this interleukin in *Giardia*-infected mice treated with inulin meanwhile Marciano at al. (2015) and Zhang et al. (2018) observed a decrease of IL-6 concentration in rats administered inulin (3%) and inulin/oligofructose (10%), respectively. This apparent inconsistency between studies could be due to a differential effect of these substances depending on the dose in which they are given to the animals. In this sense, Song et al. (2018) reported that, in chickens, whereas the use of a low dose of inulin (0.25%) was translated into a reduction of IL-6 gene expression, a high dose (2%) was associated with an increase. However, as we did not determine the levels of this interleukin in our animals, we cannot confirm if the increase observed in Pig-MAP with a 5% inclusion level of inulin + OF, was or not related with a down-regulation of IL-6 expression.

Regarding IEL, these cells are part of the gut-associated lymphoid tissue (GALT) and it is believed that they can have a suppressant activity in the development of oral tolerance (Trejdosiewicz, 1992). There are numerous works supporting the effect of prebiotics (between them, inulin and OF) on the GALT. Some of them are compiled by Schley et al. (2002) that suggest different hypotheses about the way prebiotics can cause these effects, but, in all cases, effects would be mediated by an increase in the lactic acid bacteria population promoted by prebiotics. Mechanisms proposed would include: direct contact of lactic acid bacteria (or bacterial products) with immune cells in the intestine; synthesis of short-chain fatty acids by the microbiota; and modulation of mucin production. However, from our results, it is difficult to confirm any of these hypotheses although it is true that the treatments including the prebiotic were those that modified the molar proportion of SCFA in colon in a greater extent. A complementary analysis of the microbiota composition would have help to clarify the possible role of microbial shifts busting local immune response.

Together with changes in immunity markers, the PRE treatment was also able to limit the colonization of the gut by *Salmonella* considering that this was the only treatment in which *Salmonella* numbers in digesta were always below countable levels. Other authors have also described the potential of non-digestible oligosaccharides to control the presence of this pathogen in the gut by different mechanisms. For example, oligofructose has been shown to reduce *Salmonella enteriditis* counts in the cecum of laying hens (Adhikari et al., 2018) and also to reduce *Salmonella* adhesion to HT-29 cells in a 50% (Wang et al., 2015), effect that was directly related to its concentration. Moreover, Kanjan et al. (2017), testing the efficacy of inulin in a proximal colon model, also observed a competitive exclusion of *Salmonella* due to nutrient limitation and antibacterial metabolites produced by stimulated bifidobacteria. In our case we could hypothesize that any of these mechanisms could be behind the lower colonization levels found with the PRE treatment although we cannot provide evidences.

Despite beneficial effects found in this work against *Salmonella* with the two-strain-probiotic or with the inulin+OF mixture, we could not find any synbiotic behaviour when both strategies were combined. No benefits were found in *Salmonella* prevalence in colon, nor in immunity response compared to CTR-. The negative impact of the challenge on faecal consistency or on the villus/crypt ratio was similar for SYN and CTR-. Despite this, it is remarkable the lower weight gains reported for this treatment during the post-challenge period. Whereas in formula-fed infants it might be desirable a moderate weight gain due to their tendency to a rapid growth curve (Appleton et al., 2018), in challenge animal models it is an unmistakable sign of unwellness. This can be partially explained by the drop in feed intake registered in the post-challenged period, particularly in the acute phase (0-4), that only was significant with the SYN treatment. Nevertheless, the decrease in ADG is higher than the expected for this diminished ingestion suggesting that other factors would be involved. A diminished digestibility or profitability of the diet could have been associated to an increased transit time of digesta with this treatment. In this regard, the inclusion of inulin-type prebiotics has been reported to be responsible of stool softening and increased defecation frequency (Euler et al., 2005; Kapiki et al., 2007) and in our study numerical increases were seen in faecal scores few days after the challenge with the SYN treatment. However, differences were of little magnitude and not significant when compared to the rest of the challenged groups. An impaired digestibility with the SYN diet could also be due to the decreased ileal villous height registered after the challenge, more marked with the SYN treatment in the 0-4 PI period. Nevertheless, like faecal consistency, differences with the rest of challenged groups were not neither significant to fully explain growth impairment. Another explanation for the retarded growth would be a possible overgrowth of the probiotic along the small intestine, boosted by the administration of the prebiotic, competing for nutrients with the host. However, these phenomena would have also taken place during the adaptation week before the challenge, and results obtained do not support this fact. Finally, it should not be discarded that the lower gains registered for this treatment would have been a random effect associated to a limited

number of replicates for assessing effects on performance (N = 6 pens), even more critical in challenging scenarios when a higher residual variability is expected.

What is true is that the probiotic and prebiotic combination tested in the present study did not exhibit any synbiotic effect against the oral *Salmonella* challenge. Previous studies of other authors evaluating the effect of synbiotic combinations have, contrarily, demonstrated positive results. For example, Baffoni et al. (2011) observed a reduction of campylobacter shedding in chicken challenged with this pathogen and treated with *Bifidobacterium longum* subsp. *longum* and fructo-oligosaccharides. Naqid et al. (2015) also were capable of reducing *Salmonella* Typhimurium excretion in infected pigs using a combination of *Lactobacillus plantarum* and lactulose. Different outcomes could be due to the distinct synbiotic combinations tested but also to many other factors like the diets in which are included or the dosage as, for example, the prebiotic was included in those studies in much smaller percentages.

To sum up, the probiotic strains *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001 and the prebiotic mixture of inulin enriched with oligofructose can provide benefits in a *Salmonella* Typhimirium infection scenario. Whereas the two-strain probiotic appears to speed-up the clearance of the pathogen probably by competitive-exclusion mechanisms, the tested prebiotic mixture reduced colonic colonization possibly by modulation of the local and systemic immune response. However, these desirable effects are not synergistic when the two compounds are administered in a synbiotic combination. More studies are required to determine the impact of these treatments on the intestinal microbiota ecosystems and better understand the mechanisms involved.

Chapter 6. Effects of the administration of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001 and their synbiotic combination with galacto-oligosaccharides against enterotoxigenic *Escherichia coli* F4 in an early weaned piglet model

6.1 INTRODUCTION

Diarrhoea is the second infectious cause of death among children younger than five years, only preceded by pneumonia (Liu et al., 2015). The main pathogenic agents responsible for these deceases are viruses, rotavirus and calicivirus, producing a 38% and 13% of the cases respectively, followed by *Escherichia coli*, enteropathogenic (EPEC) and enterotoxigenic (ETEC), contributors to the 12% and 8% of the deaths (Lanata et al., 2013). Although this bacteria forms part of the normal microbiota of the intestine, some strains, like the above mentioned, have developed pathogenic mechanisms to cause intestinal or extraintestinal disease (Clements et al., 2012). Due to the increasing development of antibiotic resistances in this organism (Poirel et al., 2018) new strategies for prevention and therapy need to be implemented. Probiotics are defined by the FAO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". These advantageous effects are strain dependent and can include gastrointestinal disorders prophylaxis and treatment, immune system enhancement, cancer prevention, cholesterol normalization, among others (Kechagia et al., 2013). Regarding the potential of probiotics to fight ETEC diarrhoea, different strains of bifidobacteria and lactobacilli have been proven to have an antagonistic activity against *E. coli* in vitro (Vazquez-Gutierrez et al., 2016; Fijan et al., 2018; Song et al., 2019) with also positive results when tested *in vivo* (Romond et al., 1997; Kumar et al., 2016). Previous works of our group showed that the particular strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 can reduce ileal colonization by ETEC, improving local immune response in a piglet model (Barba-Vidal et al., 2017). Moreover, *Lactobacillus rhamnosus* HN001 has been demonstrated to reduce enterohemorrhagic *Escherichia coli* translocation and to increase IgA concentration and blood leucocyte phagocytic activity in mice (Shu et al., 2001). Bearing this in mind we hypothesized that the efficacy of these combined probiotic bacteria may be even increased by the addition of fermentable carbohydrates that would promote their growth and activity in the gut.

Galacto-oligosaccharides (GOS) are obtained from lactose by transgalactosylation reactions catalyzed by β-galactosidases, resulting in a chain of galactose units with a terminal glucose unit (Tzortzis, 2009). This prebiotic has been associated with increments of lactobacilli and bifidobacteria *in-vitro* and also in human clinical trials (Grimaldi et al., 2016; Canfora et al., 2017; Paganini et al. 2017). Moreover, GOS are considered to be highly similar to oligosaccharides from human milk (HMO) turning it into a prebiotic of election when designing synbiotics with *Bifidobacterium longum* subsp. *infantis* or other probiotic strains isolated from the infant intestine. Particularly for *B. logum* subsp. *infantis* it has been described genomic adaptations for HMO utilization (Sela et al., 2008). Furthermore, GOS has been shown to interfere with *E. coli* adhesion to tissue culture cells (Shoaf et al., 2006). Hence, the objective of this work was to evaluate if combining a multi-strain probiotic composed by *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, with galacto-oligosaccharides could improve its activity against enterotoxigenic *Escherichia coli* F4 in a weaned piglet model.

6.2 MATERIALS AND METHODS

The experiment was performed to evaluate efficacy of a mixture of *Bidifobacterium longum* spp. *infantis* CECT7210 and *Lactobacillus rhamnosus* HN001, galacto-oligosaccharides and their synbiotic combination against an oral challenge with enterotoxigenic *Escherichia coli* F4. The trial was performed at the Experimental Unit of the Universitat Autònoma de Barcelona (UAB) and received prior approval (Permit No. CEEAH: 4026 DMAH: 10118) from the Animal and Human Experimental Ethical Committee of this Institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to European Union Guidelines *(*Directive 2010/63/EU*, European Commission, 2010)*. All efforts were made to minimize animal suffering.

6.2.1 Animals, housing and experimental design

This trial was carried out as Level 2 High-Risk Biosecurity procedures and involved personnel received appropriate training. A total number of 96 male piglets [Landrace x Large White] x Pietrain of 21 (± 2) days of age, weighting 5.04 (± 0.32) kg, were used. All animals came from a high-sanitary-status farm in which mothers were not vaccinated against *Escherichia coli*.

Piglets were transferred to the experimental unit located in the UAB, which consisted of four boxes of eight pens each (thirty-two pens, three animals per pen). Each pen of $2m^2$ consisted of a feeder and water nipple to provide feed and water *ad libitum*. All weaning rooms were equipped with automatic heater, forced ventilation and each pen had an individual heating light.

At arrival, animals were distributed according their initial body weight to ensure a homogeneous average body weight between treatment groups. Trial was conceived as a completely randomized design that included five experimental groups: non-inoculated control (CTR+) and inoculated control (CTR-), probiotic combination (PRO), prebiotic (PRE) and synbiotic (SYN). These last four groups were orally challenged and distributed equally in three of the four rooms, meanwhile one full room was kept for non-inoculated control pigs, being the design hence unbalanced. Each experimental group had six replicates except for the non-challenged groups which had eight replicates instead. In the challenged rooms, probiotic and synbiotic treatments were distributed within four pens on one side of the room, and the control and prebiotic pens were on the other side of the room, separated by a corridor in between to avoid cross-contamination.

6.2.2 Probiotic strains, prebiotic and diets

Tested probiotics were *Bifidobacterium longum* subsp. *infantis* CECT 7210, supplied by Ordesa S.L., and *Lactobacillus rhamnosus* HN001 (Danisco USA Inc.) strains. Both strains were provided lyophilized containing 5 x 10^{10} and 3 x 10^{10} colony-forming units [cfu] per gram of product respectively in a

maltodextrin base. The lyophilised probiotics were daily mixed into the feed for a final dosage of 5.5 x 10^7 and 3.3 x 10^7 cfu/g, respectively. The feed was totally replaced daily. Previous to the trial it was confirmed the viability of the probiotics in the dry feed along the day.

Table 6.1. Ingredient and nutritional composition of the diets.

Ingredients (g/kg FM)	**Control**	**Synbiotic**
Maize	206.7	196.3
Wheat	179.4	170.4
Barley 2 row	169.4	160.9
Extruded soybean	148.6	141.2
Sweet whey-powder (cattle)	99.6	94.7
Fish meal	59.8	56.8
Soy bean meal 44	79.7	75.7
Whey-powder 50% fat	24.9	23.7
Mono-calcium phosphate	6.8	6.4
Calcium carbonate ($CaCO_3$)	3.9	3.7
L-Lysine HCL	5.3	5.1
Vit-Min Premix*	4.0	3.8
Sodium chloride (marine salt)	2.5	2.4
DL-Methionine 99	3.9	3.7
L-Threonine	2.8	2.7
L-Tryptophan	0.8	0.8
L-Valine	2	1.9
Prebiotic	0	50
Analysed composition (g/kg FM)	**Control**	**Synbiotic**
Dry matter	920.2	916.1
Ashes	49.6	48.2
Crude fat	64.1	61.6
Crude protein	203.2	191.1
Neutral detergent fiber	92.7	91.0
Acid detergent fiber	32.7	32.4

***Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D3, 39.9 mg vitamin E, 3 mg vitamin K3, 2 mg vitamin B1, 2.3 mg vitamin B2, 3 mg vitamin B6, 0.025 mg vitamin B12, 20 mg calcium pantothenate, 60 mg nicotinic acid, 0.1 mg biotin, 0.5 mg folic acid, 150 mg Fe, 156 mg Cu, 0.5 mg Co, 120 mg Zn, 49.8 mg Mn, 2 mg I, 0.3 mg Se*

The experimental galacto-oligosaccharides (GOS) was in syrup form and was manually mixed into the diet up to final concentration of 5% (w/w). When mixed in the SYN diet, GOS was previously mixed to the probiotics.

