Universitat Autònoma de Barcelona

EVALUATION OF PROBIOTIC STRATEGIES IN THE PREVENTION OF PIGLET POST-WEANING GASTROINTESTINAL DISORDERS

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TESIS DOCTORAL PRESENTADA PER: **EMILI R. BARBA VIDAL**

SOTA LA DIRECCIÓ DE LES DOCTORES:

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Susana M. Martín Orúe i **Lorena Castillejos Velázquez**, investigadores del departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

Certifiquen:

Que la memòria titulada **"Evaluation of probiotic strategies in the prevention of piglet post-weaning gastrointestinal disorders"**, presentada per Emili R. Barba Vidal 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.

I perquè consti als efectes oportuns, signen la present a Bellaterra, 12 de Gener de 2017.

Dra. Susana M. Martín Orúe

Dra. Lorena Castillejos Velázquez

The role of the infinitely small in nature is infinitely great

Louis Pasteur

Agraïments/Acknowledgements

A aquestes alçades, podria resumir el procés de la tesis amb dos conceptes:

a) Muntanya russa: tants pots trobar-te a dalt, com a baix, com a vegades... de cap per baix. Pot marejar una mica, prepareu la *Biodramina*!
b) Curiós: vaig entrar amb l'objectiu de respondre una pregunta. Després de dedicar-hi pràcticament 4 anys, surto amb la pregunta resposta en una "situació determinada" i moltes més preguntes de les que tenia quan vaig entrar!

En fi, sigui com sigui, la veritat és que en el procés d'aquesta tesis he après molt. He après molt amb l'encert, però més l'error, amb la feina que m'ha fet progressar i amb el dubte que m'ha fet reflexionar. Una de les conclusions a les que he arribat és que la paciència és la mare de la ciència (segurament no calia fer una tesi per arribar aquí però mira, hi ha gent que ens costa més...). Una paciència que si bé és cert que en una petita part l'he hagut de cultivar jo, no és menys cert que la gran majoria l'he fet cultivar jo a les altres persones. Començant per les meves directores, als meus companys del departament, a l'Olga i a la Mònica en el laboratori, la meva família... a tots els que em deixo, gràcies per haver tingut paciència amb mi! M'heu fet una mica més científic!

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Taula agraïments. Ingredients i composició nutritiva de la dieta experimental *as-fed basis*, g/kg.

Ingredients	
Directores [1]	280.8
Empreses: Ordesa i Norel [2]	170.0
Famílies [3]	150.0
Becaris [4]	122.4
Professors i investigadors [5]	100.0
Serveis d'anàlisis [6]	50.0
Grangers [7]	50.0
Quotidians [8]	30.3
Canada [9]	21.3
Amics [10]	10.6
Porquets [11]	6.8
Gossos [12]	4.8
Sort [13]	3.0
Analyzed composition	
Tesis	911.0
Experiments	174.9
Laboratori	59.4
Granja	92.7
Viatges	72.5
Cerveses i una mica de rauxa	34.8

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11. **Porquets:** No seria just que no tingués en compte tots els animals que han nodrit els nostres resultats. Gràcies.

12. **Gossos:** No sé si hauria aguantat segons quins dies sense la meva dosis de cani-cross i llepades. Gràcies!

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Summary

The main objective of the Thesis was to evaluate the potential of new probiotics treatments in weanling pigs to enhance gut health at early life stages and to fight intestinal pathogens, giving insights regarding possible modes of action. Secondarily, the Thesis also pursued the development of new sensible tools to evaluate the response of the animals to probiotics under experimental models.

To achieve the objectives, four trials were designed. In Trial 1 and Trial 2, the potential of the probiotic strain *Bifidobacterium longum* subsp. *Infantis* CECT 7210 was evaluated in weanling piglets in front of a *Salmonella* Typhimurium and an ETEC K88 oral challenge respectively. Trial 3 evaluated the response of the combination of the previous strain with *Bifidobacterium animalis* subsp. *lactis* BPL6 in a *Salmonella* Typhimurium challenge. Finally, in Trial 4 the probiotic *Bacillus licheniformis* CECT 4536 was assessed in piglets experimentally challenged with *Salmonella* Typhimurium.

A similar protocol was used in all Trials. In brief, 72 weanling piglets were transported to the university facilities from commercial farms. For Trials 1-3 animals were distributed in 24 pens in a 2 x 2 factorial design; treated or not with the probiotic and challenged or not with the pathogen. In Trial 4, two treatments were evaluated in the same facility, the probiotic and sodium butyrate; with 6 extra piglets used as a challenge negative control. After an adaptation period, animals were orally challenged with the corresponding pathogen and one animal per pen was euthanized at Day 4 and 8/9 post-inoculation (PI). For all experiments, main parameters assessed were animal performance, clinical signs, pathogen excretion, fermentation profile, immune response and intestinal morphology. Moreover, in Trial 3 a panel of blood biomarkers was analyzed and in Trial 4 a systematic behavior analysis was performed.

The probiotic strain *Bifidobacterium longum* subsp. *Infantis* CECT 7210 increased intraepithelial lymphocytes and reduced *Salmonella* shedding (Trial 1) and ileum coliform colonization (Trial 2). In non-challenged animals it increased butyric acid and the villus:crypt ratio at Days 8/9 PI. Effects on feed intake, microbial fermentation and intestinal architecture showed a differential pattern between challenged and non-challenged animals.

When the previous strain was combined with *Bifidobacterium animalis* subsp. *lactis* BPL6, the beneficial effects of the probiotics were seen in both groups, challenged and non-challenged; increasing voluntary feed intake, decreasing diarrhea scores of the PI period, improving fermentation profiles at Day 8 PI, stimulating intestinal immune response by increasing intraepithelial lymphocytes and improving villus:crypt ratio. The probiotic combination also reduced fecal excretion of *Salmonella* and decreased rectal temperature to similar levels to non-challenged animals 48 hours PI.

Regarding the blood biomarkers tested, micro-minerals zinc and cupper were useful descriptors of pig performance. Blood electrolytes (sodium, chloride and potassium) and acid base indexes (bicarbonate, total carbon dioxide and base excess in the extracellular fluid compartment) enabled to detect the most distressed animals. Finally, biochemical parameters assessed (glucose, hematocrit and hemoglobin) were good descriptors of health status.

In Trial 4, the probiotic *Bacillus licheniformis* CECT 4536 reduced shedding of *Salmonella*. No significant effects of the probiotic were seen for performance, rectal temperature, fecal consistency, fermentation profile, inflammatory response and histological parameters. However, a positive effect of the probiotic on behavioral displays was observed. Particularly exploring, feeding and other active behaviors in the morning period.

The results exposed in this thesis have highlighted that the use of probiotics may be a way to improve response of piglets to early weaning and pathogen pressure. Furthermore, the Thesis demonstrated that the analysis of blood parameters and behavior can be sensible tools to be considered in feed additive research.

Resumen

La presente tesis tiene como objetivo evaluar el potencial de nuevos tratamientos probióticos en lechones recién destetados para mejorar la salud intestinal y combatir patógenos, indagando en posibles mecanismos de acción. Secundariamente, la tesis persigue el desarrollo de nuevas herramientas sensibles para evaluar la respuesta de los animales a los probióticos bajo modelos experimentales.

Para lograr los objetivos, se diseñaron cuatro pruebas. En las pruebas 1 y 2, se evaluó el potencial de la cepa probiótica *Bifidobacterium longum* subsp. *Infantis* CECT 7210 en lechones destetados frente a un desafío oral con *Salmonella* Typhimurium y ETEC K88 respectivamente. La prueba 3 evaluó la respuesta de la combinación de la cepa anterior con *Bifidobacterium animalis* subsp. *Lactis* BPL6 en un desafío de *Salmonella* Typhimurium. Finalmente, la prueba 4 evaluó el probiótico *Bacillus licheniformis* CECT 4536 en un desafío con *Salmonella* Typhimurium.

Se utilizó un protocolo similar en todas las pruebas. Brevemente, 72 lechones recién destetados fueron transportados de granjas comerciales a las instalaciones de la universidad. Para las pruebas 1-3, los animales se distribuyeron en 24 corrales en un diseño factorial 2x2; tratados o no con el probiótico y desafiados o no con el patógeno. En la prueba 4, se evaluaron dos tratamientos, el probiótico y el butirato sódico; con 6 lechones adicionales usados como control negativo del desafío. Después de un período de adaptación, los animales fueron desafiados oralmente con el patógeno correspondiente y un animal por corral fue sacrificado los días 4 y 8/9 post-inoculación (PI). En todos los experimentos se evaluó el rendimiento productivo, signos clínicos, excreción de patógenos, perfil de fermentación, respuesta inmune y morfología intestinal. Además, en la prueba 3 se analizó un panel de biomarcadores sanguíneos y en la prueba 4 se realizó un análisis sistemático del comportamiento.

La cepa probiótica *Bifidobacterium longum* subsp. *Infantis* CECT 7210 aumentó los linfocitos intraepiteliales, redujo la excreción de *Salmonella* (prueba 1) y la colonización de coliformes en el íleon (prueba 2). En animales no desafiados aumentó el ácido butírico y la ratio vellosidad:cripta los días 8/9 PI. Los efectos sobre el consumo de alimento, la fermentación microbiana y la arquitectura intestinal mostraron un patrón diferente entre animales desafiados y no desafiados.

Cuando la cepa anterior se combinó con *Bifidobacterium animalis* subsp. *Lactis* BPL6 los efectos beneficiosos del probiótico fueron observados en ambos grupos, desafiados y no desafiados; aumentando la ingesta voluntaria de alimento, disminuyendo la consistencia fecal del periodo PI, mejorando los perfiles de fermentación a día 8 PI, estimulando la respuesta inmune intestinal con un aumento de los linfocitos intraepiteliales y mejorando la relación vellosidad:cripta. La combinación probiótica también redujo la excreción fecal de *Salmonella* y disminuyó la temperatura rectal a niveles similares a los animales no desafiados 48 horas PI.

Respecto a los biomarcadores sanguíneos, los micro-minerales fueron útiles para describir el rendimiento de los cerdos. Los electrolitos y los índices de bases ácidas permitieron detectar los animales más afectados. Finalmente, los parámetros bioquímicos fueron buenos descriptores del estado de salud.

En la prueba 4, el probiótico *Bacillus licheniformis* CECT 4536 redujo la excreción de *Salmonella*. No se observaron efectos significativos del probiótico en el resto de parámetros evaluados. Sin embargo, se observó un efecto positivo del probiótico en el comportamiento, particularmente en relación a la exploración, alimentación y otros comportamientos activos durante el período de la mañana.

Los resultados de esta tesis indican que los probióticos pueden ser una forma de mejorar la adaptación de los lechones al destete y a la presión de patógenos. Además, demuestran que el análisis de parámetros sanguíneos y comportamiento pueden ser herramientas sensibles a considerar en la investigación de aditivos alimentarios.

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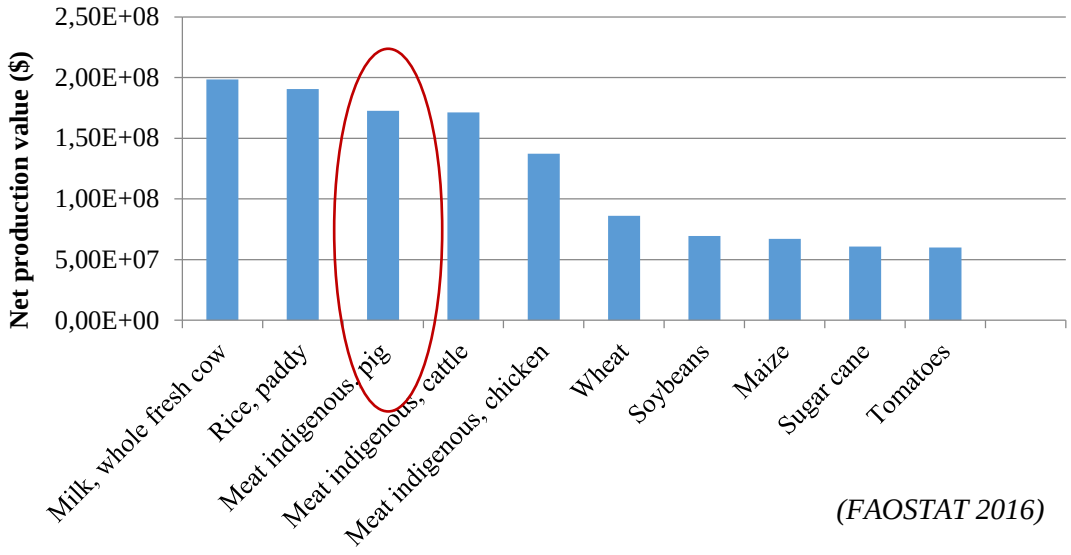
Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
AFB1	Aflatoxin B1
AHLs	Acyl homoserine lactones
AIs	Auto-inducers
Angptl4	Angiopoietin-related protein 4
ANOVA	analysis of variance
APC	Antigen presenting cells
BW	Body weight
CF	Crude fiber
CFU	Colony forming units
CNS	Central nervous system
CP	Crude protein
DCs	Dendritic cells
DFM	Direct feed microbials
DM	Dry matter
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EHEC	Enterohemorragic *Escherichia coli*
ENS	Enteric nervous system
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FDA	Food and Drug Administration
FM	fresh matter
FOS	Fructo-oligosaccharides
G:F	Gain feed ratio
GABA	Gamma-aminobutyric acid
GC	Goblet Cells
GF	Germ-free
GIT	Gastrointestinal tract
Gpr	G-protein-coupled receptor
HRV	Human rotavirus
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
IPEC	Porcine intestinal epithelial cells
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
M cells	Microfold cells
MAMPs	Micro-organism-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
mRNA	Messenger RNA
MRS	Man Rogosa Sharpe
MUC	Colonic mucin
NF-κB	Nuclear factor-κB
NOD-like receptors	Nucleotide-binding oligomerization domain-like receptors
pBD-2	Porcine beta-defensin-2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	Post-inoculation
PRRs	Pattern recognition receptors
qPCR	quantitative PCR
QPS	Qualified Presumption of Safety
QQ	Quorum quenching
QS	Quorum sensing
RNA	Ribonucleic acid
SCFAs	Short chain fatty acids
SPF	Specific pathogen-free
TGFβ	Transforming growth factor-β
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
UAB	Universitat Autònoma de Barcelona
XLT4	Xylose-Lactose-Tergitol-4

Chapter 1. General Introduction

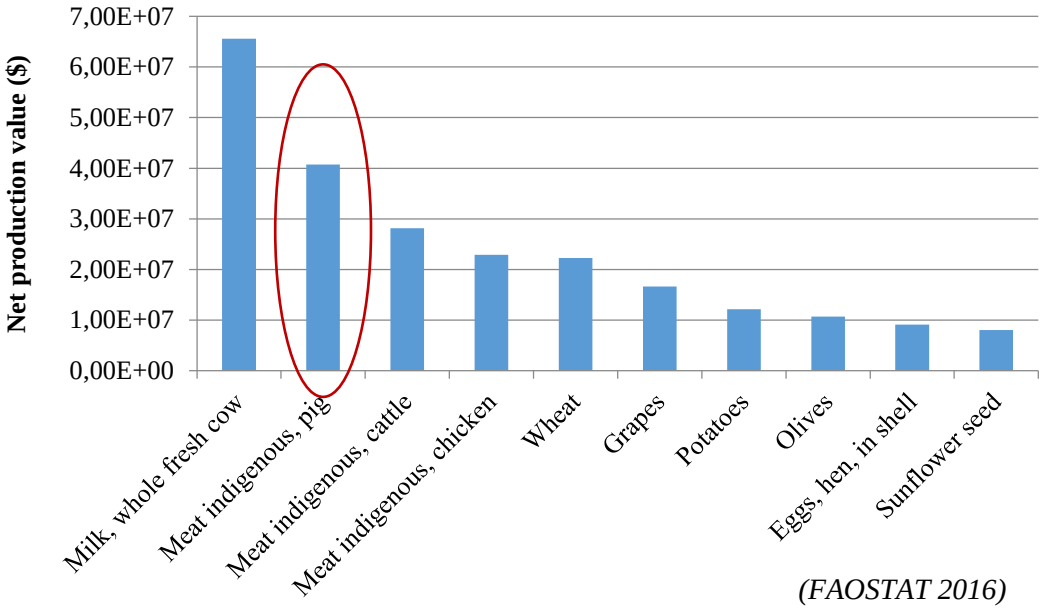
Pig meat production is the third most valuable product yearly produced when considering food and agricultural commodities worldwide and the second most valuable when just considering the European Union (EU) (see Figures 1.1 and 1.2).

Figure 1.1. Top 10 most valuable commodities produced worldwide in 2013.



(FAOSTAT 2016)

Figure 1.2. Top 10 most valuable commodities produced in Europe in 2013.



(FAOSTAT 2016)

Due to its importance, powerful forces of economic and social change are rapidly transforming the livestock sector in many developed and developing countries. Production

of livestock, especially pigs and poultry, is becoming more intensive, geographically concentrated, vertically integrated and linked with global supply chains.

Likewise, animal-health and food-safety standards are improving in order to elevate public health and in this context, a challenging horizon is emerging for the swine industry with two main problems to be solved.

On the one hand, increase food-safety standards and reduce *Salmonella* zoonoses in meat and meat products. *Salmonella* spp. is a ubiquitous bacterium which is able to infect a broad-host-range serotype of animals including humans, causing gastrointestinal infections (Majowicz et al., 2010). Salmonellosis is one of the top 10 most common diseases in weaning and grower/finisher pigs (Haley et al., 2012). Nonetheless, most importantly in the EU over 100.000 human clinical cases of salmonellosis are reported each year and animal food products play an important role in it (Forshell and Wierup, 2006). Latest European Food Safety Authority (EFSA) estimations report that after the establishment of efficient control methods in laying hens and broilers, 56.8% of zoonotic human cases are attributable to pork (EFSA, 2012; EFSA, 2013). These numbers are closely related to the prevalence of this pathogen in farms; it has been estimated that between 25% and 48% of the USA swine herd is colonized by *Salmonella* on the farm (Davies et al., 1999) and around 20% in Spain (Vico and Mainar-Jaime, 2012). Imminent action in order to prevent this situation is needed as it is expected that standards at slaughter and processing will become more stringent as new EU policies are being made with the aim of establishing a future control plan similarly to what has been already implemented in poultry. Multidisciplinary strategies to control this pathogen will be needed involving biosecurity, hygiene, management measures and where nutrition can play an important role as well.

On the other hand, antibiotic use as growth promoters has been banned in the EU (Regulation (EC) No 1831/2003) and is increasingly questioned in other markets due to the potential risk of antibiotic resistance (Gallois et al., 2009; Pugh, 2002). Furthermore, everyday more preoccupied customers worldwide, in addition to EU regulations, are pressing in order to diminish therapeutic use of antibiotics or ideally eliminate them from food production. For this reason, swine industry must find an alternative to antibiotics in situations where they have been broadly used such as a successful outcome to early weaning. Early weaning (21-28 days), much earlier than it would have occurred if animals were in the wild (10 weeks), is current in commercial situations and survival and growth of

piglets in the post-weaning period is one of the most critical moments for the intensive pig production systems (Jensen and Recén, 1989; Weary et al., 2008). In brief, piglet suffers the tremendous stress of being abruptly separated from the sow; a change from a milk based to a less digestible dry cereal based feed diet, introduction to new social partners and new physical environments. As a consequence, the period after weaning is characterized by sub-optimal growth performance (e.g.: low feed intake, deteriorated feed efficiency, body weight loss) and all this process causes marked changes in gastrointestinal tract (GIT) physiology, microbiology and immunology (Pluske et al., 1997; Weary et al., 2008). Epithelial damage also decreases nutrient digestibility which provides more substrates for pathogen proliferation, being Enterotoxigenic *Escherichia coli* (ETEC) one of the most common opportunistic bacteria affecting this age (Jensen et al., 2006; Madec et al., 2000). To sum up, high incidence of intestinal disturbances often occurring with diarrhea are a common cause of morbidity and/or mortality (Heo et al., 2013; Pluske et al., 1997).

Researchers in animal nutrition have the mission reinforce the natural defenses of the animals in front of pathogens, creating a healthier and more productive microbial ecosystem as mean to prevent these digestive disorders and reduce bacterial pathogen colonization. In a healthy animal, the presence of a stable gut microbiota greatly influences the normal development of physiological, immunological and morphological parameters in the gastrointestinal tract. Therefore, it has been suggested, the dietary manipulation of the intestinal microflora could be a potential alternative to prevent the mentioned disorders (de Lange et al., 2010). Up to date, one of the most direct and promising approaches to obtain a desirable microflora and improved gut balance is the use of probiotics; which have demonstrated to be able to improve resistance to pathogenic bacteria colonization and an enhanced host mucosa immunity (Gaggìa et al., 2010; Yang et al., 2015).

It therefore seems opportune to conduct a comprehensive study about the role of probiotics in pig production and its effect on the animal performance and gut health, taking into account the direct interaction with the host, as well as indirect actions through changes in gastrointestinal ecosystem.

Chapter 2. Literature Review

2.1. History and definition of Probiotics

Eli Metchnikoff, the Russian born Nobel Prize recipient working at the Pasteur Institute at the beginning of the last century was the first person who suggested some selected bacteria played a positive role in our bodies GIT (Metchnikoff, 1907). At this same time, Henry Tissier, a French pediatrician, observed that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar, Y shaped morphology. These "bifid" bacteria were, on the contrary, abundant in healthy children (Tissier, 1906) and he suggested that these bacteria could be administered to patients with diarrhea to help them restore a healthy gut flora.

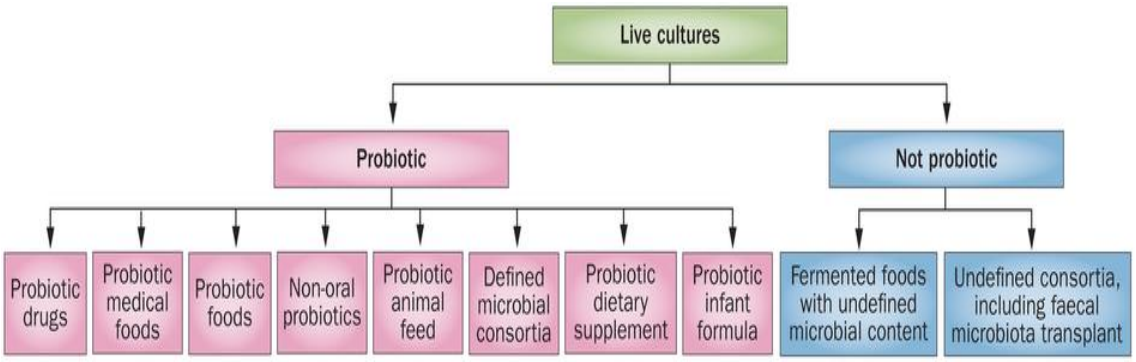
Although Metchnikoff and Tissier were the first to make scientific suggestions about the probiotic use of bacteria, the word "probiotic" meaning "for life" was not coined until 1965 (Lilly and Stillwell), as opposite to the word antibiotic, to name substances produced by microorganisms which promoted the growth of other microorganisms. Since this first definition, successions of redefinitions were made. For instance, Parker (1974) described the term as organisms and substances which contribute to intestinal microflora balance. Fuller (1989), in order to point out the microbial nature of probiotics, redefined the word as "A live microbial food/feed supplement which beneficially affects the host animal by improving its intestinal balance".

Unfortunately, probiotic history has been controversial among the scientific community. In the beginning, the observations of Metchnikoff and Tissier were so appealing that commercial exploitation immediately followed their scientific works and results were not always positive. Moreover, early studies often suffered from a lack of rigorous study design or characterization of probiotic strains. The probiotic concept was therefore regarded as scientifically unproven and it received minor interest for decades, with some research involving animal feeding in order to find healthy substitutes for growth promoting agents. In the last 20 years, however, research in the probiotic area has progressed considerably and significant advances have been made in the selection and characterization of specific probiotic cultures and substantiation of health claims relating to their consumption.

Hence nowadays, the most widely used definition for the probiotics is the proposed by the United Nations and World Health Organization Expert Panel, as "live micro-organisms which when administered in adequate amounts confer a health benefit on the

host" (FAO/WHO, 2001); implying that a health benefit must be demonstrated for the probiotic. Figure 2.3 represents an overall framework for use of the term "probiotic", encompassing diverse end uses (Hill et al., 2014). It must be taken into account that the development of metabolic by-products, dead microorganisms, or other microbial-based, non-viable products have demonstrated potential; however, these do not fall under the probiotic definition.

Figure 2.3. Overall framework for use of the term "probiotic".



From Hill et al. (2014).

2.2. Probiotics in pig production

2.2.1. Characteristics of probiotics

In general, probiotic bacteria must have the characteristics proposed in Table 2.1. However, it must be considered that characteristics may vary between countries and targeting species.

Table 2.1. Expected characteristics of probiotics.

- ✓ Non-toxic and non-pathogenic
- ✓ Accurate taxonomic identification
- ✓ Normal inhabitant of the targeted species
- ✓ Survival, colonization and being metabolically active in the targeted site, which implies:
  - Resistance to gastric juice and bile
  - Persistence in the GIT
  - Adhesion to epithelium or mucus
  - Competition with the resident microbiota
- ✓ Production of antimicrobial substances
- ✓ Antagonism towards pathogenic bacteria
- ✓ Modulation of immune responses
- ✓ Ability to exert scientifically-supported health-promoting properties
- ✓ Genetic stability and lack of transmissible antibiotic resistance genes
- ✓ Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
- ✓ Viability at high populations
- ✓ Desirable organoleptic and technological properties when included in industrial processes
- ✓ Efficacy in other host species

Adapted from Gaggìa et al. (2010).

2.2.2. Applications of probiotics in porcine

2.2.2.1. Use of probiotics as zootechnical additives

High productivity in farming industry may involve the use of diets, installations or management constraints and the apparition of opportunistic diseases (Rauw et al., 1998). Altogether, these physiological and psychological stressors can affect negatively the animal's intestinal balance creating a dysfunction of the intestinal barrier and increasing intestinal permeability. Consequently, alterations on gut microbial composition and a higher susceptibility to enteric pathogens may appear (Gareau et al., 2010).

It is well-known that a healthy gut microbiota contributes to the overall development and metabolic needs of the animal. It provides the host with many beneficial functions including production of volatile fatty acids, re-cycling of bile salts, production of vitamin K, cellulose digestion, and development of immune system (Guarner and Malagelada, 2003; Heo et al., 2013; Kim and Isaacson, 2015). Moreover, a complex microbiota (many different biotypes) may confer advantages to the hosts by allowing rapid adaptation to environmental changes and resistance to pathogens (Fouhse et al., 2016; Marchesi and Shanahan, 2007).

Probiotics in porcine production, aim to establish a healthy gut microbiota and thereby improve the health and wellbeing of the animals (Cho et al., 2011; Kenny et al., 2011). When using probiotics in pigs, high productivity in addition to better quality and safer products are expected. They are used in all stages of porcine production: sows during pregnancy and/or lactation, pre-weaning and post-weaning piglets or growing pigs up to market weight. However, the target of using probiotics is different in every situation. Main applications of probiotics in different productive stages can be seen in Table 2.2 and will be briefly discussed further on.

Table 2.2. Main applications attributed to probiotics in the swine industry.

Phase	Main applications attributed to probiotics	References
Sows	Mitigation of frequently associated problems such as stress and constipation.	Chaucheyras-Durand and Durand, 2009
	Reduction of clinical signs of the uterus and/or udder disease	Alexopoulos et al., 2004; Apic et al., 2015
	Increase in feed consumption during last pregnancy stages or lactation	Alexopoulos et al., 2004; Bohmer et al., 2006; Hayakawa et al., 2016; Jeong et al., 2015; Kritas et al., 2015
	Improvement of body condition at the end of lactation	Bohmer et al., 2006; Jeong et al., 2015; Kritas et al., 2015
	Reduction of the weaning–estrus interval due to energy mobilization	Alexopoulos et al., 2004; Bohmer et al., 2006; Hayakawa et al., 2016; Kritas et al., 2015; Wang et al., 2014
	Improvement of colostrum quality, milk quality and quantity	Alexopoulos et al., 2004; Scharek-Tedin et al., 2015
	Reduction of gut pathogens in sows and/or piglets	Baker et al., 2013; Kritas et al., 2015
	Modulation of litter immunity	Scharek-Tedin et al., 2015; Siepert et al., 2014
	Enhancement of litter size	Alexopoulos et al., 2004; Apic et al., 2015; Baker et al., 2013; Jeong et al., 2015; Taras et al., 2005; Taras et al., 2006

Sows	Enhancement of growth rates of the piglets	Alexopoulos et al., 2004; Apic et al., 2015; Baker et al., 2013; Hayakawa et al., 2016; Kritas et al., 2015; Taras et al., 2005; Wang et al., 2014
	Reduction of clinical signs of diarrhea in piglets	Alexopoulos et al., 2004; Apic et al., 2015; Taras et al., 2006
	Delivery of probiotics to piglets	Kenny et al., 2012; Scharek et al., 2005; Scharek-Tedin et al., 2015
Piglets	Modulation of stress response	Le Bon et al., 2010; Gan et al., 2014
	Modulation of piglet's gut microflora	Ahmed et al., 2014; Bhandari et al., 2008; Bomba et al., 2002; Le Bon et al., 2010; Krause et al., 2010; Scharek-Tedin et al., 2015; Upadhaya et al., 2016; Zhang et al., 2009; Zhang et al., 2016
	Protection against pathogenic bacteria, gastrointestinal disorders and diarrhea	*(see Table 2.3)*
	Enhancement of intestinal barrier function	Guerra-Ordaz et al., 2014; Le Bon et al., 2010; Lessard et al., 2009; Lodemann et al., 2006; Putaala et al., 2008; Roselli et al., 2007; Yang et al., 2016
	Modulation of immunity	Daudelin et al., 2011; Lessard et al., 2009; Naqid et al., 2015; Scharek et al., 2005; Scharek-Tedin et al., 2015; Shu et al., 2001; Siepert et al., 2014; Wang et al., 2009; Yang et al., 2016; Yin et al., 2014; Zhang et al., 2009; Zhang et al., 2016; Zhou et al., 2015; Zhu et al., 2014
	Improvement of digestibility, enhanced growth and feed conversion ratio	Ahmed et al., 2014; Yu et al., 2008

Piglets	Improvement of productive parameters in piglets	Ahmed et al., 2014; Bhandari et al., 2010; Casey et al., 2007; Chen et al., 2006; Davis et al., 2008; Hayakawa et al., 2016; Konstantinov et al., 2008; Krause et al., 2010; Kritas et al., 2015; Mallo et al., 2010; Shu et al., 2001; Taras et al., 2005
	Supplementation of targeted nutrients	Gan et al., 2014
Fattening pigs	Improvement of meat quality	Černauskienė et al., 2011; Meng et al., 2010
	Improvement of digestibility	Černauskienė et al., 2011; Chen et al., 2006; Giang et al., 2011; Meng et al., 2010; Yan and Kim, 2013; Yang et al., 2015; Zhang and Kim, 2015
	Reduction of contamination by decreasing fecal NH_3- N	Chen et al., 2005; Chen et al., 2006; Wang et al., 2009b; Yan and Kim, 2013
	Reduction of subclinical pathogenic infections or zoonoses	Gebru et al., 2010 + *(see Table 2.3)*
	Reduction of mortality	Davis et al., 2008
	Improvement of weight gain	Černauskienė et al., 2011; Chen et al., 2005; Davis et al., 2008; Gebru et al., 2010; Meng et al., 2010; Wang et al., 2009b; Yan and Kim, 2013
	Improvement of gut health	Yan and Kim, 2013

Sows

Sows management in pig production is complex as many times producers have to take decisions in between what is "good for the sows" and "good for the piglets" (Barnett et al., 2001). Many physiological and welfare issues have been described in sows, mainly due to physical confinement and dietary restrictions in gestation. Some of these problems have been addressed by legislation, for example with the EU ban on the pregnancy-long housing of sows in gestation stalls, in full effect since January, 2013 (Council Directive 2008/120/EC.). However, further efforts should be done in mitigating the situation as these issues may affect the stress response of the sow, her health and injury status, with probable direct or indirect effects on piglets (Barnett et al., 2001).

In this context, probiotics are used in sows to enhance their health, well-being and reproductive performance. Several in-field studies have pointed out that supplementation of sows may increase feed consumption during last pregnancy stages or lactation. As a consequence, improved body condition at the end of lactation and reduction of the necessity of the energy mobilization at lactation, with a reduction of the weaning–estrus interval have also been reported with probiotics. This process is especially important in young sows after their first litter to prevent 'starvation sterility', caused by reduced feed intake during lactation with high mobilization of body tissue (Bohmer et al., 2006). In addition, it has been suggested that probiotics can be useful for mitigation of frequent problems such as constipation associated to diet restrictions and stress due to cage confinement in late-gestation.

A vast amount of reproductive-performance related benefits have been reported with the use of probiotics in sows. For example, an increase of the number of piglets or higher piglet growth rates with higher body weight (BW) at weaning. In this sense, the increasing popularity of hyper-prolific sows in commercial situations clearly opens a window for probiotic use as their lower weight piglets require refined management, environmental and nutritional conditions in order to survive and develop healthily (Martineau and Badouard, 2009).

A reduction of clinical signs of the uterus and/or the udder disease and less clinical signs of diarrhea in piglets have also been described. Finally reduction of gut pathogens in sows and on piglets have been reported although literature at this point is divergent,

as other authors reported no effects on microbial populations (Bohmer et al., 2006; Wang et al., 2014) by giving probiotics to sows.

Kenny et al. (2012) postulated that the most efficient way to deliver probiotics to piglets may be to dose sows before and during farrowing in order to saturate her and her environment with desirable organisms. Like this, piglets could start acquiring them as part of its natural development even before eating dry feed, opening a route for early modulation of piglets physiology and immune system. However, there is a high disparity on the dosing methods observed in literature. Time length to supplement sows varies from full gestation and lactation, the last gestation days and lactation (starting supplementation when sows are moved to farrowing cages) or just lactation period. Moreover, the majority of studies only tested the effect of supplementing one productive cycle although there is evidence that administering probiotics for more than one cycle is beneficial (Kritas et al., 2015). There is also no consensus in literature whether to supplement piglets creep feed with probiotic treatment (Hayakawa et al., 2016; Kritas et al., 2015; Taras et al., 2005, 2006) or not (Alexopoulos et al., 2004; Apic et al., 2014; Baker et al., 2013; Bohmer et al., 2006; Wang et al., 2014). However, even when not supplementing piglets, it seems that several mechanisms may act in order to provide piglets with the benefits of probiotic supplementation of sows. For instance, Taras et al. (2005) detected probiotic bacteria administered to sows in piglet feces and digesta without administering them the probiotic treatment, indicating a second route of uptake besides diet and Alexopoulos et al. (2004) found higher milk fat and protein content at mid suckling period with the probiotic supplementation.

To conclude, administering probiotic treatments to sows has demonstrated its potential. Nevertheless, a better knowledge of the best way to seed the piglet gut with beneficial bacteria could be obtained with trials where supplementation in gestation is compared to supplementation in lactation and/or in the creep feed for piglets (Bosi and Trevisi, 2010). Finally, homogenization on the dosing protocols would be necessary in order to select most effective probiotic strategies at this stage.

Piglet

The neonatal period is a critical time where the GIT and immune system have not fully developed yet. These deficiencies result in low disease resistance in piglets and make them vulnerable to stress reactions or invasion by pathogenic microorganisms, which may seriously affect individual healthy development (Konstantinov et al., 2006; Levast et al., 2014). In addition, in commercial situations piglets have to face an early weaning, with a considerable amount of psychological and physiological stress induced by changes in feed and the environment. The existence of all these challenges at this stage is concerning, as in this same period gut microbiota plays a critical role in "educating" the neonatal gut immune system to generate functional adult systems for recognizing pathogens and dealing with novel food antigens (Heo et al., 2013; Lallès et al., 2007).

The main application of probiotics in weaning is to improve weaning outcome by relieving stress, preventing diarrhea, re-establishing microbiota balance after the transient drop in favorable bacteria, protecting against pathogenic bacteria, enhancing intestinal barrier function and stimulating immunity (Ahasan et al., 2015). A vast body of literature that appeals to probiotic capacities to achieve positive interactions with the host by enhancing productive parameters in piglets. However, only a few of the published articles attempted to measure the increase in nutrient bioavailability in piglets due to a probiotic treatment. The rest of the studies did not investigate whether these improvements could be due to a direct action of the probiotic achieving a higher bioavailability of feed nutrients, an indirect gut health modulation (relieve weaning stress, prevent diarrhea, improving the intestinal microbiota profile, etc.) or perhaps a combination of both. Finally, another strategy that has demonstrated a positive impact in piglets is to supplement the probiotic treatment with targeted nutrients and increase its bioavailability.

Fattening pigs

Interest of using probiotics in growing pigs is mainly to enhance productivity and meat quality. It's been speculated that as growing-finishing pigs have a mature GIT, with high digestive enzyme activity, immune capacity and disease resistance, the influence of probiotics in growing-finishing pigs is relatively limited (Yang et al., 2015). Nevertheless, probiotic supplementation in this stage pigs has demonstrated positive

results in many studies. This lack of agreement between published research may be because effects can vary depending on the age of the animals or diets where they are used. Scientific literature published until now would support the idea that older pigs have more developed immunity and capacity to resist intestinal disorders. However, there is still margin for probiotic to act and potentiate growth, especially in early growing phases or high-density diets.

Another aspect is that the use of probiotics in this productive stage is rather complex. Although enzymes produced by probiotics may increase feed digestibility; counter effects of the probiotic treatment should be expected such as reducing the availability of dietary components for their own metabolism or stimulation of immunity. Therefore, we will only see positive effects when these advantages are able to balance and surpass the energetic costs for the pigs. Kenny et al. (2012) speculated that when probiotics can fully demonstrate a beneficial response is in commercial conditions, especially in low sanitary conditions where subclinical diseases may be compromising animals. In this case, administering probiotics may prevent subclinical diseases, mainly due to the immunity enhancement. This situation would pay off energetic costs probiotic treatments have for the animals in terms of mounting the immune response or reducing the availability of dietary nutrients. On the other hand, when pigs are raised in high sanitary and welfare conditions (as used in research centers) costs in terms of energy may exceed the treatment benefits and profit of the probiotics are rarely seen.

2.2.2.2. Use of probiotics against pathogens

As already mentioned, one of the most important targets for probiotic use is to reduce pathogen pressure. Interest in this application has increased since the use of antibiotics as growth promoters was banned in the EU (Regulation (EC) No 1831/2003) and pressure is exerted to limit their therapeutic use due to the potential risk of antibiotic resistance (Gallois et al., 2009; Pugh, 2002). It is a critical task especially in early life immature stages, where prevention for clinically relevant pathogens such as ETEC is vital to prevent disease states post-weaning. Moreover, there is a necessity to reduce pathogens such as *Salmonella* spp. in growing-finishing phases as their zoonotic potential is a major public health issue.

A vast amount of research is yearly published in relation to probiotic capacities to fight digestive affections. It is worth to mention that the interest of finding probiotic strategies not only exists in animal production, but is also present in human medicine; which in many cases use the pigs as a human model (Meurens et al., 2012). This fact enriches the amount of information available. Certain aspects of this work are useful for the pig industry, particularly studies focused on the mechanism of action or interaction with host mucosal surfaces or pathogenic bacteria (Kenny et al., 2011). However, several considerations should be taken to adapt the probiotic use in a practical and viable way at farm, mainly related to dosing, management and stability on a farm environment. Furthermore, in some cases, parameters measured do not reflect the efficacy of probiotics in pig production (Kenny et al., 2011).

Table 2.3. Pig in vivo scientific works evaluating the use of probiotics against digestive bacterial pathogens.