Pre-starter diets were formulated in concordance with the nutrient requirement standards for pigs (NRC, 2012) and given in a mash form. The possible amino acid dilution in the PRE and SYN diets due to incorporation of the prebiotic was compensated by the addition of synthetic amino acids: 0.5 g L-valine, 0.9 g L-lysine HCL, 1.2 g DL-methionine, 0.5 g L-threonine and 0.2 g L-tryptophan per kg of feed. Details for their ingredient and chemical composition is given in ***Table 6.1***.

6.2.3 Escherichia coli strain

The bacterial strain of enterotoxigenic ETEC F4 used was isolated from faeces of 14-week old pigs and provided by the Infectious Diseases Laboratory (Ref. 30/14) of the UAB. This strain presented the following virulence factors: F4ab, F4ac, LT, STb and EAST1 and was negative for K99, F6, F18, F41, STa, VT1, VT2 y EAE. The oral inoculum was prepared by a 12-hour overnight incubation at 37°C in Brain Heart Infusion broth (Oxoid; Hampshire, England) with slow agitation (250 rpm) in an orbital incubator. A total volume of 6 mL from the culture was given directly to the animals which final concentration was 1 x 10^9cfu/mL. Inoculum concentrations were also determined before the inoculation by McFarland standards and were plated in Luria Agar (LA) (in-house made: tryptase, yeast extract, NaCl, agar, Oxoid; Hampshire, UK) the same day for manual plate counting.

6.2.4 Experimental procedure

The experiment had a duration of fifteen days. After an adaptation period of 7 days, animals were challenged orally with the pathogen. One animal of each pen was euthanized on days 4 and 8 post-inoculation (PI).

Following the week of adaptation, the inoculum containing the pathogenic bacteria culture was given to the challenged groups via oral route: one 6 mL dose (6 x 10^9 cfu) of enterotoxigenic *Escherichia coli* F4. The same amount of sterile broth was administered to non-challenged piglets. For the purpose that the animals had a full stomach in the moment of the oral challenge, pigs were starved for a period of 12 hours and feed was reintroduced 30 minutes before the inoculation.

From the challenge onwards, animals were checked daily to determine clinical signs and evaluate their post-inoculation status (i.e, dehydration, anorexia, apathy, general behaviour and faecal score), always by the same person. Faecal score was measured using a scale: 1 = solid and cloddy, 2 =soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Rectal temperature was assessed with a digital thermometer (Accuvet, Sanchung City, Taiwan) on days 1 and 2 PI.

Performance of animals was monitored: individual body weight was registered at arrival and on days 0,4 and 8 PI and feed intake was monitored daily, associated to the regular feed change to maintain probiotic viability. The average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F) were calculated by pen. Mortality rate was also registered and no antibiotic treatment was given to animals.

For microbiological analysis, faecal samples were collected aseptically after spontaneous defecation or by digital stimulation at arrival and on the day of the inoculation (0 PI). Faecal samples were obtained from the largest animal of each pen (N = 32).

On days 4 and 8 PI one pig per pen was euthanized. On day 4 PI, the selected animal was the one with the medium initial BW, while on day 8 PI, the chosen piglet was the heaviest one of each pen. Animals were euthanized and sequentially sampled during the morning of each day (between 8:00 and 13:00 hours). An intramuscular injection containing 20 mg/kg of ketamine (Ketamidor; Wels, Austria) and 2 mg/kg of xylazine (Xilagesic; Les Franqueses del Vallès, Spain) was given to the animals to induce deep sedation. Prior to injection of euthanasic drug, 10 mL sample blood was taken from each animal via venipuncture of the cranial cava vein using 10 mL blood collection tubes without anticoagulant

(Aquisel; Madrid, Spain). Right after blood sampling, pigs were administered a lethal dose injection of sodium pentobarbital intravenously (140 mg/kg BW; Euthasol, Le Vet B.V.; Oudewater, Netherlands). Once dead, animals were bled, abdomen immediately open and gastrointestinal tract extracted.

A faecal sample taken from rectum was kept in ice and used for traditional microbiology, being analysed within the first 4 hours.

Afterwards, digesta of ileum and proximal colon was collected and homogenized prior to pH determination with a pH-meter calibrated on each day of use (Crison 52–32 electrode, Net Interlab; Barcelona, Spain). Subsamples of colonic and ileal digesta were preserved for different analysis. One aliquot of colonic content was kept frozen at −80°C for ETEC F4 quantification by qPCR. A set of ileal and colonic digesta samples were conserved frozen at -20°C in H_2SO_4 solution (3 mL of content plus 3 mL of 0.2 N H_2SO_4) for ammonia (NH_3) determination and am additional set (~10 g) was also frozen (−20°C) until analysis for short-chain fatty acids (SCFA) and lactic acid was performed.

Five-cm-long sections of distal ileum were collected from each animal, washed thoroughly with sterile PBS, opened longitudinally and scraped with a microscopy glass slide to obtain the mucosa scraping to determine the number of enterobacteria and coliforms attached to the intestinal mucosa.

For the histological study, 1-cm sections from the ileum were removed, opened longitudinally, washed thoroughly and carefully with 4% formaldehyde solution (Panreac; Castellar del Vallès, Spain) before fixing them by immersion in the same solution.

Blood samples were centrifuged (3,000 × g for 15 min at 4°C) after blood coagulation and the serum obtained, which was stored frozen at -20°C.

6.2.5 Analytical procedures

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

International, 1995). Neutral detergent fibre and acid-detergent fibre were determined according to the method of Van Soest et al. (1991).

For enterobacteria and coliform counts, samples were serially diluted in Lactate Ringer Solution (Sigma-Aldrich; Madrid, Spain) and proper dilutions seeded in MacConkey agar (Oxoid; Madrid, Spain) and eosin methylene blue agar (Scharlab; Barcelona, Spain). Plaques were incubated for 24h at 37°C and colonies were manually counted. Presence of ETEC F4 in colonic digesta and ileal scrapings was determined by real-time PCR. To extract the DNA from these samples the commercial QIAmp DNA stool minikit (Qiagen; West Sussex, United Kingdom) was used. After the process, several aliquots of DNA eluted in Qiagen buffer AE (total volume; 200 µL) were stored frozen at
-80°C. A qPCR targeting the gene coding the F4 fimbria of *E. coli,* using the SYBR green dye, was performed according to the procedure described by Hermes et al. (2013). The qPCR results were scored in to five levels according to the number of gene copies per gram of fresh matter (FM). Scores were defined as following: negative = less than 4 logarithmic units of gene copies per gram of fresh matter; low = 4 – 5.5 logarithmic units of gene copies per gram of fresh matter; medium = 5.5 – 7 logarithmic units of gene copies per gram of fresh matter; high = 7 – 8.5 logarithmic units of gene copies per gram of fresh matter and very high = more than 8.5 logarithmic units of gene copies per gram of fresh matter.

Short-chain fatty acids and lactic acid analyses were performed by gas liquid chromatography, after the samples underwent acid-base treatment prior an ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide (MBTSTFA) plus 1% tert-butyldimethylchlorosilane (TBDMCS) agent, using the method of Richardson et al. (1989), modified by Jensen et al. (1995).

Ammonia concentrations were assed using a gas-sensitive electrode (Hatch Co.; Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain) and following a procedure described by Hermes et al. (2009), which was adapted from Diebold et al.

(2004). Samples were diluted (1:2) in 0.16 M NaOH and, after homogenization, were centrifuged at 1500 x g for 10 minutes. Once the ammonia was released, it was measured in the supernatants as a change in voltage in mV.

Serum concentrations of Tumor Necrosis Factor-α (TNF-α) were determined by Quantikine Porcine TNF-α kits (R&D Systems; Minneapolis, USA) and pig major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A.; Segovia, Spain) according to the manufacturer's instructions.

For histological study, tissue samples were dehydrated and embedded in paraffin wax, sectioned 4-µm thickness and stained with haematoxylin and eosin. Measurements of ten different villus-crypt complexes per sample were considered including counting of intraepithelial lymphocytes (IEL), Goblet cells (GC) and number of mitosis of each complex. Analyses were performed with a light microscope (BHS, Olympus; Barcelona Spain) following the procedure described by Nofrarías et al. (2006).

For Mucin4 (MUC4) gene polymorphism determination, hair containing follicles were collected from 81 pigs. DNA was extracted following the procedure described by Luise et al. (2019). For genotypification, a Restriction Fragment Length Polymorphism PCR (PCR-RFLP) was performed following the guidelines described by Jørgensen et al. (2003). Pigs were classified in two groups: susceptible allele carriers if they had $MUC4^{GG}$ or $MUC4^{CG}$ genotype; or non-carrier animals if they were $MUC4^{CC}$homozygotes.

6.2.6 Statistical analysis

Results are expressed as means with their standard errors unless otherwise stated. Microbiological counts were transformed [log] for analysis. A two-way ANOVA was used to examine the effect of the five experimental treatments and the MUC4 gene polymorphism with the following model:

$Yijk = \mu + treat_i + MUC4_j + treat*MUC4_{ij} + \varepsilon_{ij}$

Where Y_{ijk} relates to each observation of the outcome variable, μ is the global mean, $treat_i$ is the main effect of treatment, $MUC4_j$ is the main effect of MUC4 gene polymorphism and $treat*MUC4_{ij}$ corresponds to the interaction between treatment and MUC4 gene polymorphism. Finally, ε_{ij} is the experimental error term. Regarding MUC4 effect and interaction term, they were removed from the model when found to be not significant.

All analysis were done using a generalized linear model (GLM) or mixed effects model of R software. Fisher´s exact test was used when analysing frequencies. When treatment effects were established, means comparison was adjusted by Tukey–Kramer test. The considered experimental unit was the pen. The α-level used for the determination of significance was P = 0.05. The statistical trend was considered for P > 0.05 and < 0.10. Version 3.5.1 of R statistical analysis software was used (R Development Core Team; New Jersey, USA).

6.3 RESULTS

The course of the experiment went as expected without any remarkable incidence. Feed intake was within normal values guaranteeing that piglets received, the planned doses of the probiotics and the prebiotic in the corresponding treatments.

Following the oral challenge with the pathogenic bacteria, animals developed moderate clinical signs of diarrhoea that began to resolve spontaneously at the end of the study. Eight spontaneous casualties occurred (3 CTR-, 3 PRE, 1 PRO and 1 SYN groups) and no humanitarian euthanasia was required.

Regarding the analysis of MUC4 polymorphism the distribution of animals between treatments was the following: CTR+: 14 non-carriers / 6 carriers; PRO: 10non-carriers/ 7 carriers; PRE: 8 non-carriers/ 7 carriers; SYN: 7 non-carriers/ 10 carriers; CTR-: 7 non-carriers/ 5 carriers.

6.3.1 Performance parameters

Results obtained for body weight (BW), average daily feed intake (ADFI) and average daily weight gain (ADG) are summarized in ***Table 6.2***.

During the adaptation week animals of all experimental groups had a similar feed intake. However, after the pathogen inoculation, all challenged groups showed numerical reductions in intake, although differences only reached statistical significance in groups CTR- and SYN (P = 0.002).

Table 6.2.Effects of experimental treatments on feed intake and weight gain.

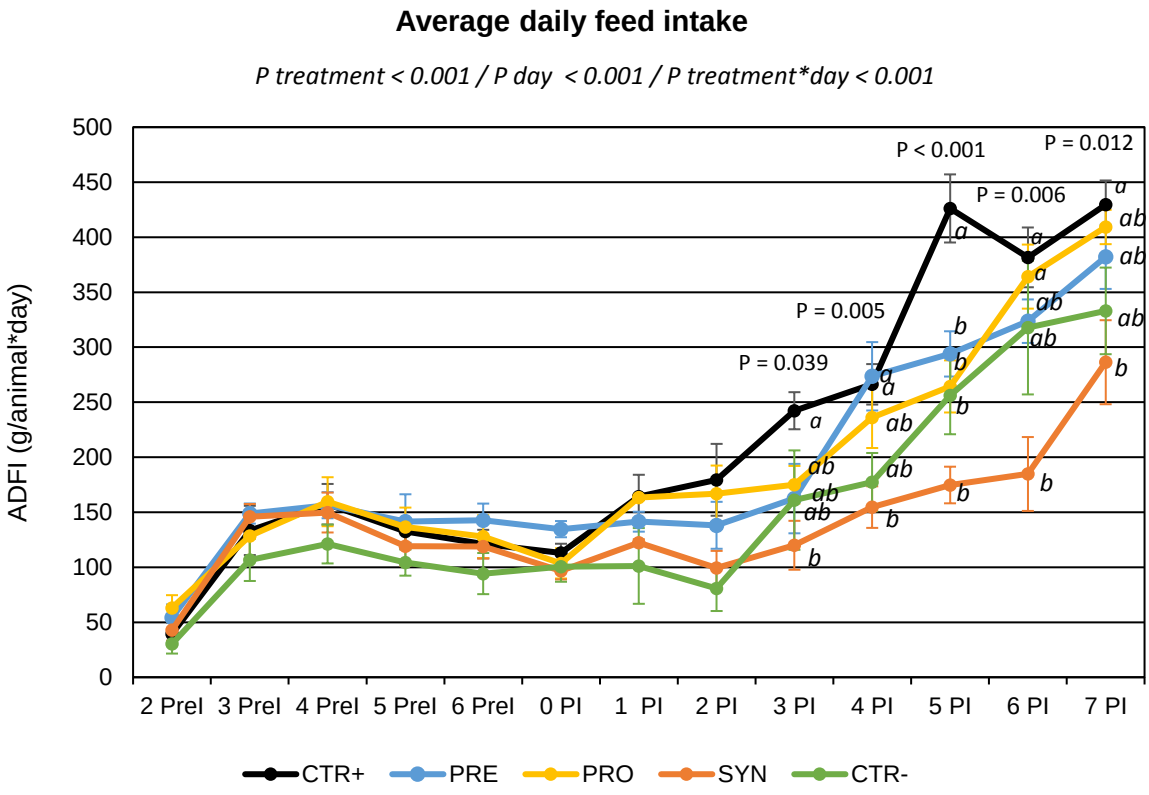
	Treatments					***RSD***	***P-value***
	CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
BW (kg)							
Initial	5.02	5.09	5.01	5.05	5.03	*0.142*	*0.880*
Final	7.24	6.89	7.25	6.48	6.58	*0.726*	*0.223*
ADFI (g)							
Adaptation	105.1	111.8	118.8	102.3	83.9	*27.86*	*0.285*
0-4 PI	213.0^{a}	185.3^{ab}	179.0^{ab}	124.1^{b}	130.1^{b}	*42.35*	*0.002**
4-8 PI	404.8^{a}	350.5^{a}	330.9^{ab}	207.7^{b}	306.2^{ab}	*70.83*	*<0.001**
ADG (g)							
Adaptation	40.7	46.2	64.6	52.0	34.4	*38.93*	*0.707*
0-4 PI	155.4^{a}	74.2^{ab}	26.7^{b}	-13.7^{b}	-44.7^{b}	*71.50*	*<0.001**
4-8 PI	317.6^{a}	329.3^{a}	333.0^{a}	198.5^{b}	282.5^{b}	*82.15*	*0.047**
G:F							
Adaptation	0.34	0.04	0.36	0.54	0.50	*0.665*	*0.727*
PI	0.76	0.52	0.75	0.54	0.44	*0.328*	*0.300*
Total	0.68	0.5	0.65	0.59	0.51	*0.185*	*0.298*