Reference	Probiotic strain	Pathogen	Prod. Phase	Benefits	Main results
De Cupere et al., 1992	a) *Bacillus cereus* b) *Lactobacillus* spp. c) *Streptococcus faecium*	ETEC	Piglets	No	No improvements on clinical symptoms or mortality. No improvements on fecal *E.coli* shedding.
Shu et al., 2001	*Bifidobacterium lactis*	ETEC	Piglet	Yes	Reduced diarrhea scores and fecal shedding of *E.coli.* Improved animal performance. Increased T-cell differentiation and pathogen-specific antibody titers.
Bhandari et al., 2008	*Bacillus subtilis*	ETEC	Piglet	Yes	Reduced diarrhea scores and mortality. Modulated microbial diversity.
Lessard et al., 2009	a) *Pediococcus acidilactici* b) *Saccharomyces cerevisiae* c) *P. acidilactici + S. cerevisiae*	ETEC	Piglet	Yes	Before challenge: a) increased T-cell differentiation. After challenge: a, b, c) Reduced bacterial translocation. b) Increased ileal immunoglobulins.
Zhang et al., 2009	*Lactobacillus rhamnosus*	ETEC	Piglet	Yes	Reduced diarrhea scores and fecal coliform shedding. Modulated microbial diversity. Increased jejunal immunoglobulins. Modulated systemic inflammatory cytokines.
Bhandari et al., 2010	*Escherichia coli*	ETEC	Piglet	Yes	Reduced ETEC in ileum. Improved animal performance.

Reference	Probiotic strain	Pathogen	Prod. Phase	Benefits	Main results
Wang et al., 2009	*Lactobacillus fermentum*	ETEC	Piglet	Yes	Increased T-cell differentiation and ileum cytokine expression
Konstantinov et al., 2008	*Lactobacillus sobrius*	ETEC	Piglet	Yes	Reduced levels of ETEC in the ileum, improved performance and increased diarrhea.
Krause et al., 2010	*Escherichia coli*	ETEC	Piglet	Yes	Increased animal performance and microbial diversity. Reduced diarrhea scores (in presence of raw potato starch).
Daudelin et al., 2011	a) *Pediococcus acidilactici* b) *Saccharomyces cerevisiae* c) *P. acidilactici* + *S. cerevisiae*	ETEC	Piglet	Yes	a,b) Reduced ETEC attachment to intestinal mucosa. a,c) Induced ileum cytokine expression.
Trevisi et al., 2011	*Lactobacillus rhamnosus*	ETEC	Piglet	No	Reduced animal performance. Increased diarrhea scores. Reduced serum immunoglobulins. Tended to a worse histomorphology.
Li et al., 2012	High and low dose *Lactobacillus rhamnosus*	ETEC	Piglet	Yes	High and low dose reduced fecal coliform shedding and improved diarrhea scores (low dose was more effective).
Guerra-Ordaz et al., 2014	*Lactobacillus plantarum*	ETEC	Piglet	Yes	Improved ileal histomorphology. Reduced systemic inflammatory cytokines. Improved fermentation profile in ileum and colon.

Reference	Probiotic strain	Pathogen	Prod. Phase	Benefits	Main results
Zhu et al., 2014	High and low dose *Lactobacillus rhamnosus*	ETEC	Piglet	Yes	Both doses improved diarrhea scores. Modulated ileal T-cell differentiation. High dose increased serum cytokine expression.
Zhou et al., 2015	High and low dose *Bacillus licheniformis* + *B. subtilis*	ETEC	Piglet	Yes	Increased serum and ileal T-cell differentiation. Low dose: increased jejunal cytokine expression.
Yang et al., 2016	*Bacillus licheniformis* + *B. subtilis*	ETEC	Piglet	Yes	Increased intestinal cytokines and epithelial barrier integrity.
Zhang et al., 2016	Low, moderate, or high doses *Bacillus licheniformis* + *B. subtilis*	ETEC	Piglet	Yes	Modulated microbiota and improved histomorphological parameters.
Casey et al., 2007	5 strain combination: *Lactobacillus murinus* (x2 strains), *L. salivarius, L. pentosus* + *P. pentosaceous*	*Salmonella* Typhi-murium	Piglet	Yes	Reduced diarrhea scores. Increased animal performance. Reduced fecal *Salmonella* shedding.
Szabó et al., 2009	*Enterococcus faecium*	*Salmonella* Typhi-murium	Piglet	No	Increased colonization and fecal shedding of *Salmonella*. Increased serum immunoglobulins.
Gebru et al., 2010	*Lactobacillus plantarum*	*Salmonella* Typhi-murium	Growing pigs	Yes	Increased animal performance. Reduced fecal *Salmonella* shedding.

Reference	Probiotic strain	Pathogen	Prod. Phase	Benefits	Main results
Kreuzer et al., 2012	*Enterococcus faecium*	*Salmonella* Typhi-murium	Piglet	No	Reduced animal performance. No effect on fecal *Salmonella* shedding. Increased pathogen translocation.
Yin et al., 2014	Fermented feeds with: a) *Lactobacillus zeae* b) *Lactobacillus casei*	*Salmonella* Typhi-murium	Piglet	Yes	a,b) Improved diarrhea scores. Decreased rectal temperature, serum haptoglobin concentrations and fecal *Salmonella* shedding
Naqid et al., 2015	*Lactobacillus plantarum*	*Salmonella* Typhi-murium	piglets	Yes	Increased serum immunoglobulins.
Broadway et al., 2016	*Lactobacillus* spp.	*Salmonella* Typhi-murium	Piglet	No	Increased *Salmonella* translocation and provoqued no effect on fecal *Salmonella* shedding.
Upadhaya et al., 2016	a) *Bacillus subtilis* b) *Bacillus methylotrophicus*	*Salmonella* Typhi-murium	Piglet	Yes	a,b) Decreased *Salmonella* fecal shedding. Modulated microflora, serum systemic inflammatory cytokines and stress biomarkers.
Spiehs et al., 2008	a) *Bacillus licheniformis* + *B. subtilis* b) *Enterococcus faecium*	*Salmonella* Typhi-murium and ETEC	Piglet	No	a, b) No effect on diarrhea scores. No effect on systemic inflammatory cytokines and immunoglobulins.

Reference	Probiotic strain	Pathogen	Prod. Phase	Benefits	Main results
Walsh et al., 2012	*Enterococcus faecium* + *Bacillus subtilis* + *B. licheniformis*	*Salmonella* Typhi-murium and ETEC	Piglet	No	Increased coliform shedding, no effect in *Salmonella* scores. Prevented decrease in animal performance.
Ahmed et al., 2014	a) *Lactobacillus reuteri* b) *Bacillus subtilis* + *B.licheniformis*	*Salmonella* Typhi-murium and ETEC	Piglet	Yes	a, b) Increased animal performance. Reduced *Salmonella* and *E.coli* shedding

2.2.3. Authorized probiotics in animal feed

The use and characteristics of probiotic bacteria in feed production are regulated by the EFSA who must award the Qualified Presumption of Safety (QPS), presumption being defined as "an assumption based on reasonable evidence" and qualified to allow certain restrictions (EFSA, 2007). Similarly, the Food and Drug Administration (FDA) considers probiotics Direct Feed Microbials (DFM) and they must be granted a "GRAS" status ("Generally Regarded As Safe") to be used in the US.

The discussion of these regulatory mechanisms are out of the scope of this review, but European approach will be briefly exposed to illustrate how safety is actually affronted. According to the QPS concept, putative microorganisms must be identified to strain level, demonstrate no association with any infection in humans or animals and must not harbor transferable antibiotic resistance genes (EFSA, 2007). If these microorganisms are of certain predetermined taxonomic groups, either do not pose any safety risk or the risk can be clearly defined and eliminated, the group can be designated as a group with QPS status and may not be the subject of a detailed pre-market safety assessment other than satisfying predetermined specific qualifications. With this strategy micro-organisms with proven reasonable safety are prioritized but microorganisms with uncertain risk status will need to undergo a detailed pre-market safety assessment (EFSA, 2007). Once they are accepted, they are assigned to the category zootechnical additives and classified in a functional group depending on intended use: digestibility enhancers, gut flora stabilizers or other zootechnical additives.

In terms of different probiotic bacteria, *Lactobacillus* and *Bifidobacterium* species are generally considered the safest choice as probiotics, with very rare cases of infections. Although some *Bacillus* species have been granted with QPS status, the use of viable spores of *Bacillus* is more questioned as several cases of food poisoning have been reported and still in some cases the absence of toxigenic activity needs to be verified. As for *Enterococcus* species, although some strains have a long history of safe use in both animals and humans, these bacteria have also been associated with several infections and the presence of transferable antibiotic resistance determinants. For this reason the QPS status has not been given to this genus and despite the fact that there are many commercial probiotic products available in the market with *Enterococcus* bacteria, safety assessment must be done in a case-by-case basis (EFSA, 2007, 2013, 2016).

As technology is constantly evolving and information related to a specific strain may vary within time, a revised list is published every year by the EFSA with the microorganisms that have been granted a QPS status. The current authorized list of feed additives can be found at the Regulation (EC) No 1831/2003. European Union Register of Feed Additives. Edition 246. Annex I – 05.12.2016 and can be accessed by in the following link or QR-code.

https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-eu-reg-comm_register_feed_additives_1831-03.pdf

2.3. Potential modes of action of probiotics

A description of the state of the art of the potential modes of action of probiotic treatments will be done in the following section. With the objective putting some order in the complex matrix by which the probiotics may act, direct or indirect effects of a probiotic treatment will be discussed separately. When bacteria or their components interact directly with the animal metabolism, for instance affecting digestibility, immune response, gene-expression or gut-brain axis; they will be considered direct effects. On the other hand, changes in gastrointestinal ecosystem that produce a beneficial effect, for instance promoting a healthy microbiota or interfering in mechanisms of bacterial communication; will be considered indirect effects.

In order to discuss the latest advances published in the field of probiotics, the large amount of mechanistic studies recently carried out in human beings or animals as a "human models" will be considered. Nevertheless, in many cases further characterization in swine production will be needed to fully assess their potential in pigs and in a farm environment (Chaucheyras-Durand and Durand, 2010; Meurens et al., 2012).

2.3.1. Direct effects in the host

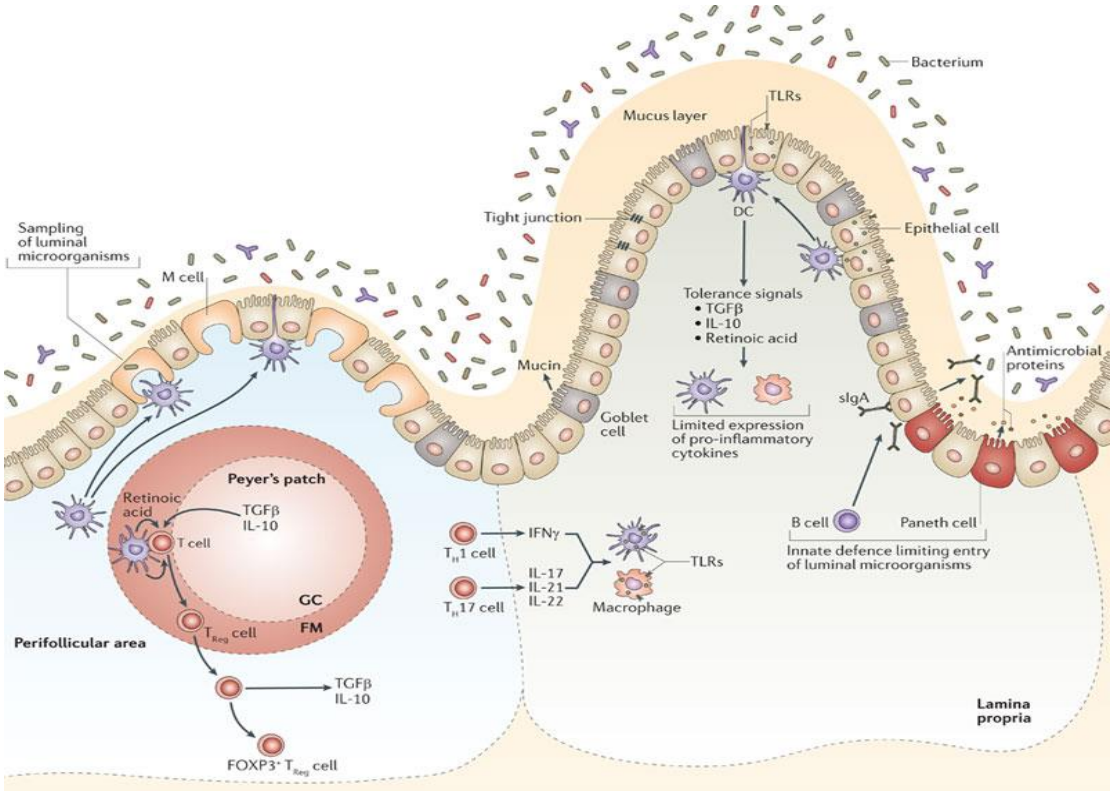
Direct effects in the host are the ones obtained by direct action of the probiotic (or their components) in the host cells. It must be remembered that metabolic by-products, dead microorganisms, or other microbial-based, non-viable products do not fall under the probiotic definition (Hill et al., 2014). Nevertheless, in many occasions, these substances have demonstrated potential and they will be considered in this section to thoroughly discuss probiotic mechanisms of action.

2.3.1.1. Modulation of immune response

Modulation of host immunity is one of the most commonly reported benefits of the consumption of probiotics, hence the name of *immunobiotics* is increasingly being used (Villena and Kitazawa, 2013). The immune response in the host sometimes should be stimulated (for example in infections) while it should be restricted in other cases (for example in allergies). It is an extremely important function as it is closely integrated with other vital functions such promoting a favorable microbial population, absorption of water or nutrients, energy metabolism and eventually performance (Brown, 2011; Reimert et al., 2014). New molecular tools are increasingly being developed and nowadays we are starting to understand intrinsic probiotic mechanisms. A graphic display of the known mechanisms of host tolerance or defence against intestinal microorganisms can be seen in Figure 2.4.

This section will give a brief insight of well-established and recently reported effects of probiotics in intestinal immunity. Moreover, the mechanisms how they modulate their actions will also be addressed.

Figure 2.4. Host mechanisms of defence and tolerance against intestinal microorganisms.



Extracted from Bron et al. (2011).

2.3.1.1.1. Innate Immune response

To start with, the intestinal range of non-specific anti-bacterial weapons that are constitutively produced by enterocytes, or specialist cell types, are an attractive target by which probiotics may exercise strong influence.

2.3.1.1.1.1. Barrier function enhancement

Specific probiotics may enhance epithelial barrier function and protect against pathogen-induced disruption of membrane barrier by several mechanisms. Firstly, restoration of disrupted epithelial barrier has been described with probiotic *Escherichia coli* strains, by changing protein kinase C signaling resulting in an amplification of expression and redistribution of *zonula occludens* protein 1 and *zonula occludens* protein 2, which are important proteins for preservation and maintenance of tight-junction function (Putaala et al., 2008; Zyrek et al., 2007). This function may be most important in counteracting the effects of pathogens, which often exert gastrointestinal symptoms by weakening the junctions between cells. Loose junctions allow translocation of the pathogens and activation of inflammatory signals or establishment of local inflammatory lesions (Kenny et al., 2011).

Secondly, they also protect barrier function by preventing close contact of microorganisms with the proliferative cells in the crypts and shaping the composition of microbiota at mucosal surfaces (Schroeder et al., 2011). Probiotic treatments have been reported to increase activity of these antimicrobial molecules and potentiate branches of the innate and adaptative response through toll-like receptor (TLR) 2 or TLR4 signaling (described later) or cell-mediated immune system cells (Linde et al., 2008; McCracken and Lorenz, 2001). Enhancement of host cells antimicrobial peptides such as lysozymes and defensins, produced by Paneth cells among other immune cells including neutrophils and macrophages have been reported. Schlee et al. (2008) demonstrated the capacity of different probiotic lactobacilli strains to upregulate production of human beta-defensin-2 through the inductions of pro-inflammatory pathways including nuclear factor-κB (NF-κB) and AP-1 as well as MAPKs. As for swine, pathogenic bacteria such as *Salmonella* have demonstrated their ability to upregulate porcine beta-defensin-2 (pBD-2) expression *in vitro* (Veldhuizen et al., 2009). Still, to our knowledge, the capacity of stimulating pBD-2 has not been described for any probiotic in porcine yet. Further investigation should be encouraged in this research line as pBD-2 broad antimicrobial activity has been supported *in vitro* and *in vivo* (Tang et al., 2015; Veldhuizen et al., 2008).

Thirdly, Goblet cells (GC) produce mucins forming a protective layer on the epithelium preventing direct epithelial contact with luminal microorganisms and these production may also be enhanced by probiotic treatments (McCracken and Lorenz, 2001). A *Lactobacillus plantarum* strain was shown to increase messenger RNA (mRNA) expression of colonic mucin (MUC) 2 and MUC3 in HT29 intestinal cells, and this led to inhibition of adhesion of enteropathogenic *E. coli* (EPEC) (Mack et al., 2003). Moreover, Caballero-Franco et al. (2007) tested the capacity of a probiotic complex of Lactobacilli, Bifidobacteria, and Streptococci strains in rats where it significantly stimulated mucin secretion and MUC2 gene expression; however, MUC1 and MUC3 gene expression were only slightly elevated (Caballero-Franco et al., 2007).

Finally, probiotics have also been described to protect barrier function by providing beneficial effects on intestinal cell homeostasis in an environment of pro-apoptotic cytokines. A *Lactobacillus rhamnosus* strain or its metabolic products were described to prevent cytokine-induced apoptosis *in vitro* by activating the anti-apoptotic Akt/protein kinase B and inhibiting pro-apoptotic p38/mitogen-activated protein kinase by Tumor

Necrosis Factor (TNFα), interleukin 1(IL1), or gamma-interferon (IFNγ) (Yan and Polk, 2002).

2.3.1.1.1.2. Innate bacterial recognition: Toll-like receptor signaling

Micro-organism-associated molecular patterns (MAMPs) from gut commensal microbiota (including probiotics) are detected by various pattern recognition receptors (PRRs) in order to develop and train immune system (Bron et al., 2011; Corthésy et al., 2007). PRRs include TLRs, nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) and C-type lectin receptors (Artis, 2008). Among them, TLRs are increasingly being studied due to the ubiquitous nature of TLR mRNA expression in pigs (Shimosato et al., 2005; Thomas et al., 2006; Tohno et al., 2006) and the evidence that TLR play a pivotal role in the protective effects of probiotics. As TLRs have received much attention in scientific research recently and will probably gain more relevance in the near future, deeper insight of the field will be given.

Thirteen mammalian TLRs have been identified so far. They are expressed in diverse cell types including gut epithelial cells, B cells, mast cells, dendritic cell, macrophages, neutrophils and T regulatory cells (Sutmuller et al., 2006). Cell surface expressed *TLRs* (*TLR1*, *TLR2*, *TLR4*, *TLR5* and *TLR6*) have been described to recognize predominantly bacterial, fungal and parasite ligands such as probiotic cell wall components (peptidoglycan and teichoic acids) or other strain-specific conserved probiotic molecules (Akira et al., 2006; Bron et al., 2011). In the other hand, *TLR3*, *TLR7*, *TLR8*, and *TLR9* are expressed within endosomes and recognize single and double-stranded RNA and DNA (Akira et al., 2006).

Table 2.4. Main TLRs and their ligands.

TLRs	Ligands
TLR1, TLR2, TLR6	Lipoproteins
TLR3	Double strand RNA (dsRNA)
TL4	Lipopolysaccharide (LPS)
TLR5	Flagellin
TLR7, TLR8	Single strand RNA (ssRNA)
TLR9	Cytosine-phosphate-guanine dinucleotides (CpG)

Adapted from Cario (2005)

Probiotics have been reported to modulate several TLR, for instance *B. longum* and *L. plantarum* were shown to inhibit inflammatory cytokine expression via a TLR2 and TLR4 activation and inhibiting intestinal bacterial glycosaminoglycan degradation in an experimental model of colitis with mice (Grangette et al., 2005; Lee et al., 2009). Signaling through TLR5 was reported to occur by the commensal *E. coli* strain and its associated flagellin, which would trigger pro-inflammatory responses in enterocytes both *in vitro* in human epithelial cells and *ex vivo* in murine ileal models (Bambou et al., 2004). Moreover, DNA of a probiotic mixture has been reported to have a systemic anti-inflammatory effect in contrast to DNA from pathogenic bacteria which induced an inflammatory reaction via TLR9 in murine experimental colitis (Rachmilewitz et al., 2004).

Toll-like receptor signaling has been shown to be involved in three important mechanisms that are crucial for maintaining a healthy epithelial barrier: epithelial cell proliferation and maintenance of tight junctions, expression of antimicrobial factors and modulation of immune responses (Villena and Kitazawa, 2013). Ligation of TLR starts signaling cascades that involve the activation of the transcription factor NF-κB and subsequent up-regulation of stimulatory molecules as well as inflammatory cytokines and chemokines (Kumar et al ., 2009). Briefly, during infection, TLR signaling is up-regulated at first leading to inflammation while some intracellular regulators attenuate the TLR response in a negative feedback loop with the aim of maintaining the immune balance. Overall, in a healthy individual, intestinal colonization stimulates these mechanisms that in turn contain the microbiota within the intestinal lumen and neutralize MAMPs to protect the host from the systemic translocation of bacteria or bacterial products (Cerf-Bensussan and Gaboriau-Routhiau, 2010). As an example, TLR4 signaling pathway and it's regulatory mechanisms can be seen in Figure 2.5 (adapted from Villena and Kitazawa (2014)). For further information, regulation of TLR signaling pathways were extensively revised by Villena and Kitazawa (2013).

Probiotic modulation of TLRs in pigs

Wachi et al. (2014) demonstrated that both, a strain of *Lactobacillus delbrueckii* and its extracellular polysaccharides attenuate ETEC-induced inflammatory response in porcine intestinal epithelial cells (IPEC) by downregulating TLR4-dependent NF-κB and mitogen-activated protein kinase (MAPK).

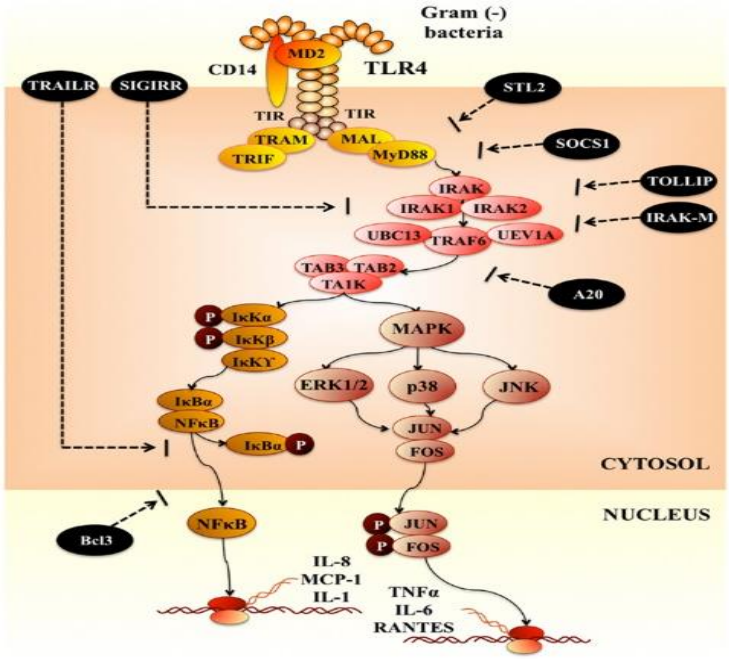


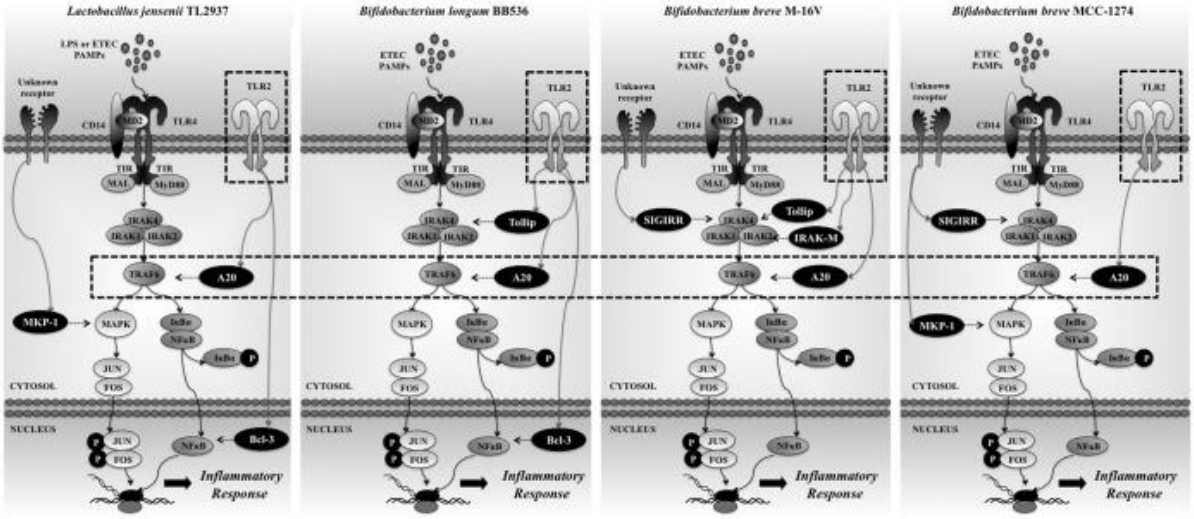
Figure 2.5. TLR4 signaling pathway and its regulatory mechanisms.

Adapted from Villena and Kitazawa (2014).

Similarly Shimazu et al. (2012) and Villena et al. (2012) observed that *Lactobacillus jensenii* attenuated the expression of pro-inflammatory cytokines and chemokines triggered by ETEC or by Lipopolysaccharides (LPS) via TLR2 and 4 in IPEC cells and antigen presenting cells from porcine Peyer's patches. With this same strain, Suda et al. (2014) carried out *in vivo* experiments with weaning piglets of 3 weeks of age. Results demonstrated that it significantly reduced blood complement activity and C reactive protein concentrations, while no changes were observed in blood leukocytes, ratio of granulocytes to lymphocyte numbers, macrophage activity and antibody levels. Alternatively, Finamore et al. (2014) reported upregulated expression of A20 by a *Lactobacillus amylovorus* strain isolated from unweaned pigs, which counteracted the inflammatory status triggered by ETEC in intestine through inhibition of the TLR4 signaling pathway (modulated by TLR2). Moreover, Tomosada et al. (2013), reported that a *Bifidobacterium longum* and *B.breve* strain significantly downregulated levels of IL8, monocyte chemotactic protein (MCP)-1 and IL6 in IPEC cells challenged with heat-killed ETEC *in vitro*.

Overall, several strains with immunoregulatory capabilities use a common mechanism to induce tolerance, TLR2-TLR4 dependent and where ubiquitin-editing enzyme A20 has a key role (see Figure 2.6). This is of interest because it not only shows a common mechanism for the anti-inflammatory activity of several probiotics, but also provides a potential biomarker for the screening and selection of new immune-regulatory strains (Tomosada et al., 2013).

Figure 2.6. Several strains with immunoregulatory capabilities use a common mechanism to induce tolerance.



From Tomosada et al. (2013).

A different TLR modulation was reported by Good et al. (2014), who reported that UV-inactivated *Lactobacillus rhamnosus* produced a TLR9 mediated attenuation of mucosal cytokine response and improved histological morphology in newborn mice and premature piglets. Moreover, DNA of this strain reduced the extent of pro-inflammatory signaling in cultured enterocytes and in samples of resected human ileum *ex vivo*, suggesting that *Lactobacillus rhamnosus* DNA is sufficient for its protective effects Good et al. (2014).

In general, by studying TLR responses, a better knowledge of the mechanism of action of probiotics is achieved. Therefore, aspects such as probiotic treatment interactions and its dose-response nature can be studied to improve the rather empiric use is done nowadays. Interestingly, Li et al. (2012) studied the effect of two doses of *Lactobacillus rhamnosus* (10^{10} colony forming units (cfu)/d or 10^{12} cfu/d) in weaning piglets challenged with ETEC F4+. Unexpectedly, high-dose probiotic administration increased the incidence of diarrhea before an ETEC challenge, despite the fact that both doses ameliorated ETEC induced diarrhea. Expression of jejunal TLR2, ileal TLR9, NOD1 and TNFα mRNA was upregulated only in the low-dose piglets after ETEC challenge. Piglets pretreated with high-dose *L. rhamnosus* had stronger downregulation of ETEC-induced jejunal TLR4, IL8 and ileal pBD-2 mRNA expression and failed to upregulate TLR2, TLR9, NOD1 and TNFα mRNA expression post-challenge. Overall, this data would suggest that pretreatment with a low dose of *L. rhamnosus* might be more effective than a high dose. Probably the exposure to a larger primary *L. rhamnosus* dose may induce hypo-responsiveness to the

ETEC challenge. Hence, a safe threshold for preventative use of probiotics in clinical practice may exist and when overpassed, a decrease of the prophylactic benefits against potential enteric pathogens could be seen.

Study of *B. animalis* effects on TLR of weaning piglets was done by Trevisi et al. (2008). He reported that TNFα gene expression was positively correlated with TLR4 and TLR2 gene expressions, and negatively correlated with bifidobacteria DNA. However, the activation of TLR2 in the lymph nodes was only done when fructo-oligosaccharides (FOS) were added to the diet, suggesting a higher translocation rate. Overall, the tolerance observed in the *B. animalis* treatment with basal diet and further TLR2 activation with FOS may suggest that other bacteria promoted by FOS or another underlying mechanism fomented by the prebiotic could enhance the physiological translocation of bacteria seen in the post-weaning phases. This contrasts with the trial reported by Lessard et al. (2009) where translocation of ETEC to mesenteric lymph nodes was reduced in post-weaning piglets with dietary supplementation of probiotic *Pediococcus acidilactici* after an ETEC challenge. The impact of bacterial adhesion on translocation across the epithelium represents a recurrent question and presumably is central to the development and activation of the intestinal immune system (Corthésy et al., 2007). Considering that some strains may be capable of modulating tight junctions and thereby crossing the epithelium, work in this field should be expanded and screening for this capability should be done in a wide range of well-characterized and labeled probiotic strains to control an aspect that has been undetermined up until today.

As for viral infections, the capacity to promote immune homeostasis of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* was studied in a human rotavirus (HRV) challenge with neonatal gnotobiotic pigs (Vlasova et al., 2013). Anti-inflammatory (TLR2 and 4 down-regulation) and antiviral (TLR3 up-regulation) actions were observed in addition to increased frequencies of intestinal and systemic apoptotic mononuclear cells pre-challenge. Post-challenge, significantly decreased mononuclear cells proliferation was observed, suggesting that probiotics control excessive lymphoproliferative reactions upon HRV challenge and thereby can regulate immune hyper-reactivity and induce tolerance.

To conclude, it is interesting to point out that Darfour-Oduro et al. (2016) recently reported that pathogen-mediated selective pressures among European pig populations, have derived into positive selection in pigs of a specific TLR2 gene variant. Future experimental

functional analyses are required to determine how such variant affect porcine immune response nowadays. Nevertheless, this homogeneity opens a window to find probiotic strategies that can be used efficiently in a population level to enhance immunity.

2.3.1.1.2. Adaptive immune response

The development of an adaptive response is dependent of the antigen presenting cells (APC). Briefly, three main mechanisms have been described by which antigenic material is processed and presented to underlying immune cells. Firstly, epithelial cells which can facilitate vesicular bacterial/antigenic transfer across the barrier by receptor–mediated pinocytosis, and present antigenic material in a major histocompatibility complex molecule together with co-stimulatory molecules. Secondly, dendritic cells (DCs) sample luminal contents and prime immune activation or tolerance. Thirdly, microfold (M) cells located within the epithelial monolayer above areas of follicular lymphoid tissue (referred to as Peyer's Patches), may shuttle macromolecules and microorganisms to other effector cells such as DCs and macrophages present (Feng and Elson, 2011).

In general, if these APCs present safe commensal/probiotic peptides, tolerogenic mechanisms driven by TGFβ, IL10 and retinoic acid are initiated. This results in suppression of T effector responses (Th1, Th2, Th17, Tc) and Immunoglobulin A (IgA) production. IgA secreted by plasma cells and transported by intestinal epithelial cells into the lumen may sequester most resident bacteria in the lumen through its ability to bind mucins and dramatically reduce the microbial burden of the epithelium (Feng and Elson, 2011). Probiotic strains such as *Lactobacillus rhamnosus*, *Bifidobacterium lactis* (Rautava et al., 2006) and *Saccharomyces boulardii* (Rodrigues et al., 2000) have been demonstrated to enhance IgA production and secretion through alteration of the cytokine milieu in the gut mucosa (Roselli et al., 2007). On the other hand, if APCs present pathogenic peptides, the default setting of tolerance is bypassed and as a result of the immune stimulatory cytokine environment, effector responses are initiated: Th1 for intracellular pathogens, Th17 for fungal infection and Th2 for humoral responses to extracellular pathogens (Hardy et al., 2013; Holscher et al., 2012).

In piglets, probiotic *Lactobacillus fermentum* has been reported to modulate immune function, by inducing an increase in the pro-inflammatory cytokines IFNγ and TNFα in the

ileum, and an increase in the percentage of CD4+ lymphocyte subset in blood (Wang et al., 2009). In addition, higher cellular proliferation and depressed apoptosis have also been reported with this strain (Wang et al., 2012). Similarly, probiotic containing *Pedicoccus acidilactici* and *Sacharomyces cerevisiae* subsp. *boulardii* increased T cells in ileum and IgA secretion in post-weaning piglets challenged with ETEC (Lessard et al., 2009).

In contrast, some studies have shown immune-suppressive action of probiotics in the host. An *Enterococcus faecium* strain was reported to have an immune suppressive effect, delaying early immune response to antigens in post-weaning piglets (Siepert et al., 2014). This strain provoked a reduced proliferation of blood mono-nuclear cells in response to *Salmonella* Typhimurium antigen during 1 to 3 days post-infection, which was followed by a similar proliferative response with or without the probiotic 7 days post-infection (Siepert et al., 2014). In an earlier study Scharek et al. (2005), supplemented pregnant sows and piglets with the same probiotic strain *E. faecium* or *Bacillus cereus* and showed no effect on the lymphocyte populations in the jejunal Peyer's patches. However, levels of cytotoxic T cells (CD8+) in the jejunal epithelium of piglets at day 14 and the serum level of IgG during the post-weaning period (28-56 days) were reduced. In this trial, the total anaerobe and coliform bacterial populations were not significantly affected by the probiotic treatment, either in sows or in the piglets. Nevertheless, a remarkable decline in the frequency of β-haemolytic and O141 serovars of *Escherichia coli* was observed in the intestinal contents of piglets treated with probiotic (which could partly explain the reduction in cytotoxic T-cell populations). Increased levels of fecal IgA were seen in the group given *B. cereus* compared to the *E. faecium* and controls. Furthermore, in a murine model, Peña and Versalovic (2003) reported that *L.rhamnosus* GG specifically inhibited TNFα production and reduced TNFα/IL10 ratios in macrophages with contact-independent effect requiring only the presence of a soluble *L. rhamnosus* GG immunomodulin for complete anti-inflammatory activity. This latter study is interesting because although bacterial adhesion has been postulated as a requirement for the probiotic to exert beneficial effects (Boyle et al., 2006), some reports demonstrated that soluble factors secreted by probiotics are enough to modulate the production of cytokines and therefore, to modulate the immune system.

Duncker et al. (2006) also reported that the distribution of intestinal immune cells (granulocytes, mast cells, CD4+, CD8+, CD25+ and IgA+ lymphocytes) and the mucosal

expression of cytokines (IFNγ, TNFα, TGFβ and IL10) of young pigs was not changed by *E. coli* Nissle administration; so, in conclusion, probiotics have been reported to play all the possible roles. In fact, differences with the same probiotic can be seen by using different dosing concentrations. Interestingly, Wen et al. (2012) observed that low concentrations of *L. acidophilus* (10^6 cfu × 5 doses) significantly increased the population of the antiviral interferon IFNγ producing T cells and reduced the regulatory T cells and production of TGFβ1 and IL10 in intestinal lymphoid tissue of gnotobiotic piglets compared with untreated animals. However, on the other hand, when the same probiotic was administered at a high dose rate (up to 10^9 cfu/dose × 14 doses) regulatory T cells were increased.

To conclude, the presented studies demonstrate that it is possible to modulate immune response by using probiotics. Nevertheless, the true efficacy of probiotics bursting immunity of animals in a porcine production commercial situation still remains unclear. Immune benefits of administering probiotics cannot be generalized and many times are difficult to interpret. As already mentioned, immunity enhancement may help by preventing subclinical diseases but at the same time has an energetic costs for the host. Contrary, immune-suppressive action of probiotics may be beneficial for animal performance but can have a detrimental effect in certain challenge situations. Further reports of systematic investigation of host interaction to distinct MAMPs of probiotic strains are necessary to put some light on molecular and cellular processes occurring. A better understanding of the underlying cross-talk between these nonpathogenic bacteria and the host will probably answer many questions in the near future (Brown, 2011; Koropatnick et al., 2004; O'Hara et al., 2006).

2.3.1.2. Improvement in the nutrient bioavailability

The action of microorganisms, in fermented feeds or in the digestive tract, has been largely known to improve the quantity, availability and digestibility of some dietary nutrients into the intestinal lumen. One of the main mechanisms is bacterial enzymatic hydrolysis (such as amylase, lipase, phytase and protease activity), which may enhance protein, fat, short

chain fatty acids (SCFAs) and lactic acid bioavailability (Gomes and Malcata, 1999; Kim et al., 2007). SCFAs contribute to the available energy pool of the host (Scheppach, 1994; Velázquez et al., 1997) and their acidic properties may also protect against pathological changes in the colonic mucosa (Boyen et al., 2008; Leopold and Eikeler, 2000).

Microbial population can also derive essential amino acid carbon from dietary carbohydrate, which in case of lysine can account for up to 1 g/day (Torrallardona et al., 2003) and they can be absorbed and incorporated into host proteins being net contributors to balance the amino acidic metabolic requirements (Metges, 2000; Metges et al., 1999, 2006). The genome analysis of some probiotics have recently reported potential biosynthetic capabilities to synthesize amino acids which can complement the already present biosynthetic genes of the gut microbiome (Abubucker et al., 2012; Neis et al., 2015). Nevertheless, these capacity appears to be strongly strain dependent as Pridmore et al. (2004) reported that a strain of *Lactobacillus johnsonii* lacked genes encoding a biosynthetic pathways for amino acids while Altermann et al. (2005) did find capability to synthesize at least three amino acids (cysteine, serine and aspartate) de novo in a *Lactobacillus acidophilus* strain.

In piglets, an increase in the availability of free amino acids has been described with probiotic treatments. For instance, Zhang and Kim (2015) reported *Enterococcus faecium* increased apparent ileal digestibility of several indispensable aminoacids. However, this work did not differentiate if they were due to *de novo* microbial production or changes in the gut microbiota profile, leading to a different bacterial enzymatic hydrolysis of dietary nutrients. Moreover, Yu et al. (2008), demonstrated that *Lactobacillus fermentum* colonized and adhered to the GIT epithelium and successfully increased average daily gain (ADG) and crude protein (CP) apparent digestibility. Another probiotic combination studied by Giang et al. (2011) based on *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*; increased feed intake and weight gain and improved feed conversion. Improvements were obtained by a healthier colonic fermentative environment (higher lactic and acetic acid), increased ileal apparent digestibility of CP, crude fiber (CF) and organic matter, as well as higher total tract apparent digestibility of CP and CF. However, to the best of our knowledge, there is still no studies evaluating the potential increment of microbial amino acid production *de novo*

with a probiotic treatment *in vivo.* Hence, the net amino acid contribution of a probiotic treatment remains uncertain.

Growing pigs, have a more developed digestive system, immunity, and capacity to resist intestinal disorders but there is still margin for probiotic to act and potentiate growth, especially in growing phases or high density diets. For instance, Meng et al. (2010) studied the effect of supplementing pigs in the growing and finishing phase (from 47± 1.1 kg BW to finish) with a combination of probiotics (*Bacillus subtilis* and *Clostridium butyricum)* in two levels of energy and nutrient density diets (low vs. high). Although probiotic supplementation increased growth performance throughout the entire experiment, an increase in gain feed ratio (G:F) was not observed in the finishing phase. Interestingly, an interaction between probiotic supplementation and the nutrient density of the diet was observed in digestibility, meat quality and feed intake. A greater improvement in digestibility (N and energy) was achieved in response to probiotic supplementation in the growing phase with the high nutrient density. Moreover, Zhang et al. (2015) tested an *Enterococcus faecium* strain in different energy and crude protein density diets (low vs. high) on ileal amino acid digestibility in growing-finishing pigs (75 ± 3.4 kg BW). The coefficient of apparent ileal digestibility on CP, gross energy, and most of the indispensable amino acids (Lys-Met-Thr-Leu-Ile-Cys-Gly) were increased in both *E. faecium* treatments, but the increase was of a higher magnitude in the high density diet. Yan and Kim (2013) saw a similar result when supplementing growing pigs (25 ± 0.6 kg BW) with *E. faecium* with two nutrient density levels. The probiotic increased ADG, average daily feed intake (ADFI), G:F, the apparent total tract digestibility of dry matter (DM), N, energy and also reduced faecal H_2S and NH_3 content as well as increased fecal *Lactobacillus* spp. concentration. An interaction between energy and nutrient density of the diet and probiotic was observed on the ADG and G:F ratio, faecal *Lactobacillus*, apparent total tract digestibility and fecal noxious gas content; being the probiotic effects enhanced in high nutrient density diets.

The bioavailability to the host of minerals such as calcium, iron, zinc, manganese, selenium, copper and phosphorus can also be enhanced by probiotics. On the one hand, by lowering the pH, the ionization of the minerals is facilitated, which is a requirement for absorption (Arunachalam, 1999). On the other hand, the ability to bind, uptake and bio-transform metal ions from the medium has been reported for several probiotic lactic acid

bacteria (LAB) and yeasts (Bomba et al., 2002a; Mrvčić et al., 2012). This property has important physiological, technological and nutritional implications. The high binding of iron by bifidobacteria and lactobacilli, has been suggested to reduce the availability of iron to pathogenic microorganisms (Bomba et al., 2002b) with consequent antimicrobial effects, as iron is an essential nutrient for all microbes. Moreover, enrichment of these bacteria with selected heavy metals such as selenium (Se) can be used to improve health and obtain more bioavailable selenium compounds like selenocysteine, selenomethionine and methylated selenium (Pophaly et al., 2014). Applications in pig production are very interesting as Se is an essential trace element nutrient for mammalian animals. Se plays a key role in redox regulation, antioxidant defense and immune function through glutathione peroxidase enzymes that catalyze the removal of the excess potentially damaging radicals produced during stress situation (Maggini et al., 2007). For instance, supplementation of a selenium enriched probiotic treatments (based on *Lactobacillus acidophilus* and *Sacharomyces cerevizae*) has been tested with positive results in animal growth performance, antioxidant status, immune function and some selenoprotein mRNA expression, in piglets raised under high ambient temperature (Gan et al. 2014). These results would suggest the Se enriched probiotic is useful to prevent heat stress, a major health problem in pigs which reduces the antioxidant capacity and immunity (Xiang-hong et al., 2011). Alternatively, another example of the functionality of enriching probiotics with Se would be research published by Su et al. (2009), who reported that the sperm motility rate, acrosome integrity rate and activity of glutathione peroxidase, were higher in stock boars supplemented with selenium-enriched probiotics than that in control stock boars fed a basal diet.

In addition, *Bifidobacterium* probiotics have also been reported to synthesize vitamins from the B complex (folic acid, niacin, thiamine, riboflavin and pyridoxine), vitamin C (ascorbic acid) and vitamin K which are frequently obtained as natural ingredients in foods, but probiotic supplementation can more effectively help to meet metabolic requirements (Arunachalam, 1999).

Finally, probiotic treatments may have the ability to alleviate symptoms of intestinal malabsorption as well as to induce suppression of putrefactive bacteria of the gastrointestinal tract. This would enable to stop losses of nutrients and apparition of toxic compounds (among other health related benefits) (Metchnikoff, 1907; Parvez et al., 2006).

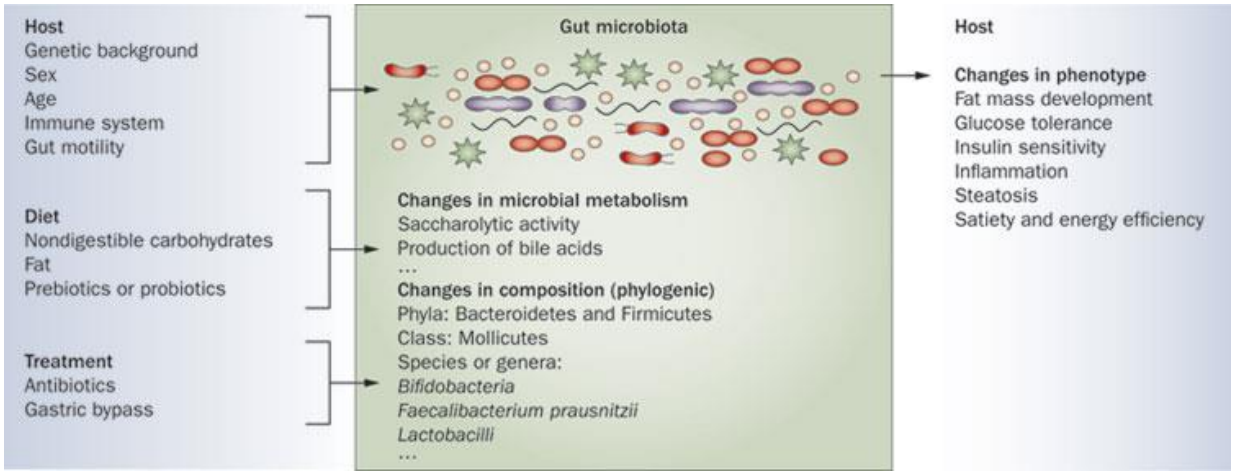
To conclude, many potential mechanisms of probiotics to increase nutrient bioavailability have been described: enzymatic hydrolysis, production of SCFA, synthesis of aminoacids, increase mineral bioavailability, synthesis of vitamins and reduction of detrimental stimulus such as putrefactive bacteria or malabsorption.

2.3.1.3. Epigenetic modulation

It is now generally accepted that the 'central genome dogma' (i.e. a causal chain going from DNA to RNA to proteins and downstream to biological functions) should be replaced by the 'fluid genome dogma', that is, complex feed-forward and feed-back cycles that interconnect organism and environment by epigenetic programing (Shenderov and Midtvedt, 2014). The epigenetic programing is the net sum of interactions derived from own metabolism and microbiota as well as external factors such as diet, pharmaceuticals, environmental compounds, and so on that can affect the host phenotype (see Figure 2.7, Delzenne et al., 2011). Diet and gut microbiota are the two most important environmental factors playing imperative roles in epigenomic programing, most importantly in pregnancy and early in life (Jiménez-Chillarón et al., 2012; Kotloff et al., 2013; Salminen and Isolauri, 2008).

Among all the different ways to influence gut microbiota, probiotics are increasingly gaining popularity for their capacity to influence not only in the gut, but also in the metabolism of cells in tissues outside of the intestines. They have been reported to modulate lipid (adipose tissue) and glucose (liver) homeostasis, as well as systemic inflammation in the host (Arora et al., 2013). Hence, they can become effective tools for both programming and, eventually, reprogramming gene expression to improve swine health and productive parameters at specific developmental points.

Figure 2.7. Epigenetic influences on the host.



From Delzenne et al. (2011) evaluating epigenetic influence on the occurrence of metabolic disorders associated with obesity.

2.3.1.3.1. Epigenetic influence on immunity by probiotics

The influence of probiotic treatments in the regulation of immune-relevant genes is still largely unknown. However, new high-throughput molecular and metagenomic tools are increasing the amount of studies assessing probiotic mechanisms of actions and more information will be available in the near future (Kreuzer-Redmer et al., 2016). Although probiotic effects in immune response have already been discussed, this section will focus on the latest advances that evidence that immune response may be regulated by the expression of certain genes in which probiotics could clearly interact.

Specific probiotics are capable to affect the host gene expression and modulate immune response. For instance, Van Baarlen et al. (2009) reported that *Lactobacillus plantarum* supplementation affected duodenal gene expression of cellular pathways and mucosal gene expression patterns; potentiating the establishment of immune tolerance in healthy adults (Van Baarlen et al., 2009). Moreover, supplementation of piglets with the same strain (Hulst et al., 2015) also showed a higher expression level of several B cell-specific transcription factors/regulators. The probiotic upregulated expression of genes that repress NF-κB and PPARG-mediated transcription. Consequently, the expression of the enzyme adenosine deaminase (responsible for the breakdown of the anti-inflammatory mediator adenosine) was downregulated. Chytilová et al. (2014) in an *in vivo* experiment with gnotobiotic pigs and Taranu et al. (2015) *in vitro* with porcine IPEC-1 cells found evidence that *L. plantarum* and other related *Lactobacillus* strains modulated TLR and NF-κβ gene expression in an ETEC challenge. Altogether, results suggested the existence of a cross-

talk where *L. plantarum* may modulate gene expression tempering inflammation in piglets even under pathogenic challenges.

Supplementation of sows and piglets with *Enterococcus faecium* has also been reported to affect intestinal immune-associated gene expression (Siepert et al., 2014; Starke et al., 2013) and promote changes in CD14 expressing cells of the sows' milk (Scharek-Tedin et al., 2013). Interestingly, it has been reported that *E. faecium* strain did not have beneficial effects in piglets challenged with *Salmonella* (Kreuzer et al., 2012; Szabó et al., 2009). For this reason, Siepert et al. (2014) compared the relative gene expression levels of both pro- and anti-inflammatory genes, as well as T cell activation (CD86) and inhibitory molecules (CTLA4) of piglets supplemented or not with *E. faecium* and challenged with *Salmonella*. An initial inflammatory response accompanied by an anti-inflammatory or immuno-suppressive situation was observed (significantly reduced levels of IL8, IL10 and the co-stimulatory molecule CD86 mRNA). Authors suggested that this immuno-suppressive situation may delay the host response to infections, and provide pathogenic bacteria such as *Salmonella* with a "window of opportunity", leading to the increased bacterial loads and shedding observed in challenge trials.

2.3.1.3.2. Epigenetic influence on energy metabolism, absorption or secretion

Host genes expressed in the intestine may control energy metabolism, absorption, oxidation or storage and can be modulated in a direct or indirect way by gut microbiota and eventually probiotics (Lodemann et al., 2008; Turnbaugh et al., 2006). There is evidence in scientific literature suggesting probiotics are capable to modulate gene expression and achieve either a positive or a negative energetic balance. Nevertheless, it is worth to mention that up until today, much of the research has been done targeting humans and aiming to achieve a negative energetic balance in order to decrease obesity.

Samuel et al. (2008) performed a study in G-protein-coupled receptor (Gpr41) deficient mice and suggested that the activation of GPR41 by SCFAs is responsible for the release of the gut hormone PYY. This peptide has been shown to enhance nutrient absorption by decreasing the intestinal motility and inhibiting electrolyte secretion in the small intestine and colon. The capacity of probiotics to modulate fermentation and influence SCFA ratio (Gibson and Fuller, 2000) could also influence energy metabolism as, in addition to their role as energy substrates, SCFA have been proposed to bind to specific receptors (GPR41

and GPR43) (Delzenne et al., 2011). Forssten et al. (2013) also reported that some *Lactobacillus* strains were able to modify the increase of satiety hormones PYY, producing satiety when given to rats.

Moreover, specific probiotics have been used to modulate adiposity by controlling the angiopoietin-related protein 4 (Angptl4). This is a potent lipoprotein lipase inhibitor, which inhibits the uptake of fatty acids from circulating triglyceride-rich lipoproteins in white adipose and muscle tissues, and promotes fatty acid oxidation, both in skeletal muscle cells and in adipocytes (Mandard et al., 2006; Yoshida et al., 2002). Overexpression of Angptl4 in white adipose tissue reduces fat mass (Mandard et al., 2006) and conversely Angptl4-deficient mice exhibit increased lipoprotein lipase activity and adiposity (Bäckhed et al., 2004). Aronsson et al (2010) observed that germ-free (GF) mice monocolonized with *Lactobacillus paracasei* presented an increased level of Angptl4. Moreover, an increase in Angpt14 could also contribute to a decrease in fat mass observed in normal mice that were fed a high-fat diet supplemented with *L. paracasei* (Aronsson et al., 2010). Bjerg et al. (2014) reported that the probiotic *L. paracasei* strain increased mRNA of the glucagon encoding gene in piglets and glucagon-like peptide-1 secretion in an isolated pig intestine. When a high dose of this probiotic was tried in humans, it negatively affected energy intake in an *ad libitum* meal.

Microbiota and probiotics can also influence inflammation-induced alterations in intestinal ion transport. Hence, modulation of the host microbiota may influence not only susceptibility to infection but also reduce symptom severity through modulation of colonic ion transporters (Ghosh et al., 2011). Briefly, *Citrobacter rodentium* induced colitis in susceptible C3H/HeOuJ mice resulted in a marked reduction the gene expression of apical transporters in comparison with non-susceptible C57BL/6 mice. However, susceptible C3H/HeOuJ mice with the microbiota of non-susceptible C57BL/6 donor mice displayed a significantly reduced change in expression of transporters following infection with *C. rodentium* (Ghosh et al., 2011). *Enterococcus faecium* (Lodemann et al., 2006) and to a lesser extent *Bacillus cereus* var. *toyoi* (Lodemann et al., 2008) have been reported to increase Na+/glucose transport and Na+ coupled L-glutamine transport. This effect appears to be strongly dependent to bacterial metabolites, as metabolites produced by *Lactobacilli* dramatically increased glucose absorption in acute exposure with Caco-2 colonic epithelial cells (Rooj et al., 2010).

Furthermore, probiotic treatments to influence energy metabolism have generally been centered in the gut. However, different strategies have demonstrated their potential effect beyond. One of the arms of energy balance (i.e., energy intake) has been shown to be regulated centrally through the hypothalamic appetite centers (Sousa et al., 2008). Intracerebroventricular administration of *L. acidophilus* supernatants was reported to decrease body weight with no apparent effect on food intake, which was attributed to the increased expression of leptin in the cerebral cortex, thalamus, hypothalamus, and choroid plexus (Sousa et al., 2008).

To sum up, a growing body of evidence now suggests that the host microbiota and probiotics can modulate host gene expression. Genes related with immune response, intestinal function and other metabolic processes can be regulated with the use of probiotics. Nevertheless, nowadays there is still a big variability in the results presented, probably attending to a high inter-individual variation depending on the genetic background of the host. Regulatory mechanisms are very complex, with many interactions and we are only beginning to understand some of them. Further research and cross-over designs will be necessary to eliminate the mentioned variability and achieve more consistent results. Although we are still in the first steps, "-omic" technologies will undoubtedly help us to understand in depth epigenetic modulation of probiotics.

2.3.1.4. Modulation of gut brain axis function.

Back in 1833, Beaumont, an army surgeon monitored gastric secretions through a fistula in a patient's stomach and noted an association between mood and gut function. It was the first time that bidirectional communication between the brain and the gut was recognized, showing that emotional states can alter the function of the gastrointestinal tract and vice versa. Although this line of research has been far from mainstream during the 19th and the 20th century, things changed due to the flourishment of our understanding of the complexity and diversity of the microbiome in the past decade. Nowadays we can see an expanding volume of evidence to support the view that cognitive and emotional processes can be altered by a metabolically complex intestinal microbiota through the brain-gut axis and research in this field is becoming increasingly popular (Cryan and O'Mahony, 2011; Diaz Heijtz et al., 2011; Dinan and Cryan, 2016).

The intimate relation between microbiome, immunity and mental well-being is now generally acknowledged in humans (Forsythe et al., 2010). An attractive line of research is to modulate the development of the brain and behavior with the use of probiotics as therapeutic agents through the gut-brain axis (Collins and Bercik, 2009; Dinan and Cryan, 2016; Mayer et al., 2014). In this line, the *psychobiotic* term has been coined to refer to live organisms that when ingested in adequate amounts produce a health benefit in patients suffering from psychiatric illness (Dinan et al., 2013).

2.3.1.4.1. Gut microbiota and nervous system

The enteric nervous system (ENS), sometimes referred to as "the second brain", comprises intrinsic primary afferent neurons, motor neurons, and glial cells along the entire length of the gut and is particularly known to play an essential role in normal intestinal and metabolic function (Forsythe et al., 2014; Furness, 2012). The use of GF animals has provided one of the most significant insights into the role of the microbiota in regulating the development of the ENS and the gut–brain axis function. Both structural and functional abnormalities in the gut and immune system are observed in GF mice compared to specific pathogen-free (SPF) mice, mice colonized with altered Schaedler flora and normally colonized mice (Collins et al., 2014; Luczynski et al., 2016). Interestingly, probiotic feeding has been reported to have a different modulation of the myenteric neuronal function depending on the strain. *Lactobacillus reuteri* increased excitability in myenteric neurons and decreased the duration of the inhibitory slow after hyperpolarization in rats (Kunze et al., 2009), while perfusion of an *in situ* preparation of mouse myenteric plexus neurons with *Bifidobacterium longum* fermented medium decreased the excitability of ENS neurons (Bercik et al., 2011).

While the ENS can operate independently from the central nervous system (CNS), microbiota–ENS communication also influences CNS signaling systems (Kunze et al., 1995; Perez-Burgos et al., 2014). Bacteria within the gut not only produce antimicrobial peptides, SCFAs and vitamins, but also most of the common neurotransmitters found in the brain. Neuronal processes of ENS neurons terminate in the gut epithelial lining and can respond directly to luminal contents or indirectly to these neurochemicals produced by luminal bacteria or enteroendocrine cells (Dockray, 2013; Perez-Burgos et al., 2014).

Bidirectional communication has been described through the *vagus* nerve, a crucial pathway implicated in microbiota–ENS–CNS-brain communication (Forsythe et al., 2014; Zhou and Foster, 2015). By this mechanism, immune system and nervous system are in continuous communication in order to maintain a basal homeostatic state of activation. This situation can be altered by metabolic dysfunctions or both psychological and physical stress (Dinan, 2009), but modulation of intestinal microbiota and efficient use of *psycobiotics* can play an effective role in maintaining a healthy equilibrium (Zhou and Foster, 2015). For instance, Desbonnet et al. (2014) reported that microbiota is essential for the social development of the mouse. In his study, GF mice showed social impairments in behavioral tests who spent an increased amount of time engaged in repetitive self-grooming behavior during a social interaction test. However, following a normal post-weaning bacterial colonization of the germ-free mice gut, these behaviors turned to normal.

Probiotic effects on brain affecting behavior, mood, cognition, emotion and pain have been well-documented in clinical studies with humans and pre-clinical studies using murine models (Barrett et al., 2012; Bravo et al., 2011; Hall et al., 2010; Leyer et al., 2009; Messaoudi et al., 2011; Neufeld et al., 2011; Rao et al., 2009). In case of the pig, it has been used to acquire knowledge in gut-brain axis and novel potential therapies, mainly as an animal model for humans (Bannai and Torii, 2013; Díaz-Güemes et al., 2007; Malbert, 2013; Wang and Donovan, 2015). A briefing of the most important findings with potential implications in pig production will be described as follows.

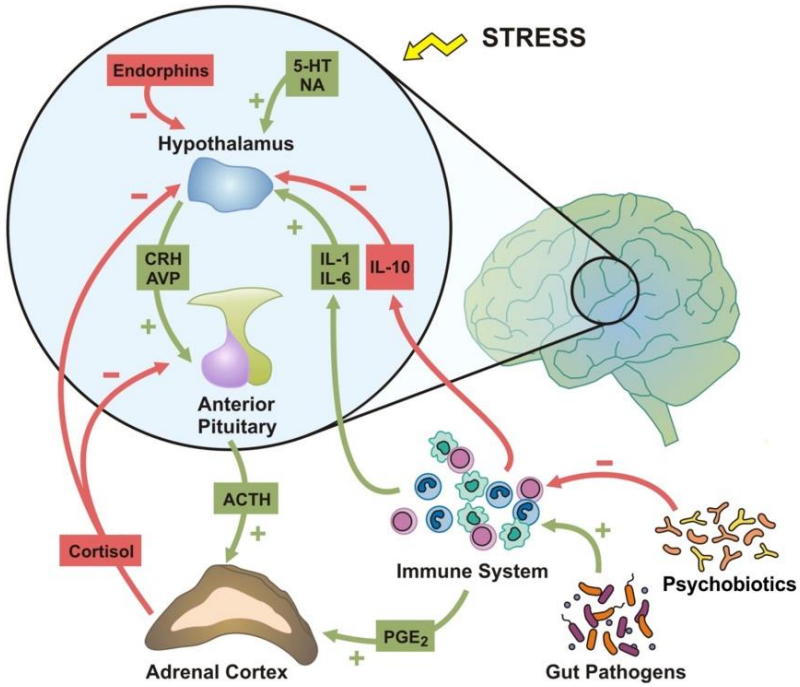
2.3.1.4.2. Gut-brain axis modulation: potential implications in pig production

Commercial management implies stressful situations and in particular early life stress such as premature maternal separation of piglets (Weary et al., 2008). These circumstances can have a lifelong impact on the microbial content of the intestine (Borre et al., 2014; Zhao et al., 2015), and permanently alter immune functioning with several neuroendocrine changes leading to inflammation and dysfunction (see Figure 2.8). In addition, repeated social stress has been shown to decrease the relative abundance in bacteria of the genus *Bacteroides*, while increasing the relative abundance of bacteria of the genus *Clostridium* in the cecum (Louis, 2012). Stressors also can increase circulating levels of IL6 and MCP-1, which are significantly correlated with stressor-induced changes to three bacterial genera (i.e., *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea*) (Finegold et al., 2002; Louis, 2012).

Several situations such as mixing in transition and confinement of sows in cages before farrowing could reasonably induce these type of affections.

Figure 8. Neuroendocrine changes with stress leading to inflammation and dysfunction.

From Dinan et al. (2013).



Probiotics may improve gut-barrier function and have immune-regulatory effects to help animals to deal with these stressful situations. Some authors have described effects through the generation of T regulatory cell populations, consequent synthesis and secretion of IL10, decrease of pro-inflammatory cytokines and suppression hypothalamic-pituitary-adrenal axis activity (Hardy et al., 2013; Zhou and Foster, 2015). IL10 has potent anti-inflammatory properties and it also has been suggested to have broad neuro-immune effects potentially affecting illness behavior (Forsythe et al., 2016; Lavasani et al., 2010; Levkovich et al., 2013).

Besides, Barrett et al. (2012) reported the ability of intestinally derived strains of *Lactobacillus* and *Bifidobacterium* to produce gamma-aminobutyric acid (GABA) from monosodium glutamate. GABA is the main inhibitory neurotransmitter in the brain regulating many physiological and psychological processes, playing an important role in the regulation of movement, blood pressure, heart rate and pain perception; and has been implicated in anxiety and depression (Cryan and Kaupmann, 2005; Mody et al., 1994; Schousboe and Waagepetersen, 2007).

Serotonin (5-HT) is a metabolite of the amino acid tryptophan and plays an important role in the regulation of a number of bodily functions and well-being. It has been shown that the plasma serotonin levels of conventional mice are significantly higher than GF mice, who have no intestinal microbiota (Collins and Bercik, 2009), demonstrating the capacity of the microbiota to influence levels. Furthermore, oral ingestion of *Bifidobacterium infantis* increased levels of the serotonin precursor, tryptophan, in the plasma of rats. This finding suggests that the strain may have potential as an antidepressant (Desbonnet et al., 2008). In pigs, some studies have linked serotonin metabolism to abnormal behaviors such as tail-biting (Valros et al., 2015) or high tryptophan levels with reduced aggressive behavior in piglets and sows (Li et al., 2006; Poletto et al., 2010), suggesting potential beneficial effects from these treatments.

Eventually, the list of potential *psychobiotics* is considerably large *Bifidobacterium, Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* produce serotonin; *Escherichia*, *Bacillus* and *Saccharomyces* produce norepinephrine while *Bacillus* and *Serratia* have the potential to produce dopamine (Desbonnet et al., 2008; Lyte, 2011; Schousboe and Waagepetersen, 2007).

Sticking to swine production, behavior response in stressful situations altering gut homeostasis such as bacterial challenges have been documented (Rostagno et al., 2011; Ahmed et al., 2015) and it has also been suggested that gut microbiota may play an important role in the apparition of abnormal behaviors such as tail-biting (Brunberg et al., 2016). However, there is no studies yet assessing the impact of probiotics in gut-brain axis and pig behavior.

To conclude, there are sufficient data in human and preclinical studies to support that gut brain axis plays a relevant role in probiotic treatment. Therefore, the use of *psycobiotics* in swine could be indicated to improve animal well-being and reduce effects of common stressors nowadays present in commercial-intensive production. We hope to see some interesting reports in swine production in the near future, as with the development of new technologies, the study of the neuroendocrine response of probiotic treatments affecting the gut-brain axis will get more cost-effective.

2.3.2. Indirect changes in gastrointestinal ecosystem

Many health benefits of the probiotics may not be due to a direct action of the bacteria on the host but to an indirect effect promoted by modulations of the gastrointestinal microbial ecosystem. The gastrointestinal tracts of mammals are colonized by a wide array of bacteria, yeasts and viruses (Sears, 2005) which in humans are speculated to outnumber the total of eukaryotic cells in the body by an order of magnitude (Luckey, 1972). This bacterial component present in the gut host has been described as an indispensable organ (Forsythe et al., 2010), which contributes an array of gene products not native to the host (Kim et al., 2007; Neis et al., 2015). The effects of a probiotic treatment aiming to establish a healthy and functional microbiota in the host will be described in the following section.

2.3.2.1. Promotion of a healthier microbiota.

Probiotics may influence microbial composition in many ways eventually creating a more favorable microbial population and environment for the host. With a healthy microbial population in the GIT, pigs may achieve enhanced performance, more efficient digestion and improved immunity. Moreover, establishment of a heterogenic and resistant microbiota (many different biotypes) may confer advantages to the hosts by allowing rapid adaptation to environmental changes and enhanced resistance to pathogens (Fouhse et al., 2016; Marchesi and Shanahan, 2007).

2.3.2.1.1. Modifying nutrient availability to other bacteria.

A well-characterized mechanism used by probiotics is to influence the nutrient availability for other microbial groups. Bacteria in the proximal colon have a large supply of nutrients, provided by dietary residues transiting from the small intestine, whilst those occupying the distal region of the colon have more limited substrate availability (Fooks and Gibson, 2002). Some probiotic strategies aim the decrease of a specific nutrient in order to compromise the growth of other bacteria and shift the balance to beneficial microbes (Cho et al., 2011). This mechanism is explained as competition for nutrients (Gerritsen et al.,

2011) or by a higher nutrient adequacy (Underwood et al., 2015) of probiotics for a certain nutrient.

Fooks and Gibson (2002) suggested an increase in lactobacilli/bifidobacteria numbers by way of a probiotic may thereby decrease the substrate available for other bacterial populations. Later on, a recent line of interest in human nutrition is to treat children with the probiotic *Bifidobacterium longum* subsp. *Infantis*; as among other immune-enhancing related benefits, it has been described to be able to degrade most-efficiently human-milk oligosaccharides (Arboleya et al., 2016; Underwood et al., 2015). Therefore, substrate available in distal colon is utilized and growth of pathogenic bacteria is prevented (Arboleya et al., 2016; Garrido et al., 2011; Sela et al., 2008; Underwood et al., 2015).

Alternatively, probiotics also provide metabolic products as specific nutrient for other bacteria an effect known as *cross-feeding*. For instance, it has been proposed that cross-feeding of lactate or acetate produced by bifidobacteria can stimulate the growth of other bacteria within the gut community, which may enhance gut health by the formation of butyrate (Belenguer et al., 2006; Van der Meulen et al., 2006; De Vuyst and Leroy, 2011). Furthermore, changes in fermentative environment and pH have also been reported to influence gut microbiota profile. Lactic acid bacteria, which are able to produce lactic acid as the major metabolic end-product of carbohydrate fermentation, are extensively used in swine production with antioxidative, immunomodulative, and bactericidal effects (Yang et al., 2015).

2.3.2.1.2. Interfering with bacterial communication (Quorum sensing)

A cell to cell communication within bacteria through secretion of chemical signals, called auto-inducers (AIs) has been described (Hughes and Sperandio, 2008). These AIs molecules accumulate during growth in the surrounding environment of the bacteria, enabling single cells to sense the density of bacteria surrounding and the different signaling molecules. Once these AIs reach a critical concentration, bacterial population as a whole, can make a coordinated response (de Kievit and Iglewski, 2000) and can activate or repress a number of target genes.

This process of bacterial cell-cell communication has been called quorum sensing (QS) and is used for communication between bacteria and also with their host (Hughes and

Sperandio, 2008). Several putative QS systems have been discovered. Their mechanisms are out of the scope of this review, but it can be broadly resumed that Gram-positive bacteria use peptide-like molecules while Gram-negative bacteria use acyl homoserine lactones (AHLs). Altogether, they regulate miscellaneous functions as diverse as motility, virulence, sporulation, antibiotic production, DNA exchange, and development of more complex multicellular structures such as biofilm (Hughes and Sperandio, 2008; Miller and Bassler, 2001; Schuster et al., 2013).

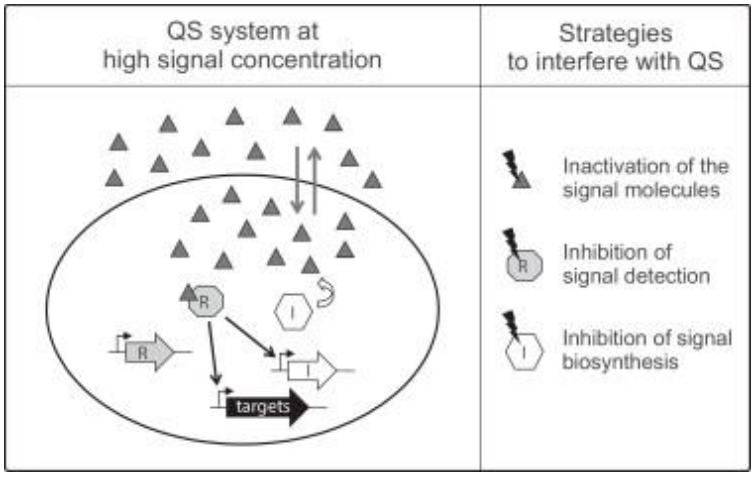


Figure 9. Simplified general scheme of a quorum sensing system of Gram-negative bacteria together with targets and strategies to interfere with QS.

From Fetzner (2015).

The capacity to behave collectively as a group has advantages for bacteria. For example the ability to migrate to a more suitable environment, better nutrient supply (de Kievit and Iglewski, 2000) and to adopt new modes of growth, such as sporulation in *Clostridium acetobutylicum* (Steiner et al., 2012) or biofilm formation in *E. coli* (González Barrios et al., 2006), which may afford protection from deleterious agent.

In turn, it has been postulated that the disruption of QS signaling, also termed quorum quenching (QQ), might offer new avenues to prevent and/or treat bacterial infections. On the one hand, probiotic bacteria can degrade the AIs of pathogenic bacteria by enzymatic secretion or production of AIs antagonists (Brown, 2011; Cerda-Cuellar et al., 2009). On the other hand, the discovery that QS signaling is a regulatory mechanism controlling gene expression in a number of pathogenic bacteria, has also provided intriguing new drug targets (Otto, 2004; Suga and Smith, 2003) capable to reduce pathogenesis via inhibition of virulence factor expression and biofilm formation (Fetzner, 2015; Zhu and Kaufmann, 2013). Several probiotics have been reported to be able to modulate virulence of pathogens. For instance, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* strains have demonstrated to inhibit on the growth, adhesion activity, and biofilm formation of

enteroaggregative *Escherichia coli* (Lazăr et al., 2009; Miyazaki et al., 2010). Yun et al. (2014) demonstrated that *Lactobacillus acidophilus* modulated the growth and virulence of *Clostridium difficile* and Cotar et al. (2010) demonstrated the capacity of several probiotic strains to modulate pathogenesis and virulence of *Pseudomonas aeruginosa*. These anti-virulence strategies have attracted significant attention in the last few years and it has been postulated that probiotics can play a prominent role in future therapies by appeasing pathogens as well as modulating host's ecosystem either as a single agent approach or part of a combination therapy (Fetzner, 2015; Grandclément et al., 2016; van den Nieuwboer et al., 2016; Shenderov, 2011; Yeo et al., 2015).

Problems nowadays present in swine production such as post-weaning ETEC opportunistic piglet infection can be ameliorated with probiotics via QS and QQ. Liu et al. (2015) did an *ex vivo* study where intestinal loops were inoculated with EHEC 0157:H7. Loops that presented lesions had an upregulation of QS genes while animals that had a naturally present bacteria with probiotic properties, *Veillonella caviae,* did not present this upregulation. A different approach was used in our group by González-Ortiz et al., (2013), who fed piglets a *Bacillus cereus* var. *Toyoi* strain, obtained sterile supernatants of ileal content and co-incubated different strains of *E. coli* and *S.* Typhimurium with the sterile supernatants. A significant reduction in the invasiveness of *E. coli* to IPEC-J2 cells was observed for the animals that received the probiotic in respect to the control diet. Cerda-Cuellar et al. (2009) reported a possible mechanism of this probiotic strain for those positive effects was the enzymatic degradation of N-AHLs. However, recently (González-Ortiz et al., 2016) reported *in vitro* studies where the co-incubation of ETEC K88 with the probiotic strain was able to reduce gene expression of the fimbrial adhesin (F4) and when AHLs were added in the medium, the expression of fimbrial adhesin (F4) and heat-labile enterotoxin (LT) was reduced. Altogether, results obtained until now suggest that *B. cereus* var. *Toyoi* can act on the QS systems of ETEC-K88 by different complex mechanisms including enzymatic degradation of lactones and modulation of gene expression.

Interestingly, some pathogenic bacteria have been reported to use several common QS systems, not only for cross-talk between cell–cell communication but also to communicate with the host mammalian hormones, process known as inter-kingdom communication (Forsythe et al., 2016; Holban et al., 2013; Hughes and Sperandio, 2008). For example, it has been reported that *E.coli* and *Salmonella* use the *luxS*/AI-2 to achieve inter-cellular

signaling and *QseC*/AI-3/epinephrine/norepinephrine to recognize both bacterial inter-cellular signaling, adrenergic and stress sensing (Holban et al., 2013; Walters and Sperandio, 2006). Bearson and Bearson, (2008) reported that when interacting with the host, the presence of norepinephrine up-regulated genes in the flagellar and chemotaxis regulon of *Salmonella* Typhimurium. Altogether, these adaptive processes may explain opportunistic behaviors of the pathogens, such as immune evasion and stress-induced recrudescence of *Salmonella*, during fluctuations of host hormone levels (Bearson and Bearson, 2008; Walters and Sperandio, 2006). Rasko et al. (2008) identified a molecule, N-phenyl-4-{[(phenylamino)thioxomethyl]amino}-benzenesulfonamide (LED209) capable to interact with *QseC* receptor and inhibit activation of virulence gene expression in *S.* Typhimurium, both *in vitro* and *in vivo* in a mice model. Further characterization of probiotic mechanisms of action in the near future will probably reveal they play an important role capable to interfere in this QS inter-kingdom communication.

To conclude, QS and QQ strategies are presented as promising tools in order to modulate microbial ecosystem and prevent or fight bacterial pathogens. Hence, further investigation should be performed to assess the potential of different probiotic bacteria QQ mechanisms. However, it must also be considered that it is an emerging field of recent apparition and up until today virtually all the evidence has been reported *in vitro.* Further *in vivo* trials should be performed in order to confirm and assess the real potential.

2.3.2.1.3. Production of antimicrobial compounds

Probiotics have also been reported to produce several substances with a direct antimicrobial effect. They include toxic metabolites of oxygen, the lactoper-oxidase-thiocyanate system, organic acids, and bacteriocins (Bomba et al., 2002a).

Organic acids, mainly SCFA antimicrobial effects have been largely acknowledged (Jensen, 1998). These SCFA are a fermentation product of many probiotic bacteria. Probiotic bacteria have been described to increase the production of SCFA by pig caecal bacteria by accelerating the breakdown of carbohydrates that are resistant to indigenous bacteria (Sakata et al., 2003).

The probiotic production of bacteriocines also has an interesting capacity to reduce pathogen affections, either in an indirect way potentiating host immunity (described in

previous sections) or with a direct antimicrobial effect. Some *Lactobacillus* spp. have been largely studied for its potential production of bacteriocins. For instance, *Lactobacillus plantarum* sole bacteria has been described to produce several bacteriocins with different spectrum (Diep et al., 2009). *In vitro* trials demonstrated high efficacy against *Lactobacillus* spp. and close related Gram-positive (e.g. *Pediococcus pentosaceus* and *P. acidilactici*) (Anderssen et al., 1998; Diep et al., 2009). Efficacy, although with a higher minimum inhibitory concentration, was also demonstrated for *Enterococcus faecalis* and *Listeria innocua* (Maldonado-Barragán et al., 2009). Nevertheless, *Lactobacillus plantarum* bacteriocins have not shown to be active against other Gram-positive pathogens, such as *Staphylococcus aureus* or *Listeria monocytogenes* (van den Nieuwboer et al., 2016). *Lactobacillus sakei* and *L. acidophilus* have also been described to prevent enterohemorragic *E.coli* (EHEC) *in vitro* and *in vivo* in mice models (Park et al., 2014; Zeinhom et al., 2012). .

In relation to pig production, a five-strain probiotic mixture composed of several *Lactobacillus* spp. and a *Pediococcus pentosaceus* strain improved the clinical and microbiological outcome of *Salmonella* infection in pigs (Casey et al., 2007). It was subsequently established that the only bacteriocin producer was a *L. salivarius* strain, which dominated over strains co-administered with it in both the ileum digesta and mucosa of weaned pigs (Walsh et al., 2008). The authors suggested that the superior ileal survival of this strain could be attributed to bacteriocin production, indicating that this antimicrobial confers a competitive advantage over the other co-administered probiotics (Walsh et al., 2008).

2.3.2.1.4. Inhibition of pathogen adhesion

It is commonly accepted that many intestinal pathogens must adhere to the intestinal epithelium if they are to colonize in the intestine and produce diseases (Walker, 2000). Therefore, some bacterial strains have been chosen as probiotics for their ability to inhibit pathogen adhesion.

Several mechanisms of probiotics have been described in this line. Firstly, probiotics may adhere themselves to the gut epithelium and thus compete with pathogens for adhesion receptors, a mechanism known as competitive exclusion (Savage, 1969). Secondly, an

expected effect of the addition of probiotics to the gastrointestinal tract is to increase normal and beneficial microflora colonization, which will also contribute to inhibit adhesion of harmful pathogens on the intestinal epithelium (Cho et al., 2011). Thirdly, their cell wall fragments have been described to adhere to specific receptors and inhibit adhesion of specific bacteria, probably due to a mechanism of steric hindrance where the bulk of the probiotic organisms on the cell surface prevent access of pathogens (Ouwehand and Conway, 1996). Furthermore, probiotic metabolic products present in sterile supernatants can prevent adhesion. In this case the exact mechanisms of action has not been reported yet, although it has been described that it is not pH dependent and the metabolic products present in supernatants are not affected by proteolytic enzymes (Hynönen et al., 2014; Tsai et al., 2016). Alternatively, probiotics may also coaggregate bacteria diminishing pressure of potential pathogens (García-Cayuela et al., 2014; Reid, 1999) or produce receptor analogues and bind pathogenic bacteria, inhibiting their implantation in the host epithelial cells (Badia et al., 2012). Finally, as mentioned in the immune section probiotics may modulate host immune system and inhibit pathogen adhesion by the induction of mucin production, the degradation of carbohydrate receptors by secreted proteins or upregulation of chemokine and cytokine production (Feng et al., 2016; Oelschlaeger, 2010; Rajput and WeiFen, 2012; Roselli et al., 2006).