Body weight (BW) (kg), average daily feed intake (ADFI) (g/day), average daily gain (ADG) (g/day) and feed efficiency (gain:feed ratio, G:F) for the pre-inoculation period (adaptation: days 1-8), acute post-inoculation period (0-4 PI: days 8-11), chronic post-inoculation period (4-8 PI: days 11-15), total post-inoculation period (PI: days 8 – 15) and whole trial (total: days 1-15). CTR+: Non-inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N= 6 for all groups except for non-challenged animals, N= 8.

Similarly, average daily gain (ADG) was not different between treatments along the first week but decreased after the challenge. During the first period after the inoculation (0-4 PI) all challenged groups showed significant decreases except for the animals receiving the PRO treatment in which numerical reductions weight gains did not reach statistical significance. In the second phase (4-8 PI), animals belonging to PRO and PRE groups showed a fast recovery of weight gain reaching similar levels (even numerically higher) than non-inoculated piglets. However, animals receiving SYN diet showed a tendency towards lower weight gains compared to CTR+ (P = 0.083).

Figure 6.1 shows the evolution of feed intake along the experimental trial.

Figure 6.1. Evolution of feed consumption for the experimental groups along the entire experimental period.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. Bars correspond to standard error.

Statistical differences between groups were found from day 3 onwards. SYN group was clearly the most affected being the treatment that consistently showed the lowest intakes, being significantly different from CTR+ at days 3, 4, 5, 7 and 8 PI.

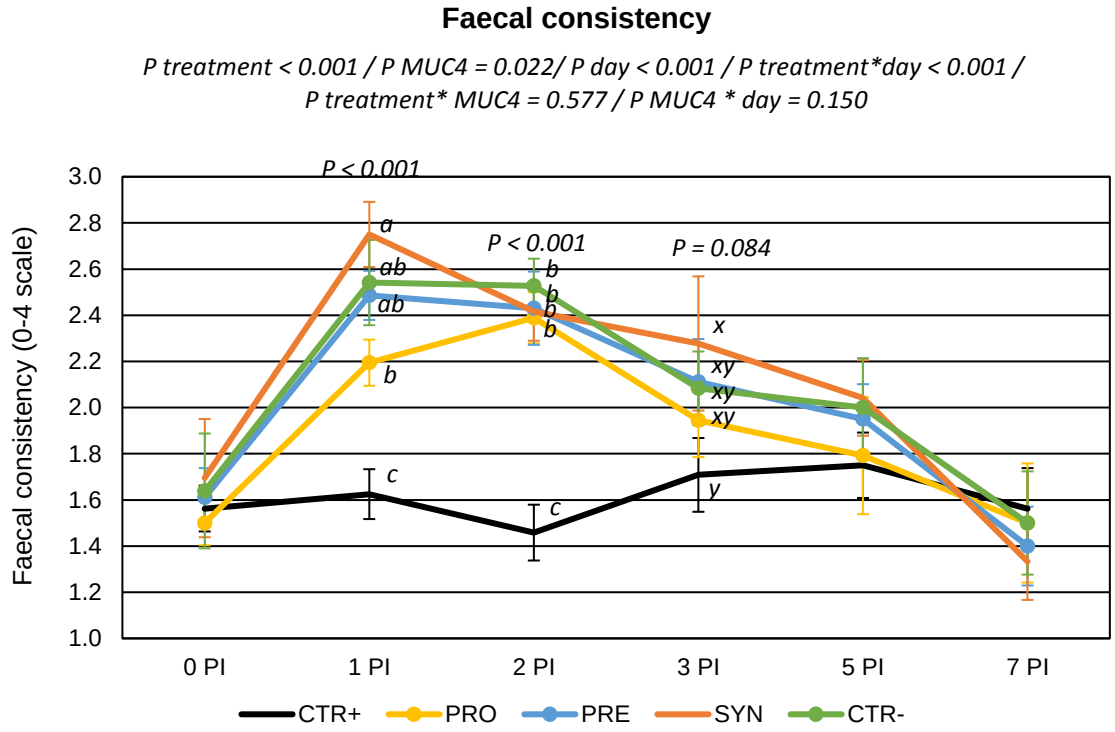
6.3.2 Clinical signs

Evolution of faecal consistency along the post-infection period is represented in ***Figure 6.2***.

ETEC F4-challenged animals showed higher faecal scores (lower consistency of faeces) immediately after the inoculation, being these differences significant on days 1 and 2 PI and recovering from day 3 PI onwards. MUC4 polymorphism played an important role on faecal consistency, having animals carrying the susceptible allele worse scale numbers than those that do not on days 1 and 2 PI (2.03 vs 2.47; P = 0.009 and 1.95 vs 2.34; P = 0.013 for not carriers and carriers on days 1 and 2 PI, respectively). Between challenged groups it was possible to find differences one day after the challenge, showing the PRO group the better consistency and SYN the worst (P < 0.001). On day 3 PI, an interaction was found between the experimental treatments and the MUC4 gen. Whereas no difference between treatments was observed among non-carriers pigs, in carriers animals, SYN group showed more liquid faecal consistency compared to CTR+ (1.33^{b}, 1.84^{ab}, 2.43^{ab}, 2.80^{a} and 2.60^{ab} for CTR+, PRO, PRE, SYN and CTR- respectively; P = 0.010).

Rectal temperature was not affected by the challenge, experimental diets nor MUC4 gene and were within normal values (not fever).

Figure 6.2. Evolution of average faecal scores for the different experimental groups in the post-inoculation period.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. MUC4 represent the effect of polymorphism of MUC4 gene.
Bars correspond to standard error.

6.3.3 Microbiological analysis

Table 6.3 shows enterobacteria and coliforms plate counts from faecal samples taken at the arrival of the animals, before the oral challenge and at days 4 and 8 PI. The table also includes plate counts from ileal scrapings sampled at day 4 and 8 PI.

As expected, there were no differences between experimental groups in plate counts the day of arrival. However, after one week receiving the diets, animals fed SYN showed lower values of faecal enterobacteria (P = 0.001) or coliforms (P < 0.001) than animals receiving the probiotics or prebiotic

separately. On day 4 PI, SYN was the only group with lower counts of faecal coliforms compared to CTR- (P = 0.023). On day 8 PI only PRE and CTR- groups remained with higher plate counts compared to non-challenged pigs (P < 0.001 for both groups).

Table 6.3. Effects of experimental treatments on enterobacteria and coliform counts in faecal samples and ileal scrapings.

	Treatments					**RSD**	***P-value***
	CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
Enterobacteria (log cfu/g FM)							
Faeces							
Arrival	9.35	9.76	8.10	8.91	7.87	1.259	*0.242*
Day 0 PI	8.82^{ab}	10.93^{a}	10.76^{a}	7.55^{b}	10.12^{ab}	1.685	*0.001**
Day 4 PI	6.41	6.63	6.92	6.29	8.52	1.501	*0.043**
Day 8 PI	5.40^{b}	5.34^{b}	6.85^{a}	5.79^{b}	6.75^{a}	0.441	*<0.001**
Ileal scrapings							
Day 4 PI	7.59	7.61	7.54	7.46	7.41	*0.201*	0.393
Day 8 PI	6.62^{b}	6.62^{b}	7.62^{a}	7.59^{a}	7.24^{ab}	*0.423*	<0.001*
Total coliforms (log cfu/g FM)							
Faeces							
Arrival	9.18	9.70	8.09	8.81	7.87	1.325	*0.335*
Day 0 PI	8.34^{b}	10.83^{a}	10.66^{a}	5.86^{b}	9.72^{ab}	1.248	*<0.001**
Day 4 PI	6.35^{ab}	6.31^{ab}	6.82^{ab}	5.90^{b}	8.48^{a}	1.380	*0.008**
Day 8 PI	5.31^{b}	5.22^{b}	6.83^{a}	5.71^{b}	6.62^{a}	0.441	*<0.001**
Ileal scrapings							
Day 4 PI	7.48	7.46	7.46	7.42	7.31	*0.222*	*0.642*
Day 8 PI	5.90^{c}	6.55^{bc}	7.18^{ab}	7.65^{a}	7.29^{ab}	*0.576*	*<0.001**

CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained by an ANOVA using the generalized linear procedure in R software.

MUC4 gene also had an impact on faecal counts of enterobacteria and coliforms on day 4 PI, having non-carrier animals lower counts than carriers (enterobacteria 6.27 vs 7.44 cfu/g; P MUC4 = 0.015; coliforms 6.23 vs 7.22 cfu/g; P MUC4 = 0.010). Interaction was not found significant except for faecal

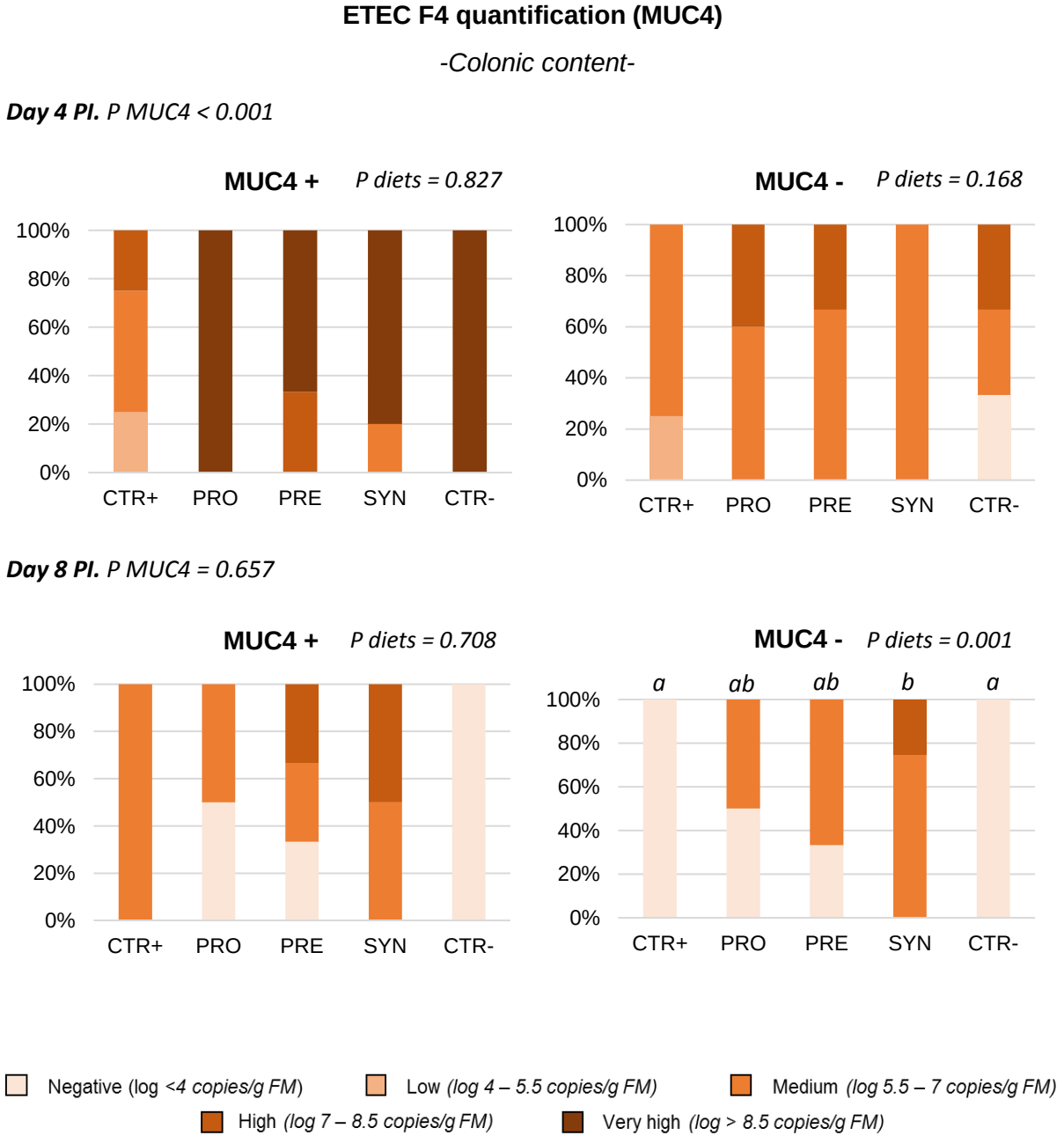
enterobacteria before the challenge, when differences between diets were found only in non-carrier pigs showing the lowest number of enterobacteria in the animals receiving the synbiotic (9.17, 10.86, 10.77, 5.92, 10.05 cfu/g for CTR+, PRO, PRE, SYN and CTR-, respectively; P interaction = 0.003).