All these mechanisms are difficult to assess *in vivo*. Hence, probiotic bacteria able to adhere to epithelial cells *in vitro* and block adherence of pathogens are normally selected and their probiotic effect is extrapolated to the host (Oelschlaeger, 2010). Several probiotics have demonstrated *in vitro* their capacity to compete and inhibit the adhesion of pathogens. For instance, *E. faecium* inhibited *E. coli* adhesion and it was suggested to be by a mechanism of steric hindrance of their cell wall fragments (Jin et al., 2000). *S. cerevisiae* var. *boulardii* is highly rich in galactomannan residues and it has been described to bind to ETEC K88 adhesins (Badia et al., 2012). *Bifidobacterium animalis* and *Lactobacillus rhamnosus* strains and their supernatant fractions inhibited *E. coli* adhesion (Roselli et al., 2006). Collado et al., (2006) reported several *Bifidobacterium* strains had ability to competitively exclude *Salmonella* Typhimurium, *Escherichia coli*, *Listeria monocytogenes, Enterobacter sakazakii*, and *Clostridium difficile;* and Tsai et al. (2016) reported multiple *Lactobacillus* strains were able to reduce adhesion of *Salmonella* Cholerasuis. These latter articles could not discern whether the inhibition capacity was due

to competitive exclusion of the binding sites or the secretion of factors not affected by proteolytic enzymes.

However, in contrast to the vast amount of scientific reports suggesting the protective effects of probiotics from pathogen adhesion, there is some evidence in scientific literature where probiotics increased the adhesion of intestinal pathogens. Strikingly, some *Lactobacillus* (Collado et al., 2007c; Gueimonde et al., 2006; Tuomola and Ouwehand, 1999), *Bifidobacterium* (Collado et al., 2005, 2007c) and *Enterococcus* (Rinkinen et al., 2003) strains have been reported to increase pathogen adhesion. The exact mechanisms by which these probiotics enhanced pathogen adhesion has not been elucidated, however Rinkinen et al. (2003) reported it may be correlated with high adhesive capacity of the probiotic bacteria. No direct evidence has been reported until now relating apparition of clinical infections with the increase in pathogen adhesion with probiotics. Nevertheless, the presence of these pathogens in mucus will increase the number of carriers that may seed pathogens as well as increase the potential risk of pathogen invasion.

In resume, probiotics can alleviate pathogen pressure by inhibiting pathogen adhesion. Still, the ability to inhibit the adhesion of pathogens appears to depend on the specific probiotic strain, the pathogens and host; therefore, it needs to be studied case-by-case.

2.3.2.2. Reduction of health detrimental components in the gut

Probiotic microorganisms can be applied to reduce the health risks from hazardous components. In this aspect, the capacity of some probiotics to reduce mycotoxins, produced from fungi on a wide variety of crops, is especially important for feed production. The mode of action often relates to the absorption of the compound to microbial biomass and therefore reduce their bioavailability for the host. For example, aflatoxin B1 (AFB1) has been shown *in vitro* to be bound by a probiotic strain of *Lactobacillus rhamosus* or a probiotic mixture of *L. rhamnosus* and *Propionibacterium freudereichii* strains (Gratz et al., 2004) and *in vivo* by *Saccharomyces cerevisiae* in a murine model (Madrigal-Santillán and Madrigal-Bujaidar, 2006). Another mode of action may be direct degradation by probiotic strains. *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* spp. have been shown to degrade ochratoxin A and AFB1 in milk by

fermentation (Galvano et al., 2001). A final mode of action is more indirect and is related to the above mentioned probiotic modulation of gut microenvironment. For example, the production of organic acids by probiotic microorganisms was reported to negatively affect the production of Shiga-toxin 2 from EHEC O157:H7 (Mohsin et al., 2015).

Regarding swine industry, mycotoxins are a major concern as they can negatively affect health and productivity (Iheshiulor et al., 2011). Probiotic strategies are being investigated in order to degrade mycotoxins in swine feed (Lei et al., 2014) and i*n vivo* experimental research is increasingly undertaken in order to evaluate probiotic strategies to mitigate their effects, although results reported are still far from conclusive. Meth et al. (2015) found no effectiveness with a *Bacillus subtilis* treatment in growing pigs eating aflatoxin contaminated diets, despite the *in vitro* activity for this strain has been previously demonstrated (Gao et al., 2011; Kimura and Hirano, 1988). Besides, Van Le Thanh et al. (2015) studied a commercial probiotic preparation (live bacteria, yeasts and plant extracts) in weanling pigs with diets contaminated with deoxynivalenol and although no difference were obtained in digestibility and histological parameters, a higher retention of Ca and P was observed with the probiotic treatment.

2.3.2.3. Effects of colonization patterns on gut maturation

After birth, facultative anaerobic populations start growing in the piglet gut consuming the oxygen over the first few days and shortly after populations of anaerobes increase to become dominant (Thompson et al., 2008). This neonatal microbiota is relatively dynamic, greatly influenced by the immediate environment (dominated by the mother's microbiota) and compositionally distinct to that of post-weaned (maternally independent) young. This first two-week period has been reported as a "developmental window" by Thompson et al., (2008), in which microbial dynamics and host-gut microbiota modulation are less stable and more susceptible to disturbance and/or microbial therapies (Wang et al., 2013). Afterwards fecal microbial community gets more stable to the extent that a significant correlation was observed with the microbiota of >30 days old piglets on a very sudden onset, after 14 days and before 21 days of age (Thompson et al., 2008). This finding suggests that although important differences in the porcine gut community are associated with weaning (Bian et al., 2016; Konstantinov et al., 2006), early environmental microbial

exposure within a specific time period is a major determinant of adult microbial community structure.

The fact that gut microbiota of animals is critically determined at the very earliest stages after birth, has been described by several authors as 'microbial imprinting' (Konstantinov et al., 2006; Mach et al., 2015). This gut microbiota plays a critical role in the "education" of the neonatal gut to generate functional adult systems (Lewis et al., 2012). The profound impact of the microbiota on gut maturation is best illustrated by comparison of GF with the conventionally-colonized gut. Poorly developed immune system, low cell turnover rate associated to a deficient villus-crypt architecture and altered secretions have been described in GF animals (Luczynski et al., 2016; Taylor-Pickard et al., 2008). Despite, association with normal microflora or certain selected microorganisms has been shown to be effective in the generation of a functional and immune competent gut in GF animals (Collins et al., 2014; Forsythe et al., 2010).

Another important point to consider is the "hygiene hypothesis" (Strachan, 1989) which postulates that the growing incidence of immune-mediated diseases is the consequence of reduced infection and exposure to microbes during early childhood. Few attempts have been done to observe these effects in swine. For instance, Mulder et al. (2009) compared genetically-related piglets bred in outdoor, indoor and isolation conditions and observed that pigs bred in outdoor conditions had a greater and healthier microbial diversity, with more *Lactobacillus spp.* and less potential pathogenic species. Moreover, in a similar approach comparing piglets nursed by their mother on a commercial farm with isolator-reared siblings, Lewis et al. (2012) observed a higher level of immune development in farm reared animals resulting in a significantly higher development of T-regulatory systems. As a consequence, isolator-reared piglets had an increased serum IgG response to novel dietary soya protein relative to farm-reared piglets.

Considering the importance of early gut microbiota, colonization patterns present in nowadays pig production should be re-considered. From a practical standpoint, the neonatal period is the most efficient time to deliver probiotics, which may ensure the establishment of life-long health benefits and produce a robust microbiota, resistant to adverse ecological shifts at challenges such as weaning (Kenny et al., 2011). The use of "creep feed", nutritional strategies to introduce dry feed consumption (sometimes supplemented with probiotic species) are usual in commercial conditions, however, piglets usually ingest small or null quantities of them (Pajor et al., 1991). Microbial transmission from mother to child has been broadly studied in humans (see Figure 2.10), but in pig production research in this area is still scarce.

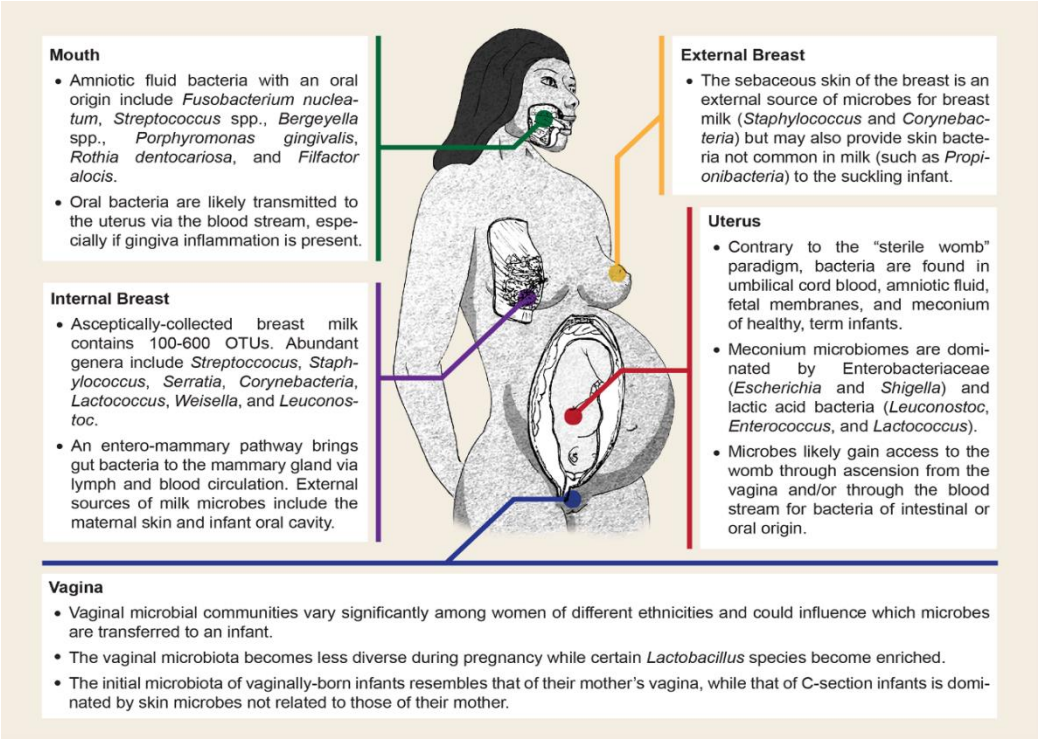


Figure 2.10. Sources of microbial transmission in humans from mother to child

From Funkhouser et al. (2013).

However, as just mentioned, early gut colonization is important to achieve a healthy gastrointestinal environment. Thus, alternative methods of early deliver of probiotics to pigs will be discussed in the following paragraphs.

2.3.2.3.1. **Birth and mother environment**

In this first moment, the pattern of colonization is influenced by host genetics, type of birth and neonatal environmental factors. In relation to birth, Huurre et al. (2008) reported that human infants born by caesarean delivery generally exhibit reduced numbers of *Bacteroides*, *Bifidobacterium*, and *Escherichia coli* and increased colonization of clostridia compared with vaginally delivered infants. Similarly, in the pig specie, Wang et al. (2013) reported a higher microbial diversity as well as a higher ratio of butyrate and propionate in vagina delivered animals than in cesarean delivered animals. In relation to probiotic use, the passage of a probiotic treatment based on *Lactobacillus acidophilus* has been detected at birth via vagina in sows, rat and mice models (Buddington et al., 2010).

Secondly, 'environmental' bacteria are then ingested from the feces, skin and the early-life environment (McLoughlin and Mills, 2011). For instance, *B. cereus* var. *toyoi* has been described to be conferred to piglets by contact with maternal feces (Jadamus et al., 2001; Taras et al., 2005). In addition, it is likely that all the maternal fecal bacterial community are transferred to the piglets and influence on their microbiota (Bian et al., 2016; Schmidt et al., 2011; Taras et al., 2005). Alternatively, other potentially influential factors such as exposure to antibiotics have profound effects on the adult gut mucosa-adherent microbiota and immune development in the pig (Mulder et al., 2009). Moreover, Thompson et al. (2008) reported that cohoused piglets suddenly develop very similar communities. Reasons for this are still not clear. It has been speculated that in addition to coprophagy, a translational period exists where environmental changes selectively advantages a subset of the communities (Inoue et al., 2005; Thompson et al., 2008). This hypothesis would be supported by the fact that the immune system undergoes considerable postnatal development and may result in a decrease in the potential for environmental microbes to colonize as the infant develops (Thompson et al., 2008).

Finally, genetic host traits also appear to affect colonization pattern in sows. Bian et al. (2016) reported that in a cross-fostering model with an obese Chinese breed (Meishan) and a lean Western breed (Yorkshire) specific bacteria taxa were associated with the different breeds. Altogether, data suggests that there is an interesting opportunity to manipulate resultant profiles of gut communities by feeding probiotics to sows during gestation-lactation period and modulating their fecal microbiota.

2.3.2.3.2. **Mother milk. Entero-mamary route**

Milk and in particular colostrum milk intake has been identified as a key point in gut formation. It harbors bacteria that can be necessary for a healthy gut in the adult phase as well as passive immune factors, growth factors and hormones; which are known to be important for the immune defense and the development of the intestinal epithelial cells of the offspring (Levast et al., 2014; Salmon et al., 2009).

In human infants, differences in gut microbiota between breast-fed and formula-fed infants are generally reported (Harmsen et al., 2000) and the same differences have been reported between sow-fed and formula-fed piglets (Li et al., 2012a; Poroyko et al., 2010). Milk components such as oligosaccharides, fat and proteins (Bian et al., 2016; Chatelais et al., 2011; Leonard et al., 2010; Li et al., 2012a) affect the gut microbiota, health and performance of piglets. In this sense, in a trial with spores of *B. licheniformis* and *B. subtilis*, Alexopoulos et al. (2004) observed modifications in milk quality with consequent increased health scores and body weights in piglets.

In addition, the mammary and entero-mammary route must be considered as an important pathway to fulfill newborns gut with probiotic bacteria (Fernández et al., 2013; Scholtens et al., 2012). Interestingly, human breast milk microbiome has been reported to change over time. Milk produced immediately after birth harbored more LAB along with *Staphylococcus*, *Streptococcus*, and *Lactococcus*, while breast milk after six months of lactation had a significant increase in typical inhabitants of the oral cavity, such as *Veillonella*, *Leptotrichia*, and *Prevotella* (Cabrera-Rubio et al., 2012), perhaps to prime the infant for the switch to solid food. Martín et al. (2004) was the first to hypothesize that maternal bacteria could translocate through the intestinal epithelial barrier and migrate to the mammary glands via an endogenous cellular route (entero-mammary pathway). Subsequently, entero-mammary delivery of probiotics via breastfeeding has been reported for *Lactobacillus* species (Abrahamsson et al., 2009; Arroyo et al., 2010; Jiménez et al., 2008) and even for gut-associated obligate anaerobes (*Bifidobacterium breve)* (Jost et al., 2014). In addition, Treven et al. (2015) reported in a rat model that two probiotic *Lactobacillus* strains (*rhamnosus* and *gasseri*) orally delivered altered the whole mesenteric lymph nodes and mammary gland microbiota.

As for sows, naturally occurring probiotic bacteria (mainly lactobacilli) have been isolated from their milk (Martín et al., 2009). In addition, dietary inclusion of prebiotic treatments

during lactation and gestation resulted in increased levels of bifidobacteria compared to lactobacilli in sow milk (Gyawali et al., 2015). However, to our knowledge, no entero-mammary delivery of probiotics has been described yet in swine species.

Undoubtedly, breast milk may be a valuable source of probiotic bacteria and might open a window of opportunity for early delivery of probiotics (Chassard et al., 2014). Hopefully, as we gain understanding of the diversity and function of maternally transmitted microbes in sows, more complete and effective probiotic strains will be produced to enhance piglet gut health by feeding sows.

2.3.2.3.3. **First meals**

Weaning in piglets is accompanied with a significant change in the bacterial composition and its metabolites. Introduction of solid feed and subsequent weaning are major events, that dominate succession of the gut microbiota in the early life of piglets and modulate microbiota in an adult-like profile (Bian et al., 2016; Konstantinov et al., 2006).

Major microbiological changes such as a significant diminution of the lactobacilli population have been detected during the weaning process (Konstantinov et al., 2004). Early studies have demonstrated that the porcine intestinal *Lactobacillus* community can decrease in time (Sghir et al., 1998). However, the exposure of the animal to stress factors such as mixing with other piglets, suboptimal feed intake and transportation are factors that contribute to a faster decrease of lactobacilli, precluding it's beneficial effects on stress modulation (Bian et al., 2016; Bravo et al., 2011; Konstantinov et al., 2006).

For this reason, strategies to facilitate adaptation to weaning, prevent gut dysbiosis and encourage a prompt colonization of a healthier gut population are desirable. As already mentioned, the introduction of probiotic strategies via "creep feed" is increasingly being studied (Alexopoulos et al., 2004; Giang et al., 2010; Shim et al., 2005). However, results of these experiments are largely variable mainly because piglets usually ingest small or null quantities of them (Pajor et al., 1991). Alternatively, the administration of probiotics in a water suspension, in milk fermentate or milk suspension have can be considered. Gebert et al. (2011) supplemented a milk replacer with a *Lactobacillus* probiotic strain and saw positive effects on pre-weaning animals. However, the commercial use of these

strategies has financial and management counterparts that limit their regular application in industry.

Lastly, as reviewed previously, one of the main applications for probiotics in pigs nowadays is their inclusion of probiotics in pre-started diets. There is a vast amount of experimental research appealing to positive effects of probiotic treatments, improving the weaning response of the animals (see Table 2.2).

To conclude, early interventions in microbiota are a potential target to reduce GI tract disturbances of piglets and these benefits will be dragged up to adult animals. Further research should be made in this line, comparing supplementation in gestation, lactation, creep feed and/or milk replacers in order to gain better knowledge of which are the optimum strategies and strains to supplement neonatal piglets.

2.4. Present and future of probiotics in feed production

2.4.1. Actual probiotic market

Animal production sector demands safer methods to obtain high productivity. Consequently, interest and demand for in-feed probiotics is continuously increasing all over the world and global animal feed probiotics market is projected to reach $4.71 billion by 2021 (Markets and Markets, 2016).

However, there is still a large way to go, as probiotic use in swine production nowadays is still rare. This contrasts with reports with positive effects of probiotics on the performance of pigs, which are now quite frequent. Bosi and Trevisi (2010) suggested that empiric usage of probiotics, leading to variable outcome, probably preclude probiotic in feed compounds to be widespread. How can the probiotic industry overcome this situation?

In this section, we have performed a *strengths*, *weaknesses*, *opportunities*, and *threats* (SWOT) analysis to have a better view of how the probiotic use will potentially progress in the near future.

Table 2.5. Strengths, weaknesses, opportunities, and threats (SWOT) analysis of probiotics in the actual market.

	Positive	Negative
Internal Origin	**STRENGTHS** Strong evidences of the potential of probiotics to modulate host health and productivity. Identification of possible modes of action of probiotics.	**WEAKNESSES** Safety not fully guaranteed. Scarce viability of many probiotic strains in the feed/water (i.e. LAB). Lack of universal effects.
External Origin	**OPPORTUNITIES** Development of new technologies ("-omic" techniques). New knowledge on the role of the microbiome. Increasing legislative pressure to reduce the use of antibiotics in the feed industry. Consumers in favor of this more "natural" and antibiotic free methods.	**THREATS** Too exigent regulation requirements. Empiric use with high variability has made probiotics be regarded as inconsistent. Not enough background for a knowledge-based design of probiotic therapies.

2.4.1.1. Strengths and opportunities

Progression from health to disease seem to be associated with microbiome alterations. Hence, the capacity of modulating host health status with probiotic interventions, together with the positive results reported in performance, make them attractive investments. In addition, nowadays we have a sufficient amount of quality trials to start to investigate the reasons for different outcomes of probiotic usage.

Until today, probiotic benefits have been described in the context of physiological or clinical improvement. However, a more complete understanding of the interactions between genetic, microbial and environmental influences within animals is getting increasingly available. This context will hopefully enable to define a "healthy microbiome" in a near future, providing a target for therapeutical interventions (Sanders et al., 2013). Furthermore, this deep understanding will probably allow the capacity to forecast the effects of a specific probiotic intervention depending on the microbial ecology.

All this knowledge is getting increasingly available with the rapid evolution and cost reduction of the new "-omic" technologies, which are contributing greatly to determine the mechanisms of action of probiotics. Nowadays the cost of sequencing has dropped very

fast, allowing them to be used by virtually everyone. However, a bottleneck has been created in the capacity and cost of computation. Hopefully, in a near future, improved algorithms, increased personnel trained in analysis of microbiome data, and access to free or inexpensive computing power such as cloud-based resources will make this techniques easily available (Huttenhower et al., 2014).

Other technologies are also getting increasingly available for improving probiotic efficacy. For instance, genetic manipulation of probiotics has been experimentally used with successful results (Whelan et al., 2014). This fact will open a window to obtaining tailored probiotic treatments fitting to customer needs in the future. Besides, great advances in protection of susceptible strains such as LAB have also been made (Sewell, 2016). This new methods will be able to guarantee probiotic stability in a farm environment, enabling probiotic therapies to display its maximum potential. However, high costs of the methods nowadays still preclude them to be generalized.

Finally, probiotics also have on their side that regulators and consumers are in favor of this promising "new" products in the market, because they offer a possibility to decrease antimicrobial usage with safer therapeutic and preventive health benefits (Ahasan et al., 2015). Moreover, general public is everyday more against the use of "chemicals" in meat production and would welcome the use of "friendly" and sustainable animal production strategies instead.

2.4.1.2. Weaknesses and threats

In a fist instance, safety of probiotics is an issue. As mentioned before, food-safety authorities worldwide have designed generic risk assessment tools to enable a tailored assessment, depending on the characteristics of the treatments. For instance, QPS by the EFSA in Europe and GRAS by the FDA in United States. This, safety assessment evolves together with technology and everyday measures undertaken are more stringent, including publication of the genomic sequence, antibiotic resistance profiling, toxicological studies (including toxin production) (Kumar et al., 2015). In this context, data will probably never be absolute but high safety standards can be guaranteed.

When focusing in probiotics used in animal production, serious risk posed by probiotic microbes in feed are:

- Transfer of antibiotic resistance due to the presence of transmissible antibiotic resistance genes in some probiotic bacteria (Bajagai et al., 2016). Multi-drug resistant pathogens are becoming one of the greatest threats to public health around the world (Sengupta et al., 2013). Therefore, strict measures are recommended in order to use only probiotic microorganisms with proven absence of transferable antibiotic resistance genes. An example would be the regulations adopted by the European Commission, which suspended the existing authorizations of the additive Toyocerin® (*B. toyonensis*), because it posed a risk for the spread of genes coding for resistance to tetracycline and chloramphenicol, antibiotics of human and veterinary importance.
- Risk of infection from the probiotic micro-organisms. Probiotics are generally selected to have good adherence to the intestinal mucosa, as this is considered important for their mechanism of action. Despite, adherence to the intestinal mucosa may also increase bacterial translocation and virulence. So concern exists that the most potent probiotics may also have increased pathogenicity (Boyle et al., 2006). Probiotic sepsis normally occur in hosts with underlying immune compromise or debilitation, and usually resolves with simple antibiotics (Hammerman et al., 2006). Although physiological translocation has been reported in weaning piglets (Trevisi et al., 2008), no disease reports have described sepsis related to probiotic use in otherwise healthy animals. This may be because even if bacteria do translocate in healthy individuals, they are usually trapped and killed by the immune system and detrimental effects are rare (Liong, 2008).
- Excessive immune stimulation or under-stimulation may be another important issue especially in hosts with already compromised immunity (Boyle et al., 2006). Therefore, even though a health challenge may be an indicated moment to use probiotics, depending on the case, a detrimental response can be obtained (Bosi and Trevisi, 2010).
- Toxic effects in the host, due to the production of enterotoxins by the micro-organisms contained in probiotics (Anadón and Martínez-Larrañaga, 2006; Isa et al., 2016; Musa and Seri, 2009).
- Safety of handlers of animals an animal feed. Infection (gastro-intestinal or systemic) or skin and/or eye and/or mucus membrane sensitization is a potential risk of their workplace (Bajagai et al., 2016).

Overall, microorganisms used as probiotics in animal feed are generally considered as safe. However, the evidence available up to date is not enough to address all the safety issues, and precludes a declaration of probiotics as universally safe or unsafe (Hempel et al., 2011). In addition, the possibility of probiotics used in animal feed entering the human food chain cannot be ruled out. Hence, precautions should be taken to protect animals, humans and the environment. This context has led some authors to suggest safety of probiotics should be discussed in general terms and not specifically to those used in animal feed (Bajagai et al., 2016; Doron and Snydman, 2015).

Secondarily, probiotic viability in feed and water has to be considered. Probiotic treatment should deliver viable bacteria in the intestine (FAO/WHO, 2001; Hill et al., 2014). However, when probiotics are used in the feed manufacturing, stresses and application processes may have critic effects on probiotic viability and desirable characteristics (Collins et al., 1998). Moreover, storing conditions in farms and feed-mills may be harsh for probiotic survival with high and variable temperatures, moisture, long shelf-life periods, etc. Probiotic producers should ensure quality preparations and guarantee that at the recommended storage conditions, the product maintains the stated compositions of the labels until the "best-before" date of the product, with a decrease of one or two logarithmic units at maximum (Czinn and Blanchard, 2009). Altogether, these criteria are difficult to meet. It has often been reported that the number, quantity, type and quality of organisms in commercial preparations of probiotic bacteria was below the one declared or even absent (Marcobal and Underwood, 2008; Wannaprasat and Koowatananukul, 2009).

Lactic acid bacteria and in particular *Bifidobacteria* have short shelf lives if not maintained carefully. In ordinary dried form they are unprotected against the chemical and physical stresses of feed manufacturing such as pelleting, which can seriously preclude their viability (Yang et al., 2015). On the other extreme, although not original from porcine gut, spore producing bacteria are attractive for producing probiotic compounds, as spores are extremely robust and stable, yet non-replicating under normal storage conditions (Cutting, 2011). This natural protection makes *Bacillus* suitable for all types of feeds as they can withstand massive strains during feed production and storage and protects them from low pH values in the stomachs of monogastric animals (Cutting, 2011; Gaggìa et al., 2010; Mazza, 1994). Finally, in case of yeast cultures, these yeasts are living fungi and are made dormant by drying, as their external surface is more stable and less permeable in this state.

This process may enable them to survive feed production and storage undamaged. Once in the digestive tract, the presence of sufficient moisture and warmth allows them to regain their metabolic activity (Ahasan et al., 2015; Lessard et al., 2009).

Thirdly, as mentioned thoroughly during the review, probiotic effects are context specific and depend on the host-related physiological parameters (e.g. health status and genetics) and environment (e.g. sanitary status and diet). Therefore, probiotic strains deemed to be useful in a certain farm may not have a positive outcome in another one (Trevisi et al., 2008). The influence of so many parameters anticipates that obtaining fully reproducible outcomes may be extremely complex. To guarantee beneficial effects, a tailored assessment of the probiotic use should be made for every situation. This requires a high amount of knowledge of probiotic intrinsic capacities to adapt to different situations (e.g. sanitary levels, different genetics, farm management…) that sometimes is still unavailable. Due to lack of understanding of probiotic mechanisms, probiotic have been used empirically in swine industry for many years. This fact has made probiotics treatments to be regarded as inconsistent. Hence, considering the low benefit margin actually present in swine production, many farmers or feed producers are unwilling to invest in them considering them less "reliable".

2.4.2. Future horizons for probiotic use

This section aims to recover the latest research and the current trends in probiotic usage in order to address the future probiotic use.

2.4.2.1. Multi-species or multi-strain probiotic consortium

It is reasonable to think that a combination of probiotics can have a potentially higher efficacy, as it can integrate the effects of the different individual strains. In fact, back in the early nineties, a consensus of probiotic experts concluded 'Different strains can be targeted toward different disorders and can be blended into one preparation' (Sanders, 1993). Generally speaking, these combinations appear to be effective in a number of diseases (Chapman et al., 2011). However, these effects are not always consistent and some of the research published recently revealed no benefits or even sometimes a worse outcome with combinations (Spiehs et al., 2008; Walsh et al., 2012a). The use of probiotic combinations

is getting increasingly popular (Vandenplas et al., 2014; Yang et al., 2015), and should be evaluated thoroughly. Differences between single strain or combined probiotic therapies are exposed in Table 2.6 (adapted from Timmerman et al., (2004)) and will be briefly discussed further on.

Table 2.6. Overview of differences between single strain probiotics and probiotic combinations.

Monostrain probiotic	Probiotic combinations
Successful colonization	

Survival depends on the properties of one specific strain.
• Has to overcome alone:
  ○ Host barriers
  ○ Endogenous microflora
	Different strains with different characteristics have an enhanced chance of colonization.
	• More strong points enhance chance of survival.
	• Can improve chances of successful colonization of the other strains, through:
	  ○ Reduction of antagonistic activity of the endogenous microflora
	  ○ Induction of optimal pH range
	  ○ Creation of an anaerobic niche
	  ○ Enhanced adhesion

Health effects exerted by the probiotic preparation	
Probiotic effect is limited to the strain specific properties.	Probiotic effect enhanced due to combination • Additive effect of specific strain properties • Synergistic effects of different strains. Positive interrelationships between strains that enhance biological activity. • Symbiosis between different strains, e.g. due to exchange of different metabolites

Adapted from Timmerman et al. (2004).

It must be considered that probiotic combinations can be multi-strain probiotics, containing more than one strain of the same species or closely related species (for instance *Lactobacillus acidophilus* and *L.casei*), or multispecies probiotics, containing strains of different probiotic species that belong to one or more genera (e.g. *L.acidophilus, Bifidobacterium longum* and *Enterococcus faecium*) (Timmerman et al., 2004). This difference has implications, as it has been suggested that the greater variety of probiotic genera present within a mixture may reduce its effectiveness, through mutual inhibition by the different species, antimicrobial compounds or competition for either nutrients or binding sites (Chapman et al., 2011, 2012). However, multispecies probiotics have also been related to a broader spectrum of activity (for example, inhibition of a wider variety

pathogenic bacteria), and if well-designed, a greater amount of synergism and symbiosis when different probiotic effects are combined (Timmerman et al., 2004). Symbiosis may enhance certain probiotic characteristics like growth or metabolic activity of strains. For example, growth of *L. acidophilus* is described to be enhanced by the presence of *B. animalis*, possibly due to the production of acetate (Kailasapathy and Chin, 2000). In relation to synergistic effects, the combination of strains *B. lactis* and *L. rhamnosus* have been described to enhance each other's adhesion in the large intestinal mucus of pigs (Collado et al., 2007a).

In pigs, probiotic combinations have demonstrated to be successful when used as a dietary supplement (Giang et al., 2011) or in fermented feeds (Choi et al., 2011). Several combinations have also demonstrated their ability to ameliorate a pathogen challenge (Ahmed et al., 2014; Casey et al., 2007; Daudelin et al., 2011; Yang et al., 2016; Zhang et al., 2016; Zhou et al., 2015). Still, regrettably the majority of the studies do not compare the effect of the combination with the single strains that compose them. One exception is Daudelin et al. (2011), who compared the administration of *Pediococcus acidilactici*, the yeast *Saccharomyces cerevisiae boulardii* and their combination in an ETEC challenge. Upregulation of pro-inflammatory cytokines was observed in the *P. acidilactici* group and the combination, but not in the *S. cerevisiae boulardii* group, suggesting that the effect obtained when the two probiotics were combined was primarily due to *P. acidilactici*. This result is illustrative that due to the lack of knowledge of the effects of combining probiotics, in many cases nowadays multistrain probiotics functionality might be underestimated (Timmerman et al., 2004). To sum up, the combination of probiotic strains has an attractive potential, but we are just starting to understand the mechanistic interactions present when probiotics are given together. Future research should be aimed to obtaining symbiotic or synergistic combinations to maximize the positive benefits of these probiotic combinations.

2.4.2.2. Knowledge-based probiotic strain selection

For the probiotics to represent a real and effective treatment, it is necessary to ensure their consistent efficacy. A fundamental step to achieve it is to encourage basic research in order to identify and further characterize existing probiotics strains, determine optimal doses needed for each strain and asses their stability through processing and digestion (Musa and

Seri, 2009). Hence, future selection of probiotics should be based on the scientific literature published until the moment, and should take in consideration the host and environmental characteristics to take the maximum advantage probiotic treatments. Following from this, selection of the probiotic species should be less empiric and focused on specific targets in relation to the bacteria used. For example, strains targeting M cells should be identified for applications that seek to boost intestinal immunity enhancing development of secretory IgA (Corthésy et al., 2007). Targeting hypothalamic-pituitary-adrenal axis could be done to improve animal well-being and reduce effects of common stressors (Hardy et al., 2013; Zhou and Foster, 2015). Lastly, probiotic strains adapted to the colonic environment can be good candidates to fight gut dysbiosis if possessing anti-inflammatory properties (Corthésy et al., 2007), or can enhance productive performance by bacterial enzymatic hydrolysis (Kim et al., 2007) or biosynthetic pathways for amino acids (Pridmore et al., 2004). Moreover, the regions of the gastrointestinal tract that are most immunologically responsive to ingested probiotic strains are another key consideration that is undefined at present. This is an issue that should be addressed in the future to further segment and maximize probiotic effects (Corthésy et al., 2007).

2.4.2.3. Potentiation of probiotic treatments

Research and development should be aimed to enhance probiotic weak points. In this line, encountering methods that will ensure the maximum efficacy of probiotics at the time of their consumption is essential (Bomba et al., 2002a). As already mentioned, LAB have short shelf lives if not maintained carefully (Yang et al., 2015). However, they can be used on feed types which place little technical stress on the microorganisms (for example in milk replacers) or can be protected against mechanical and heat impacts during feed manufacturing, transport and storage (Ahasan et al., 2015). In fact, protective coating using technological procedures such as microencapsulation or micro-sphering are getting increasingly available, and may permit these non-spore bacteria by-pass feed manufacturing and storage constraints, reaching the intestinal site intact (B Haffner et al., 2016; Mitropoulou et al., 2013). For instance, a recently developed *Lactobacillus plantarum* (Lactoplan®, Nutraferma, IA, USA) (Sewell, 2016) strain is able to withstand pelleting temperatures of 95 ºC and maintain cell counts for up to one year of storage. Heat-stable and shelf-stable strains will probably have good acceptance in the sector.

Alternatively, fermented liquid feeds have been postulated as a specific context where probiotics may be reliably delivered (Kenny et al., 2011). Lactic acid bacteria are associated by their common metabolic and physiological characteristics, such as producing lactic acid as the major metabolic end-product of carbohydrate fermentation. This trait has linked LAB, in special lactobacilli, with direct and indirect actions against spoilage, health promotion, pathogen reduction and nutritive value of an animal feed; making them attractive candidates to be used in feedstuffs (Caplice and Fitzgerald, 1999; Missotten et al., 2015; Moran et al., 2006; Plumed-Ferrer et al., 2005; van der Wolf et al., 2001). A reasonably high amount of viable organisms would be delivered in pigs eating fermented liquid feed, overpassing many handicaps of when probiotics are used as "in feed" treatments. However, relatively complex and expensive agro-technical fermentation and delivery systems are necessary for this method of probiotic delivery to achieve its potential (Kenny et al., 2011).

Furthermore, genetic manipulation offers the potential to enhance the existing probiotic properties of an organism or to load an organism with "extra" beneficial properties (Steidler, 2003). For instance probiotics could be engineered to sequestrate toxins, prevent enzyme deficiencies or target specific interventions in the immune system (Behnsen et al., 2013; Steidler, 2003). This strategy is gaining interest in human therapeutic use (de Moreno de LeBlanc et al., 2015; Piñero-Lambea et al., 2015; Whelan et al., 2014) but animal production could also greatly benefit of it. Although research in pig production applications are still incipient, a few very attractive innovations have been presented. Bjerre et al. (2016) generated tryptophan-overproducing *Bacillus subtilis* strains for in-situ aminoacidic production and use in pigs. Moreover, a genetically engineered *L. plantarum* producing porcine lactoferrin was produced by Xu et al. (2016), which effectively improved the growth performance, intestinal morphology and immunological indices of weaned piglets.

2.5. Literature Review Resume

To sum up, this literature review has comprehensively studied the use of probiotics in swine production. It has been reported that probiotics are extended in all the different productive phases of swine production. Moreover, they are used for multiple purposes, such as improving performance, mitigating diseases or increasing quality of the product. This is possible because a wide range of direct and indirect mechanisms of action can be activated by probiotics. Direct mechanisms would be to influence on host immune response, improve nutrient bioavailability, epigenetic modulation or gut-brain axis modulation; whereas indirect mechanisms involve a creation of a healthier microbiota, reduction of detrimental components in the gut and stimulation of gut maturation. Furthermore, it has been discussed that probiotic effects are closely related to the specific strain used and the context in which they are applied.

A SWOT analysis has also been performed to analyze the probiotic use in swine production. Many elements can be seen in favor of probiotics, such as the capacity to modulate health, the fact that they are considered “natural” alternatives to antibiotics or a great technological development during the last decade. However, aspects such as the lack of universal effects and their not fully guaranteed safety counter back their use. Finally, it has been stated that future for probiotics in swine production relies on enhancing their positive effects. For this purpose, carefully selected probiotic combinations will probably gain popularity, as they can be more effective and robust. In addition, the development of new formulations, encapsulation and packaging methods will probably allow the maximum viability of the bacterial species utilized and maybe, in a future, the use genetically engineered probiotics will open a window to tailor-made probiotic treatments.

Chapter 3. Objectives and Experimental Design

Increasing antimicrobial resistance in pathogenic bacteria has created the need for the development of novel preventive and therapeutic agents in the animal industry.

In the last years, our research group (Animal Nutrition, Management and Welfare group) has been involved in several projects with the aim of studying different feeding strategies, intended to fight intestinal pathogenic bacteria (AGL2005-07428-C02-01/GAN; AGL2007-60851/GAN; AGL2009-07328/GAN and AGL2012-31924). As a follow-up to this line, the project of this Thesis has the main objective of evaluating different probiotic strategies aimed to fight two of the most relevant pathogens for the pig industry nowadays: ETEC K88 and *Salmonella* Typhimurium. The project has been funded by two research agreements signed with the Agro-food Industry: Ordesa S.L. (CDTI-IMPRONTA: Proyecto INCOMES: Ref. IPT-20111008) and NOREL S.A. (CDTI. Ref. IDI-20140262). NOREL S.A. is one of the main Spanish companies developing, manufacturing and commercializing feed additives for the animal nutrition industry (http://www.norel.net/). Ordesa S.L. is a Spanish company leader in the market of baby foods and milk formulae (http://www.ordesa.es/). It is therefore expected that many of the advances in the scientific knowledge of this Thesis will be of applicability to humans, and particularly to babies, considering that the pig is an excellent animal model for this kind of studies.

The main objectives of the Thesis are the following:

A. To evaluate in weanling pigs the potential of new probiotics treatments: a) *Bifidobacterium longum* subsp. *Infantis* CECT 7210, b) the combination of *Bifidobacterium longum* subsp. *Infantis* CECT 7210 and *Bifidobacterium animalis* subsp. *lactis* BPL6 and c) *Bacillus licheniformis* CECT 4536; to enhance gut health at early life stages and to fight intestinal pathogens. It is also aimed to give new insights regarding possible modes of action of these strains.

B. As a secondary objective, the Thesis also pursues the development of new sensible tools to evaluate the animal response to probiotics under experimental models, particularly different blood biomarkers and the animal behavior analysis.

To achieve these three objectives, the following trials were done and included in Chapters 4 to 7.

- **Trial 1:** Assessment of the effect of the novel probiotic strain *Bifidobacterium longum* subsp. *Infantis* CECT 7210 in weanling piglets experimentally challenged with *Salmonella* Typhimurium. In this experiment, main parameters assessed were animal performance, clinical signs, pathogen excretion, fermentation profile, immune response and intestinal morphology.
- **Trial 2:** Assessment of the effect of the novel probiotic strain *Bifidobacterium longum* subsp. *Infantis* CECT 7210 in weanling piglets experimentally challenged with ETEC K88. Main parameters assessed were the same as in Trial 1.
- **Trial 3:** Assessment of the effect of the probiotic combination of *Bifidobacterium longum* subsp. *infantis CECT 7210* and *Bifidobacterium animalis* subsp. *lactis* BPL6 to improve health status of weaning piglets in a *Salmonella* Typhimurium oral challenge. Main parameters assessed were the same as before, together to panel of blood biomarkers analyzed by using an iSTAT® System and also blood minerals (Zn, Fe & Cu) by Inductively Coupled Plasma Optical Emissions Spectrophotometer (ICP-OES).
- **Trial 4:** Assessment of the effect of the probiotic strain *Bacillus licheniformis* CECT 4536 in piglets experimentally challenged with *Salmonella* Typhimurium. Main parameters assessed were the same as in Trial 1. Moreover, the suitability of a systematic behavior analysis was evaluated.

Chapter 4.

Potential of the probiotic strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 to improve health status and fight digestive pathogens

4.1. Introduction

Gastroenteritis due to enteric infections occur globally each year, and enterotoxigenic *Escherichia coli* spp. and *Salmonella* spp. are among the most common bacterial causes of diarrhea-associated morbidity and mortality (CDC, 2013; Lanata et al., 2013), especially in children up to five years of age (Kotloff et al., 2013; Liu et al., 2012; Payment P., 2002). The estimated annual mortality from illness due to *Salmonella* spp., considering the global population, rises to 155,000 deaths (Majowicz et al., 2010), and 157,000 deaths are annually associated with ETEC only in children from 28 days to 5 years of age (Bourgeois et al., 2016); this represents, on the whole, a considerable burden in both developing and developed countries.

It has largely been demonstrated that breast-feeding prevents gastrointestinal diseases in infants and can also confer protection from translocation of intestinal pathogens across the gut mucosa (Wold and Adlerberth, 2000). However, lifestyle in developed countries is leading to a drastic decrease in breast-feeding and, although milk formulae have been very much improved during the last few decades, they are still far from emulating the multitude of biological functions of mother's milk.

There are well-documented benefits of administering probiotic microorganisms in milk formulas which include improvements in several infections, allergic disorders, diarrhea, and inflammatory diseases (Bin-Nun et al., 2005; Minocha, 2009). Furthermore, probiotics and their metabolites have been suggested to have an important role in the formation or establishment of a well-balanced, indigenous intestinal microbiota in new-born infants and adults (Gill, 2003; Salazar et al., 2009) and to be remarkably beneficial in improving microbiota in hospitalized pre-term infants (Schwiertz et al., 2003).

In relation to the efficacy of probiotics to fight infectious diseases, various probiotic strains, mainly from the *Lactobacillus* spp. and *Bifidobacterium* spp. genus have demonstrated their potential to inhibit or ameliorate the outcome of these infections in humans and animal-models (Gill et al., 2001; Spinler et al., 2008; Tanner et al., 2016; Weizman et al., 2005). In particular, *Bifidobacterium longum* subsp. *infantis* CECT 7210, brand name *B. infantis* IM1, which was isolated from infant feces, has previously demonstrated a protective effect against rotavirus infection *in vitro* and in a murine model (Moreno et al., 2011).

Pig *in vivo* models have usually been selected as an excellent animal model to study various microbial infectious diseases due to its greatest similarity to humans in terms of anatomy, genetics and physiology (Meurens et al., 2012). Moreover, the domestic pig is susceptible, like humans, to ETEC and non-typhoidal *Salmonella enteritidis* serovars such as Typhimurium and Enteritidis, as these pathogens have broad host specificity (Blanco et al., 1991; Gal-Mor et al., 2014).

The objective of this work is, therefore, to demonstrate the potential of a probiotic strain, *Bifidobacterium longum* subsp. *infantis* CECT 7210, to enhance gut health at early-life stages and to fight diarrhea-related diseases caused by ETEC K88 or *Salmonella* by using the weaning piglet as a model.

4.2. Materials and methods

Two different experiments were performed to evaluate efficacy of the probiotic against an oral challenge with *Samonella* Typhimurium (Trial 1) or ETEC K88 (Trial 2). Both trials were performed 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 the European Union Guideline (Directive 2010/63/EU).

4.2.1.Animals, housing and experimental design

The two trials were conducted as a Level 2 High-Risk Biosecurity Procedure, with appropriate training of the personnel involved. A total of 144 male piglets were used: 72 Large White x Landrace piglets weaned at 24 (±4) days of age and 7.9 (±0.05) kg BW for the first trial and 72 Landrace piglets weaned at 21 (±2) days of age and 6.8 (±0.19) kg BW for the second. In both cases, animals were obtained from high-sanitary-status farms, in the first trial from mothers serologically negative to *Salmonella*, and, in the second trial, from mothers that did not receive any *E. coli* vaccination.

The UAB facilities available for these studies were an experimental unit with three rooms of eight pens each (twenty-four pens, three animals per pen). In each trial, animals were distributed by taking initial BW into account for a similar average BW within pens. The pens were allocated to four treatment groups following an unbalanced 2 x 2 factorial arrangement (factors being probiotic and pathogen challenge), with 8 replicates per

treatment for the challenged animals and 4 replicates for the non-challenged group. The treatments were, therefore: 1) no challenge + no probiotic (NN); 2) no challenge + probiotic (NP); 3) challenged + no probiotic (CN) and 4) challenged + probiotic (CP). Two rooms were challenged with pathogens and one was left unchallenged. In each room, probiotic treatment was distributed within 4 pens on one side of the room, and the 4 control pens were on the other side of the room, separated by a corridor in between.

Pigs were maintained under a 14:30 h light/ 9:30 h dark lighting regimen. Each pen (2 m^2) had a feeder and a water nipple to provide feed and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating, forced ventilation and an individual heat-light per pen. The trials were conducted during the spring season (March for the first trial and May for the second), with an average room temperature of 26ºC (± 4ºC).

4.2.2.Probiotic strain and diets

The probiotic treatment was supplied by Ordesa S.L. and consisted of a daily dosage (10^9 cfu) of *Bifidobacterium longum* subsp. *infantis* CECT 7210, which was supplemented in a 2 mL solution, while the control group received a solution of the same amount of carrier as placebo. During the experimental period, pigs received the treatment orally and individually, in a daily pattern using disposable syringes without needle. The probiotic tested was a unique batch of lyophilized bacteria, which was re-suspended every day no more than 1 h prior to administration. A pre-starter diet without additives (Table 4.1) was formulated to satisfy the nutrient requirement standards for pigs (NRC, 2012) and was given in a mash form.

4.2.3.*Salmonella* and ETEC strains

In the first trial, 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 (mainly enteric and with sporadic septicemia), which was provided by the *Infectious Diseases Laboratory* (*Ref. 301/99)* of the UAB. The oral inoculum was prepared by 24 h incubation at 37ºC in buffered peptone water (Oxoid; Hampshire, UK) and diluted (1:20) with sterile phosphate buffered saline (PBS) (Sigma-Aldrich; Madrid, Spain). Final concentrations of the inoculums were $1x10^9$ cfu/mL for the first inoculation day and $3x10^9$ cfu/mL for the second day. Inoculum concentrations were determined before the inoculation by

MacFarland standards and were plated the same day in order to check them by manual plate counting.

Table 4.1. Ingredient and nutrient composition of the experimental diets as-fed basis, g/kg.

Ingredients	
Maize	280.8
Wheat	170.0
Barley 2 row	150.0
Extruded soybean	122.4
Sweet whey-powder (cattle)	100.0
Fishmeal	50.0
Soybean meal 44	50.0
Whey-powder 50% fat	30.3
Mono-calcium phosphate	21.3
Calcium carbonate (CaCO3)	8.2
L-Lysine HCL	4.5
Vitamin-Mineral Premix [A]	4.0
Sodium chloride (marine salt)	3.0
DL-Methionine 99	2.4
L-Threonine	2.3
L-Triptophane	0.9
Analyzed composition	
DM	911.0
CP	174.9
CF	59.4
NDF	92.7
ADF	34.8
Ash	72.5

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D_3. 39.9 mg vitamin E, 3 mg vitamin K_3, 2 mg vitamin B_1, 2.3 mg vitamin B_2, 3 mg vitamin B_6, 0.025 mg vitamin B_{12}, 20 mg calcium panthotenate, 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.

In the second trial, the bacterial strain of ETEC K88 used (serotype O149:K91:H10 [K-88]/LT-I/STb) was isolated from a colibacillosis outbreak in Spain (Blanco et al., 1997). It

was kindly donated by the Dr. Blanco *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela, Lugo (Reference FV12048). The oral inoculums were prepared by an overnight incubation at 37ºC in Brain Heart Infusion broth (Oxoid; Hampshire, England) with slow agitation (1 x *g*) in an orbital incubator. For the first inoculation, the culture was given directly, and for the second inoculation day, bacteria were concentrated by centrifuging (2,000 x *g*, 10 min and 4ºC), and supernatant was eliminated. Final concentration of the inoculums was 9 x 10^8 cfu/mL for the first inoculation day and 8 x 10^9 cfu/mL for the second day. Inoculum concentrations were also determined before the inoculation by MacFarland standards and were plated the same day for manual plate counting.

4.2.4.Experimental Procedure

The duration of the study was 16 days for the *Salmonella* Trial and 14 days for the ETEC K88 Trial. After an adaptation period of 7 days for Trial 1 or 4 days for Trial 2, the animals were orally challenged with the pathogen. One animal of each pen was euthanized on Days 4 and 8 post-inoculation (PI) in Trial 1 and on Days 4 and 9 PI in Trial 2.

Fecal samples for microbiological analysis were obtained from random animals at their arrival, and individual body weight and pen feed consumption were registered during the adaptation time. After the adaptation period, the pathogenic bacteria culture was administered to the challenged group by oral gavage: two 2 mL doses (2 x 10^9 cfu and 6 x 10^9 cfu) of *Salmonella* Typhimurium on Days 8 and 10 in the first trial and two 6 mL doses (5 x 10^9 cfu and 5 x 10^{10} cfu) of ETEC K88 on Days 5 and 6 in the second trial. The same amount of sterile broth was given to the non-challenged animals. In order to ensure that the stomach was full at the time of inoculation, and thus facilitate bacterial colonization. Feed withdrawal was performed at 21:00 h of the previous day and provided again 15 minutes before inoculation.

From the first challenge onwards, animals were checked daily for clinical signs to evaluate their status post-inoculation (i.e., dehydration, apathy and fecal score), always by the same person. Fecal 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 (Thermoval Rapid, Hartmann; Spain) on Days 1, 2 and

3 PI. Mortality rate was also registered, and no antibiotic treatment was administered to any of the animals in the trials.

Body weight was recorded on Days 0, 4 and 8 PI (9 PI in the case of ETEC K88), while feed consumption on Days 0, 1, 2, 4, 6 and 8 PI (9 PI in the case of ETEC K88*).* The ADG, ADFI and G:F were calculated by pen.

For microbiological analysis, on the inoculation day (0 PI) fecal samples were taken aseptically from 24 animals after spontaneous defecation associated with the manipulation of the animal or by digital stimulation. In the case of the *Salmonella* trial, fecal samples were taken from the animal with the highest initial BW of each pen (N=24), while in the ETEC K88 challenge, fecal samples were obtained from the animal with the medium body weight of each pen (N=24). For the *Salmonella* trial, additional fecal samples were collected on Days 1, 3, and 7 PI from the same animal.

On Days 4 and 8 PI (9 PI in the ETEC K88 challenge), one pig per pen was euthanized. On Day 4 PI, the animal selected was the one with the intermediate initial BW, while on Day 8/9 PI, the heaviest was selected. Animals were euthanized and sequentially sampled during the morning (between 08:00 and 12:00 h). Prior to euthanasia, a 10 mL sample of blood was obtained by venipuncture of the cranial vena cava using 10 mL tubes without anticoagulant (Aquisel; Madrid, Spain). Immediately after blood sampling, piglets received an intravenous, lethal injection of sodium pentobarbital (200 mg/kg BW; Dolethal, Vetoquinol S.A.; Madrid, Spain). Once dead, the animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract excised.

In the ETEC K88 trial, fecal samples were obtained directly from the rectum for traditional microbiology. For both trials, digesta (approximately 50 mL) from the ileum and proximal colon (considered to be 0.75 m from the ileocecal junction) was collected and homogenized. The pH of the contents was immediately determined with a pH-meter calibrated on each day of use (Crison 52-32 electrode, Net Interlab; Barcelona, Spain).

From colonic digesta various subsamples were taken for different analysis. One aliquot was stored at -80ºC for ETEC K88 quantification by qPCR. To determine the presence of the probiotic in the gut, additional subsamples were taken. Bacterial isolation was performed in these samples before storing at -80ºC with *Geniul* commercial protocol

(Terrassa, Spain). Briefly, 1 g of colonic content sample was weighed in a 15 mL Falcon tube and diluted 1:10 with enriched Man Rogosa Sharpe (MRS) broth (Oxoid; Madrid, Spain) + 0.25% cysteine (Sigma-Aldrich; Madrid, Spain) + 2% Tween 80 (Sigma-Aldrich; Madrid, Spain). Ten glass spheres (5 mm diameter) were added to the tube and vortex (1 min) to homogenize the suspension. Two-hundred-fifty µL of the sample suspension were transferred to an Eppendorf tube with 250 µL of enriched MRS broth. Three centrifugation (13,000 x g for 5 min at 4ºC) and re-suspension (500 µL of enriched MRS broth) steps were performed and, finally, the bacterial pellet was re-suspended in 200 µL of sterile PBS and stored at -80ºC for DNA extraction.

A set of ileal and colonic digesta samples was also preserved in a H_2SO_4 solution (3 mL of content plus 3 mL of 0.2 N H_2SO_4) for NH_3 determination and was kept frozen at -20°C. An additional ileal and colonic sample set (approximately 20 g) was also frozen (-20°C) until analyzed for SCFA and lactic acid.

Bacteria attached to the intestinal mucosa were also analyzed in the ETEC K88 trial. For that, 5-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.

For the histological study, 3-cm sections from the ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a 4% formaldehyde solution (Carlo-Erba Reagents; Sabadell, Spain).

Blood samples were centrifuged (3,000 x *g* for 15 min at 4ºC) after 4 h refrigeration, and the serum obtained was divided into different aliquots and stored at -20ºC.

4.2.5.Analytical Procedures

For *Salmonella* bacteria counts (Trial 1), all samples were transferred (1:10) to buffered peptone water. Quantitative assessment was made by seeding serial dilutions of the samples 10^{-2}, 10^{-4} and 10^{-6} in Xylose-Lactose-Tergitol-4 plates (Merck; Madrid, Spain). Randomly chosen positive isolates were identified by means of the API20E system (Bio-Mérieux; Barcelona, Spain). With this scheme, animals were given a level as following: negative, for animals with no *Salmonella* growing at 10^2 dilutions (<10^3 cfu/g); low, for

animals with counts from 10^3 cfu/g to 10^4 cfu/g; medium, for animals with counts from 10^5 cfu/g to 10^6 cfu/g, and high, for counts from 10^7 cfu/g to 10^8 cfu/g.

For enterobacteria and coliform counts (Trial 2), samples were serially diluted in Lactated Ringer's Solution (Sigma-Aldrich; Madrid, Spain) from 10^{-4} up to 10^{-8} and seeded in MacConkey agar (Oxoid; Madrid, Spain) and eosin methylene blue agar (Scharlab; Barcelona, Spain). The plates were incubated for 24 h at 37ºC, and colonies were manually counted.

Moreover, in the second trial, *E. coli* K88 was quantified in colonic digesta by real-time PCR (quantitative PCR [qPCR]) using SYBR green dye. DNA from colonic samples (approximately 250 mg) was extracted and purified using the commercial QIAamp DNA stool minikit (Qiagen; West Sussex, United Kingdom). The recommended lysis temperature was increased to 90ºC, and a posterior incubation step with lysozyme was added (10mg x mL^{-1}, 37ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 mL of Qiagen buffer AE and stored at -80ºC until use. A SYBR green qPCR targeting the gene coding the F4 fimbria of *E.coli* K88 was performed according to the procedure described by Hermes et al. (2012). Results are expressed as cfu/g of fresh matter (FM) and log of F4 gene copies/g FM.

For probiotic detection, DNA was extracted from previously pre-treated colonic samples with a commercial kit v-DNA reagent following the manufacturer´s instructions (Geniul; Terrassa, Spain. Doc. Code 450000112). Briefly, samples were re-suspended in 1 mL of v-DNA buffer and centrifuged (13,000 x g for 5 min at 4ºC). After that, incubation (90ºC, 10 min) with 200 µL of v-DNA reagent was performed in a shaking incubator and finally DNA was suspended in 600 µL of v-DNA buffer. DNA obtained from pure cultures of the probiotic was used for construction of the standard curves. Cultures of bacteria were grown overnight in anaerobiosis with MRS broth, and serial 1:10 dilutions were performed in sterile MRS. DNA from dilution -1 (7.5 x 10^7 cfu/mL) to -6 (7.5 x 10^2 cfu/mL) was extracted with QIAamp DNA minikit (Qiagen) and used as standard curve. For the qPCR, a strain-specific probe designed for the 16s RNA gene was used ([6FAM]CCGGTTAGTCCTCTACCGTACGCAAGC[TAM]) (Sigma-Aldrich; Madrid, Spain) and an amplicon of 234 bp was obtained by the following primers (Forward: 5'-CGCCGGTGCCAGTCA-3'; Reverse 5'-CACAGCGGGCAGATCGGTAT-3') (Sigma-

Aldrich; Madrid, Spain). The master mix used was 5x Hot Firepol Probe qPCR Mix Plus (no ROX) (Solis BioDyne; Tartu, Estonia) and reaction conditions for amplification of DNA were 95ºC for 15 min and 45 cycles of 95ºC for 15 s and 60ºC for 1 min. Real-time PCR was performed with the ABI 7900 HT Sequence Detection System (PE Biosystems) using optical-grade ninety-six-well plates. The minimum level of detection was established in 3.3 x 10^3 cfu/g of colonic sample.

The SCFA and lactic acid analyses were performed by gas chromatography, after the samples were submitted to an acid-base treatment followed by an ether extraction and derivatization with *N*-(*tert*butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1% *tert*-butyldimethylchlorosilane (TBDMCS) agent, using the method of Richardson et al. (1989), modified by Jensen et al. (1995).

The concentrations of NH_3 were determined with the aid of a gas-sensitive electrode (Hatch Co.; Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A.; Barcelona, Spain). Three grams of acidified content were diluted (1:2) with 0.16M NaOH, after homogenization samples were centrifuged (1,500 x *g*) for 10 min. The ammonia released was measured in the supernatants as a different voltage in mV according to a procedure previously described in Hermes et al. (2009), which was adapted from Diebold et al. (2004).

Serum concentrations of Tumor Necrosis Factor-α (TNFα) were determined by Quantikine Porcine TNFα kits (R&D Systems; Minneapolis, USA) according to the manufacturer's instructions. 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) as described in Saco et al. (2011). In the *Salmonella* trial, serological antibodies of *Salmonella* were tested by ELISA *Salmonella* Herdcheck (Idexx; Hoofddorp, Netherlands), and the cut-off for positivity was established in optic density ≥40%.

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned 4-µm thickness and stained with hematoxylin and eosin. Measurements of ten different villus-crypt complexes per sample were performed with a light microscope (BHS, Olympus; Barcelona Spain) using the technique described in Nofrarias et al. (2006).

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

4.2.6.Statistical Analysis

Results from both trials are expressed as lsmeans 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 probiotic treatment, as well as the interaction between the two (only included when significant). The general linear and mixed models of SAS (SAS Institute Inc.; Cary, NC, USA) were used to analyze the effect of experimental treatments as well as Fisher's exact test on microbiological data, to analyze the frequencies of positive animals as contingency tables.

When treatment effects were established, treatment means were separated using the probability-of-differences function adjusted by Tukey–Kramer. The pen was considered the experimental unit for analysis, and random effect was used to account for variation between pens. The α-level used for the determination of significance for all of the analysis was $P = 0.05$. The statistical trend was also considered for $P < 0.10$.

4.3. Results

In general, the trials proceeded as expected. Animals used in both studies showed a good health status at the beginning of the experiment. In the *Salmonella* trial, none of the animals seeded *Salmonella* in feces on arrival, and serological analysis confirmed that animals had not been exposed to *Salmonella* prior to the day of inoculation, all being sero-negative during the whole trial. In the second trial (ETEC K88), one death was registered in the CN group on Day 4 (before the challenge). Death was attributed to post-weaning stress, as the animal was the smallest pig of the pen and had previously shown symptoms of apathy. No antibiotic treatment was administered to any of the animals in the trial.

4.3.1.Probiotic detection

The ability of the probiotic strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 to colonize the gut was evaluated by analyzing the probiotic strain by qPCR in the colonic

content on Day 8/9 PI. The bacteria was detected in 70% of the treated animals (2.66 x 10^5 cfu/g for NP and 4.11 x 10^4 cfu/g for CP in Trial 1; 4.05 x 10^4 cfu/g for NP and 5.57 x 10^4 cfu/g for CP in Trial 2) and in none of the non-treated ones (NN and CN). Levels quantified were near the detection limit of the method (established at 3.4 x 10^3 cfu/g). No significant differences were seen related to the challenge in the number of positive animals or detected concentrations.

4.3.2.Animal performance

Effects of the experimental treatments on BW, ADG and ADFI are shown in Table 4.2.

Table 4.2. Animal performance in *Salmonella* and ETEC K88 trials.

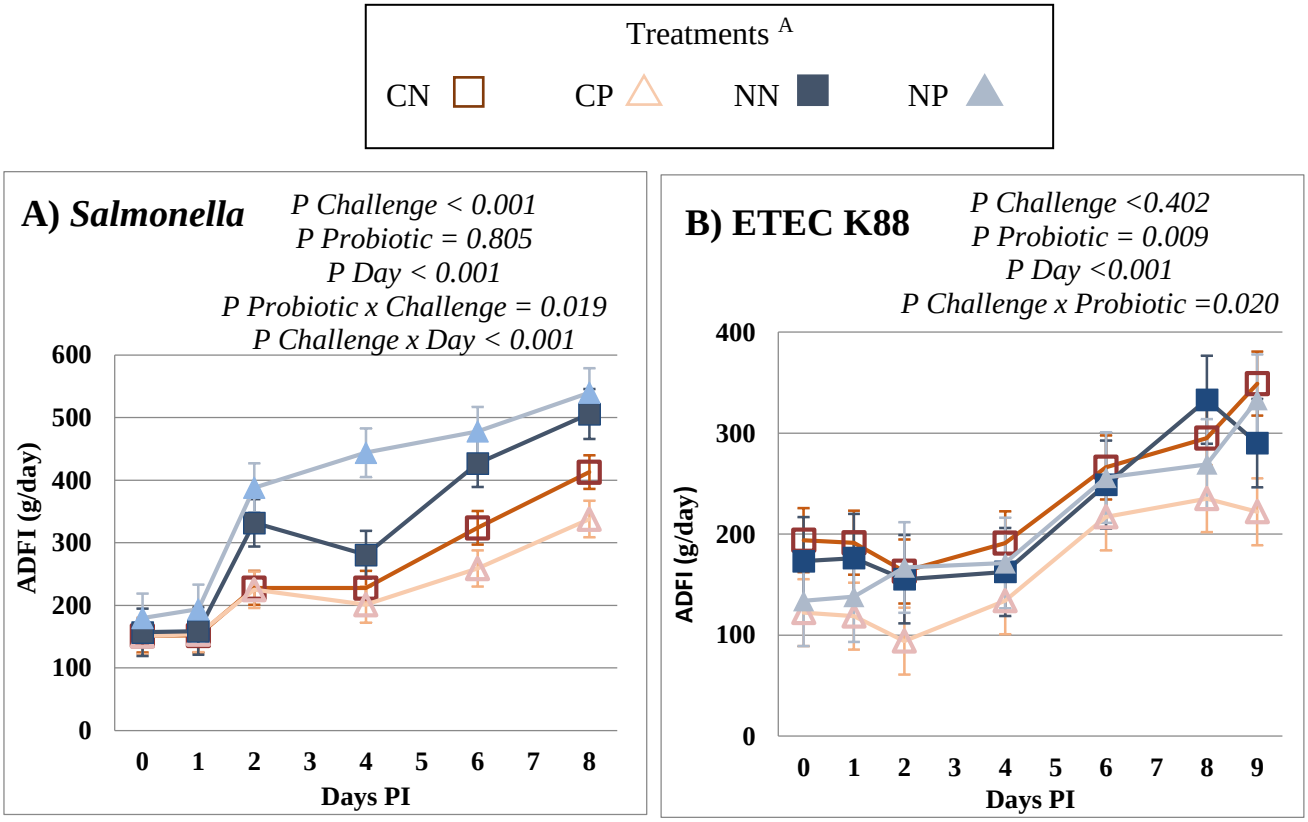
	Treatments [A]					***P*-value**		
	CN	**CP**	**NN**	**NP**	**RSD [B]**	***Challenge***	***Probiotic***	***Interaction***
Trial 1. *Salmonella*								
BW [C] (kg)								
Initial	7.88	7.88	7.95	7.87	*0.131*	*0.590*	*0.502*	*0.516*
Final	9.77	9.44	10.91	11.15	*0.645*	*<0.001*	*0.861*	*0.318*
ADFI [D] (g/d)								
Pre-inoculation [E]	179	170	183	183	*42.0*	*0.659*	*0.812*	*0.812*
Post-inoculation [F]	353	330	458	513	*70.0*	*<0.001*	*0.598*	*0.216*
ADG [G] (g/d)								
Pre-inoculation [E]	94	58	90	103	*43.8*	*0.290*	*0.539*	*0.214*
Post-inoculation [F]	156	145	288	345	*62.0*	*<0.001*	*0.399*	*0.215*
Trial 2. ETEC K88								
BW [C] (kg)								
Initial	6.80	6.77	6.72	6.78	*0.553*	*0.883*	*0.973*	*0.855*
Final	8.74	7.78	8.25	8.35	*1.078*	*0.937*	*0.184*	*0.271*
ADFI [D] (g/d)								
Pre-inoculation [E]	151	84	115	130	*42.1*	*0.787*	*0.165*	*0.035*
Post-inoculation [F]	283	231	270	278	*75.3*	*0.610*	*0.510*	*0.378*
ADG [G] (g/d)								
Pre-inoculation [E]	36	-11	23	10	*50.5*	*0.865*	*0.186*	*0.434*
Post-inoculation [F]	200	119	163	173	*76.3*	*0.808*	*0.294*	*0.183*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. [C] Body weight. [D] Average Daily Feed Intake. [E] Experimental Days 0 to 7 for Trial 1 and 0 to 4 for Trial 2. [F] Experimental Days 8 to 16 (0 to 8 PI) for Trial 1 and 5 to 14 (0 to 9 PI) for Trial 2. [G] Average Daily Gain. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

The *Salmonella* challenge negatively affected final BW, ADFI and ADG in the post-challenge period, while the ETEC K88 challenge did not significantly modify any of those parameters. Probiotic treatment did not show significant effects on the studied parameters despite an interaction (P = 0.035) observed in the ETEC K88 trial for ADFI during the adaptation period. Before the challenge, the CP group unexpectedly showed a lower ADFI than did its control (CN).

Daily registers of ADFI during the post-challenge period are shown in Figure 4.1. As seen before, the oral challenge with *Salmonella*, but not the ETEC K88 challenge, promoted a significant reduction in the intake. In both trials, a significant interaction challenge x probiotic (P = 0.019 for Trial 1 and P = 0.020 for Trial 2) was recorded, the probiotic promoting a higher feed intake in the non-challenged animals and a decrease in the challenged ones.

Figure 4.1. Average daily feed intake of the post-inoculation period.

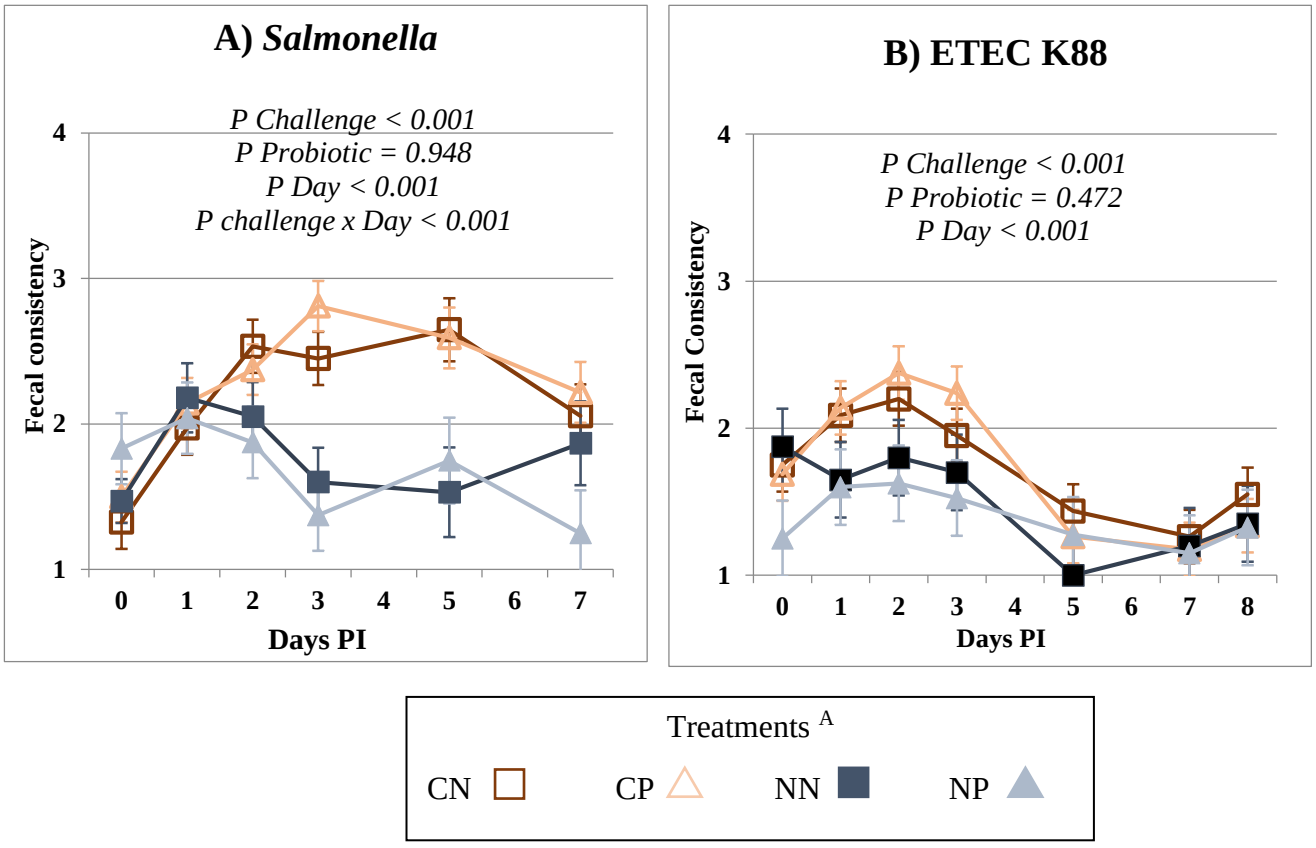


Experimental Days 8 to 16 (0 to 8 PI) for Trial 1 and 5 to 14 (0 to 9 PI) for Trial 2. [A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. n = 8 for groups CN and CP, n = 4 for groups NN and NP. Interactions only included when significant.

4.3.3.Clinical signs

Figure 4.2 shows the evolution of fecal consistency after the challenge in both trials. Pathogen inoculation significantly affected fecal scores, with more liquid feces in both trials (*P* challenge < 0.001). However, whereas the *Salmonella* challenge promoted diarrhea with scores between 2-3, the ETEC K88 challenge only promoted a slight fecal inconsistency, with scores that were rarely above 2. Moreover, there was a clear, differenciated pattern response after the inculation day between challenged and non-challenged animals, as in the *Salmonella* trial the interaction between challenge and day was significant (*P* < 0.001), but the pattern was very similar in the ETEC K88 trial. Regarding the probiotic treatment, no significant differences in fecal consistency were found in any of the trials.

Figure 4.2. Evolution of the mean fecal scores in the different experimental groups during the post-challenge period.



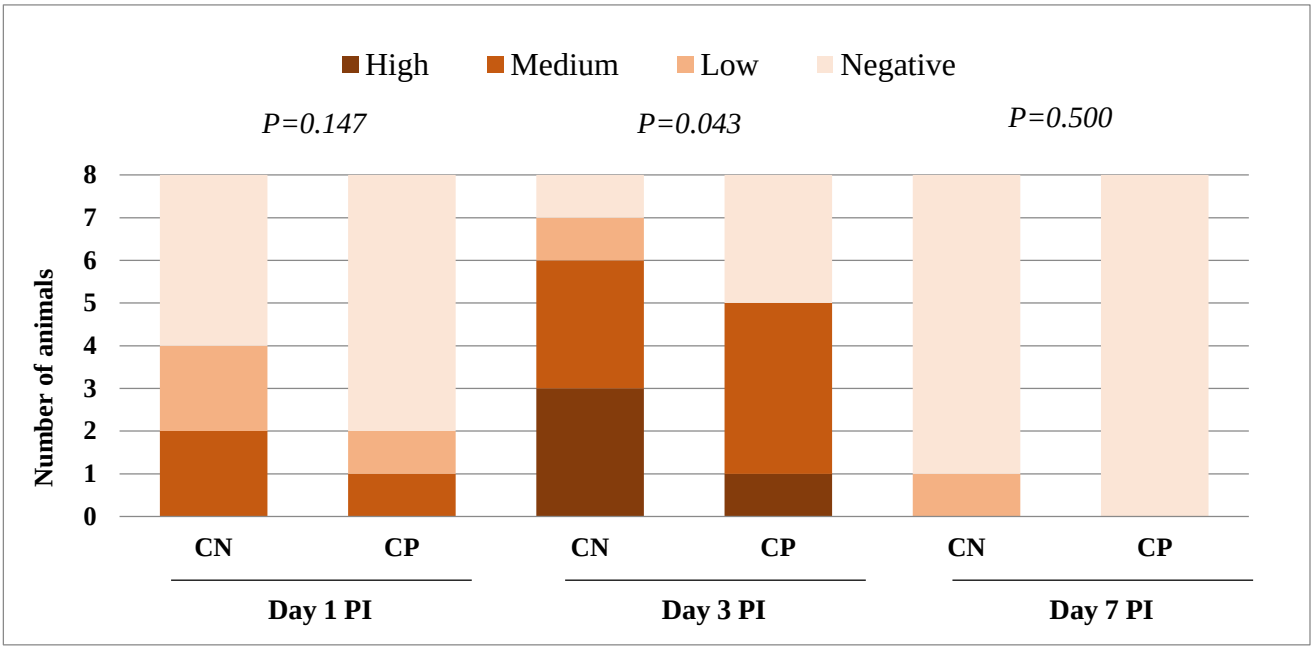
[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. n = 8 for groups CN and CP, n = 4 for groups NN and NP. Interactions only included when significant.

A moderate increment in rectal temperature was seen due to the *Salmonella* challenge (39.3ºC ±0.05ºC vs. 39.6ºC ±0.04ºC, *P* = 0.005), while this increment was not significant in the ETEC K88 trial (38.9ºC ±0.05ºC vs 39.1ºC ±0.06ºC, *P* = 0.180). No differences were detected due to probiotic treatment.

4.3.4.Microbial analysis

In the first trial, none of the animals seeded *Salmonella* on arrival, and non-challenged piglets remained negative along the whole study. Figure 4.3 shows the evolution of *Salmonella* counts along the post-challenge period in the challenged group. Challenged animals treated with probiotic had lower fecal excretions of *Salmonella* on Day 3 PI (*P* = 0.043).

Figure 4.3. Number of animals in the different range levels of fecal excretion of *Salmonella* at 1, 3 and 7 days post-challenge.



Negative 0 - 10^2 cfu/g, Low 10^3-10^4 cfu/g, Medium 10^5 - 10^6 cfu/g and High 10^7 - 10^8 cfu/g. CN (challenged + no probiotic) and CP (challenged + probiotic). n = 8 for groups CN and CP.

In the ETEC K88 trial, no significant differences were seen on the arrival day or before the inoculation, in fecal enterobacteria or coliform plate counts (data not shown). Table 4.3 shows the microbiological analysis on Days 4 and 9 PI in feces and colon digesta. No significant differences related to the experimental treatments were found in enterobacteria or coliform plate counts in feces. Nevertheless, the qPCR aiming to target the inoculated

ETEC K88 bacteria in colonic content did show an increase of copy numbers of the F4 fimbria K88 gene in the challenged groups on Day 4 PI (P = 0.029). These differences were not maintained on Day 9 PI, when levels decreased in the challenged group near the detection limit of the method (log 3.974 gene copies/g). No differences were found related to probiotic administration. Regarding the microbial plate counts in the ileal scrapings, around 30% of the samples on Day 4 PI and 50% on Day 8 PI were below the minimum level of detection (10^5 cfu/g).

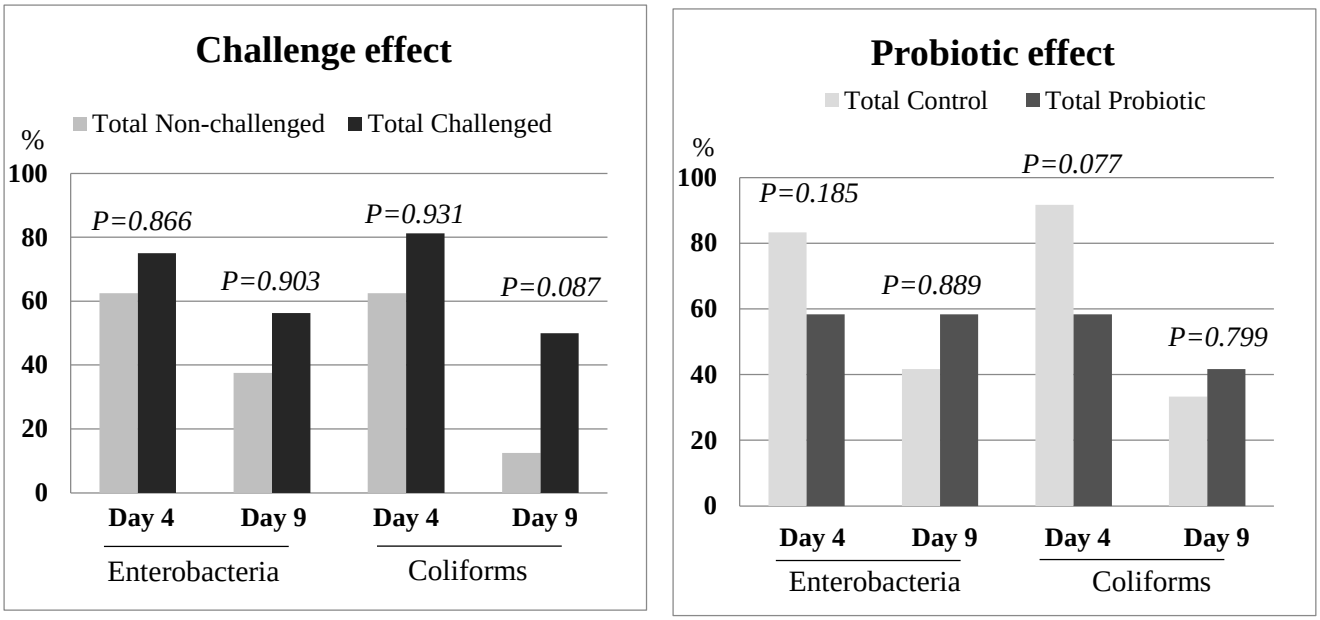
Table 4.3. Effects of ETEC K88 trial on microbiological analysis.