Regarding ileal scrapings, differences between treatments were only seen on day 8 PI ($P < 0.001$) being the PRO treatment the only not different from CTR+.

Together to plate count, ETEC F4 was also specifically quantified by real-time PCR in colonic digesta and ileal scrapings. MUC4 polymorphism had a clear impact on the prevalence of ETEC F4 in colonic digesta at day 4 PI with a much clearer effect of the challenge in carrier animals (P MUC4 < 0.001) (***Figure 6.3***). At day 8 PI an interaction MUC4*treat was found with a higher prevalence of the pathogen with the SYN diet but only in the non-carrier animals. No significant impact of the MUC4 polymorphism was found in ileal scrapings counts at days 4 nor 8 PI.

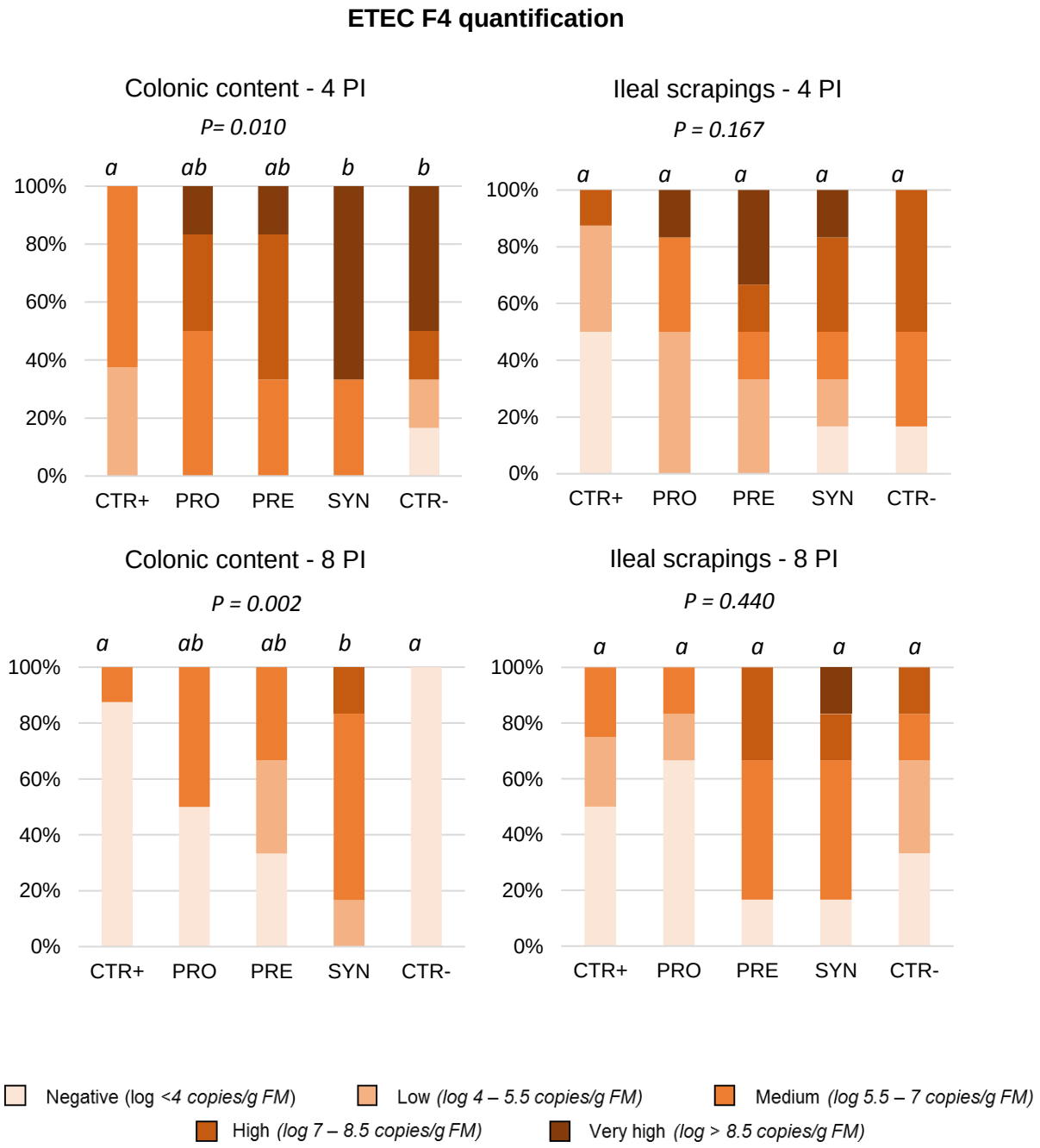
When considering all the animals, regardless of MUC4 polymorphism (***Figure 6.4***), no differences were found in ETEC F4 in ileal scrapings. Regarding colonic digesta, on day 4 PI, SYN group showed a greater prevalence of ETEC F4 compared to CTR+ and similar to CTR-; PRO and PRE showed intermediate levels ($P = 0.010$). On day 8 PI, SYN was the only group that maintained animals with high excretion levels (SYN vs. CTR+; $P = 0.002$), while animals from the CTR- recovered from the challenge turning negative, PRO and PRE showed intermediate levels.

Figure 6.3. Effect of MUC4 polymorphism on the percentage of animals with different levels of ETEC F4 counts in colonic digesta on days 4 and 8 post-inoculation. Different animals were sampled on day 4 PI and 8 PI.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals. N = 8. P- values were obtained using Fisher's Exact Test on R software.

Figure 6.4. Effect of experimental treatments on the percentage of animals with different levels of ETEC F4 counts in colonic digesta and ileal scrapings on days 4 and 8 post-inoculation. Different animals were sampled on day 4 PI and 8 PI.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N= 6 for all groups except for non-challenged animals, N= 8. P- values were obtained using Fisher's Exact Test on R software.

6.3.4 Intestinal fermentation

Table 6.4 shows values of intestinal pH, ammonia, lactic acid and short chain fatty acid concentrations in ileal and colonic content for the different experimental treatments.

At ileal level it was observed the highest pH values in animals receiving PRE group at day 4 PI, being different from CTR+ and PRO, but not from the other challenged groups. Furthermore, SYN showed numbers similar to CTR+ ($P < 0.001$). However, at day 8 PI, it was observed the opposite trend being the PRE treatment that with the lowest pH values ($P = 0.044$). Ammonia concentrations diminished at day 8 PI in PRO, PRE and SYN compared to animals that received the control diet regardless if they were challenged or not ($P = 0.044$).

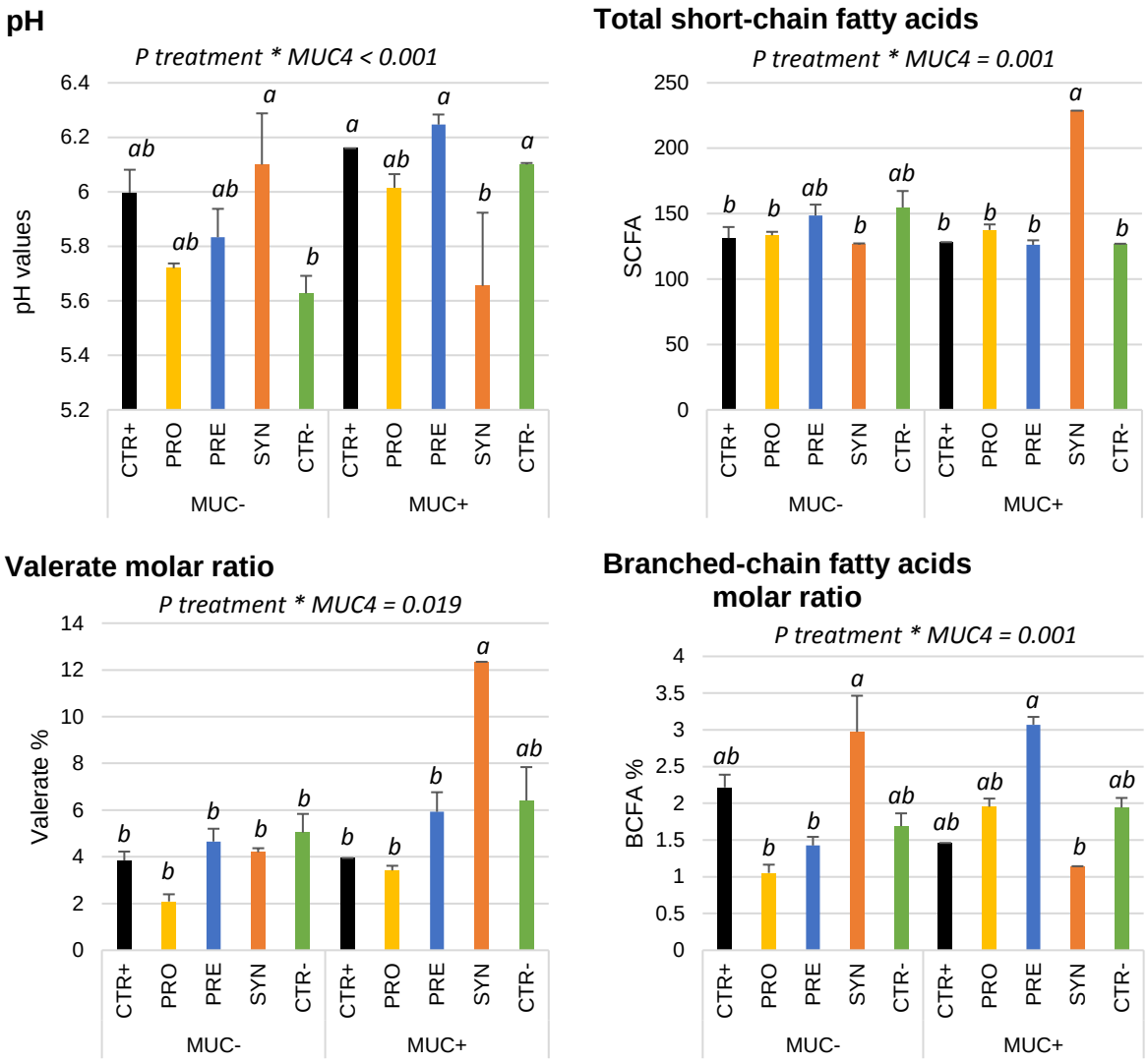
At colonic level, pH was numerically increased at day 4 PI in all challenged groups although differences with CTR+ were only significant for SYN and CTR- (P diet = 0.041). In addition PRE was the only treatment with reduced ammonia values at day 4 PI compared to CTR+ (P diet = 0.007). No differences were seen in lactic acid concentration. Regarding total short chain fatty acids, it was observed a trend for a decreased concentration in SYN and CTR- treatments ($P = 0.085$) at day 4 PI but not at day 8 PI. At day 8 PI it was found a marked increase in lactic acid concentration with CTR- although differences with other treatments did not reach statistical significance ($P = 0.119$). The molar ratios of different SCFA showed some changes related to the experimental treatments. On day 4 PI it could be seen a numerical trend for an increased acetic acid percentage with the challenge (P diet = 0.057) and at day 8 PI, the PRO group showed higher acetic acid concentration compared to the rest of inoculated piglets ($P = 0.004$). PRO showed lower percentages of branched chain fatty acids compared to SYN group ($P = 0.049$) and valeric acid was reduced in PRO, PRE and SYN compared to CTR+ at day 4 PI (P diet = 0.047).

Table 6.4. Effects of experimental treatments on ileal and colonic fermentation. In this table are included values corresponding to pH, ammonia concentration (NH_3) (mmol/L of FM), lactic acid (mmol/kg of FM), total short-chain fatty acids (SCFA) (mmol/kg of FM) and molar ratio of these SFCA.

	Treatment						RSD	P-value
	PI Day	CTR+	PRO	PRE	SYN	CTR-		
ILEUM								
pH	4	6.49^{c}	6.58^{bc}	6.94^{a}	6.70^{abc}	6.86^{ab}	*0.169*	*<0.001**
	8	6.53	6.52	6.41	6.56	6.57	*0.110*	*0.044**
NH_3 *(mmol/L)*	4	1.99	1.86	0.99	1.84	1.53	*0.973*	*0.390*
	8	2.69^{a}	1.38^{b}	1.52^{b}	1.59^{b}	2.04^{ab}	*0.835*	*0.044**
Lactic acid *(mmol/kg)*	4	35.8	18.1	5.6	8.3	16.7	*27.48*	*0.368*
	8	19.8	24.5	27.6	23.1	12.5	*19.60*	*0.742*
SCFA *(mmol/kg)*	4	3.85	3.59	2.33	4.71	1.99	*2.306*	*0.288*
	8	2.67	3.07	2.59	3.37	3.47	*1.662*	*0.849*
COLON								
pH	4	6.04^{c}	6.11^{bc}	6.35^{abc}	6.66^{a}	6.54^{ab}	*0.389*	*0.041**
	8	6.02	5.92	6.04	5.95	5.79	*0.307*	*0.149*
NH_3 *(mmol/L)*	4	16.4^{a}	10.6^{ab}	7.2^{b}	10.6^{ab}	12.7^{ab}	*4.86*	*0.007**
	8	8.84	6.63	7.15	9.02	9.16	*3.01*	*0.477*
Lactic acid *(mmol/kg)*	4	2.57	0.56	2.71	0.26	0.27	*3.542*	*0.550*
	8	0.85	0.43	0.10	0.00	7.27	*4.877*	*0.119*
SCFA *(mmol/kg)*	4	124.1	105.7	100.6	75.5	78.1	*33.72*	*0.085*
	8	130.9	136.2	137.3	127.4	145.4	*32.07*	*0.442*
Molar ratio of SCFA (%)								
Acetic	4	50.7	58.6	59.6	52.8	55.4	*5.99*	*0.057*
	8	50.3^{ab}	55.3^{a}	48.7^{b}	46.4^{b}	48.7^{b}	*3.44*	*0.004**
Propionic	4	26.6	25.1	26.7	28.5	28.1	*3.68*	*0.518*
	8	26.1	25.9	29.7	28.2	25.8	*3.76*	*0.317*
Butyric	4	16.1	12.9	10.2	12.6	11.1	*5.48*	*0.332*
	8	17.6	14.2	14.1	16.7	18.2	*3.33*	*0.118*
Valeric	4	4.20^{a}	1.70^{b}	1.72^{b}	2.30^{b}	2.37^{ab}	*1.665*	*0.047**
	8	3.85^{ab}	2.97^{b}	5.28^{ab}	6.23^{a}	5.49^{ab}	*2.018*	*0.011**
BCFA	4	2.30^{ab}	1.70^{b}	1.74^{ab}	3.73^{a}	2.95^{ab}	*1.119*	*0.031**
	8	2.12	1.65	2.24	2.51	1.77	*0.743*	*0.097*

CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8.
P- values were obtained by an ANOVA using the generalized linear procedure in R software.
BCFA= branched-chain fatty acids.

Figure 6.5. Interactions between MUC4 gene polymorphism and diets on pH values, total short-chain fatty acids, valerate molar ratio and branched-chain fatty acids molar ratio on day 8 post-inoculation.



CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N= 6 for all groups except for non-challenged animals, N = 8. Bars correspond to standard error.

These differences promoted by diets on the fermentation profile were however influenced by the MUC4 polymorphisms when analysed at day 8 PI. Interactions found are shown in ***Figure 6.5***. SYN treated animals showed lower colonic pH and higher amount of total SCFA in colon but only in the carrier pigs (P interaction < 0.001). Regarding valerate, SYN group presented a sharped increased in molar ratio but only in the carrier pigs (P interaction = 0.019) and BCFA molar ratio was increased by

SYN in non-carrier animals but by PRE in carrier animals. Finally, resistant-genotyped piglets showed increased ammonia levels in colon on day 4 PI (13.31 vs 9.79 mmol/L; P MUC4 = 0.006) but no interaction was found with treatments.

6.3.5 Immune response

Values corresponding to serum levels of the pro-inflammatory cytokine TNF-α and acute phase protein Pig-MAP are shown in ***Table 6.5***.

Concentration of TNF-α, on day 4 PI, was higher in the SYN treatment when compared to CTR- and PRE, differences did not reach significance with the rest of the groups (P diet = 0.023). At day 8 PI SYN group showed the lowest values (P = 0.050).

Table 6.5. Effect of experimental treatments in serum levels of acute-phase protein Pig-MAP and TNF-α.

	Treatment					***RSD***	***P-value***
	CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
TNF-α (pg/mL)							
Day 4 PI	85.7^{ab}	97.6^{ab}	75.3^{b}	118.2^{a}	76.7^{b}	*23.83*	*0.023**
Day 8 PI	83.6	74.3	83.1	70.2	93.8	*13.68*	*0.050*
Pig-Map (mg/mL)							
Day 4 PI	0.59^{b}	0.76^{b}	0.72^{b}	1.49^{ab}	2.42^{a}	*0.986*	*0.013**
Day 8 PI	0.51^{b}	0.57^{b}	0.56^{b}	2.43^{a}	0.62^{b}	*0.752*	*0.003**

CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained by an ANOVA using the generalized linear procedure in R software.

On day 4 PI Pig-MAP values were higher in CTR- compared to CTR+. Animals treated with PRO and PRE had levels closer to the CTR+ group and SYN treatment showed intermediate levels (P diet = 0.013).

On day 8 PI, Pig-MAP concentration was clearly higher in animals belonging to SYN group whereas the rest of challenged groups resembled levels of CTR+ group (P diet = 0.003).

No effects due to MUC4 gene polymorphism were observed.

6.3.6 Intestinal histological structure

Effects of the experimental treatments on ileal villous height, crypt depth and mitosis number are shown in ***Table 6.6***.

Table 6.6. Effects of treatments on ileal histomorphological parameters on days 4 and 8 post-inoculation.

	PI Day	Treatment					*RSD*	*P-value*
		CTR+	**PRO**	**PRE**	**SYN**	**CTR-**		
Villous height (μm)	**4**	311.8^{a}	241.4^{b}	246.8^{b}	245.3^{b}	220.9^{b}	*34.76*	*<0.001**
	8	336.8^{a}	291.8^{ab}	272.1^{b}	269.0^{b}	266.5^{b}	*28.59*	<0.001*
Crypt depth (μm)	**4**	272.4^{ab}	271.9^{ab}	282.5^{a}	251.6^{ab}	241.8^{b}	*22.19*	*0.020**
	8	271.3	274.0	271.3	250.5	278.3	*36.17*	*0.710*
Villous height/ crypt depth ratio	**4**	1.14^{a}	0.88^{b}	0.87^{b}	0.97^{ab}	0.91^{b}	*0.114*	*<0.001**
	8	1.24^{a}	1.06^{b}	1.00^{b}	1.09^{ab}	0.96^{b}	*0.099*	*<0.001**
IEL (Cell no./100 μm)	**4**	0.37	0.50	0.52	0.59	0.38	*0.207*	*0.285*
	8	0.69	0.56	0.66	0.60	0.71	*0.293*	*0.885*
GC (Cell no./100 μm)	**4**	2.38	1.96	2.39	2.95	2.08	*1.031*	*0.513*
	8	1.94	1.77	2.28	2.07	2.76	*0.790*	*0.250*
Mitosis (Cell no./100 μm)	**4**	0.16^{a}	0.24^{ab}	0.32^{b}	0.30^{b}	0.31^{b}	*0.103*	*0.036**
	8	0.23	0.22	0.27	0.22	0.26	*0.098*	*0.896*

CTR+: Non-Inoculated animals receiving placebo; PRO: Inoculated animals receiving the probiotics; PRE: Inoculated animals receiving the prebiotic; SYN: Inoculated animals receiving the synbiotic; CTR-: Inoculated animals receiving placebo. N = 6 for all groups except for non-challenged animals, N = 8. P- values were obtained by an ANOVA using the generalized linear procedure in R software.

The impact of the ETEC F4 oral challenge was evidenced on the structure of the ileal epithelium. On day 4 PI all challenged groups showed a reduction of the villous height (P < 0.001), which was still

significant on day 8 PI except for PRO group (P diet < 0.001). When analysing crypt depth on day 4 PI, CTR- had the lowest values whereas PRE, the highest, reaching the difference between the two treatments the statistical significance (P = 0.028). According to this, the ratio villous height/crypt depth was modified by the inoculation on both sampling days (P < 0.001), with reductions of the ratio in all challenged groups except for SYN, in which reductions did not reach statistical significance compared to CTR+ (P = 0.064).

The challenge also was associated to a higher number of mitosis (P diet =0.036) although this increase was not significant in animals treated with PRO when compared to CTR+ (P = 0.649). Regarding Goblet cells and IEL, no significant differences were detected related to the experimental treatments.

MUC4 gen polymorphism had an impact on mitosis number on day 4 PI, having carrier animals less mitosis than non-carrier animals (0.23 vs 0.29cell number/ 100 µm; P MUC4 = 0.049). Furthermore, the ratio between villous height and crypt depth on day 8 PI showed and interaction effect (P = 0.004) as synbiotic-treated animals exhibit higher villous:crypt ratio than the rest of challenged-groups, but only in the carrier animals (1.33, 1.07, 1.01, 1.32, 0.95 for CTR+, PRO, PRE, SYN and CTR-, respectively).

6.4 DISCUSSION

A piglet model of ETEC F4 colibacillosis was used in this study to evaluate the efficacy of *Bifidobacterium longum* subsp. *Infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001, galacto-oligosaccharides and their combination. This experimental model of colibacillosis, has been successfully used in our research group to test the efficacy of different in-feed additives (Barba-Vidal et al., 2017; Guerra-Ordaz et al., 2014). The model pretends to promote a mild course of diarrhoea appropriate to evidence the potential of feeding strategies to prevent and/or combat the disease. In the current experimental trial, effects caused by the pathogen challenge could be appreciated. In all challenged groups a decrease of feed intake was registered followed, consequently, by a drop in weight gain. Piglets also showed an impairment of faecal consistency immediately after the

inoculation with evident effect son the intestinal epithelium structure and in the Pig-MAP response. Furthermore, it is documented that, susceptibility of pigs to attachment of ETEC F4 fimbriae to receptors on intestinal brush is determined by MUC4 gene polymorphism (Peng et al., 2007; Trevisi et al., 2009). As expected, heterozygote and homozygote animals encoding for the susceptible allele showed an increment of enterobacteria, coliforms and, also ETEC F4 presence in some of the sampling days, as well as a worsened faecal consistency due to this susceptibility to infection. These results support the usefulness of this candidate gene as marker for genetic selection of farming pigs towards pigs with increased resistance to diarrhoea induced by *E. coli* (Liu et al., 2015). Despite it, it is also fair to remark that some authors have not found necessarily direct associations between MUC4-gene susceptibility and performance (Fontanesi et al., 2012), evidencing the complexity of the pathogeny in which probably other determinants will be also involved.

Regarding the potential of the probiotic to fight the disease, animals receiving the probiotic combination of *B. infantis* CECT 7210 and *L. rhamnosus* HN001 alone showed a reduced impairment of weight gain immediately after the inoculation (0-4 days PI) being the only challenged group that was not significantly different from CTR+. Moreover, in the 4-8 PI period, PRO group also showed improved weights gains reaching similar levels to CTR+. This improved response could have been the result of a competitive exclusion of the pathogen by the probiotics. Actually, this group showed at day 4 PI an ETEC F4 colonic prevalence with intermediate values between CTR+ and CTR- groups. Moreover, it was observed a lower number of faecal plate counts of enterobacteria and coliforms compared to the CTR- and also a reduced number of attached bacteria to the ileal mucus, being the only experimental diet that was not different from the CTR+. Other authors have reported beneficial effects against pathogenic agents of these two probiotic strains but separately. Barba et al. (2017) showed, in a similar piglet model, that *Bifidobacterium longum* subsp. *infantis* CECT 7210 tended to reduce the percentage of animals with ileal countable coliforms and also was able to decrease faecal *Salmonella* excretion after an ETEC F4 or a *Salmonella* Typhimurium oral challenge, respectively. Moreover, this strain has also be demonstrated to have an antiviral activity when tested against

Rotavirus in mice (Moreno-Muñoz et al., 2011) and also antidiarrheic properties in healthy infants (Escribano et al., 2018). Similarly, *L. rhamnosus* HN001 has been proved to be successful against pathogens, like ETEC and *Staphylococcus aureus* (Gopal et al., 2001; Inturri et al., 2016; Eggers et al., 2018).This putative reduction in the entero pathogen challenge promoted by the PRO treatment, could also had explained the intermediate levels of mitosis observed between CTR+ and CTR- at day 4 PI and the faster recovery of villi height at day 8 PI. These effects could also be associated to the reduced response observed in acute phase protein Pig-MAP at day 4 PI for this treatment that ranged 0.43-1.50 mg/mL values being clearly below2 mg/mL considered as normal in the weanlings (Piñeiro et al., 2009: normal (<1 mg/mL), borderline (1-2 mg/mL) high levels (>2 mg/mL)). Pig-MAP is commonly induced by IL-6 (González-Ramón at al., 2000) that, at the same time, is stimulated by nuclear factor kappa B (NF-κB) activation (Brasier, 2010).NF-κBis a protein complex which controls the expression of genes implicated in inflammation process (Baker et al., 2012). It is described in the literature that *B. infantis* and *L. rhamnosus* can also modify it (Khailova et al., 2014; Gamallat et al., 2016; Ishizuka et al., 2016), which is consistent with our results. A possible modulation of the immune response by this probiotic should also be considered. In this regard previous works with the *B. infantis* CECT 7210 showed consistent increases in ileal IEL (Barba-Vidal et al., 2017) and *L. rhamnosus* HN001 was attributed immune-modulating properties (Gill et al., 2001). However, we could not find such an effect in the present study when combined with *L. rhamnosus* HN001.

When galacto-oligosaccharides were supplemented alone in the piglets' diet it also brought favourable outcomes. Like the probiotics alone, PRE treatment was able to improve weight gain in the 4-8 PI period, reaching similar values to the CTR+ being even numerically higher. In this case supplementing diets with the prebiotic alone, did not exert any effect on enterobacteria or coliform populations and neither in the ileal histomorphometry, although, like PRO, prevalence of colonic ETECF4 showed intermediate levels between CTR+ and CTR- groups. With this treatment it was also found at day 4 PI the lowest levels of Pig-MAP compared to the other challenged groups being the only one significantly different from CTR- and similar to CTR+. In fact, GOS could have led towards an

alleviation of inflammatory state as shown in several studies (Vulevic et al., 2013; Verheijden et al., 2015). Wang et al. (2018) attributed these modulatory effects to the ability of GOS to increase anti-inflammatory cytokine IL-10 while decreasing IL-8 by modulating, once again, NF-κB protein complex. Regarding fermentation, the prebiotic encouraged a drop of colonic ammonia both in ileum and colon, suggesting a possible shift towards a less proteolytic and beneficial microbiota due to its inclusion, as, for example, ammonia may buffer SCFA and block their activity (Davila et al., 2013; Shen et al., 2015).