	Treatments [A]					***P*-value**		
	CN	**CP**	**NN**	**NP**	**RSD** [B]	***Challenge***	***Probiotic***	***Interaction***
Feces								
Enterobacteria (cfu/gFM)								
Day 4 PI	8.24	8.31	8.48	9.09	*1.339*	*0.390*	*0.561*	*0.644*
Day 9 PI	7.75	8.31	8.21	8.12	*1.114*	*0.635*	*0.784*	*0.511*
Total coliforms (cfu/gFM)								
Day 4 PI	8.13	7.83	8.26	8.15	*1.009*	*0.613*	*0.639*	*0.824*
Day 9 PI	7.5	7.89	8.21	8.08	*1.064*	*0.345*	*0.787*	*0.581*
Colon Digesta								
***E.coli* K88 (log copies/gFM)**								
Day 4 PI	5.38	6.00	4.25	4.14	*1.464*	*0.029*	*0.692*	*0.571*
Day 9 PI	4.70	5.11	4.78	4.90	*0.556*	*0.786*	*0.285*	*0.556*

Plate counts of total coliforms and enterobacteria in fecal samples (log cfu/g fresh matter [FM]) and F4 gene copy numbers of *E. coli* K88 in colon digesta (log of F4 gene copies/g FM). [A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

When analyzing the frequencies of animals with countable numbers of enterobacteria or coliforms in ileal scrapings by main effects (challenge effect or probiotic effect, Figure 4.4), there was a trend for a higher percentage of animals with countable coliforms on Day 9 PI in the challenged group (P = 0.087), and the probiotic trended to diminish the percentage of animals with countable coliforms on Day 4 PI (P = 0.077*)*. No interaction effects were found.

Figure 4.4. Percentage of animals in ETEC K88 trial with countable plate counts of enterobacteria or coliforms in ileal scrapings (> 10^5 cfu/g).



Main effects: challenge (n=16 for Total challenged animals, n=8 for Total non-challenged animals) and probiotic (n=12 for probiotic and control animals).

4.3.5.Changes in fermentative activity

Table 4.4 shows the changes promoted by the experimental treatments on the main colonic fermentation products of both trials.

The *Salmonella* challenge increased ammonia concentration in ileum on Day 4 PI (1.5 vs 2.3 mmol/l; P = 0.033) and showed a trend to increase it in the colon (P = 0.094 and P = 0.097 for Days 4 PI and 8 PI, respectively). No significant differences related to the experimental treatments were detected in any of the rest of ileal parameters analyzed (pH, SCFA and lactic acid). In colonic content, a significant decrease in the total concentration of SCFA (P < 0.001) was seen with the challenge on Day 4 PI, more pronounced in animals receiving the probiotic treatment (P challenge x probiotic = 0.008). This pattern was also found on Day 8 PI with numerical differences (P challenge x probiotic = 0.131). Molar ratios of SCFA (data not shown) were not significantly modified by any of the treatments although trends for interactions were seen on Day 4 PI for propionic acid (P challenge x probiotic = 0.068) and butyric acid (P challenge x probiotic = 0.092). In both

cases, the molar proportions in the challenged animals that received the probiotic remained closer to the mean values registered for the non-challenge animals.

Table 4.4. Colonic pH values, ammonia concentration and fermentation products for Days 4 and 8 post-inoculation (PI) in *Salmonella* and ETEC K88 trials.

		Treatments [A]					*P*-value		
	Days PI	**CN**	**CP**	**NN**	**NP**	**RSD [B]**	***Challenge***	***Probiotic***	***Interaction***
Trial 1. *Salmonella*									
pH	4	5.81	5.88	5.78	5.72	*0.193*	*0.285*	*0.912*	*0.433*
	8	5.61	5.63	5.627	5.75	*0.175*	*0.384*	*0.359*	*0.507*
NH_3	4	12.44	10.46	9.34	7.3	*4.051*	*0.094*	*0.271*	*0.987*
(mmol/L)	8	12.5	12.49	10.19	7.03	*5.165*	*0.097*	*0.488*	*0.489*
SCFA	4	116.5	90.2	123.3	141.3	*17.49*	*<0.001*	*0.587*	*0.008*
(mmol/kg)	8	142.3	129.9	131.5	142.1	*16.95*	*0.925*	*0.905*	*0.131*
Lactic acid	4	2.48	4.48	2.45	2.3	*6.926*	*0.717*	*0.762*	*0.723*
(mmol/kg)	8	1.18	3.59	0.9	3.58	*4.982*	*0.948*	*0.251*	*0.949*
Trial 2. ETEC K88									
pH	4	5.92	6.07	5.89	5.93	*0.12*	*0.103*	*0.071*	*0.238*
	9	5.88	5.91	6.08	5.94	*0.17*	*0.138*	*0.459*	*0.257*
NH_3	4	14.3	13.5	20.3	14.0	*4.67*	*0.122*	*0.093*	*0.197*
(mmol/L)	9	15.2	27.6	16.7	18.5	*11.52*	*0.457*	*0.168*	*0.300*
SCFA	4	130.7	124.2	113.9	130.4	*14.57*	*0.409*	*0.435*	*0.082*
(mmol/kg)	9	147.3	142.0	123.5	136.0	*22.00*	*0.133*	*0.711*	*0.363*
Lactic acid	4	9.05	4.14	7.65	3.02	*7.37*	*0.698*	*0.151*	*0.965*
(mmol/kg)	9	2.87	1.65	7.02	2.97	*3.75*	*0.107*	*0.120*	*0.392*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

In the ETEC K88 trial, pathogen inoculation significantly affected ileal fermentation on Day 9 PI; pH was reduced (6.4 vs 6.1, $P < 0.001$) while acetic acid and lactic acid (the main fermentation products) tended to be increased (3.4 vs 9.6 mmol /kg for acetic acid, $P = 0.051$ and 12.7 vs. 37.3 mmol/kg for lactic acid, $P = 0.076$). In colonic digesta, ETEC K88 provoked a numerical increase in pH ($P = 0.103$) on Day 4 PI, with an increase in the molar ratio of propionic (25.8% vs. 29.0%; $P = 0.015$) and a decrease in branched-chain fatty acids (1.69% vs. 1.07%; $P = 0.042$). The probiotic administration significantly increased ileal pH on Day 4 PI (6.32 vs. 6.18, $P = 0.024$) and also trended to increase pH in the colon ($P = 0.071$). Moreover, a trend to reduce ammonia concentration was also seen with the probiotic on Day 4 PI in the colon ($P = 0.093$) and on Day 9 PI in the ileum (1.07 vs. 0.79 mmol/L, $P = 0.058$). Regarding changes in molar proportions with the probiotic, a significant increase of the branched-chain fatty acids molar percentage was seen with the probiotic treatment on Day 9 PI (1.35% vs. 1.99%; $P = 0.039$). Different interactions were registered on Day 4 PI. In the ileum, probiotic trended to decrease lactic acid in challenged animals and to increase it in non-challenged animals (P challenge x probiotic = 0.052). In colonic content, the probiotic trended to increase the total SCFA concentration only in the non-challenged animals (P challenge x probiotic = 0.082) with increases in the acetic acid proportion in the challenged animals and decreases in the non-challenged ones (P challenge x probiotic = 0.017). A contrary interaction was seen for molar ratios of propionic and butyric acids (P challenge x probiotic = 0.099 and $P = 0.056$, respectively).

4.3.6.Immune response

Table 4.5 shows the serological concentrations of the acute-phase proteins Pig-MAP and the pro-inflammatory cytokine TNFα. In the *Salmonella* trial, both indexes were significantly increased by the challenge on Days 4 ($P < 0.01$) and 8 PI ($P < 0.03$), while in the *ETEC K88* trial only TNFα responded to the challenge on Day 4 PI ($P = 0.003$). The probiotic treatment did not significantly modify any of the parameters, although in the ETEC K88 trial a significant interaction ($P = 0.022$) was found in Pig-Map values on Day 4 PI, when the probiotic treatment increased this index in the challenged animals but decreased it in non-challenged ones.

Table 4.5. Effects on serum levels of pro-inflammatory cytokine TNFα and acute-phase protein Pig-MAP on Days 4 and 8 post-inoculation in *Salmonella* and ETEC K88 trials.

		Treatments [A]					*P*-value		
	Days PI	**CN**	**CP**	**NN**	**NP**	**RSD [B]**	***Challenge***	***Probiotic***	***Interaction***
Trial 1. *Salmonella*									
Pig-Map	4	1.65	2.57	0.87	0.74	*0.907*	*0.003*	*0.326*	*0.194*
(mg/ml)	8	1.33	1.68	0.80	0.64	*0.762*	*0.027*	*0.788*	*0.449*
TNFα	4	146.2	155.0	95.8	84.5	*37.01*	*0.001*	*0.938*	*0.542*
(pg/ml)	8	128.1	134.1	87.2	82.7	*27.95*	*0.001*	*0.950*	*0.673*
Trial 2. ETEC K88									
Pig-Map	4	0.80 [a,b]	0.92 [a]	0.87 [a,b]	0.67 [b]	*0.147*	*0.163*	*0.551*	*0.022*
(mg/ml)	9	0.84	1.14	0.77	0.75	*0.599*	*0.379*	*0.603*	*0.539*
TNFα	4	83.9	79.2	64.0	49.9	*16.85*	*0.003*	*0.218*	*0.536*
(pg/ml)	9	92.3	92.2	90.1	77.2	*17.97*	*0.284*	*0.516*	*0.427*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

4.3.7.Intestinal morphology

The histomorphological results of the ileum on Day 4 PI are summarized in Table 4.6. Results for Day 8/9 PI are not shown in the table. The *Salmonella* challenge caused a decrease in villus height ($P < 0.001$), deeper crypt depth ($P = 0.007$) and worse villus:crypt ratio ($P < 0.001$) on Day 4 PI. A significant interaction was seen on Day 8 PI in crypt depth that was increased by the probiotic in the non-challenged animals and decreased in the challenged ones (212, 240, 229 and 203 μm NN, NP, CN and CP, respectively; $P = 0.017$). Moreover, other trends for similar interactions were seen in the villus:crypt ratio on Day 4 PI ($P = 0.107$) and Day 8 PI ($P = 0.091$). The *Salmonella* challenge increased intra-epithelial lymphocytes (IEL) both days PI ($P = 0.042$ for Day 4 PI and 1.71 vs. 2.18, $P = 0.046$ for Day 8 PI) and mitosis in crypts on Day 4 PI ($P = 0.035$). Probiotic also had significant effects on Day 8 PI when it increased IEL (1.80 vs. 2.09 cel/100 μm, $P = 0.002$) and decreased goblet cells (GC) (1.31 vs. 1.05 cel/100 μm, $P = 0.025$).

Table 4.6. Histological determinations in ileum on Day 4 PI in *Salmonella* and ETEC K88 trials.

	Treatments [A]					*P-value*		
Day 4 PI	**CN**	**CP**	**NN**	**NP**	**RSD [B]**	***Challenge***	***Probiotic***	***Interaction***
Trial 1. *Salmonella*								
Villus height (µm)	186	141	250	296	*46.9*	*<0.001*	*0.992*	*0.361*
Crypt depth (µm)	244	245	206	218	*24.5*	*0.007*	*0.562*	*0.581*
Ratio Villus:Crypt	0.78	0.58	1.22	1.37	*0.242*	*<0.001*	*0.818*	*0.107*
IEL [C] (Nº cel/ 100 µm)	2.09	2.03	0.75	1.44	*1.027*	*0.042*	*0.482*	*0.407*
GC [D] (Nº cel/ 100 µm)	1.48	1.37	1.08	0.98	*0.561*	*0.119*	*0.661*	*0.982*
Mitosis[E] (Nº cel/ 100 µm)	0.40	0.45	0.31	0.27	*0.136*	*0.035*	*0.900*	*0.481*
Trial 2. ETEC K88								
Villus height (µm)	224	223	252	260	*49.7*	*0.152*	*0.886*	*0.818*
Crypt depth (µm)	205	213	187	207	*20.7*	*0.194*	*0.144*	*0.504*
Ratio Villus:Crypt	1.11	1.05	1.34	1.25	*0.258*	*0.066*	*0.524*	*0.877*
IEL [C] (Nº cel/ 100 µm)	2.64	2.74	1.79	2.36	*0.420*	*0.004*	*0.091*	*0.224*
GC [D] (Nº cel/ 100 µm)	1.08	1.48	1.69	1.45	*0.488*	*0.192*	*0.709*	*0.152*
Mitosis[E] (Nº cel/ 100 µm)	0.34	0.38	0.33	0.29	*0.120*	*0.354*	*0.934*	*0.472*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP. [C] IEL= Villus intraepithelial lymphocytes; [D] GC= Villus goblet cells/100 µm; [E] Number of mitosis in crypts.

In the E*TEC* K88 trial, the challenge tended to decrease the villus:crypt ratio on Day 4 PI (P = 0.066), increased IEL both days (P = 0.004 Day 4 PI and 2.08 vs. 2.49, P = 0.104 Day 9 PI) as well as GC on Day 9 PI (0.91 vs. 1.22 cel/ 100, P = 0.093). The probiotic treatment trended to increase IEL on Day 4 PI (P = 0.091), and an interaction was seen on Day 9 PI in villus height (P = 0.017) and villus:crypt ratio (P = 0.006) with lower values in CP and increased ones in NP. No differences related to the experimental treatments were detected in mitosis.

4.4. Discussion

The aim of this study is to determine if the administration of the probiotic strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 is able to enhance health at early life stages and, moreover, if it confers protection against common opportunistic digestive pathogens such as *Salmonella* Typhimurium or ETEC K88. To assess this objective, the probiotic was tested in two different trials, one for each pathogen, and a different clinical outcome was obtained for each challenge. In the *Salmonella* trial, nearly all parameters evaluated responded significantly to the challenge, with the animals presenting clear clinical signs of acute self-limiting diarrhea whereas, in the ETEC K88 challenge, effects were milder and no severe cases were seen, the evaluated parameters being weakly affected. Hence, at the end, we were able to test the probiotic in a wide range of intestinal disease.

Many *in vitro* studies have demonstrated the ability of bifidobacteria to inhibit the growth of potential pathogens, particularly *Salmonella* and *E. coli* (Bielecka et al., 1998; Gagnon et al., 2004; Tanner et al., 2016). However, it is not so easy to find well-designed *in vivo* studies able to demonstrate the potential of a probiotic strain to fight a particular pathogen. Regarding *Bifidobacterium* strains, Shu et al. (2000 and 2001) observed, in a piglet model, that *Bifidobacterium lactis* conferred protection against *E. coli* and *Salmonella* among other intestinal pathogens although it did not completely reduce the pathogenic colonization. In addition, Knol et al. (2007) associated an increase of bifidobacteria with reductions of the presence of clinically relevant pathogens in formula-fed pre-term infants. These protective effects against pathogens have mainly been attributed to a pluripotent stimulatory effect on the immune system (Akahashi et al., 2013; Gill et al., 2001; Medina et al., 2007), although some other modes of action could explain their antimicrobial activity like the production of organic acids (Saulnier et al., 2009), bacteriocines and bacteriocine-like substances (Cheikhyoussef et al., 2008), and the capacity to inhibit the pathogenic adhesion to enterocytes or prevent bacterial translocation (Gagnon et al., 2004; Searle et al., 2009).

In the *in vivo* study herein presented, the administration of a single daily dose (10^9 cfu) of *Bifidobacterium longum* subsp. *infantis* CECT 7210 was not able to fully prevent pathogen colonization; nevertheless, it was able to reduce pathogen loads, particularly the load of

Salmonella in the fecal content and the number of animals with high numbers of coliforms attached to their ileal mucosa.

The probiotic also showed some particularly beneficial effects on the animals. A clear effect on intestinal immune function was seen with an increase in ileal IEL in both challenges. IEL is a functionally heterogeneous population that contains cells with antitumor activity, natural killer activity, allospecific cytotoxic T lymphocytes or their precursors and mast cells. It is well established that they have a relevant role in the gastrointestinal immune system, playing an important role in the regulation of the immune response (Ogra et al., 2012). In particular, other immune effects have been described with the same strain. Moreno et al. (2011) reported an increment of the IgA antibody levels in feces by the inclusion of the probiotic in a murine model and also increases in anti-inflammatory IL-10 have been recorded (unpublished data). Ewaschuk et al. (2008) reported that *Bifidobacterium infantis* immunomodulation could at least be partially regulated by bioactive peptides, which can retain their biological activity even without *B. infantis* being present. In that sense, recent works with our strain have reported the production of peptides with protease activity able to hydrolyze β-casein and produce functional peptides with antirotaviral activity (Chenoll et al., 2015 and Chenoll et al., 2016).

Effects of the probiotic also were seen in the first trial for GC numbers, with a significant reduction on Day 8 PI. These results were somehow unexpected, as probiotics have been suggested to promote mucus secretion as one mechanism to improve barrier function and exclude pathogens (Ohland et al., 2010) and GC are responsible for the production and maintenance of the protective mucus layer (Specian and Oliver, 1991). However, it would also be possible to consider a reduction in GC as a good sign, considering that challenged animals normally have higher numbers of GC (in our case, a trend was recorded on Day 8 PI and numerical differences the rest of the days), probably as a response to dysbiosis and deterioration of the mucous layer.

Effects of the probiotic treatment on other parameters evaluated in this study were variable, depending on whether animals were challenged or not, and should be analyzed separately.

In general terms, the effects of the probiotic in weight gains and feed intakes were scarce. Nevertheless, in both trials, during the first week post-challenge, an interaction could be

seen in the ADFI, with enhanced feed consumption in the non-challenged animals receiving probiotic and diminution in the challenged ones. Moreover, at the end of the ETEC K88 trial, the challenged animals receiving the probiotic showed almost 1 kg of BW less than their counterparts, despite this difference not being significant. This apparent worse performance of the challenged animals with the probiotic could be explained in the ETEC K88 trial by a random, worse adaptation of the CP group to weaning, as this group unexpectedly decreased their intakes during the first week before the challenge. Nonetheless, this explanation cannot be given for the *Salmonella* trial, which suggests a differential effect of the probiotic in challenged or non-challenged animals.

Regarding colonic fermentation, it could be observed in both trials, on Day 4 PI, that animals treated with the probiotic showed an increase in the SCFA concentrations if they were not challenged, but if they were orally inoculated with the pathogen, SCFA concentration was reduced. In addition, a favorable fermentative environment was detected in non-challenged animals receiving probiotic, with a tendency to increase butyric acid but not in the challenged ones. Although *Bifidobacterium* spp. are not butyrate producing bacteria, it has been proposed that cross-feeding of lactate or acetate produced by bifidobacteria can stimulate the formation of butyrate by other bacteria within the gut community (Belenguer et al., 2006; Van der Meulen et al., 2006; De Vuyst and Leroy, 2011). For instance, Falony et al. (2006) co-cultured fermentations of *Bifidobacterium longum* and two acetate-converting, butyrate-producing colonic bacteria with oligofructose as the sole energy source and observed interspecies interactions. Due to the high complexity of the colon ecosystem, we could not evaluate this metabolic process in our *in vivo* assay. Nevertheless, the higher presence of butyrate reported for the NP group in both trials and the diminution of acetic acid in the same animals registered in the ETEC K88 trial would suggest a possible cross-feeding phenomenon present only in the non-challenged animals. In the challenged animals, gut dysbiosis caused by the challenge had probably disturbed all of these microbial interactions so much that it precluded the probiotic to promote butyrogenic effects. Moreover, histomorphological findings reinforce this theory as probably a higher butyrate presence in the NP group, being the preferred energy source for the colonocyte and a potent differentiating agent (Scheppach, 1994), contributed to the observed increase of the villus:crypt ratio of the non-challenged animals treated with the probiotic.

Furthermore, although statistical significance was only achieved in the ETEC K88 trial, Pig-MAP also responded differentially, with increases in the challenged animals receiving probiotic and decreases in the non-challenged ones on Day 4 PI. In swine, Pig-MAP is a major acute-phase protein, and higher serum concentrations have been related to acute inflammatory processes and also to the extent of tissue injury, expressing strong and protective responses to bacterial infections (Piñeiro et al., 2009). As mentioned above, our strain has been reported to have anti-inflammatory effects (Moreno et al., 2011), but no evidence of this effect was seen in the CP group.

All of these results suggest that our probiotic interacted differently in each situation, as probiotics are subject to the influence by many internal and external factors which can affect their efficacy (de Lange et al., 2010). A better comprehension of these interactions (such as gut health conditions, bacterial populations and their connections) could bring to light improved application protocols to get over the inconsistent results reported nowadays in scientific literature.

This possible differential behavior of a probiotic under a disease condition is particularly relevant if we wish to use the probiotic therapy to treat intestinal disease. Guaranteeing the safety of a new probiotic is therefore essential in this circumstance. It is not the first case in scientific literature in which uncertainty regarding probiotic use is reported, as it has been recorded that probiotics can be useful in a healthy situation, but detrimental when the intestinal barrier is affected, especially if the patient is in an immuno-compromised situation; which could lead to bacterial translocation and sepsis (Liong, 2008). In humans, these bacterial sepsis for probiotic treatments have been mainly reported for *Lactobacillus* spp. and *Bacillus* spp., as reviewed by Boyle et al. (2006) and Liong (2008) but recently bifidobacterial sepsis have been recorded in preterm infants treated with probiotic (Bertelli et al., 2015; Jenke et al., 2012; Zbinden et al., 2015) and even in an elder man undergoing chemotherapy treatment (Weber and Reynaud, 2015). Trevisi et al. (2008) tried different concentrations of a *Bifidobacterium animalis* in weaning piglets with and without a FOS-based prebiotic and found a positive correlation of the *B. animalis* administration and translocation in mesenteric lymph nodes when FOS was supplemented. The authors speculated about the possibility of an increase in other bacteria promoted by FOS that could also be translocated and thus contribute to the reported results.

Regarding the safety of the probiotic strain tested in this study, Moreno et al. (2011) tested a 10^9 cfu dose in an acute ingestion study with immunosuppressed mice, and no bacterial load of *Bifidobacterium* spp. was found in blood, liver, spleen or mesenteric lymph nodes. Bifidobacteria are generally considered safe or even favorable for gut health, and a vast body of literature appeals to protection exercised by these probiotics in avoiding pathogen translocation. Nevertheless, we speculate that in our challenged animals, gut dysbiosis caused by the bacterial challenge, in addition to the weaning syndrome, characterized by stress, depressed feed intake plus altered structure and function of the gut (Beers-Schreurs and Vellenga, 1992), might have led the probiotic to have a detrimental effect despite the observed reductions in pathogen loads. More research should be addressed to study any possible effect of this probiotic on the barrier integrity and bacterial translocation to discard any possible risks and uncertainties in its use to treat intestinal pathologies.

Finally, although the pig has been described as an excellent model for humans (Meurens et al., 2012; Wang and Donovan, 2015), certain limitations of the model should be taken into account when interpreting results. Firstly, the host-specific nature of *Bifidobacterium longum* subsp. *infantis*, as this bacteria is one of the first and most predominant gut colonizers in infants (Underwood et al., 2015) but not in pigs. This bacteria has co-evolved with humans (Arboleya et al., 2016) and this would explain why *B. infantis* grow better in the presence of human-milk oligosaccharides than in lactose. When human-milk oligosaccharides are present, *B. infantis* increases their adhesion rate to intestinal cells and expression of selected cytokines (Chichlowski et al., 2012). Secondly, gastrointestinal disorders are multifactorial, and therefore it is difficult to fully emulate them in controlled experimental conditions (Rossi et al., 2012). A moderate-high controlled dose of a single intestinal pathogen (even in multiple doses as in our experiment) achieved significant challenge effects (diarrhea, enhancement of inflammatory cytokines, histomorphological affections…) and allowed us to test the probiotic in different controlled ranges of intestinal affections. However, this way to be exposed to the pathogen is quite different from what a natural process would be where a low and continuous exposure is normally expected. Lastly, the way the probiotic is administered can also determine differences in the response. In this study, the probiotic was given in a single bolus every morning at the peak of eating activity, aiming to ensure that piglets received the stated concentration of viable bacteria and that the stomach was full in order to favor probiotic viability. Nevertheless,

the effects could have been different if we had given the probiotic in a continuous pattern, as it would be if added to a milk formula. Considering that the pig is not the natural host for these bacteria, the probiotic probably had few opportunities to persist in the gastrointestinal tract. This could be the explanation for the low numbers of probiotic cells found in the colon, considering that the animals were sampled more than 24 h after the last dose.

To sum up, various consistent effects of the probiotic in both experiments were detected although a different response pattern was seen between challenged and non-challenged animals. The potential of the probiotic was demonstrated by clearly producing a beneficial response in non-challenged animals and by improving their post-weaning situation. Nevertheless, the response of challenged animals treated with probiotic was not always positive although the probiotic consistently reduced the pathogen loads. More in-depth investigation should be performed to better assess the mechanisms for the different response patterns observed and improve probiotic therapeutic protocols. Limitations in the model must be considered when evaluating experiment results and extrapolating the potential of the probiotic to children.

4.5. Conclusions

The probiotic *Bifidobacterium longum* subsp. *infantis* CECT 7210 had a positive effect against pathogens by reducing the fecal excretion of *Salmonella* Typhimurium and the mucosal colonization of coliforms in the ETEC K88 trial. In addition, it produced a stimulation of the intestinal immune system by increasing IEL. Different responses to the probiotic in challenged and non-challenged animals were also recorded, mainly in feed intake, SCFA concentrations and the villus:crypt ratio that would suggest a distinct effect of the probiotic intervention, depending on the structure of the microbiota and on the integrity of the intestinal barrier. More research is needed for fully guarantee the safety and efficacy of this strain for its use in children with gastrointestinal disorders.

Chapter 5.

Potential of the probiotic combination *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Bifidobacterium animalis* subsp. *lactis* BPL6 to improve health status of weanling piglets in a *Salmonella* Typhimurium oral challenge

5.1. Introduction

Salmonella spp. enteric infections are among the most common diarrhea associated causes of morbidity and mortality (CDC, 2013), especially in children up to five years (Lanata et al., 2013). In fact, newborn babies are considered to be especially vulnerable as their immune system is still not fully developed and may be prone to infections by opportunistic pathogens (Lanata et al., 2013; Thanabalasuriar and Kubes, 2014). Altogether, *Salmonella* infections are estimated to be responsible of up to 155,000 deaths considering the global population (Majowicz et al., 2010) and over 100,000 human clinical cases are reported each year only in the EU with an estimated overall economic burden of human salmonellosis of 3 billion euro a year (EFSA, 2013).

Probiotics and their metabolites have been suggested to have an important role in the formation or establishment of a well-balanced, indigenous, intestinal microbiota in new-borne infants and adults (Gill, 2003; Salazar et al., 2009). Furthermore, the administration of probiotic microorganisms in milk formulas has well documented benefits including improvements in infections, diarrhea, allergic disorders and inflammatory diseases in children (Bin-Nun et al., 2005; Minocha, 2009). For instance, remarkable beneficial effects against *Salmonella* have been documented by *Bifidobacterium* spp. genus, with well documented research *in vitro* (Liévin et al., 2000; Tanner et al., 2016) and in *in vivo* using animal models (Shu et al., 2000; Silva et al., 2004; Zacarías et al., 2014). In particular, one of the strains conforming the probiotic combination tested in this study *Bifidobacterium longum* subsp. *infantis* CECT 7210 has demonstrated a reduction on *Salmonella* fecal shedding in an *in vivo* model with weanling pigs (Barba-Vidal, Unpublished Data) and protective effects against a rotavirus infection *in vitro* and in a murine model (Moreno et al., 2011).

Multi-strain and multi-specie probiotic combinations have been suggested to have greater efficacy than single strains, as complementary or even synergistic effects can be achieved on the host when given together in comparison to giving them separately (Chapman et al., 2011; Collado et al., 2007b; Timmerman et al., 2004). Nevertheless, studies and reviews evaluating them are still limited and further research is needed. The objective of this work was therefore to demonstrate the potential of a probiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *B. animalis*

subsp. *lactis* BPL6 to enhance gut health at early life stages and to ameliorate the outcome of a *Salmonella* challenge using the weaning piglet as a model.

5.2. Materials and methods

The experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona 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) and all efforts were done to minimize animal suffering.

5.2.1.Animals and Housing

The trial was conducted as a Level 2 High Risk Biosecurity Procedure, with appropriate training of the personnel involved. A total of 72 male piglets (Large White x Landrace) from a high-sanitary-status farm and from mothers serologically negative to *Salmonella* were used. Animals were weaned at 28 (±3) days of age, 7.7 (±0.28) kg body weight (BW) on average, and were transported to the UAB facilities, where they were placed in three rooms of eight pens each (24 pens, three animals per pen) taking initial BW into account for a similar average BW within pens. The pens were allocated to four treatment groups following an unbalanced 2 x 2 factorial arrangement (factors being probiotic and pathogen challenge), with 8 replicates per treatment for the challenged animals and 4 replicates for the non-challenged group. The treatments were, therefore: 1) no challenge + no probiotic (NN); 2) no challenge + probiotic (NP); 3) challenged + no probiotic (CN) and 4) challenged + probiotic (CP). Two rooms were challenged with pathogens and one was left unchallenged. In each room, probiotic treatment was distributed within 4 pens on one side of the room, and the 4 control pens were on the other side of the room, separated by a corridor in between.

Pigs were maintained under a 14:30 h light/ 9:30 h dark lighting regimen. Each pen (2 m^2) had a feeder and a water nipple to provide feed and water for ad libitum consumption. The weaning rooms were equipped with automatic heating, forced ventilation and an individual heat-light per pen. The experiment was conducted during the spring season (April), with an average room temperature of 26ºC (±4ºC). The experimental treatments were distributed evenly among the three rooms.

5.2.2.Experimental Products and Diets

The probiotic treatment was supplied by Ordesa S.L. and consisted on a daily dosage (10^9 cfu) of a combination of *Bifidobacterium longum* subsp. *infantis CECT 7210 (B. infantis)* and *Bifidobacterium animalis* subsp. *lactis* BPL6 (*B. animalis*), supplemented in a 2 mL solution. The control group received, as placebo, the same amount of carrier. During the experimental period pigs received the treatment orally and individually, in a daily pattern using disposable 2 mL syringes without needle. Probiotic tested was a unique batch of lyophilized bacteria, which was re-suspended and administered every day, in less than 1 h.

A pre-starter diet without additives (Table 5.1) was formulated to satisfy the nutrient requirement standards for pigs of this age (NRC, 2012) and was given in a mash form.

5.2.3.Bacterial Strain

The bacterial strain used in the present study was a *Salmonella* Typhimurium var. Monophasic (formula: 4,5,12:i:-, resistance profile: ACSSuT-Ge, Fagotype: U302) that was isolated from a salmonellosis outbreak (mainly enteric and with sporadic septicemia) of fattening pigs in Spain, and was provided by the Infectious Diseases Laboratory (Ref. 301/99) of the UAB. The oral inoculum was prepared by 24 h incubation at 37ºC in BPW (Oxoid, Hampshire, UK) and diluted (1:20) with sterile PBS (Sigma-Aldrich, Madrid, Spain) to reach a final concentration of 2.5 x 10^8 cfu/ml.

5.2.4.Experimental Procedure

The duration of the study was 16 days, in which performance and clinical data were evaluated. After one week of adaptation to the diets (Day 8), a single 2-ml dose ($5x10^8$ cfu) of *Salmonella* Typhimurium was administered to the challenged animals by oral gavage and a single 2-ml dose of sterile BPW to the non-challenged animals (challenge control group).

Table 5.1. Ingredient composition and nutrient analysis of the experimental diets as-fed basis, g/kg.

Ingredients	
Maize	280.7
Wheat	170.0
Barley 2 row	150.0
Extruded soybean	122.4
Sweet whey-powder (cattle)	100.0
Fishmeal LT	50.0
Soybean meal 44	50.0
Whey-powder 50% fat	30.3
Mono-calcium phosphate	21.3
Calcium carbonate (CaCO3)	8.2
L-Lysine HCL	4.5
Vitamin-Mineral Premix [A]	4.0
Sodium chloride (marine salt)	3.0
DL-Methionine 99	2.4
L-Threonine	2.3
L-Triptophane	0.9
Chemical composition	
DM	903.2
Ash	74.1
Crude Fat	64.5
Crude Protein	189.3
Neutral detergent fiber	111.6
Acid-detergent fiber	35.1

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D_3. 39.9 mg vitamin E, 3 mg vitamin K_3, 2 mg vitamin B_1, 2.3 mg vitamin B_2, 3 mg vitamin B_6, 0.025 mg vitamin B_{12}, 20 mg calcium panthotenate, 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.

Body weight was recorded on Days 1, 8, 12 and 16, while feed consumption was recorded at Days 1, 7 and on a daily basis on the post-inoculation (PI) period (Days 8-16). The ADG, ADFI and G:F ratio were calculated by pen. Animals were checked daily for clinical signs to evaluate their status (i.e., dehydration, apathy and fecal score) after the *Salmonella* challenge, always by the same person. Fecal 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 (Thermoval Rapid, Hartmann, Spain) on Days 9 and 10 (1 and 2 PI). Mortality rate was also recorded and no antibiotic treatment was administered to any of the animals of the experiment.

For microbiological analysis, on Day 1 fecal samples were taken aseptically from 24 animals that were randomly selected from the total before distribution. Samples were taken after spontaneous defecation associated with the manipulation of the animal or by digital stimulation. On Days 8, 9, 11 and 15 (Days 0, 1, 3 and 7 PI), fecal samples were taken from the animal with the highest initial BW of each pen (N = 24).

At Days 4 and 8 PI (Experimental Days 12 and 16, respectively), one pig per pen was euthanized. On Day 4 PI, the animal selected was the one with the intermediate initial BW, while on Day 8 PI, the heaviest was selected.

Animals were euthanized and sequentially sampled during the morning (between 09:00 and 12:00 h). Prior to euthanasia, a 10-ml sample of blood was obtained by venipuncture of the cranial vena cava using 10-ml tubes without anticoagulant (Aquisel, Madrid, Spain). Immediately after blood sampling, selected piglets received an intravenous lethal injection of sodium pentobarbital (200 mg/kg body weight as Dolethal; Vetoquinol S.A., Madrid, Spain). Once dead, animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract was excised.

Digesta (approximately 50 ml) from the ileum and proximal colon (considered to be 0.75 m from the ileocecal junction) was collected and homogenized. The pH of the digesta was determined immediately after homogenization of the samples with a pH-meter calibrated on each day of use (Crison 52-32 electrode, Net Interlab, Barcelona, Spain). Without delay, contents collected were sub-sampled and kept on ice until analysis or being stored. Colonic samples (1 g) were plated for *Salmonella* quantification. To determine the presence of the probiotic in the gut, 2 g of digesta were sampled (only in Day 8 PI) and bacterial isolation was performed before storing them at -80ºC with GenIUL commercial protocol (Terrassa, Spain). Briefly, 1 g of colonic content sample was weighted in a 15 mL falcon and diluted 1:10 with enriched with MRS broth (Oxoid, Madrid, Spain) + 0.25% cysteine (Sigma-Aldrich, Madrid, Spain) + 2% Tween 80 (Sigma-Aldrich, Madrid, Spain). Ten glass spheres (5 mm diameter) were

added to the tube and vortex (1 min) to homogenize the suspension. Two-hundred fifty µL of the sample suspension were transferred to an eppendorf with 250 µL of enriched MRS broth. Three centrifugation (13,000 x g for 5 min at 4ºC) and resuspension (500 µL of enriched MRS broth) steps were performed and finally bacterial pellet was re-suspended in 200 µL of sterile PBS and stored at -80ºC for DNA extraction and quantification via qPCR. A set of ileal and colonic digesta samples were preserved in a H_2SO_4 solution (3 ml of digesta plus 3 ml of 0.2 N H_2SO_4) for ammonia (NH_3) determination and were kept frozen at -20°C. An additional ileal and colonic sample set (approximately 20 g) was also frozen (-20 ºC) until analyzed for SCFA and lactic acid.

For the histological study, 3-cm sections of the ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a 4% formaldehyde solution (Carlo-Erba Reagents, Sabadell, Spain).

Blood samples were centrifuged (3,000 x g for 15 min at 4ºC) after 4 h refrigeration, and the serum obtained was divided into different aliquots and stored at -20ºC to evaluate immune response.

5.2.5.Analytical Procedures

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

For probiotic detection, DNA was extracted with a commercial kit following manufacturer instructions (v-DNA reagent, Doc. Code 450000112. GenIUL, Terrassa, Spain). Briefly, samples were suspended in 1 mL of v-DNA buffer and centrifuged (13,000 x g for 5 min at 4ºC). An incubation of the bacterial pellet (90ºC, 10 min) with 200 µL of v-DNA reagent was done in a shaking incubator and finally DNA was suspended in 600 µL of v-DNA buffer. The *GenIUL Bifidobacterium spp.* qPCR kit was used for probiotic quantification (reference: 4900021000, GenIUL, Terrassa, Spain). The kit provided a *Bifidobacterium longum* CECT 4551 DNA standard for the construction of the standard curves (from 2 x 10^6 to 20 DNA copies per PCR reaction). Each reaction included 4 µl of a 5x HOT FIREPol qPCR Master mix including qPCR assay primers designed for the 16s RNA gene; 5 µl of diluted (1/10) DNA samples and a 11 µl of RNAse free water. Reaction conditions for amplification of DNA were 95 ºC

for 15 min and 45 cycles of 95 ºC, 15s for denaturation, 54 ºC 30s for annealing and 72 ºC 45s for extension and fluorescent detection. To determine the specificity of the amplification, an analysis of the product melting curve was performed after the last cycle of each amplification. The minimum level of detection of the method, considering the amount of DNA included in each reaction, was established in 6.3 x 10^3 16S ribosomal RNA gene copies/g of FM sample, compared to a non-template control dissociation curve. Real-time PCR was performed with the ABI 7900 HT Sequence Detection System (PE Biosystems) using optical-grade ninety-six-well plates.

Ammonia concentration in digesta samples was determined with the aid of a gas-sensitive electrode (Hatch Co., Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A., Barcelona, Spain). Three grams of acidified content were diluted (1:2) with 0.16M NaOH, after homogenization samples were centrifuged (1500 x g) for 10 min. The ammonia released was measured in the supernatants as different voltages in mV according to a procedure previously described in Hermes et al. (2009) that was adapted from Diebold et al. (2004). The SCFA and lactic acid analyses were performed by gas chromatography. The samples were submitted to an acid-base treatment followed by 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). For *Salmonella* bacteria counts, all samples were transferred (1:10) to BPW. Quantitative assessment was made by seeding the serial dilutions 10^{-2}, 10^{-4} and 10^{-6} of the samples in XLT4 plates (Merck, Madrid, Spain). The qualitative assessment was made by incubating samples in BPW (37ºC, 24 h), transferring them to Rappaport-Vassiliadis enrichment broth (Oxoid, Hampshire, UK) for a second incubation (42ºC, 48 h) and seeding them in XLT4 plates in order to observe H_2S positive colonies.

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned to a 4-µm thickness and stained with hematoxylin and eosin. Measurements of 10 different villous-crypt complexes per sample were performed with a light microscope (BHS, Olympus, Barcelona Spain) using the technique described in Nofrarias et al. (2006).

Serum concentrations of TNFα were determined by Quantikine Porcine TNFα kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Pig

major acute-phase protein concentrations were determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain) as described in Saco et al. (2011). Serological antibodies of *Salmonella* were tested by ELISA *Salmonella* Herdcheck (Idexx, Hoofddorp, Netherlands), and the cut-off for positivity was established at optic density ≥40%.

5.2.6. Statistical Analysis

Results are expressed as lsmeans with their standard errors unless otherwise stated. A two-way ANOVA was used to examine the effect of experimental challenge and probiotic treatment, as well as the interaction between the two (only included when significant). The general linear and mixed models of SAS (SAS Institute Inc., Cary, NC, USA) were used to analyze the effect of experimental treatments. For microbiological data, Fisher's exact test was used to analyze the frequencies of positive animals as contingency tables and odds ratio (OR) with its 95% confidence interval were calculated on basis of the fixed effects.