The interest of mixing our probiotic strains with GOS as prebiotic is based on the reported ability of *Bifidobacteria* and *Lactobacillus* to degrade and use galacto-oligosaccharides as an energy source. β-glycosidic linkages connecting saccharides that conform galacto-oligosaccharides are hydrolysed in the colon by these two genera of bacteria bearing β-galactosidases (Andersen et al., 2012;Garrido at al., 2013).Several works have revealed a rise in bifidobacteria and lactobacillus presence when tested together with different types of GOS, high-pure or not (Hong et al., 2014; Monteagudo-Mera et al., 2016; Kittibunchakul at al., 2018), although utilization of GOS varies depending on the bacterial strain and the composition of the oligosaccharide (Thongaram et al., 2017).Hence, considering the previously mentioned characteristics, it can be deduced that administering these probiotics with GOS could potentially lead to a synergistic activity and enhance benefits produced by themselves independently. However, works evaluating these combinations are limited and results are not always consistent. For example, Tanner et al. (2014) and Abrahamse-Berkeveld et al. (2016) observed good outcomes, including an enhanced growth and pathogen inhibition, when mixing a bifidobacteria strain with GOS in both, *in vitro* and *in vivo*, whereas Krumbeck et al (2018), did not find any synergy in the capacity of improvement intestinal barrier function combining bifidobacteria and galacto-oligosaccharides in humans. In our study, we observed some synergy during the first week before the challenge, particularly in the amount of enterobacteria and coliforms that were lower compared to the rest of supplemented groups. This effect could suggest a positive impact of the synbiotic on the autochthonous microbiota promoting the growth of microorganisms, which together with the probiotics, would have displaced enterobacteria. These results are particularly relevant as this first

week after weaning is one of the most critical periods in the life of pigs, in which they have to cope with numerous stressors and dysbiosis is commonly present. Shifts of microbiota towards bifidobacteria by the administration of synbiotics containing GOS (± oligofructose (OF)) and strains of *Bifidobacterium* have been reported in trials with healthy new born babies and infants (Simeoni et al., 2016; Chua et al., 2017), and, particularly, these last authors, attributes this event to the increase of endogenous bifidobacteria. However, outcomes with SYN were different after the challenge. During the acute period of the infection (0-4 PI), animals belonging to SYN group showed a higher decrease infeed intake compared to the other treatments, with similar values to those challenged pigs that were not supplemented. This depression of feed intake was even more pronounced in the 4-8 PI period. Accordingly to this reduced feed intake, SYN and CTR- were the only two groups that lost weight in the 0-4 PI period, and SYN showed even a trend for lower gains than CTR- in the 4-8 PI period (199 vs. 283 g/d, P = 0.083).Together to a reduced intake, an impaired nutrient utilization associated to the diarrhoea could also explain weight loss (Clements et al., 2012). Although in our study we were not able to find big differences in faecal score due to the dietary supplementation, the highest faecal scores on day 1 PI were reported for SYN group with values that were significantly higher than those found in PRO group. This trend for a more acute peak of diarrhoea with the SYN diet is supported by the higher prevalence of ETECF4 found at colon at day 8 PI compared to CTR + that was particularly evident in the groups of animals carrying the MUC4 gene. Moreover, concentration of inflammatory serum markers, were also higher with this diet. On day 4 PI TNF-α values were the highest with this diet, being even significantly higher than CTR- although not different from PRO or CTR+. Regarding Pig-MAP, whereas at day 4 PI, SYN showed intermediate levels between CTR+ and CTR-, at the end of the trial (day 8 PI) SYN group maintained markedly high values in contrast to the rest of the challenged groups that were able to normalize Pig-MAP levels. A higher energy expenditure associated to an inflammatory response could have also contributed to the lower performance of the SYN treated animals.

Having discussed this, it is evident that there is some reason that is modifying the impact of the ETECF4 challenge in the synbiotic-treated piglets. It seems that before the challenge SYN could have helped piglets to overcome the weaning challenge and dysbiosis but after the ETEC challenge the benefits seen against the pathogen with the probiotics and prebiotic supplemented separately are not evident.

An explanation for that could be the complex interactions between members of the intestinal microbiota that could have been disturbed by the supplemented synbiotic. It is known that a well-established and developed microbiota is characterized by an equilibrium between a complexity of microbes through cross-feeding, competitive exclusion and quorum quenching phenomena that is difficult to alter (Walter et al., 2018). However, in our case it is important to remind that early weaned piglets are in a situation of stress with a microbiota ecosystem under development that can be easily modified by external factors (Campbell et al., 2013). Thus, the administration of the synbiotic compound could have determined changes in the sequence of colonization of the gut conforming a different microbial ecosystem that could turn in less stable and more susceptible to be colonized by opportunistic pathogens like ETEC F4. In the literature, it can be found two theories though which microbiota can be reshaped that are the “founder hypothesis” and the “nutrient-niche hypothesis” (Sommer et al., 2017; Livak & Bäumler, 2019). The first one, describes that, in an immature microbiota, the firstly acquired microbes tend to be maintained with priority and, the second one, that the introduction of a new nutrient to the gut lumen can open a new niche prone to be occupied by allochthonous bacteria. In our case both phenomena could have been produced, considering that we introduced a fermentable fibre, boosting supplemented probiotic strains together to indigenous bifidobacteria. All together could have determined marked changes in the acquisition and shaping of the microbiota ecosystem. Actually, the drops promoted in ammonia concentration by PRE in both ileum and colon, are only seen in ileum when the prebiotic was administered in the SYN form suggesting that the GOS-probiotics could have been selectively consumed by the probiotics along the small intestine not being able to modify colonic fermentation in a same way. Supporting the idea that SYN treatment could have promoted a different microbial equilibrium it should be also remarked the

reduction observed in the enterobacteria and coliforms numbers after the first week of adaptation. A reduction in these groups promoted by boosted bifidobacteria population could have impaired colonization resistance against pathogenic *E. coli*. Previous authors have described how commensal Enterobacteriaceae can protect neonates against *Salmonella* colonization through oxygen competition (Litvak et al., 2019) and also by the production of bacteriocins used to eliminate the close-related competitors (Stecher, 2015). We could also hypothesize that the biodiversity of the intestinal ecosystem could have been reduced by the synbiotic combination, making it in this way the ecosystem more susceptible to dysbiosis as more niches would become available for opportunistic/pathogenic bacteria settlement. Interesting the administration of SYN produced an increment of SCFA concentration at day 8 PI together with an increase molar ratio of valeric acid but only in pigs carrying the MUC4 susceptible allele. A more acute challenge determined by the presence of the allele MUC4 could have determined this effect, suggesting that the effect of the SYN was somehow challenge-dependent. SYN supplementation could have opened niches for other valeric-producing bacteria. Bacteria from the families Acidaminococcaceae and Veillonellaceae have been associated to increments in valeric acid and, particularly, in pigs, *Megasphaera elsdenii* has been isolated in faeces (Yokishawa et al., 2018; Zhang et al., 2018; Liu et al., 2019). Van Nevel et al. (2005) observed that the inclusion of galactomannans from locust bean gum resulted in an increment of valeric acid and decreased mitotic index in crypts. In our case something similar may be happening, as in carrier animals mitosis were lower and crypts were shorter in SYN group, this could also explain the increased villous/crypt ratio found with this diet.

It is also fair to remark that this apparent higher opportunity of ETEC F4 to colonize the intestine in the SYN treated animals, could also be determined by the experimental model of disease. Undoubtedly an oral challenge with a single high dose of the pathogen is far from what use to be the natural exposition with repeated lower doses. We should not discard therefore that in a real scenario the synbiotic combination could have resulted in a different outcome and neither the synbiotic effect that it may exert in healthy animals.

Summarizing, the probiotic strains *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Lactobacillus rhamnosus* HN001 reduce growth impairment after the challenge and seems able to provide competitive exclusion for ETEC F4 as it was found a lower number of colonic enterobacteria and coliforms in the gut and a trend for a diminished pathogen carriage. This could explain the lower Pig-MAP levels and improved villi height found one week after the challenge with this treatment. The supplementation of the diets with galacto-oligosaccharides also diminishes the growth impairment induced by the challenge and is associated to lower levels of plasmatic Pig-MAP that would suggest a modulation of the inflammatory response by this prebiotic. Unexpectedly, under our experimental conditions, these beneficial effects against the pathogen are not synergistic when the probiotics and the prebiotic are administered together. More research should be performed in this field to understand the complex interactions produced in the gastrointestinal tract, with an especial emphasis in the microbiota establishment at early ages.

Chapter 7. General discussion

7.1 IS THE SYNBIOTIC STRATEGY ALWAYS POSITIVE?

The objective of this Thesis was to evaluate the effects of different synbiotic combinations as potential prophylaxis or treatment against swine digestive pathogens, such as enterotoxigenic *Escherichia coli* or *Salmonella* Typhimurium. As promising findings were already reported for the administration of the probiotic contained in all mixtures (*Bifidobacterium longum* subsp. *infantis* CECT 7210) (Barba-Vidal et al., 2017), it was expected that the addition of a carbohydrate source for fermentation could enhance the response of animals in front a pathogen challenge. However, the obtained results did not totally support this hypothesis.

First and foremost, it should be noticed that the synbiotic inclusion, although did not show the benefits previously observed for the PRE or PRO in front of the pathogens, it was not harmful for the animals and even, in some chapters, showed positive outcomes in the non-challenged animals (Chapter 4) or before the challenge (Chapter 6). In the two trials included in Chapter 4, the administration of *B. infantis* CECT 7210 and oligofructose-enriched inulin promoted an increased number of intraepithelial lymphocytes that were present in the ileum. These cells are deeply involved in immune regulation and, furthermore, associated to a function of homeostasis manutention and early immune response effect (Ogra et al.; 1994; Ismail et al., 2011; Olivares-Villagómez and Van Kaer, 2018), hence, it could be deduced that pigs receiving this treatment had a more healthful gut environment, better prepared to defeat possible challenges. Appropriately, in the ETEC F4 trial (Chapter 4) this synbiotic tended to reduce the loads of enterobacteria and coliforms in non-inoculated pigs. This was consistently repeated, and significantly, in trial 4 (Chapter 6), in which piglets receiving, in this case, a mixture containing the former bifidobacterial strain, *L. rhamnosus* and galacto-oligosaccharides had lower counts of the mentioned bacterial families during the adaptation week before the challenge with ETEC F4. Other authors have reported reductions in levels of problematic inhabitants of the gut by the use of diverse synbiotics.

Table 7.1. Effects of SYN in each trial on different parameters. For trial 1 and 2, table shows comparisons with each respective control (challenged or not), for trial 3 and 4 SYN was compared to the negative. control.

		Chapter 4		***Chapter 5***	***Chapter 6***
		TRIAL 1 (*Salmonella*)	**TRIAL 2** (ETEC F4)	**TRIAL 3** (*Salmonella*)	**TRIAL 4** (ETEC F4)
Absence of pathogen challenge	*Performance*	↓FCR	NE	NE	NE
	Clinical signs	NE	NE		
	Microbiology	NE	**(↓enterobacteria & coliforms)**		**↓enterobacteria & coliforms than PRO and PRE**
	Fermentation	↑acetate colon ↓propionate colon **↑valerate**	↑acetate ileum ↓acetate ileum ↓acetate colon **↑valerate**		
	Inflammation	NE	NE		
	Intestinal morphology	(↑ crypt depth) **↑ IEL** ↓mitosis	↓ villous height (↓ crypt depth) **(↑ IEL)** ↑mitosis		
Pathogen challenge	*Performance*	↑FCR	NE	**Loss of PRO and PRE ↑BWG**	**Loss of PRO and PRE ↑BWG** **Loss of PRO ↑ FI**
	Clinical signs	NE	NE	**Loss of PRO and PRE ↑ faecal consistency (2 PI)**	**Loss of PRO and PRE ↑ faecal consistency (1 PI)**
	Microbiology	↑ *Salmonella* clearance	**(↑ enterobacteria & coliforms)**	Loss of PRO and PRE ✓effects on *Salmonella*	Loss of PRO ↓enterobacteria & coliforms ileal scrapings **↑E. coli F4 presence** $^{(MUC4-)}$ (= PRO, PRE)
	Fermentation	↑propionate colon **↓acetate colon** **↑valerate**	**↓acetate colon** **↑valerate**	**(↑valerate)** (= PRE)	Colon: ↑ pH $^{(MUC4-)}$ (=PRO,PRE), ↓ pH$^{(MUC4+)}$ (=PRO) **(↑ valerate $^{(MUC4+)}$)** Loss of PRO and PRE ↓ BCFA$^{(MUC4-)}$ Loss of PRE ↑ BCFA$^{(MUC4+)}$ (=PRO)
	Inflammation	NE	NE	NE	↑ TNF-α, (=PRO) ↑ Pig-MAP
	Intestinal morphology	(↑ crypt depth) ↑ IEL ↓mitosis	(↓ crypt depth) ↑mitosis	Loss of PRE ↓ crypt depth and ↑ IEL	Loss of PRO effect to ↑ villous height ↑ V:C $^{(MUC4+)}$

(): trends

NE: no effects

Wang et al. (2018) found a trend to diminish amounts of *E. coli* by administering to the piglets *L. plantarum* ACCC 11016 and fructo-oligosaccharides, similarly to another synbiotic composed by a multistrain probiotic and inulin that negatively affected enterobacteria (Sattler et al., 2015). These last authors explain that all chosen probiotics had already evidenced to be effective against *E. coli* and that the added fibre produced an increment of the probiotic-related bacteria numbers (lactobacilli, bifidobacteria) thus, it can be said that a synergistic action took place. The strains used in the trials of this Thesis also had demonstrated ability to fight potential pathogens previously (Gill et al., 2001; Gopal et al., 2001; Moreno-Muñoz et al., 2011; Barba-Vidal et al., 2017), however, as no microbiota analysis of the implied animals was performed in any of the trials, it cannot be affirmed that a shift towards a desirable profile occurred like it was observed in those other works. In spite of the positive commented effects, in Chapter 4 (*Salmonella* trial) and 4 it could be observed that non-challenged animals receiving the synbiotic had a worse performance than the ones fed control diet. In Chapter 4, the SYN group showed an impaired feed conversion ratio that might be due to the inclusion of 5% of oligofructose-enriched inulin to the animal's diet, leading to a dilution of energy and limiting amino acids, as, in this trial, synthetic amino acids were not added to compensate this loss of nutrients. Other plausible reasons include changes in transit time, digestibility or host energy homeostasis (Kelly, 2009; Ley et al., 2016; Chen et al., 2017). In Chapter 5, however, the synbiotic induces a loss of appetite of the animals, producing a decrease in feed intake with a parallel loss of body weight gain. Other authors observed also reductions of feed conversion rate in pigs due to different synbiotic treatment (Guerra-Ordaz et al., 2013; Modesto et al., 2011), but it cannot be found in the literature works evidencing a reduction of feed intake. It is well known that prebiotic soluble-fibres can have a satiety effect due their ability to bind water and bulk (Kellow et al., 2014). However, as animals receiving only the prebiotic did not show any change, something else may be affecting them. These last authors also describe that gut microbes have an important role in modification of the secretion of hormones that promote satiety. Thus, it can be thought that the synbiotic may have induced a change of microbiota profile that differed from the other animals carrying this effect. Even though this reduction in ingesta

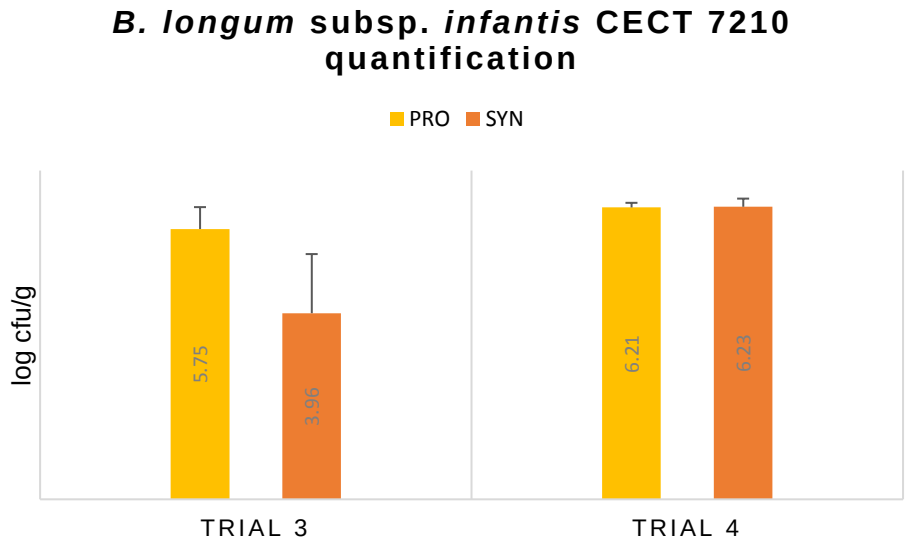
and weight gain may be considered beneficial in humans as obesity is a widespread disease (Hadi et al., 2018); in animal production, a worsened performance is not desirable and moreover during the critical post-weaning period.

All above mentioned effects were seen during the first week after weaning, before the challenge. However, when piglets were orally inoculated with the corresponding pathogen, the scenario changed, and results varied among experiments. In Chapter 4, in the *Salmonella* trial, the synbiotic combination of *B. infantis* CECT 7210 and oligofructose-enriched inulin was able to increase number of IEL, as observed in non-challenged animals, and helped in reducing the presence of *Salmonella*, making 25% of the animals receiving the compound negative in faeces. In contrast, in the ETEC F4 trial, not only the pathogenic load was not reduced, but the numbers of enterobacteria and coliforms were increased in pigs consuming the same synbiotic mixture. In Chapter 5, the same mixture plus *L. rhamnosus* was evaluated and was not effective against *Salmonella* as they were the probiotics and prebiotic by themselves and, in addition, in Chapter 6, animals receiving the same probiotic and galacto-oligosaccharides showed an increased presence of ETEC F4, more evident in animals carriers of the MUC4 susceptible gene. Nonetheless, it is the first time that these detrimental effects on orally challenged piglets are reported. Other authors like Guerra-Ordaz et al. (2014), Aluko et al. (2017), Krause et al. (2010) and Naqid et al. (2015), also using *Salmonella* or ETEC F4 challenges, described efficacy of synbiotics in reducing pathogen presence, although both, the probiotics and prebiotics tested by them were not the same as the used during the present work. As these elements have a clear impact in host's microbiome (John et al., 2018), it can be hypothesized that the changes induced by the synbiotic combinations of this Thesis may be different to those promoted in previous studies, although the lack of sequencing information does not allow a confirmation. One thing that is consistent in all Chapters, is the increase in the concentration of valeric acid with the synbiotic combinations, which might be suggesting a change in the microbiota structure with the synbiotic as has also been reported by Krause et al. (2010). In this case the authors found a more diverse microbiome with the synbiotic supplementation. However, in this Thesis, and as discussed in Chapter

6, it is hypothesized that the administration of SYN may have caused a decrease of bacterial diversity, parallel to a creation of new niches, susceptible to be occupied by new habitants (Litvak & Bäumer et al., 2019). The enhanced valerate presence could be due to the increases of particular microbial groups as for example the Veillonellaceae family members that have been shown to produce valerate from lactic acid (Marchandin et al., 2010; Yoshikawa et al., 2018) and in this context, the inoculated pathogens could have taken profit of new empty niches in the ecosystem. Related to this, in Chapter 6 SYN treated animals showed signs of an impaired health status by an enhanced proinflammatory state, that was translated in a reduction of pig's performance as ill animals are known to show decreases in feed intake and weight gain (Cornelison et al., 2018).

Another concern regarding outcomes with the SYN treatment was if a possible overgrowth of the probiotic strains induced by the prebiotic could have a detrimental impact. To assess this hypothesis and also to test the potential of the prebiotics to act as selective growth substrates, we determined the concentration of *B. infantis* CECT 7210 by quantitative real-time PCR. To do so, DNA was extracted from the colonic content samples from day 8 PI and the probiotic strain quantified by PCR using the forward (5'-CACAGCGGGCAGATCGGTAT-3') and reverse primers (5'-CGCCGGTGCCAGTCA-3') and a TaqMan probe (5'-[6FAM]CCGGTTAGTCCTCTACCGTACGCAAGC[TAM]-3'). The master mix used was "HOT FIREPol Probe qPCR Mix Plus" (Solis BioDyne; Tartu, Estonia). Results obtained from trials of Chapters 4 and 5 are expressed in the following figure. In these trials, probiotics and prebiotics were also administered to a group of the animals separately, hence, the probiotic (PRO) and synbiotic (SYN) groups can be compared:

Figure 7.1. Comparation between B.longum subsp. *infantis CECT 7210 concentration in animals treated with probiotic or synbiotic from trials 3 and 4.*



PRO- Animals receiving the probiotics; SYN- Animals receiving the synbiotic. N = 6 for both groups.

No overgrowth of *B. infantis* CECT 7210 was observed by its co-administration with oligofructose-enriched inulin nor galacto-oligosaccharides, however, in these trials there was also another strain, *L. rhamnosus* HN001, added to the feed and whose quantification was not done and, subsequently, the hypothesis of a possible overgrowth of this second strain cannot be totally discarded. Mair et al. (2010), for example, administering a multistrain probiotic containing two lactobacilli species and a bifidobacterium, among others, plus inulin, only found a significant increase of lactobacilli, but not bifodobacteria, due to treatment. *In vitro* and in pure culture, nonetheless, *B. longum* has been proved to be more capable than *L. rhamnosus* to grow in fructo-oligosaccharides and inulin mixtures and, even more in galacto-oligosaccharide containing media (Watson et al., 2013). However, this might change in a complex environment like the gut, in which a lot of relationship and cross-feed processes between all present bacteria take place (Seth & Taga, 2014; Hoek et al., 2017).

Nevertheless, and regarding the results obtained during the development of this Thesis, it should be remarked that experimental conditions are not the same that the ones taking place in commercial

farms regarding infection. Whereas in these trials animals received a great concentration of pathogen cultures orally (10^9 cfu), the natural course of the infection is not the same. The transmission is not produced by an oral ingestion of an inoculum, but for contact with contaminated faeces or materials (Fedorka-Cray et al., 1994; Dubreuil et al., 2016), meaning that the dose is much lower but continous. Hence, it can be thought that the adverse effects of the tested synbiotic combinations of *B. infantis* CECT (± *L. rhamnosus* HN001) with oligofructose-enriched inulin or galacto-oligosaccharides in infectious scenarios may be different in normal farms, being probably more similar to the observations during the first week before the challenge. During this first week after weaning, animals suffered a "natural" challenge as they arrived at new facilities with new bacterial loads, mixed with other animals and separated from the mothers, similar what normally occurs in swine industry (Rhouma et al., 2017).

7.2 IS THE WAY OF PROBIOTIC ADMINISTRATION RELEVANT?

Probiotic bacteria can be administered to the animals by different ways. The first one consists in the use of boluses than can be given in a single or repeated dose. Actually, it can be found different commercialized products designed to provide probiotics to new-born piglets by the use of oral supplements or oral paste combined with other functional ingredients to prevent diarrhoea. The advantages of this way of administration is that it can assure the ingestion of the dose and, furthermore, the probiotic viability is guaranteed as the treatment is done shortly after the preparation. However, and especially if the dosage is set as a single bolus, the effects of the probiotic treatment may be not the intended. If we consider that for an effective colonization the adhesion of the probiotic to the intestinal epithelium is required (De Melo Pereira et al., 2018), a single dose during the day could be translated into a smaller presence of viable probiotic attached to the intestinal epithelium considering that digesta transit promotes a clearance of these non-endogenous bacteria (Juntunen et al., 2001).

Another method of administration involves mixing the probiotic in the animal's feed (or water), resulting it in a constant and homogenous intake during the whole day. This is a preferable route from a practical point of view as it does not require manipulation of the animals. A high number of experimental works with probiotics in piglets use this oral route emulating the most common farming conditions (Mair et al., 2010; Guerra-Ordaz et al., 2013; Wang et al., 2018). Administered with the feed, the total amount ingested may increase compared to the boluses, and this could enhance their opportunity to colonize the gut, modulate gut ecosystem and exert their action (Kechagia et al., 2013). The main disadvantage in this case is that probiotic viability can be compromised by the exposure to the oxygen and dry conditions of the feed. Moreover, when administered into the feed the dose is not that accurate, particularly if the animals have a decreased voluntary intake.

During the development of the trials included in this Thesis, the way of the probiotics administration was changed from an oral daily bolus in trial 1 to mixing in piglet's diet from trial 2 onwards. The main reason for that was that, despite the probiotics of this Thesis are tested in weaned piglets, they are originally designed for milk-formula for human infants. As their inclusion in these milk-substitutes means that the babies are ingesting the synbiotic compound spread during the day (Braeger et al., 2011; Radke et al., 2017), more representative results were expected to be achieved by this method. To ensure a good viability of the probiotic in the feed, it was previously tested and regular feed replacements were performed.

In order to check if the way of probiotic administration had an impact on the intestinal colonization, we analysed numbers of *B. infantis* CECT 7210 quantified by qPCR as described above. The following table summarizes the results belonging to the different Chapters of this Thesis and also include data from previous trials described in the Thesis of Barba-Vidal (2017) using similar experimental models but only using the bolus administration.

Table 7.2. Percentage of animals receiving probiotic, included in each level of B. longum subsp. *infantis CECT 7210 quantification. qPCR performed using colonic content samples obtained on day 8 PI of each trial. Table includes data from trials included in this Thesis and also from Barba-Vidal (2017).*

TRIAL	ROUTE	TIME SAMPLING - ANALYSIS	Percentage of probiotic-treated animals included (%)				
			Negative	*3-4 log cfu/g FM*	*4-5 log cfu/g FM*	*5-6 log cfu/g FM*	*>6 log cfu/g FM*
Barba-Vidal (2017)							
TRIAL 1	Oral inocul.	1.5 years	33	17	42	8	0
TRIAL 2	Oral inocul.	1 year	25	0	42	33	0
Rodríguez-Sorrento (2017)							
TRIAL 1	Oral inocul.	3 years	42	0	16	0	42
TRIAL 2	Feed	2.5 years	50	0	0	44	6
TRIAL 3	Feed	1.5 years	17	8	8	25	42
TRIAL 4	Feed	1 year	0	0	0	25	75

First and foremost, it can clearly be seen how quantifications coming from the latest experiments are much higher and decrease as time passes, being a degradation of the DNA in the samples that were kept frozen at - 80°C a coherent explanation. In fact, Bahl et al. (2012), did observe different results in PCR studies between fresh and frozen stool samples, meaning that, effectively, freeze conservation does affect the quality of the results. On the other hand, authors like Donatin & Drancourt (2012) or Rapp et al. (2010) affirmed that freeze drying is the best method to preserve DNA so, to avoid these uncertain outcomes, an extraction of fresh DNA followed by a freeze dry processing may be an option to be considered.