When treatment effects were established, treatment means were separated using the probability of differences function adjusted by Tukey–Kramer. The pen was considered the experimental unit for analysis and random effect was used to account for variation between pens. 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

In general, the trial proceeded as expected. Animals showed a good health status at the beginning of the experiment. None of the animals seeded *Salmonella* in feces on the arrival and serological analysis confirmed that animals had not been exposed to *Salmonella* previously to the day of inoculation, being all animals analyzed seronegatives along the whole trial. During the PI period, 3 deaths and an euthanasia for ethical reasons were registered in the challenged groups; one from the CN group the 4th Day after the inoculation and three from the CP group on Days 3, 5 and 6 PI, all from different pens. Necropsy was made to dead animals. All of them presented fibrinohemorragic gastritis and acute diffuse fibrinous enteric-tiflo-colitis, lesions normally associated to infection of *Salmonella* Typhimurium in pigs (Wilcock and Olander, 1977). Although casualties in the CP group were more than in the CN group

(3/24 vs. 1/24), differences were not statistically significant. No antibiotic treatment was administered to any of the animals on the trial.

The ability of the probiotic strains to colonize the gut was indirectly evaluated by analyzing the total *Bifidobacterium* spp. copies in the colonic content at Day 8 PI. Mean concentrations of DNA copies / g of FM detected were 3.16 x 10^7 for CN, 2.22 x 10^7 for CP, 2.03 x 10^7 for NN and 8.11 x 10^7 for NP. Probiotic and challenge effects were not significant but a tendency was seen for the interaction challenge x probiotic (P = 0.058) where the number of DNA copies increased in non-challenged animals receiving the probiotic.

5.3.1.Animal performance

Effects of the experimental treatments on BW, ADG and ADFI are exposed in Table 5.2.

Table 5.2. Animal performance parameters.

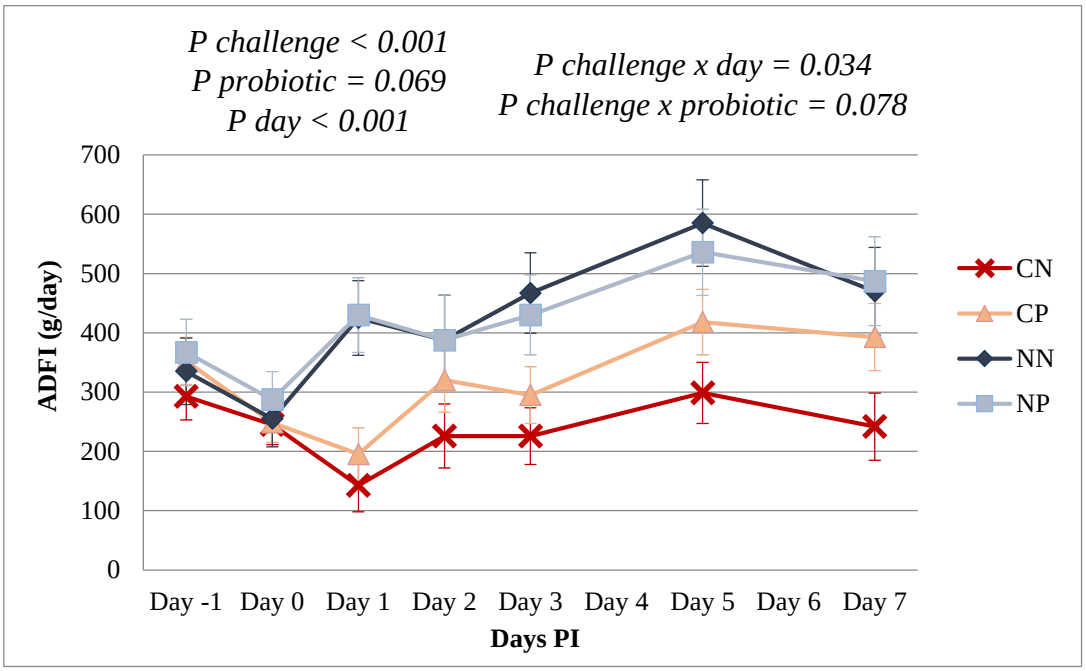
	Treatments [A]					***P*-value**		
	CN	**CP**	**NN**	**NP**	**RSD [B]**	***Challenge***	***Probiotic***	***Interaction***
BW [C] (kg)								
Initial	7.90	7.51	7.60	7.85	*0.559*	*0.929*	*0.770*	*0.195*
Final	8.78	9.34	9.82	10.35	*0.834*	*0.010*	*0.131*	*0.967*
ADFI [D] (g/d)								
Pre-inoculation [E]	259	306	273	250	*55.9*	*0.391*	*0.611*	*0.164*
Post-inoculation [F]	240	343	435	445	*114.9*	*0.008*	*0.276*	*0.368*
ADG [G] (g/d)								
Pre-inoculation [E]	53	119	78	58	*50.5*	*0.417*	*0.303*	*0.063*
Post-inoculation [F]	13	97	273	300	*122.5*	*<0.001*	*0.309*	*0.601*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. [C] Body weight. [D] Average Daily Feed Intake. [E] Experimental day 0 to 7. [F] Experimental day 8 to 16 (0 to 8 PI). [G] Average Daily Gain. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

Salmonella challenge affected negatively final BW, ADFI and ADG in the post-challenge period. Probiotic treatment did not show significant effects on the studied parameters despite a tendency to interaction (P = 0.063) was seen for the ADG before the challenge, showing the CP group a higher ADG than its control (CN).

Evolution of ADFI during the post-challenge period is shown in Figure 5.1. Feed intake was reduced by the *Salmonella* challenge ($P < 0.001$), being this effect specially manifested on day one post-inoculation (interaction challenge x day $P = 0.034$). A tendency was found for the probiotic to enhance feed consumption ($P = 0.069$), and although not significantly, this effect was more manifested in the inoculated animals (interaction challenge x probiotic $P = 0.078$).

Figure 5.1. Average daily feed intake evolution along the post-inoculation (PI) period.

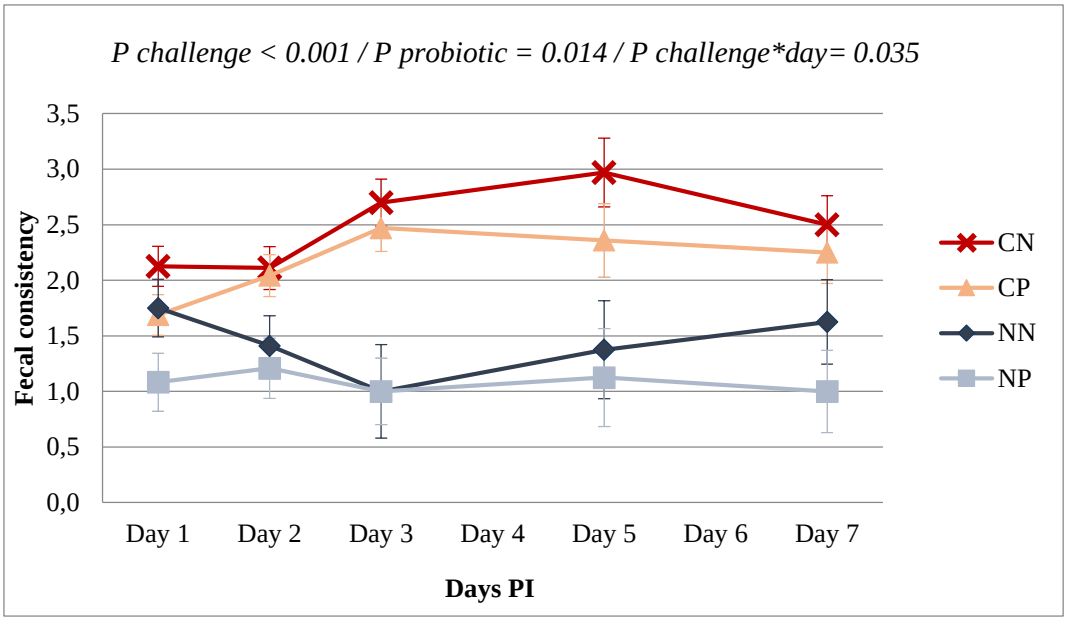


Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. n = 8 for groups CN and CP, n = 4 for groups NN and NP. Interactions only included when significant. Bars represent the standard error of the lsmeans.

5.3.2.Clinical signs

Figure 5.2 shows the evolution of fecal consistency after the challenge. *Salmonella* inoculation significantly affected fecal scores with more liquid feces (P challenge < 0.001), specially from day 3 onwards (P challenge x day = 0.035). Administration of the probiotic improved the fecal consistence with decreases in the fecal score in both, challenged and non-challenged animals (P = 0.014).

Figure 5.2. Evolution of the mean fecal scores in the different experimental groups along the post inoculation (PI) period.



Fecal score was measured using a scale from 1 (solid and cloddy) to 4 (watery or with blood). Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. n = 8 for groups CN and CP, n = 4 for groups NN and NP. P-values only included when significant. Bars represent the standard error of the lsmeans.

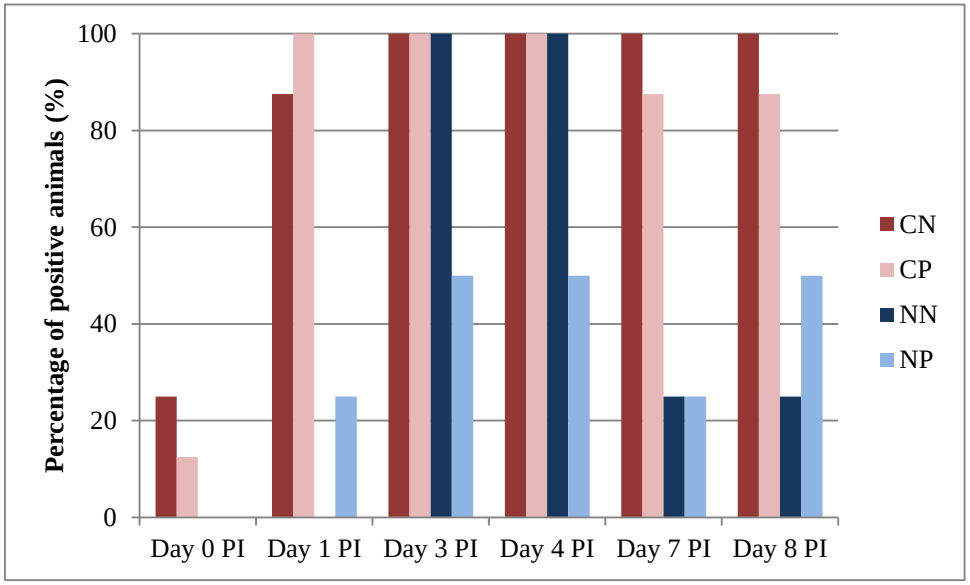
On Day 1 PI the challenged animals presented significantly higher rectal temperatures than the non-challenged animals (40.0 vs 39.3 ºC, $P < 0.001$) despite the administration of the probiotic. However, on Day 2 PI only the challenge animals not receiving the probiotic presented higher temperatures (39.9 vs. 39.2, 39.1 and 38.9 for CN, CP, NN and NP respectively) (*P* interaction probiotic*day = 0.048).

5.3.3.Salmonella analysis

None of the analyzed animals seeded *Salmonella* on the arrival. Figure 5.3 shows the prevalence of positive animals to *Salmonella* during the post-challenge period. After the oral challenge with *Salmonella*, almost all the animals that received the bacterial inoculum turned on positive in feces and kept positive all the remaining experimental period. Unexpectedly, 3 animals were positive for *Salmonella* before the inoculation (Day 0 PI) and some additional animals of the non-challenged group also became positive for *Salmonella* days onwards. However, from all samples analyzed on the PI period, a 98.7% of the samples of challenged animals were found positive (1-10^2 cfu/g)

during the PI period while a 45% of the samples of non-challenged animals were positive. No significant effects were seen with the probiotic.

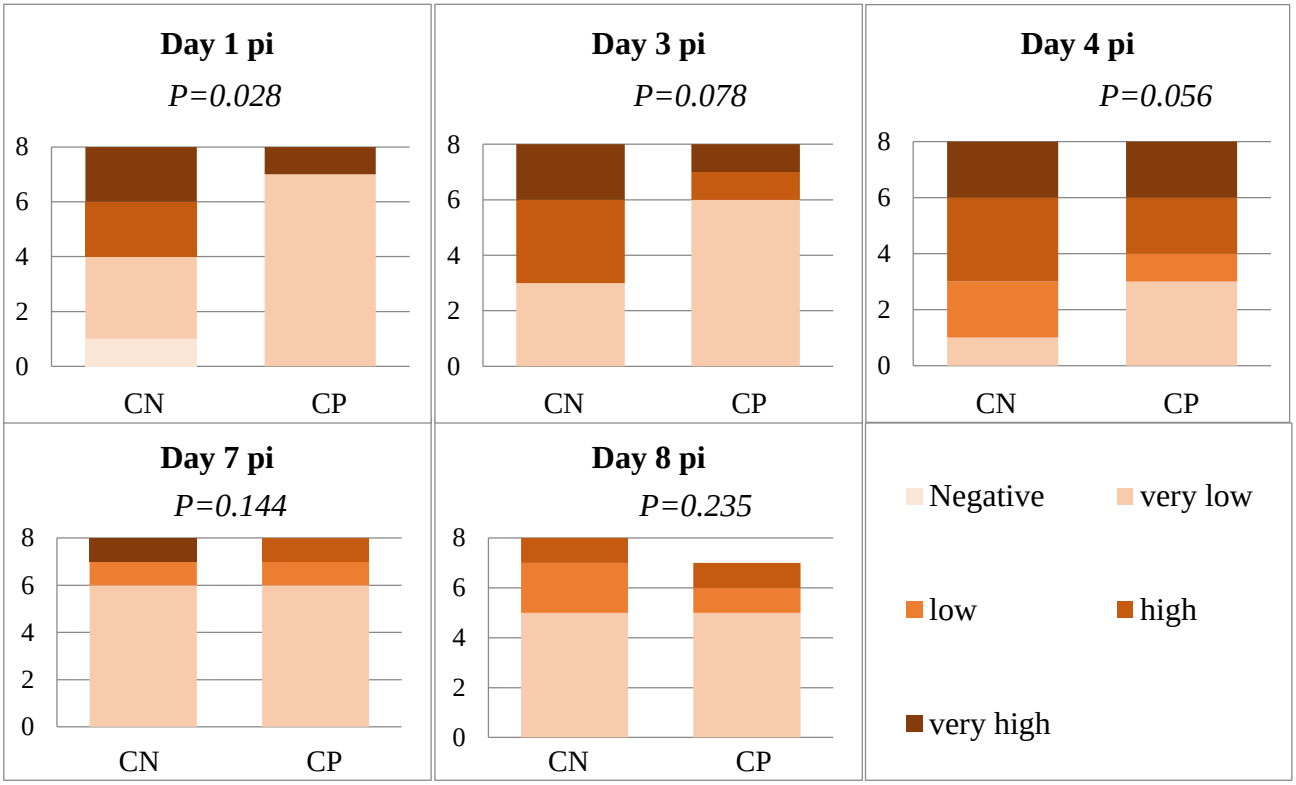
Figure 5.3. Percentage of positive (>1 cfu/g) animals to *Salmonella* spp.



In feces (Day 0, Day 1, Day 3 and 7 post-inoculation [PI]) or colonic content (Day 4 and 8 PI). Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

Figure 5.4 represents the semi-quantitative analysis of *Salmonella* in feces and colon digesta of those animals that received the pathogen inoculum. None of the non-challenged animals excreted *Salmonella* in quantifiable levels (>10^2 cfu/g) and therefore they are not represented in the figure. The probiotic administration lowered significantly the number of animals with high *Salmonella* excretion levels at Day 1 (P = 0.028) and also tended to lower them at Day 3 (P = 0.078) and 4 (P = 0.056) with an increase in the frequency of the animals with less than 10^3 cfu/g.

Figure 5.4. Number of animals in the different range levels of *Salmonella* spp.



In faeces (Days 1, 3 and 7 days post-inoculation [PI]) or colonic digesta (Day 4 and 8 PI). Range Levels: Negative (0 cfu/g), Very Low (1-10^2 cfu/g), Low (10^3-10^4 cfu/g), High (10^5 - 10^6 cfu/g) and Very High (10^7 - 10^8 cfu/g). CN (challenged + no probiotic) and CP (challenged + probiotic). n = 8 for groups CN and CP (except n=7 for CP at Day 8 PI).

5.3.4.Changes in fermentative activity

Table 5.3 shows the changes promoted by the experimental treatments on the main ileal and colonic fermentation products. In the ileum *Salmonella* challenge provoked a mild affection with a tendency to decrease ileal lactic acid concentrations at Day 4 PI (P = 0.096) and to increase pH at Day 8 PI (P = 0.099). The colon was more severely affected with a significant (P < 0.001) decrease in lactic acid concentrations at Day 8 PI and numerical decreases at Day 4 PI (P = 0.141). Significant decreases of colonic SCFA were also observed at Day 4 PI (P = 0.030) together with a tendency to increase ammonia concentrations (P = 0.104) at Day 8 PI.

Table 5.3. Colonic pH values, ammonia concentration and fermentation products for Days 4 and 8 post-inoculation (PI).

		Treatments [A]					*P*-value		
	Days PI	**CN**	**CP**	**NN**	**NP**	***RSD*** [B]	***Challenge***	***Probiotic***	***Interaction***
ILEUM									
pH	4	6.85	6.76	6.56	6.80	*0.539*	*0.608*	*0.751*	*0.515*
	8	6.83	6.76	6.66	6.28	*0.409*	*0.099*	*0.239*	*0.419*
NH3	4	4.47	2.37	2.95	5.13	*1.77*	*0.455*	*0.961*	*0.016*
(mmol/L)	8	0.81	0.71	1.09	0.74	*0.648*	*0.581*	*0.425*	*0.651*
Acetic acid	4	2.58	5.06	3.03	3.95	*4.280*	*0.887*	*0.403*	*0.706*
(mmol/kg)	8	2.73	3.89	1.60	6.15	*2.210*	*0.568*	*0.008*	*0.097*
Lactic acid	4	19.8	37.0	54.4	58.6	*34.74*	*0.096*	*0.515*	*0.691*
(mmol/kg)	8	49.1	45.7	22.6	43.1	*36.72*	*0.376*	*0.602*	*0.466*
COLON									
pH	4	6.19	6.07	5.77	6.03	*0.476*	*0.275*	*0.758*	*0.379*
	8	6.14	5.96	5.82	6.11	*0.254*	*0.473*	*0.626*	*0.058*
NH3	4	6.59	5.71	4.89	5.62	*4.398*	*0.643*	*0.967*	*0.678*
(mmol/L)	8	7.82	5.38	5.54	4.11	*2.370*	*0.104*	*0.078*	*0.635*
SCFA[C]	4	98.4	94.1	136.2	138.5	*39.42*	*0.030*	*0.957*	*0.853*
(mmol/kg)	8	115.0	140.7	140.3	123.0	*24.27*	*0.724*	*0.695*	*0.057*
Lactic acid	4	1.00	4.82	5.10	8.43	*5.644*	*0.141*	*0.170*	*0.923*
(mmol/kg)	8	0.84	1.01	12.07	8.07	*4.699*	*<0.001*	*0.363*	*0.324*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP. [C] SCFA include acetic, propionic butyric, valeric and branched-chain fatty acids.

Some beneficial changes were observed in the fermentation profile with the probiotic treatment. A significant increase in ileal acetic acid (P = 0.008) was seen at day 8 PI, being this increase of a bigger magnitude in non-challenged animals (P challenge x probiotic = 0.097). Moreover, a tendency to decrease colonic ammonia concentrations (P = 0.078) was detected at Day 8 PI. Some changes promoted by the probiotic were not the same in challenged and not-challenged animals. Surprisingly, increased ileal

ammonia levels were detected in NP group while a decrease was observed in CP animals in comparison to their control (*P* challenge x probiotic = 0.016). A trend for a similar pattern was observed for pH at colon at Day 8 PI (*P* challenge x probiotic = 0.058). On the other hand, a tendency to decrease colonic total SCFA in NP group and increase them in CP group was seen at Day 8 PI (*P* challenge x probiotic = 0.057). Molar ratios of colonic SCFA were not significantly modified by any of the treatments; mean molar ratios detected on the trial were 62.7% acetic, 23.6% propionic, 9.8% butyric, 3.0% valeric and 1.1% branched-chain fatty acids.

5.3.5.Immune response

Table 5.4 reports the serological concentrations of the acute phase proteins Pig-MAP and the pro-inflammatory cytokine TNFα. The *Salmonella* challenge provoked significant (*P* < 0.05) increases in both indexes except on Pig-Map on day 8 PI where the increase was numeric (*P* = 0.140). No significant effects were observed with the probiotic treatment although a numeric decrease of TNFα was observed at Day 8 PI (*P* = 0.121) in animals receiving the probiotic treatment.

Table 5.4. Effects on serum levels of pro-inflammatory cytokine TNFα and acute phase protein Pig-MAP in days 4 and 8 post-inoculation (PI).

	Treatments [a]					***P*-value**		
	CN	**CP**	**NN**	**NP**	***RSD*** [b]	***Challenge***	***Probiotic***	***Interaction***
Pig-Map (mg/ml)								
Day 4 PI	3.38	2.31	1.03	1.10	*1.657*	*0.024*	*0.504*	*0.446*
Day 8 PI	2.31	1.57	1.45	1.05	*1.019*	*0.140*	*0.215*	*0.708*
TNFα (pg/ml)								
Day 4 PI	151	158	87.7	77.6	*41.30*	*<0.001*	*0.927*	*0.647*
Day 8 PI	112	82.6	63.9	61.6	*21.95*	*0.002*	*0.121*	*0.182*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP.

5.3.6.Intestinal morphology

The histomorphological results of the ileum are summarized in Table 5.5. The challenge with *Salmonella* caused important decreases in villous height (*P* = 0.026 on Day 4; *P* = 0.061 on Day 8 PI), and although the crypt depth was not affected, the challenge

significantly altered the villus:crypt ratio both days ($P < 0.05$). Moreover, an increase was seen in the number of goblet cells (GC) on Day 8 PI ($P = 0.018$) and on the number of mitosis on Day 4 PI ($P = 0.022$). The probiotic administration promoted a different effect on challenged and non-challenged animals. Whereas the probiotic moderately increase the villous height in the challenged animals it decreased it in the non-challenge ones (Day 8 PI; *P* challenge x probiotic = 0.038). Regarding crypts the probiotic increased the crypt depth of the non-challenged animals until levels similar to the challenged ones (Day 8 PI; *P* challenge x probiotic = 0.011).

Table 5.5. Ileal histomorphometry at Days 4 and 8 post-inoculation (PI).

		Treatments [A]					***P*-value**		
	Days PI	**CN**	**CP**	**NN**	**NP**	***RSD*** [B]	***Challenge***	***Probiotic***	***Interaction***
Villous height	**4**	192.0	183.6	258.1	256.9	*65.94*	*0.026*	*0.869*	*0.903*
(µm)	**8**	198.3^{b}	243.4^{a,b}	303.3^{a}	237.5^{a,b}	*56.80*	*0.061*	*0.682*	*0.038*
Crypt depth	**4**	253.9	249.2	225.6	268.1	*27.24*	*0.696*	*0.125*	*0.059*
(µm)	**8**	242.5^{a}	242.7^{a}	182.8^{b}	259.1^{a}	*30.70*	*0.124*	*0.010*	*0.011*
Villous:Crypt	**4**	0.78	0.75	1.15	0.96	*0.280*	*0.027*	*0.382*	*0.509*
ratio	**8**	0.81^{b}	1.01^{b}	1.67^{a}	0.93^{b}	*0.218*	*<0.001*	*0.011*	*<0.001*
IEL [C]	**4**	1.04	1.25	1.38	1.49	*0.546*	*0.243*	*0.506*	*0.822*
(Nº cel/100µm)	**8**	0.73	1.21	0.98	1.44	*0.398*	*0.178*	*0.015*	*0.950*
GC [D]	**4**	0.74	0.69	0.71	0.66	*0.308*	*0.817*	*0.719*	*0.965*
(Nº cel/100µm)	**8**	0.96	1.04	0.66	0.44	*0.401*	*0.018*	*0.697*	*0.402*
Mitosis [E]	**4**	0.42	0.36	0.26	0.26	*0.123*	*0.022*	*0.579*	*0.579*
(Nº cel/100µm)	**8**	0.25	0.26	0.34	0.20	*0.097*	*0.765*	*0.150*	*0.103*

[A] Treatments: CN, challenged + no probiotic; CP, challenged + probiotic; NN, no challenge + no probiotic; NP, no challenge + probiotic. [B] Residual standard deviation. n = 8 for groups CN and CP, n = 4 for groups NN and NP. [C] IEL= Villous intraepithelial lymphocytes; [D] GC= Villous goblet cells/100 µm; [E] Number of mitosis in crypts

These changes were reflected in the villous:crypt ratio (Day 8 PI; *P* challenge x probiotic < 0.001). Intraepithelial lymphocytes were significantly increased by the probiotic at Day 8 PI in both groups (P=0.015).

5.4. Discussion

The aim of this study was to determine if the administration of the probiotic combination of *B. infantis* and *B. lactis* conferred protection against *Samonella* Typhimurium.

A two week trial using weanling piglets was performed where animals were challenged with *Salmonella* after 7 days of adaptation to the treatments and new environment. The challenge promoted an acute episode of diarrhea with increased fecal scores, fever response and the death (natural or euthanasia) of 4 animals that presented fibrinohemorragic lesions normally associated to *Salmonella* Typhimurium (Wilcock and Olander, 1977). Virtually all parameters studied responded significantly to the pathogen inoculation: performance parameters, fermentation products, inflammatory response and ileal histomorphology; were severely altered in comparison to non-challenged controls.

The administration of the probiotic combination was not able to prevent the infection of the animals by *Salmonella* as mostly all of them became positive in feces on day after the challenge. Despite of this, the probiotic was able to reduce the pathogen load in colon and feces, suggesting the potential of the bifidobacteria combination to exclude *Salmonella*. In this sense it is also interesting to comment that in our study unexpectedly we found some animals turning positive to *Salmonella* in feces in the non-challenged groups during the PI period, despite the seeding levels were very low ($<10^2$). This can be attributed to some fail in the biosecurity protocol, as low concentrations of *Salmonella* in the environment (10^2 to 10^3 cfu) have been reported as being able to infect exposed animals (Boughton et al., 2007; Hurd et al., 2001). The fact that all fecal samples were negative for *Salmonella* on their arrival, and that all euthanized piglets remained seronegative at the end of the study, reaffirms that these animals were not previously exposed to the pathogen in the farm of origin (Nielsen et al., 1995). In those animals, it is interesting to point out that although all NN animals were positive in two days PI, the maximum percentage of positive animals for NP was 50 % during all the week PI. This could suggest that although the probiotic was not able to prevent the infection of the animals when they are exposed to a high oral load of *Salmonella*, it could have some effect in front of a low exposure maintained in time. However,

protection in our experiment was not significant probably due to the low number of replicates on non-challenged animals (n=4).

In vitro studies have evidenced the ability of bifidobacteria to inhibit the growth of *Salmonella* (Bielecka et al., 1998; Tanner et al., 2016). A pluripotent stimulatory effect on the immune system (Akahashi et al., 2013; Gill et al., 2001; Medina et al., 2007), production of organic acids (Saulnier et al., 2009), production of bacteriocines and bacteriocine-like substances (Cheikhyoussef et al., 2008), capacity to inhibit the pathogenic adhesion to enterocytes or prevent bacterial translocation (Gagnon et al., 2004; Searle et al., 2009) have been described as the possible mechanisms of action of *Bifidobacterium* spp. for these antimicrobial effects. Regarding the strains evaluated in this study, previous works using a similar model of disease also have demonstrated the potential of the strain *B. infantis* (Barba Vidal, Unpublished data) to reduce *Salmonella* loads. Several mechanisms have been reported that could be implicated in the favorable outcome. For *B. infantis*, potential to produce peptides with protease activity (Chenoll et al., 2016), immunomodulatory capacity by increasing IL-10 (unpublished data) and IgA (Moreno Muñoz et al., 2011) has been reported. No information is available regarding the specific *B. lactis,* probiotic strain tested.

In accordance with reductions in the pathogen loads, improvement in clinical parameters were also registered with the probiotic combination. Rectal temperature of the CP group returned to levels similar to the non-challenged groups 48 hours after the pathogen inoculation and fecal scores showed less diarrhea. Regarding diarrhea scores, it is also interesting to remark that they were reduced not only in the CP group but also in the NP group. Weaning piglets suffer stress for several abrupt changes: separation from the sow, change from a milk based to a less digestible dry cereal based feed diet, introduction to new social partners and new physical environments (Weary et al., 2008) that usually promote gut dysbiosis. Our results suggest that the probiotic treatment may not only improve piglets outcome against pathogens but also may help piglets in a post-weaning period.

Differences in productive parameters are rarely reported in challenge trials evaluating probiotic treatments, as these studies are usually run in short periods and with a limited number of animals. Despite of this, we were able to evidence trends for a positive effect of the probiotic in the intake of feed in the post-challenge period, more manifested in

the CP group, and also a final live weight numerically higher ($P = 0.131$) in the animals receiving the probiotic, with more than 500 gr of difference at the end of the study. In this study this increase in feed intake and weight should be considered as a sign of better health status and adaptation to the weaning stress that allow the animal to express its genetic growth potential. These positive results should not be considered as a risk for obesity as has been clearly stated by Bernardeau and Vernoux (2013) for the extrapolation of farm animal results to humans.

Modulation of the fermentation profile was also detected with the probiotic treatment at ileal and colonic level. A general increase in ileal acetic acid concentrations (more importantly in NP group) was registered at Day 8 PI. Scientific literature reports that carbohydrate degradation by bifidobacteria exclusively takes place by the characteristic fructose-6-phosphate shunt (or bifidus pathway). Acetic acid and lactic acid are the major end-metabolites (Van der Meulen et al., 2006a; de Vries and Stouthamer, 1967) and a theoretical molar ratio of acetic acid to lactic acid of 1.5 (Van der Meulen et al., 2006a; de Vries and Stouthamer, 1968). Considering this, we can speculate that increases in acetic acid could be due to a higher bifidobacterial presence in the ileum in animals treated with probiotic. Lactic acid, as the main product of most of the inhabitants of the small intestine (Clemens and Stevens, 1979), would not have been sensible enough to reflect changes. This increase in acetic acid was more manifested in non-challenged animals at Day 8 PI reflecting a more established ileal microbiota at this time. Actually, at Day 8 PI we also were able to detect a higher number of *Bifidobacterium* spp. in colon by qPCR quantification in the non-challenged animals receiving the probiotic. However, changes in ileal fermentation were not always favorable. An interaction effect increasing ileal ammonia concentrations in NP and decreasing in CP was observed at Day 4 PI. We cannot find an explanation for that, however is worth it to mention that this effect disappear at Day 8 PI with a substantially reduction in ammonia mean values for all treatments. This evolution in ileal ammonia could be reflecting the big transitions of microbial populations that are produced during first days after weaning (Wang et al., 2013) regardless of the experimental treatment the animals received.

In relation to colonic fermentation, some benefits related to the probiotic were observed at Day 8 PI. Firstly, a reduction in ammonia concentration was reported ($P = 0.078$). This effects could be due to the ability to utilize ammonia attributed to bidifobacteria or

an indirect effect via modulation of the fermentation profile (Ahasan et al., 2015; Arunachalam, 1999) with a reduction in the proteolytic populations. Secondly, an interaction in SCFA at Day 8 PI was observed. Their concentration increase with the probiotic in the challenged animals to levels similar to the NN group, could suggest a normalization of the fermentative activity with the probiotic. However, SCFA were decreased by the probiotic in the non-challenged group. This decrease could be related to a reduced amount of fermentable substrates arriving to colon. The abrupt increase observed for acetic acid in the ileum could suggest a more active microbial population at the end of the small intestine that could have reduced the amount of substrates susceptible to be fermented in the colon.

Interesting results were also observed for ileal histomorphometry at day 8 PI. Whereas the probiotic treatment maintained villous height of challenged animals to similar levels than non-challenged, numerical reductions in the non-challenged ones were observed. Moreover, in the non-challenged animals the probiotic also promoted an increase in crypt depths up to similar values of the challenged animals. Subsequently, changes were reflected accordingly in the villous:crypt ratio. These results could be again related to a higher colonization of the ileum by the probiotic bacteria and to the presence of a higher ileal fermentative activity in these animals. In consonance, Kleessen et al. (2003) observed increased jejunal villus height and crypt depth in a rat model of human fecal flora due to fermentation of fructans by bifidobacteria. It is also important to remind the high capacity reported for bifidobacteria to adhere to enterocytes (Collado et al., 2005, 2007a; Servin, 2003) and moreover the fact that the combination of probiotic strains including *Bifidobacterium* spp. have even been demonstrated to have an increased adhesiveness than strains used separately (Collado et al., 2007a). We suspect that the increased presence of highly adhesive bifidobacteria could have contributed to the exclusion of intestinal pathogens (Collado et al., 2005) in the challenged group that more severely affect villous height (CN group). However, the adhesion of these bifidobacteria in the non-challenged animals, and the increased fermentative activity observed in ileum could had somehow compromise villous enterocytes in NP group. Nevertheless, none of the parameters analyzed in this study suggest negative effects of the probiotic in this group, moreover TNFα showed the lowest values suggesting no deleterious effects on the intestine. To our knowledge, it is the first time that such a reduction on villous height has been described due to a bifidobacterial probiotic. Still,

similar results were also found for this probiotic combination by our group in other non-published studies, probing that these effects are quite consistent. Interestingly, IEL were decreased between Day 4 and 8 PI only in the animals not receiving the probiotic despite they were challenged or not with *Salmonella*. Possibly the increased values observed at Day 4 were due to *Salmonella* or other opportunistic pathogens taking advantage of a transient dysbiosis related to weaning. At Day 8 only the animals receiving the bifidobacteria maintained those response levels suggests the ability of the probiotic strains to stimuli the immune system of the animal. It is well established that IEL have a relevant paper in gastrointestinal immune system, as they are a functionally heterogeneous population which contains cells with antitumor activity, natural killer activity, allospecific cytotoxic T lymphocytes or their precursors and mast cells (Ogra et al., 2012). As already mentioned, stimulation of the immune response is generally acknowledged to bifidobacteria (Akahashi et al., 2013; Medina et al., 2007) and has been previously demonstrated in one of the strains used in this study (Chenoll et al., 2016; Moreno Muñoz et al., 2011).

Bifidobacteria are considered to be minor colonizers of swine gut post-weaning (Konstantinov et al., 2004; Zhao et al., 2015). Colonic concentrations of *Bifidobacterium* spp. reported in piglet colon range from 10^5 to 10^8 cfu/g (Fouhse et al., 2015; Mountzouris et al., 2006) and Mountzouris et al. (2006) estimated that they contribute approximately to a 0.4 ± 0.15 % of total bacteria in the ascending colon. In our study, total *Bifidobacterium* spp. was analyzed via qPCR and despite a 10^9 daily dose of combined *Bifidobacteria* was used, only noticeable increases were observed in the non-challenged animals. Low detection levels in the challenge animals could respond to the gut dysbiosis produced by the *Salmonella* challenge that had precluded the probiotic to fully colonize the gut. Furthermore, it should be considered that at the moment the samples were taken, the animals had not received the probiotic in the last 24 h. Therefore, considering that transit time is accelerated in intestinal disorders, unless the probiotics strains had colonized the gut, it would have been very un-probable to detect it in the colonic samples. The lack of response of the qPCR numbers for *Bifidobacterium* spp. also could respond to a substitution between species, maintaining the niche a similar size. For this reason, although we cannot demonstrate the colonization of the gut by the probiotic strains, we cannot discard it either. Even in the eventual case of the strains not colonizing the gut, another possible explanation for the

effects observed with the probiotic could be that effects were mediated by metabolic products or other bioactive compound and not by the bacteria cells itself. In this regard *Bifidobacterium infantis* immunomodulation seems to be at least partially regulated by bioactive peptides which can retain their biological activity even without being *B. infantis* present (Ewaschuk et al., 2008). Moreover, recent papers demonstrate that one of the strains used, *B. infantis*, produces peptides with protease activity (Chenoll et al., 2016).

As we have seen the probiotic combination evaluated in this study demonstrated clear positive effects, not only ameliorating the *Salmonella* challenge outcome but also improving weaning response. These results improve previous ones obtained by our group for the single strain *B. infantis* (Barba-Vidal, Unpublished data), with a similar experimental design. In that study, the use of the strain *B. infantis* also diminished *Salmonella* shedding although challenged animals treated with the probiotic failed to show the significant improvements observed in this study in clinical outcomes, fermentation or histomorphometry. Other authors also have described the benefits of using combination of different strains. Particularly against *Salmonella* Typhimurium, Perdigon et al. (1990) demonstrated that a milk fermented with the mixture of *Lactobacillus casei* and *L. acidophilus* had more protective results than milk fermented with the strains separately. Authors attributed the effect to higher protective effects on mucosal tissue immunity with the strain combination. Similarly, Van Es and Timmerman (2002) compared the protection induced by a monostrain *Lactobacillus* spp. probiotic versus the one induced by multistrain combinations of the same probiotic specie in rats challenged with *Salmonella* Enteritidis and reported that post-challenge mean weight reduction was lower in the animals receiving a combination of probiotic strains. However, unluckily *in vivo* studies comparing the effect of single strains with multistrain combination are still rare (Chapman et al., 2011). In our study, reported results suggest that these two probiotics may have complementary effects and their combination allow them to better express their potential. However, synergistic mechanisms of action are still to be elucidated.

5.5. Conclusions

The probiotic combination of *Bifidobacterium longum* subsp. *infantis* CECT 7210 and *Bifidobacterium animalis* subsp. *lactis* BPL6 had a positive effect on enhancing gut health on post-weaning piglets and alleviating animals on a *Salmonella* challenge. Improvements registered on challenged animals were a reduction of the fecal excretion of *Salmonella* Typhimurium, a decrease in rectal temperature to similar levels to non-challenged animals and improvements in villous:crypt ratio. In addition, general probiotic benefits were observed in both challenged and non-challenged groups being an increase in voluntary feed-intake, decrease of diarrhea scores, healthier fermentation profiles and a stimulation of the intestinal immune system by increasing IEL.

Chapter 6.

Blood parameters as health biomarkers in weaning piglets experimentally challenged with *Salmonella* spp.

6.1. Introduction

In commercial intensive breeding systems piglets are weaned at much earlier ages (between 3-5 weeks) than those that could be expected in a natural environment (around 17 weeks (Jensen and Recén, 1989)). Moreover, weaning is considered one of the most critical periods for the swine industry in which the animals have to face multiple stressors. Piglets undergo complex social changes such as separation from their mothers and littermates, being relocated with unfamiliar counterparts. Additionally they have to adapt to abrupt changes in the feed regime and in the environment (Weary et al., 2008) leading to a variable period of hypo- or anorexia (Bruininx, 2001). All this happens in a time when the animals have still an immature immune system, low digestive capacity (Lallès et al., 2007) and a not balanced intestinal microbiota (Konstantinov et al., 2008). Weaning is therefore a time that predispose piglets to different digestive pathologies frequently related to the overgrowth of opportunistic pathogens like *Salmonella* or *Escherichia coli*.