Secondly, on the light of the results of experiments in which time elapsed between sample procurance and qPCR performance was equivalent, it is evident that the administration route may have an impact on the number of *B. infantis* CECT 7210 found in the colon of the animals. For example, when comparing the quantification obtained by Barba-Vidal in his trial 2 and the ones obtained in the trial 4 of the present Thesis, it can clearly see how inclusion of probiotics in the animal's feed did cause an increased detection of the probiotic strain in colon. A 75% of the animals that received the former strain in the feed had concentrations greater than 6 cfu per gram of content, amount that, contrarily,

piglets administered the dose as a single bolus per mouth could not reach. This fact is supported by a similar outcome when a comparison between experiment 3 of this Thesis and the 1 of Barba-Vidal is carried out. Nevertheless, the difference between feed inclusion and direct mouth inoculation is not evidenced in trials 1 and 2 of Chapter 4. This may be due to, as explained previously, a higher degradation of DNA as stool samples were stored at around 3 years before the qPCR was done.

7.3 USE OF THE PIG AS A HUMAN MODEL

As mentioned at the beginning of this Thesis the final objective of the evaluated probiotics and prebiotics is to be used in humans and particularly in lactating children. Therefore, in this work the piglet has been used as a model for humans and this would deserve some discussion. When using animal models to test the impact of a probiotic on health and their possible mechanisms of action it is important to attend to the possible limitation and advantages of the chosen model. Rodents has been generally used as a preferred animal model, main reasons include their small size and low-cost maintenance, however there are important differences between both species. Firstly, rodents are granivore, caecum-fermenters and caecotrophic animals, whereas humans are omnivorous and colonic-fermenters (Heinritz et al., 2013). The pig, despite higher costs of maintenance, appears as a better model compared to mice considering that it is a human-sized animal with characteristics that turn it in a sensitive translational model for human nutrition sharing similarities in the physiology and anatomy of the digestive tract, nutrient absorption and minimum requirements (Miller at al., 1987). Moreover, requirements are also similar and defence mechanisms of gut barrier such functional permeability remain conserved in the two species (Roura et al., 2016). However, despite similarities, there are also limitations in the pig as animal model that can be particularly relevant when evaluating probiotics and prebiotics. Although microbiota composition is similar, being mainly formed by Firmicutes and Bacteroidetes phyla members, there are some differences in genera that should be taken into account (Heinritz et al., 2013). The main genera of bacteria present in the human GIT tract are *Clostridium-Eubacterium*, *Bacteroides*, *Atopobium*, *Bifodobacterium* and *Lactobacillus* whereas in

pigs, are different: *Prevotella*, *Anaerobacter*, *Streptococcus*, *Lactobacillus* and *Coprococcus* (Heinritz et al., 2013; Kim et al., 2011). Particularly, *Bifidobacterium* is one of the five main colonizers of the human gut (with a relative abundance of 4% (Lay et al., 2005) whereas, in pigs, their number is lower (1 %) and the species are generally different from the human endogenous bifidobacteria (Leser et al., 2002; Mikkelsen et al., 2003). It means, thus, that the pig gut is not the perfect environment for human-specific bifidobacteria to attach and grow, modifying the probiotic action of the *B. infantis* given to the animals and probably not showing all its potential in our trials. Furthermore, this effect could have been exacerbated as this strain is natural colonizer of the infant gut and its preferred source of energy are the human milk oligosaccharides (HMO) which is the third most abundant solid component of human milk (Totten et al., 2012), and not the prebiotic fibres that were jointly administered. Altogether, alterations in the microbiota composition by the administration of *B. infantis* CECT 7210 with or without *L. rhamnosus* HN001 and OF-enriched inulin or galacto-oligosaccharides can possibly take a different course in humans due to the discrepancies present between the two species.

7.4 THE ROLE OF MUC4 GENE POLYMORPHISM IN THE DEVELOPMENT OF POST-WEANING DIARRHOEA.

Two enterotoxigenic *Escherichia coli* F4 experimental challenges were carried out during the development of this Thesis, which are contained in Chapters 3 (ETEC F4 trial) and 5. It is known that this strain of *E. coli* attaches to the jejunal brush border by a binding of its fimbriae to many putative receptors belonging to carbohydrates of glycoproteins shown in the intestinal epithelial cells and intestinal mucus, which differs between pigs (Van den Broeck et al., 2000; Rasschaert et al., 2007). Three antigenic variants of F4 fimbriae have been identified, namely F4ab, F4ac, and F4ad (Bakker et al., 1992), being the F4ac the most common variants worldwide (Fairbrother et al., 2005) and the F4ab & F4ac the variants used in the present Thesis.

Looking for genetic tools to select new breeds of non-susceptible pigs, several candidate genes have been identified responsible for ETEC F4ac susceptibility located on pig chromosome 13 in the q41 region (Joller et al., 2002; Jørgensen et al., 2003). Between them, the gene Mucin4 (MUC4) has been proposed as a useful genetic marker to identify susceptible genotypes (Jørgensen et al., 2003). MUC4 gene encodes for a membrane-bound-O-glycoprotein present in the mucus layer. A single nucleotide polymorphism located in this gene is believed to be the responsible of the susceptibility. The presence of the guanine nucleotide would make MUC4$^{CG/GG}$ genotypes more susceptible to ETEC F4 adhesion than the MUC4 ^{CC} (Jørgensen et al., 2003). However, this genotype does not correlate totally with the apparition of diarrhoea as animals considered "resistant" can also develop it, although that in lower percentages (Luise et al., 2019).

In the ETEC F4 trial of Chapter 4 no genotyping of the animals was performed, however, in the second trial (Chapter 6), hairs containing follicles were taken from the piglets in order to determine which alleles of MUC4 gene they were encoding for. As expected, susceptible allele-carriers piglets showed a worsened faecal consistency, as well as a lower weight daily gain and higher numbers of enterobacteria, coliforms and ETEC F4 in colon (Tables C and D) particularly in the 0-4 PI period or day 4PI.

Table 7.3. Effect polymorphism in MUC4 gene in faecal consistency on days 0, 1, 2, 3, 5 and 7 PI.

	MUC4 polymorphism			***P-values***
	RT	**SU**	**RSE**	**MUC4**
Faecal consistency (0-4 score)				
Day 0 PI	1.57	1.61	*0.631*	*0.788*
Day 1 PI	2.03	2.47	*0.612*	*0.002**
Day 2 PI	1.95	2.34	*0.593*	*0.004**
Day 3 PI	1.76	2.25	*0.755*	*0.004**
Day 5 PI	1.76	2.00	*0.528*	*0.145*
Day 7 PI	1.50	1.47	*0.588*	*0.753*

RT: animals carrying CC alleles. SU: animals carrying GC/GG alleles.
P-values calculated using an ANOVA with the generalized linear model of R.
Scale: 0= hard solid, 1 = solid and cloddy, 2 =soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood.
n=42 for RE, n=35 for SU on days 0, 1, 2 and 3 PI. n=29 for RE, n=19 for SU on days 5 and 7 PI.

Table 7.4. Effect of polymorphism in MUC4 gene on productivity, serum inflammatory markers, intestinal structure on days 4 and 8 PI.

	Animals sampled at day 4 PI		**Animals sampled at day 8 PI**		**RSE**	***P-values***		
	RT	**SU**	**RT**	**SU**		**MUC4**	**Day**	**MUC4*Day**
Average daily gain (g/day)								
Adaptation	39.8	59.1	47.0	73.5	*46.54*	*0.079*	-	-
0-4PI	126.7	-6.4	129.7	-27.3	*121.60*	*<0.001**	-	-
Inflammatory serum markers								
PigMAP *(mg/mL)*	0.82	1.52	1.00	0.78	*1.000*	*0.344*	*0.486*	*0.116*
TNF-α *(pg/mL)*	89.5	82.3	81.4	80.8	*17.23*	*0.461*	*0.229*	*0.472*
Histological parameters								
Villus height *(µm)*	253	261	290	294	*44.4*	*0.858*	*0.003**	*0.859*
Crypt depth *(µm)*	269	261	270	269	*31.7*	*0.527*	*0.628*	*0.635*
IEL *(cells/100 µm)*	0.52	0.39	0.67	0.64	*0.246*	*0.108*	*0.004**	*0.436*
GC *(cells/100 µm)*	2.26	2.45	2.02	2.32	*0.946*	*0.285*	*0.429*	*0.819*
Mitosis *(no./100 µm)*	0.29	0.24	0.25	0.23	*0.105*	*0.265*	*0.281*	*0.470*
ETEC F4 quantification (log copy F4 gene/g FM)								
ETEC F4 colon	6.05	8.33	2.30	3.63	*2.724*	*0.002**	*<0.001**	*0.508*
ETEC F4 ileum	4.50	6.02	4.44	2.73	*3.077*	*0.893*	*0.087*	*0.049**
Microbiological counts (log cfu/g FM)								
-Faeces-								
Enterobacteria	6.41	7.65	5.97	5.94	*1.226*	*0.025**	*0.003**	*0.052*
Coliforms	6.27	7.43	5.86	5.88	*1.199*	*0.027**	*0.006**	*0.074*
-Ileal scrapings								
Enterobacteria	7.53	7.51	7.43	7.40	*0.458*	*0.585*	*<0.001**	*0.766*
Coliforms	7.06	7.11	6.72	7.09	*0.634*	*0.179*	*0.001**	*0.240*

RT: animals carrying CC alleles. SU: animals carrying GC/GG alleles.
IEL= intraepithelial lymphocytes; GC= Goblet cells; ADG= Average daily gain
P-values calculated using an ANOVA with the generalized linear model of R.
n=16 for RE, n=14 for SU on day 4 PI. n=20 for RE, n=11 for SU on day 8 PI.

In this regard, Sterndale et al. (2019) also observed increased diarrhoeal episodes in MUC4-susceptible animals, although they did not find increased shedding of ETEC F4. Other authors, like Casini et al. (2003) and Trevisi et al. (2014) did find an increased excretion of ETEC F4 during the acute period after inoculation, which is consistent with the results obtained in this work. Moreover, results presented here regarding numbers of ETEC F4 in ileal content support this fact, as they were increased in susceptible animal uniquely in the samples belonging to day 4 PI. Considering these evidences, it can

be deduced that a selection of susceptible animals for experimental challenge models could help in reducing the residual variability and accordingly the number of subjects needed (Luise et al. 2019).

Nevertheless, not all evaluated parameters were influenced by the MUC4 polymorphism. In this regard, number of enterobacteria and coliforms attached to the ileal epithelium did not shown any relationship nor ileal histological measurements. This fact could be due to the section of the small intestinal tract analysed. In this Thesis we sampled ileum whereas other authors like Messori et al. (2013) or Trevisi et al. (2014) selected jejunum. As jejunum normally do not contain relevant numbers of coliforms (Dubreuil et al., 2016) probably this lower background makes it easier to find differences. Another reason to choose jejunum relies in the fact that as Trevisi et al. (2016) and Rasschaert et al. (2007) affirm, ETEC F4 attaches principally to intestinal mucosa through receptors that are located in this segment. Furthermore, another explanation for the lack of effects is that MUC4 is not the only gene involved in the susceptibility of piglets to ETEC F4 infection being its use as exclusive genetic marker controversial. Nguyen et al. (2013), testing F4 susceptibility of MUC4 resistant pigs, observed positive *in vitro* villous adhesion tests and immune response towards the pathogen, suggesting that other F4 receptors might be playing an important role. In fact, Rasschaert et al. (2007) confirmed that lack of adhesion of ETEC F4 to the villous brush borders not always is associated to the genotypic resistance. Other genes, like MUC13 or TNRC (transferrin receptor gene) have also been associated to ETEC F4 susceptibility (Zhang et al., 2008; Jacobsen et al., 2010; Ren et al.; 2012) and could explain some of these discrepancies.

Thus, and regardless of the clear relationship found in the trial of Chapter 6 between the challenge impact and MUC4 gene polymorphism, selection of animals must be done carefully taking in consideration that this might not be the only gene implied as susceptibility respond to a multifactorial process.

Chapter 8. Conclusions

1. The administration of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and oligofructose-enriched inulin to healthy weaning piglets reduces the numbers of ileal attached enterobacteria at day 7 PI (P = 0.057) and exerts a boosting effect in the local immune response by increasing numbers of intraepithelial lymphocytes in ileum. No impact is seen on growth performance or faecal consistency.

2. This combination trends to reduce caecal prevalence of *Salmonella* eight days after the pathogen oral challenge (P = 0.076), but it fails to reduce enterobacteria or coliforms when piglets are orally inoculated with enterotoxigenic *Escherichia coli* (ETEC) F4. This synbiotic is shown to have a significant effect on the colonic fermentation profile but with a different impact depending on if animals are or not challenged.

3. The combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* HN001 and oligofructose-enriched inulin does not have any significant impact on performance of piglets one week after weaning and neither on faecal consistency.

4. This combination does not have synergistic activity against an oral *Salmonella* challenge as benefits found for the multistrain probiotic and the prebiotic are lost when combined. Among them, the faster faecal clearance of the pathogen produced by the multistrain probiotic and the positive impact of oligofructose-enriched inulin on immune local response with increased numbers of intraepithelial lymphocytes in ileum at day 8 PI.

5. The combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210, *Lactobacillus rhamnosus* and galacto-oligosaccharides is capable of reducing faecal numbers of enterobacteria and coliforms in piglets one week after weaning compared to the single administration of the

multistrain probiotic or the prebiotic. No effects are seen on growth performance of faecal consistency.

6. This combination also reduces numbers of faecal enterobacteria and coliforms after an oral challenge with ETEC F4, but these reductions are concomitant with higher numbers of *E. coli* F4 in colonic digesta 8 days after the challenge and increased serum concentrations of TNF-α at day 4 PI and of Pig-MAP at day 8 PI. This combination does not show any synergism against the pathogen, losing some of the benefits obtained with the multistrain probiotic or the prebiotic. Among them, the reduction in the growth impairment associated to the challenge and the lower levels of Pig-MAP on day 4 PI.

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