In recent years, pig industry is trying to find biosecurity, management and feeding strategies to help piglets overcome the challenges of weaning, being the use of probiotics one of the most promising tools reported (Meng et al., 2010). Many reports can be found in the literature evaluating the potential of different probiotics in weaning challenges (Guerra-Ordaz et al., 2013; Prieto et al., 2014) and using experimental models of disease (Guerra-Ordaz et al., 2014; Naqid et al., 2015; Scharek-Tedin et al., 2013). Generally, animal performance and physiological parameters such as rectal temperature, fecal scores, fecal shedding of the pathogen and a set of different histo-morphologic and immunologic indicators are the most frequently evaluated parameters in these studies. Very little is known about the potential usefulness of clinical blood parameters as indicators of piglet health status. Up to our knowledge, the use of blood parameters as plasmatic Zn, Cu, Fe, acid-base or electrolyte balance parameters have not been previously considered as a valuable index in swine practice.

Portable blood analyzers provide easy access to real-time results within minutes and are used for acid-base and electrolyte balance diagnostic with therapeutic and prognostic implications in humans (Ball et al., 2001; Fencl et al., 2000) and small animals (Hopper and Epstein, 2014a, 2014b; Torrente et al., 2001), but have only been proposed recently in pigs (Kutter and Mauch, 2012) as a useful tool for the early assessment of the health status in nursery pigs (Buzzard, 2013).

Taking this in consideration, the main objective of the present study was to evaluate the potential use of different blood parameters, related to the mineral status, the acid-base and electrolyte balance in piglets experimentally challenged with *Salmonella* Typhimurium and receiving or not a probiotic combination of *Bifidobacterium spp*. We hypothesized that *Salmonella* infection may lead to dysregulation of electrolyte and acid-base balance and that these effects would be ameliorated by a probiotic supplementation, achieving a wide range of clinical responses. Moreover this work also aims to contribute to the definition of updated reference values for blood parameters of pigs considering the scarce number of published works in commercial breeds.

6.2. Materials and methods

The experiment was performed 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 strictly conformed to European Union Guideline (Directive 2010) and all efforts were made to minimize suffering. The trial was conducted following previously approved biosafety Level 2 procedures with appropriate training of the personnel involved.

6.2.1. Animals, housing and experimental design

Seventy two Large White x Landrace piglets from a high sanitary status commercial farm and weaned at 28 (±2) days of age, were brought to the experimental facilities of the UAB. Piglets were selected from sows seronegative to *Salmonella spp.* and confirmed to be microbiologically negative in feces upon arrival. They were placed in three rooms containing 8 pens (three pigs per pen) according to their body weight. The pens were allocated to four treatment groups following an unbalanced 2 x 2 factorial arrangement (factors being probiotic and *Salmonella* Typhimurium challenge), with eight replicates per treatment for the challenged animals and four replicates for the non-challenged ones. The treatments were 1) no challenge + no probiotic (NN), 2) no challenge + probiotic (NP), 3) challenged + no probiotic (CN), 4) challenged + probiotic (CP).

Pigs were maintained under a 14:30 h light and 9:30 h dark lighting regimen and had *ad libitum* access to water and food. A pre-starter diet without additives formulated to

satisfy the nutrient requirement standards for pigs (NRC, 2012) containing 18.9 % crude protein and 2,470 kcal/kg metabolizable energy (Table 6.1) was fed to all pigs.

Table 6.1. Dietary composition and nutrient analysis of the experimental diets as-fed basis, g/kg.

Ingredients	
Maize	280.8
Wheat	170.0
Barley 2 row	150.0
Extruded soybean	122.4
Sweet whey-powder (cattle)	100.0
Fishmeal LT	50.0
Soybean meal 44	50.0
Whey-powder 50% fat	30.3
Mono-calcium phosphate	21.3
Calcium carbonate (CaCO3)	8.2
L-Lysine HCL	4.5
Vitamin-Mineral Premix [A]	4.0
Sodium chloride (marine salt)	3.0
DL-Methionine 99	2.4
L-Threonine	2.3
L-Triptophane	0.9

Chemical composition	
DM	903.2
Ash	74.1
Crude Fat	64.5
Crude Protein	189.3
Neutral detergent fiber	111.6
Acid-detergent fiber	35.1

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D_3. 39.9 mg vitamin E, 3 mg vitamin K_3, 2 mg vitamin B_1, 2.3 mg vitamin B_2, 3 mg vitamin B_6, 0.025 mg vitamin B_{12}, 20 mg calcium panthotenate, 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.

6.2.2. Probiotic and bacterial inoculation

During the experimental period pigs received the probiotic treatment orally and individually, in a daily pattern using disposable syringes without needle. In the probiotic group, a daily dosage (10^9 cfu) of a combination of *Bifidobacterium longum* subsp. *Infantis* CECT 7210 and *Bifidobacterium animalis* subsp. BPL6 was supplemented in a 2 ml solution and the control group received as placebo the same amount of sterile carrier.

After a 1-week acclimation period, pigs were orally challenged with a 2ml culture of *Salmonella* Typhimurium (5 x 10^8 cfu) or received as placebo the same amount of sterile media in the non-challenged group. The *Salmonella* Typhimurium strain was proportioned by the Veterinary Laboratory of Infectious Diseases (UAB). This strain (ref. 301/99) is a *Salmonella* Typhimurium *var. Monophasic* (*formula: 4,5,12:i:-, resistance profile: ACSSuT-Ge, Fagotype: U302*) isolated from a salmonellosis outbreak (mainly enteric with sporadic septicemia) in a commercial farm of fattening pigs in Spain. The oral inoculums were prepared by 24h incubation at 37ºC in buffered peptone water (Oxoid, Hampshire, UK) and diluted (1:20) with sterile PBS (Sigma-Aldrich, Madrid, Spain) to reach a final concentration of 2.5 x 10^8 cfu/mL.

6.2.3. Sampling procedures

Animals were weighted upon the arrival, the day of the inoculation and on day 4 after the challenge. Moreover, all animals were monitored individually from day 1 up to the 4^{th} day post-inoculation (PI): rectal temperature was assessed on days 1 and 2 PI and fecal scores were given individually on days 1, 2 and 3 PI with a range from 1 for normally shaped and solid feces to 4 severe or bloody diarrhea. Fecal samples (5 g) were collected for quantitative *Salmonella* assessment on the arrival and on Day 1 PI. For *Salmonella* bacteria counts, all samples were transferred (1:10) to Buffered Peptone Water. Assessment was made by seeding the serial dilutions 10^{-2} of the samples in Xylose-Lactose-Tergitol-4 (XLT4) plates (Merck, Madrid, Spain).

Blood samples were taken at day 4 PI by venipuncture of the cranial vena cava in a sub-sample of 24 pigs (the animal with the intermediate weight of each pen). Ten milliliters of blood were obtained with a 20G syringe and stored in 10ml tubes without anticoagulant (Aquisel, Madrid, Spain). Serum was subsequently obtained after

centrifugation (2,000 x g, 10 min, 15˚C) and stored in 1.5 mL aliquots at -20ºC until use.

6.2.4. Blood analysis

Immediately after extraction, blood was used for in-site analysis of potassium (K^+), sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-), anion Gap (AG), pH, partial pressure of carbon dioxide (pCO_2), total carbon dioxide (TCO_2), base excess in the extracellular fluid compartment (BE_{ecf}), glucose (Glu), hemoglobin (Hgb) and hematocrit (Htc), using a iSTAT® System with CG8+ cartridge (Abaxis, Union City, California). Bicarbonate (HCO_3^-) and BE_{ecf} were calculated by the analyzer using the Henderson-Hasselbach formula in conjunction with the Siggaard-Anderson equation and Van Slyke equations, respectively (NCCLS., 2001). In all animals, the acid-base analysis was completed calculating the anion gap ($AG = Na^+ + K^+ – Cl^- – HCO_3^-$).

Interpretation of acid-base status was performed using the traditional approach based on the Henderson-Hasselbach equation; the parameters taken into account were pH, pCO_2, HCO_3^-, BE_{ecf}, and AG. Acid-base disorders were classified by this method using the criteria given by de Morais and Di Bartola (DiBartola, 2011). The four acid-base disturbances described were: respiratory alkalosis or acidosis, and metabolic alkalosis or acidosis. Animals with metabolic acidosis were further characterized considering AG to characterize the presence of high-AG acidosis, that is, acidosis associated with an increased concentration of unmeasured anions.

Serum sub-samples were diluted in a 0.05% p/v EDTA and 0.5% v/v NH_3 solution to analyze Zinc (Zn), Iron (Fe) and copper (Cu) by Inductively Coupled Plasma Optical Emissions Spectrophotometer (ICP-OES model Optima 4300DV, PerkinElmer Inc., Waltham, MA, USA) following the procedure described in Davin et al. (2013).

6.2.5. Statistical analysis

The experimental unit was the pig in all variables evaluated individually except for animal performance data, where the pen mean was used. The data were studied by covariance analysis that included the oral challenge and the probiotic as fixed effects and individual body weight of the pigs at the arriving day as covariate. Moreover, Pearson correlation procedure was used to study the relationship between blood parameters and weight gain. To evaluate the effects of weight lost on blood parameters, the body weight gain was categorized into four factors according to quartile range distribution. Chi-Square test was used to evaluate the association between weight loss

and fecal consistency. Multiple mean comparisons were performed using Tukey's correction. All analysis were carried out using the statistical package SAS 9.2 and alpha level for determination of significance was $P<0.05$.

6.3. Results

6.3.1. Animal performance

Final body weight (BW), average daily feed intake (ADFI) and average daily gain (ADG) was reduced by the oral challenge ($P = 0.024$, $P = 0.006$ and $P < 0.001$ respectively); that even provoked weight losses during the post-inoculation period in some animals (see Table 6.2).

Table 6.2. Effect of the experimental treatments on growth performance.

	Treatments [A]					P-value		
	NN	**NP**	**CN**	**CP**	**RSD [B]**	**Challenge [C]**	**Probiotic [D]**	**Interaction**
Average BW [E] (kg)								
Initial	**7.60**	7.85	7.90	7.51	*0.559*	*0.929*	*0.770*	*0.195*
Final	**8.87**	9.06	8.05	8.26	*0.763*	*0.024*	*0.543*	*0.977*
ADFI [F] (g/day)								
Pre-inoculation	**273**	250	259	306	*55.90*	*0.391*	*0.611*	*0.164*
Post-inoculation	**395**	398	218	276	*112.27*	*0.006*	*0.536*	*0.569*
ADG [G] (g/day)								
Pre-inoculation	**78**	58	53	119	*50.50*	*0.417*	*0.303*	*0.063*
Post-inoculation	**240**	268	-71	-25	*119.32*	*<0.001*	*0.484*	*0.858*

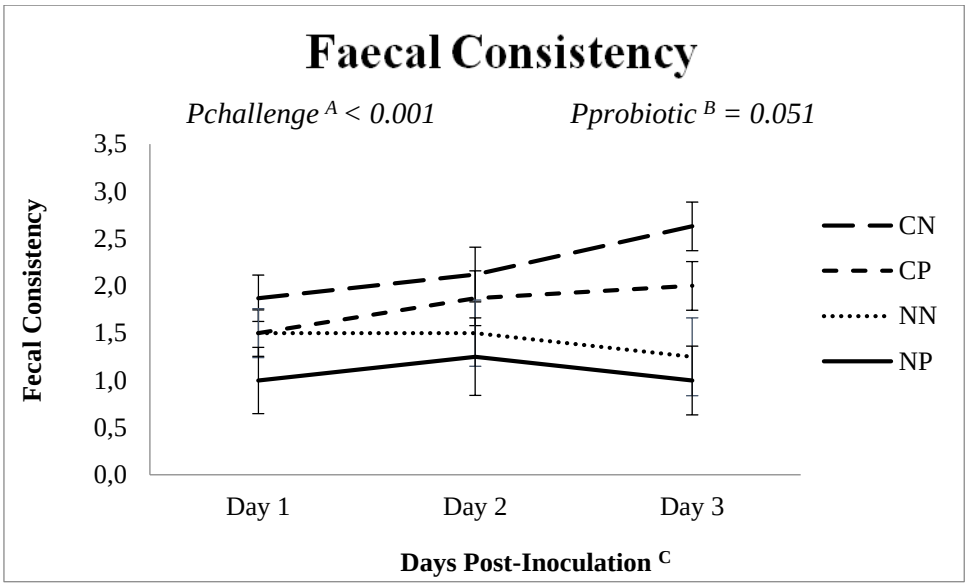
[A] Treatments: NN (no challenged + no probiotic), NP (no challenged + probiotic), CN (challenged +no probiotic) and CP (challenged + probiotic). Challenged groups n=8; non challenged groups n=4. [B] Residual Standard Deviation. [C] Challenge: main effect of the *Salmonella* Typhimurium inoculation (5 x 10^8 cfu). [D] Probiotic: main effect of a *Bifidobacterium* spp. combination (10^9 cfu). [E] Body weight (BW) (kg) at experimental days 0 and 11 (4 days post-inoculation). [F] Average daily feed intake (ADFI) (g/day) for pre-inoculation (0-7th day before the challenge) and post-inoculation period (0-4th day after the challenge). [G] Average daily gain (ADG) (g/day) for pre-inoculation (0-7th day before the challenge) and post-inoculation period (0-4th day after the challenge).

6.3.2. Clinical parameters and pathogen seeding

None of the animals seeded *Salmonella* in feces on the arrival and only challenged groups seeded *Salmonella* in countable levels (>10^3 cfu/g) at Day 1 PI (87.5% for CN and 100% for CP groups) after the challenge.

The challenged animals presented higher rectal temperatures 24 hours PI than the non-challenged animals (40.0 vs. 39.3 ºC, P = 0.010) with no changes associated to the probiotic administration. However the probiotic tended to reduce temperature in challenged animals 48 hours PI, where they presented a similar temperature to the non-challenged (39.8ºC for CN, 39.2 for CP, 39.0 for NN, 39.1 for NP; P probiotic*challenge = 0.095). The oral inoculation of the pathogen promoted moderate diarrhea in most of the animals with significant increases in the fecal score (see Figure 6.1). Administration of the probiotic showed a trend to improve the fecal consistency with decreases in the fecal score in both: challenged and non-challenged animals (1.8 ± 0.13 for control vs. 1.4 ± 0.13 for probiotic; P = 0.051 for days 1 to 3 PI).

Figure 6.1. Evolution of the mean fecal scores in the different experimental groups during the post-inoculation period.



Treatments: CN (challenged + no probiotic), CP (challenged + probiotic), NN (no challenged + no probiotic) and NP (no challenged + probiotic) (challenged groups n=8; non challenged groups n=4). Values represented are LSmeans with their standard errors. [A] Challenge: main effect of the *Salmonella* Typhimurium inoculation (5 x 10^8 cfu). [B] Probiotic: main effect of a *Bifidobacterium* spp. combination (10^9 cfu). [C] Days after the challenge with Salmonella.

In general, diarrhea was mild but severity of diarrhea was higher in animals with lower weight as was demonstrated when analyzing results of fecal scores by animal weight gain quartiles (average fecal scores 2.48, 1.96, 1.62 and 1.24 for 1st , 2nd, 3rd and 4th quartile; P < 0.001).

6.3.3. Blood parameters

Changes in blood parameters due to *Salmonella* challenge and probiotic treatment are shown in Table 6.3.

Table 6.3. Change in blood parameters in pigs orally challenged or not with *Salmonella* and receiving or not a probiotic mixture.

	Treatments [A]					P-Value		
	NN	**NP**	**CN**	**CP**	**RSD [B]**	**Challenge [C]**	**Probiotic [D]**	**Interaction**
Zn (mg/L)	0.72	0.64	0.51	0.63	*0.119*	*0.049*	*0.801*	*0.093*
Cu (mg/L)	1.33	1.41	1.72	1.76	*0.223*	*0.001*	*0.540*	*0.863*
Fe (mg/L)	7.73	3.73	3.97	8.95	*9.861*	*0.870*	*0.914*	*0.331*
Na^+ (mM)	138.6	140.9	130.5	133.4	*9.308*	*0.076*	*0.551*	*0.945*
K^+ (mM)	5.43	5.99	4.83	5.8	*0.728*	*0.233*	*0.034*	*0.540*
Cl^- (mM)	105.3	106.3	101.2	105.2	*0.76*	*0.521*	*0.553*	*0.691*
HCO_3^- (mM)	25.6	22.3	24.5	19.3	*5.12*	*0.446*	*0.110*	*0.712*
AG (mM)	13.6	17.5	14.5	14.8	*2.92*	*0.544*	*0.175*	*0.244*
pH	7.44	7.40	7.41	7.43	*0.138*	*0.990*	*0.816*	*0.673*
TCO_2 (mM)	26.7	23.2	25.7	20.1	*5.39*	*0.472*	*0.112*	*0.703*
pCO_2 (mmHg)	37.5	35.0	39.4	29.5	*11.45*	*0.758*	*0.291*	*0.517*
BE_{ecf} (mM)	1.36	-2.47	-0.11	-4.96	*6.414*	*0.555*	*0.189*	*0.874*
Glu (mg/dL)	114.7	110.4	90.9	95.4	*18.27*	*0.040*	*0.995*	*0.612*
Htc (%)	18.6	10.8	24.9	20.9	*5.32*	*0.005*	*0.030*	*0.459*
Hgb (g/dL)	6.33	3.69	8.44	7.10	*1.814*	*0.006*	0.030	0.450

Blood parameters: Zinc (Zn), copper (Cu), iron (Fe), sodium (Na^+), potassium (K^+), chloride (Cl^-), bicarbonate (HCO_3^-), anion gap (AG), pH, total carbon dioxide (TCO_2), partial pressure of carbon dioxide (pCO_2), base excess in the extracellular fluid compartment (BE_{ecf}), glucose (Glu), hematocrit (Htc) and hemoglobin (Hgb). [A] Treatments: NN (no challenged + no probiotic), NP (no challenged + probiotic), CN (challenged +no probiotic) and CP (challenged + probiotic). Challenged groups n=8; non challenged groups n=4. [B] Residual Standard Deviation. [C] Challenge: main effect of the Salmonella Typhimurium inoculation (5 x 10^8 cfu). [D] Probiotic: main effect of a daily dosage (10^9 cfu) of a combination of *Bifidobacterium longum* subsp. *Infantis* CECT 7210 and *Bifidobacterium animalis* subsp. BPL6.

The oral challenge promoted a decrease in plasmatic Zn (0.69 vs. 0.57 mg/L; $P = 0.049$) that tended to be more important in the CN group (P challenge x probiotic = 0.093) and an increase in Cu (1.37 vs. 1.74 mg/L; $P < 0.001$). A tendency to decrease Na^+ concentrations was detected (139.7 vs. 131.9 mM; $P = 0.076$) in the measured electrolytes concentrations. In relation to blood biochemistry, challenge provoked a significant decrease in Glu (112.6 vs. 93.1 mg/dL; $P = 0.040$) together with higher values of Htc and Hgb (14.7 vs 22.9 %, $P = 0.005$ for Htc and 5.01 vs 7.77 g/dL, $P = 0.006$ for Hgb).

The probiotic treatment increased blood K^+ concentrations (5.13 vs. 5.90 mM; $P = 0.034$) and decreased values of Htc and Hgb (21.71 vs. 15.85 % for Htc and 7.39 vs. 5.4 % for Hgb).

Weight loss values were correlated with the different blood parameter tested to discover the strength of the association between these two factors, regardless the experimental treatments (Table 6.4).

Many parameters showed strong correlations with weight gain. Particularly significant positive correlations were found for Zn, Na^+, K^+, Cl^- and Glu, whereas Cu, Htc and Hgb showed negative correlations. Parameters related to the electrolyte or the acid-base balance as HCO_3^-, AG, pH, TCO_2, pCO_2 or BE_{ecf} did not show any remarkable relation with weight gains. When considering exclusively non-challenged animals, a high positive correlation with weight gain was seen with Zn concentrations. On the other hand, in challenged animals positive correlations were seen with electrolytes Na^+, K^+ and Cl^- while negative correlations were seen with Cu, HCO_3^-, TCO_2 and $BE_{ecf.}$

Finally, mean values of blood parameters for the different quartiles considering the weight gain of the animals is exposed in Table 6.5. Copper responded proportionally to the weight gain while Na^+, K^+, Cl^- only showed significantly lower values in the group of animals with the lowest weights.

Table 6.4. Pearson correlation analysis between weight gain and blood parameters in weaning piglets orally challenged or not with *Salmonella* Typhimurium.

		Zn	Cu	Fe	Na^+	K^+	Cl^-	HCO_3^-	AG	pH	TCO2	pCO2	BE_{ecf}	Glu	Htc	Hgb
All Animals	**r**	0.57	-0.67	0.13	0.67	0.40	0.52	-0.27	0.18	-0.17	-0.28	-0.07	-0.29	0.47	-0.42	-0.42
(n=24)	***P*-value**	*0.006*	*0.001*	*0.547*	*0.001*	*0.055*	*0.012*	*0.231*	*0.436*	*0.444*	*0.213*	*0.755*	*0.195*	*0.024*	*0.048*	*0.048*
Non challenged (n=8)	**r**	0.74	-0.58	0.61	-0.25	0.16	0.01	0.11	-0.18	-0.19	0.11	0.53	0.02	0.67	0.22	0.23
	***P*-value**	*0.037*	*0.128*	*0.106*	*0.558*	*0.707*	*0.995*	*0.838*	*0.731*	*0.714*	*0.837*	*0.275*	*0.974*	*0.102*	*0.631*	*0.622*
Challenged	**r**	0.38	-0.56	0.08	0.71	0.44	0.65	-0.54	0.33	-0.15	-0.55	-0.25	-0.51	0.31	-0.42	-0.42
(n=16)	***P*-value**	*0.186*	*0.023*	*0.772*	*0.002*	*0.089*	*0.008*	*0.031*	*0.235*	*0.579*	*0.028*	*0.345*	*0.044*	*0.244*	*0.104*	*0.101*

Blood parameters: Zinc (Zn), copper (Cu), iron (Fe), sodium (Na^+), potassium (K^+), chloride (Cl^-), bicarbonate (HCO_3^-), anion gap (AG), pH, total carbon dioxide (TCO_2), partial pressure of carbon dioxide (pCO_2), base excess in the extracellular fluid compartment (BE_{ecf}), glucose (Glu), hematocrit (Htc) and hemoglobin (Hgb). Weight gains (kg) were calculated by difference between body weights registered at experimental days 0 and 11 (4 days post-inoculation).

Table 6.5. Comparisons between mean blood parameters in groups of pigs (n=6) stratified by body weight gain.

	Quartile Distribution [A]					
	1st Quartile	**2nd Quartile**	**3rd Quartile**	**4th Quartile**	**RSD [B]**	***P*-value**
Zn (mg/L)	0.52 [b]	0.53 [b]	0.66 [ab]	0.71 [a]	*0.113*	*0.025*
Cu (mg/L)	1.79 [a]	1.71 [a]	1.64 [ab]	1.32 [b]	*0.218*	*0.007*
Fe (mg/L)	2.08	9.15	6.21	7.40	*9.619*	*0.631*
Na (mM)	123.8 [b]	137.5 [a]	137.6 [a]	139.3 [a]	*7.27*	*0.005*
K (mM)	4.55 [b]	5.72 [a]	6.13 [a]	5.40 [ab]	*0.593*	*0.001*
Cl (mM)	94.0 [b]	108.5[a]	106.3 [a]	106.4 [a]	*5.40*	*0.002*
HCO_3^- (mM)	25.4	20.2	20.3	23.7	*5.16*	*0.281*
AG (mM)	14.0	14.8	16.2	14.6	*3.00*	*0.699*
pH	7.45	7.43	7.37	7.43	*0.135*	*0.775*
TCo_2 (mM)	26.5	21.3	21.2	24.6	*5.46*	*0.311*
pCo_2 (mmHg)	37.4	32.0	34.4	35.9	*12.06*	*0.884*
BE (mM)	1.50	-4.00	-5.00	-0.80	*6.184*	*0.303*
Glu (mg/dL)	90.7	92.8	99.8	115.2	*17.43*	*0.127*
Htc (%)	23.7	20.5	16.3	20.4	*6.47*	*0.305*
Hgb (g/dL)	8.05	6.96	5.57	6.94	*2.202*	*0.310*

Blood parameters: Zinc (Zn), copper (Cu), iron (Fe), sodium (Na^+), potassium (K^+), chloride (Cl^-), bicarbonate (HCO_3^-), anion gap (AG), pH, total carbon dioxide (TCO_2), partial pressure of carbon dioxide (pCO_2), base excess in the extracellular fluid compartment (BE_{ecf}), glucose (Glu), hematocrit (Htc) and hemoglobin (Hgb). [A] Weight gains (kg) were calculated by difference between body weights registered at experimental days 0 and 11 (4 days post-inoculation) and were stratified as following: 1st Quartile (-1.18-0.34 kg), 2nd Quartile (0.4-1.16 kg), 3rd Quartile (1.26-1.66 kg) and 4th Quartile (1.8-3.22 kg). [B] Residual Standard Deviation. Blood parameter values are means. Means with different letters are significantly different, ($P<0.05$) by Tukey- test.

6.4. Discussion

The main objective of the present study was to evaluate the potential use of different blood parameters, related to the mineral status, the acid-base and electrolyte balance as health indicators in piglets experimentally challenged with *Salmonella* Typhimurium and receiving or not a probiotic combination of *Bifidobacterium spp*.

The challenge with *Salmonella* Typhimurium succeeded in provoking an acute dysregulation of gut function, with diarrhea and the affection of several clinical parameters. On the other hand, the probiotic provoked moderate benefits in several parameters, allowing the achievement of a wide range of controlled clinical responses.

6.4.1. Plasmatic micro-minerals

In relation to micro-mineral concentrations, the experimental challenge promoted a decrease in plasmatic Zn. In humans it has been reported how pro-inflammatory cytokines during infectious diseases regulate changes in Zn hepatic reservoirs in liver cells leading to hypozincemia (Liuzzi et al., 2005). This effect could partly explain the results of reduction of Zn in pigs challenged with *Salmonella spp*., ameliorated in the challenged group receiving probiotic with similar Zn levels to the non-challenged groups. However, normal serum Zn concentrations are reported to be within the range of 0.7 and 1.5 mg/L and serum Zn concentrations associated with marginal status within the range of 0.4 and 0.8 mg/L (Puls, 1994). Therefore, values reported in this work would suggest that all our piglets, challenged and non-challenged ones, showed a marginal deficiency of Zn. This result is in consonance with other authors reporting Zn status of piglets at weaning (Carlson et al., 2007; Davin et al., 2013).

Some authors have described correlations between plasmatic Zn concentrations and weight gains (Dørup and Clausen, 1991; Giugliano and Millward, 1984) and it's been postulated that one of the first signs of mild Zn deficiency in growing animals is a reduced growth (King, 1990). To our knowledge, this is one of the few studies in which such a relationship is evidenced in weanlings receiving nutritional levels of Zn in the diet. Different reasons could explain this relationship between plasmatic Zn and growth in weanlings, such as multiple correlated conditions like infection, trauma, stress, or dietary deficiency that can reduce both plasmatic levels of Zn and growth (Davin et al., 2013; King, 2011). However, in our study this relationship was stronger when only the non-challenged animals were included in the analysis. This fact would suggest that the relationship is not just explained

by concomitant effects of stressors on plasmatic Zn and growth levels, but also due to the growth retardation described by other authors as a way to preserve body Zn (Dørup and Clausen, 1991; Giugliano and Millward, 1984). Supporting this, it's been reported that the inclusion of therapeutical levels 2000-25000 ppm can restore plasma Zn levels and improve performance in comparison to animals receiving the same diet with only nutritional levels of Zn (Case and Carlson, 2002; Davin et al., 2013; Hill et al., 2001). Although the exact mechanism has not been described yet, it may be hormonal mediated as Ninh et al. (1996) found a growth stimulating effect mediated by insulin-like growth factor I in undernourished children supplemented with Zn.

On the other hand, challenged groups also presented an increase in Cu concentrations. It has been reported that stress can promote a sustained increase in plasma Cu concentrations, that could be explained by the binding of Cu to caeruloplasmin, a widely recognized acute-phase protein (Bremner and Beattie, 1995). Besides, Cu showed an inverse correlation with weight gain increasing as the animals reduced growth. The reason for this decrease in the animals with better performance is not clear. Carlson (2007) reported decreases in the plasmatic Cu concentration during the first 2 weeks after weaning. In addition, Zn and Cu are physically and chemically similar elements that could act antagonistically in the body. Oral administration of Zn supplements usually promote decrease in the plasmatic levels of Cu due to a decrease in Cu absorption (Bremner and Beattie, 1995). The complementary responses in Cu and Zn could be the result of a different binding to organic proteins. Metallothioneins (MT) are known to be induced by exposure to heavy metal cations (Ishii et al., 2001) and specifically the expression level of MT1 in jejunum is higher with high dietary levels of Zn in piglets (Chai et al., 2014). These MT bind metals in the mucosal surface of enterocytes forming a block that prevents its movement through the cell, thereby limiting absorption of metals. However, in our case as Zn levels are on a nutritional level, it seems improbable that the decreases in plasmatic Cu were a result of interactions in intestinal absorption. In a practical sense, good correlations of Zn and Cu with weight gains could turn them into good descriptors of the performance of the animals at the end of the nursery phase.

Regarding Fe, it did not show any relationship with the weight gain or any experimental treatment. This could be the results of the a very tight regulation of cellular and systemic iron balance but also the results of the routine iron dextran administration to pig neonates (Starzyński et al., 2013).

6.4.2. Blood electrolytes

The only blood electrolyte significantly altered by the experimental treatments was K^+ concentration. The probiotic administration enhanced K^+ blood concentration restoring the concentration of K^+ to the levels registered in the non-challenged group. This could suggest a relative improvement of animals receiving probiotic and could be related to the beneficial effects observed by the probiotic treatment in fecal consistency. In relation to the challenge effects, we suspect that too mild symptoms, acuteness of diarrhea and inter-individual variability promoted by our oral challenge could explain the scarce differences found considering that changes depend on the cause of diarrhea, its severity and chronicity (Kim et al., 1994).

When related to body weight gains, blood concentration of Na^+, K^+ and Cl^- were significantly reduced only in those animals with the lowest gains suggesting a breaking point reached only by those animals seriously compromised and probably due to the loss of these electrolytes through acute diarrhea. These could be due to that they are key electrolytes with essential functions in the body. Sodium and chloride are the main extracellular cation and anion respectively in the body and influence the electrolytic balance and acid base status of animals. As for potassium, it is implicated in electrolyte balance, neuromuscular function and also acts as the monovalent cation to balance intracellular anions, as part of the Na^+/K^+ pump physiological mechanism (Glynn, 1985). Mammal organism has different ways to maintain homeostasis of essential nutrients and probably only when the health status is seriously compromised, such as in the firs quartile animals, we can find responses in these indexes.

6.4.3. Acid-base balance parameters

Anion gap evaluates the difference between the measured cations and anions in the blood. Around two-thirds of the AG comes from the negative charge of serum proteins while one-third are due the accumulation of phosphate and strong anions in serum, such as L-lactate, sulfate, and anions associated with uremia (Constable, 2000). Thus, a high-AG metabolic acidosis it is supposed to be formed by an acid that does not have chloride as its anion and a normal-AG metabolic acidosis is accompanied by an equal increase in the plasma chloride concentration to balance the decrease in plasma HCO_3^- concentration (Torrente et al., 2001). Our results were within the biologically normal range for healthy piglets (12-23

mmol/L) (DiBartola, 2011) and were not significantly different between treatments suggesting absence of disturbance caused by the *Salmonella* challenge nor the probiotic.

Respiratory acidosis and alkalosis occur when pCO_2 levels are above or below a reference range. Similarly, metabolic acidosis or alkalosis happens when HCO_3^-, and/or BE_{ecf} values are respectively below or above a reference range. Blood BE_{ecf} values near to zero are desirable (Ahmad and Mushtaq, 2006), as they reflect the maintenance of required acid-base balance for better performance (Ahmad and Sarwar, 2006). Alternatively, metabolic acidosis is characterized by reduced blood pH as a result of the accumulation of non-volatile acids or loss of serum bicarbonate (Barbosa and Alves, 2010). In our experiment, results for pH, pCO_2, HCO_3^- or BE_{ecf} were not significantly different between treatments suggesting that our challenge did not reach to disturb the acid base balance of the animals.

Remarkably, HCO_3^- concentration was not related to weight gain when all animals were considered in the analysis. However, a negative correlation was found when the challenged group was analyzed separately. This could suggest that although the appearance of metabolic acidosis was not found to be directly related to the *Salmonella* challenge; it could still be present in some of the challenged animals depending on the level of sickness. Impaired inflammatory response and anorexia in most critically ill animals could be a causing these results (Cersosimo et al., 1987; Kraut and Madias, 2010). Supporting this idea, other acid-base parameters (TCO2 and BE_{ecf}) were not correlated to weight gain when we consider all the animals, but significant negative correlations can be observed when we only consider the challenged group.

6.4.4. Chemical biochemistry

Regarding the chemical biochemistry we could detect differences related to the oral challenge on Glu, Htc and Hgb concentrations. Glucose concentrations were decreased significantly with the challenge. This fact could be due to reduced feed intake also reported with the challenge or a higher blood glucose uptake when the metabolism is responding against the *Salmonella* infection (Correa-Matos et al., 2003; Sakaguchi et al., 1979). Moreover, a positive correlation was seen in relation to weight gain. When assessing these differences by quartiles, a linear increase in Glu concentrations was seen together with weight gains although these differences were only numerical when studied by quartiles. Regarding Htc and Hgb, they were the only two blood parameters that showed significant differences related to both experimental treatments, the challenge and the probiotic. Values

of Htc and Hgb were higher in challenged pigs than in control pigs. Other authors also have described how blood Glu is decreased and Hct is increased during disease in pigs suggesting a possible dehydration and malnutrition compared to healthy animals (Buzzard, 2013). Nevertheless, it has been reported that I-stat may underestimate Htc and Hgb with hipo-proteinemia, which could be happening in challenged animals with a more severe diarrhea (Hopfer et al., 2004; Schött, 2014). This possibility must be considered because proteinemia was not evaluated in our study.

Regarding the changes observed with the administration of probiotic, as far as we know this is the first reported work that uses these indexes to evaluate the efficiency of a probiotic therapy. Similarly to probiotic effects observed in K^+, decreases observed in Htc and Hgb values with the probiotic treatment could suggest an improvement in the response of the animals against the *Salmonella* challenge reducing the severity of water losses and electrolyte in the feces (diarrhea). In addition, the probiotic was also able to decrease Htc and Hgb values in the non-challenged group. Although these last animals did not receive an oral *Salmonella* dose, it is widely known that the weaning itself is a powerful stressor that cause anorexia, growth stasis and transit dysbiosis (Lallès et al., 2007; Pluske et al., 2002; Weary et al., 2008). Htc and Hgb could be therefore a sensible index to assess gastrointestinal disorders and test the efficacy of in-feed treatments aiming to improve the adaptation of the animals to weaning.

Moreover, Htc and Hgb also showed significant negative correlations with weight gains. As discussed before, decreases in Htc and Hgb concentrations could be reflecting the level of dehydration considering that both indexes are based on whole blood and are therefore dependent on plasma volume. In this regard Balsbaugh (1986) also described an increase in the Htc values in pigs in which diarrhea was experimentally-induced concomitant with body weight loss. However, our results show that although having a significant correlation with weight gain this relationship is not linear. We suspect this results may be influenced by an underestimation of these values by the I-stat on the more severely affected animals (Hopfer et al., 2004; Schött, 2014). In this case, Htc and Hgb data measured by I-stat should be used with precaution because it may fail to detect animals in worst conditions, in contrast to blood concentration of Na^+, K^+ and Cl^- that were significantly different in animals of the lowest quartiles. This would be a serious limitation because inadequate transition to the new feed and watering regimen and common gastrointestinal disorders in the post-weaning period can compromise the hydration level and health status of the

weanlings. Therefore, encountering rapid and easy access methodologies to measure hydration level is a critical point to guarantee the survival of animals.

6.5. Conclusions

Results of this work support previous reported data that suggest marginal deficiencies of Zn in piglets at weaning and an inverse correlation with Cu concentrations. Moreover, blood parameters have demonstrated to be good descriptors of pig status. Micro-minerals Zn and Cu are good descriptors of pig performance and their study would enable a customized nutrition or husbandry practices to improve homogeneity of batches leaving the nursery period. Blood electrolytes (Na^+, Cl^- and K^+) and acid base indexes (HCO_3^-, TCO2 and BE_{ecf}) may enable to detect the most distressed animals and target them for an electrolytic therapy to improve their outcome. Finally, biochemical parameters assessed (Glu, Htc and Hgb) are good descriptors of health status and have demonstrated to be quite sensible simple indexes to be included in experimental designs aimed to evaluate health status and feed strategies in weanlings. However, they may not be useful to identify most severely affected animals.

Chapter 7.

Response to a *Salmonella* Typhimurium challenge in piglets supplemented with protected sodium butyrate or *Bacillus licheniformis* CECT 4536: effects on performance, intestinal health and behavior.

7.1. Introduction

Salmonella spp. is one of the worldwide leading causes of food-borne illnesses, contaminated pork being a significant source for human salmonellosis (EFSA, 2013; CDC, 2014). Control methods against this pathogen are needed (Andres and Davies, 2015) and probiotics and organic acids have been demonstrated to be potentially useful.

Bacillus species are widely used as probiotics in animal feeds (Cutting, 2011), with proven efficacy *in vivo* with porcine experimental models of salmonellosis (Ahmed et al., 2014; Spiehs et al., 2008; Walsh et al., 2012b). Alternatively, supplementation with organic acids or their salts has also been proposed as a possible tool to combat *Salmonella* in pigs (Creus et al., 2007) and butyrate's antimicrobial effects have been proven *in vivo* (Boyen et al., 2008).

To prove the efficacy of an in-feed additive against *Salmonella* is not easy. The best scenario would be to demonstrate its activity under natural conditions of disease, but this implies working with a high number of animals, considering that the fecal shedders may be low and intermittent (Ro and Scherer, 2008). Alternatively experimental models are used, although they also exhibit limitations for which sensible parameters are needed. Recently, it has been described how sickness behavior is a coordinated and adaptive response to illness (Weary et al., 2009) and in particular how *Salmonella* infections in pigs promote changes in animal behavior (Rostagno et al., 2011; Ahmed et al., 2015). This background suggests that behavior may be a good tool to be included in trials, potentially able to respond to treatment effects in animal health.

The objective of this work is, therefore, first to evaluate the efficacy of two different feed additives, the probiotic strain *Bacillus licheniformis* CECT 4536 and a partially protected sodium butyrate (SB) salt, using an experimental model of salmonellosis in pigs and, secondly, to explore if behavior analysis can be used as a sensible tool to evaluate in feed additives.

7.2. Materials and methods

The experiment was performed 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).

7.2.1. Animals and Housing

The trial was conducted as a Level 2 High Risk Biosecurity Procedure, with appropriate training of the personnel involved. A total of 78 male piglets (Large White x Landrace) from a high-sanitary-status farm and from mothers serologically negative to *Salmonella* were used. Animals were weaned at 24 (±4) days of age, 8.3 (±0.32) kg BW on average, and were transported to the UAB facilities. From these animals, 72 were placed in three rooms of eight pens each (24 pens, three animals per pen) taking initial BW into account for a similar average BW within pens. Each pen (2 m^2) had a feeder and a water nipple to provide feed and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating, forced ventilation and an individual heat-light per pen. The experiment was conducted during the spring season (May), with an average room temperature of 28ºC (±4ºC). The experimental treatments were distributed evenly among the three rooms. Regarding the 6 remaining piglets, they were allocated in 3 pens of a separate room (2 pigs per pen) being used as a negative control (NC) for the challenge model.

7.2.2. Experimental Products and Diets

All feed additives are commercially available and were supplied by Norel SA (Madrid, Spain): a partially protected SB based feed additive: 70% of active ingredient; 40% free and 30% protected with vegetable fats (Gustor BP70), designed with the objective of having active principle available all along the gastrointestinal tract (Mallo et al., 2012) and a probiotic with 10^9 cfu/g of *Bacillus licheniformis* CECT 4536 (Proporc).

Diets (Table 7.1) were formulated to satisfy the nutrient requirement standards for pigs (NRC, 2012). All diets were manufactured in the same batch and treatments were included in a second mixture, on top, following the manufacturer's recommended dosages. There were three experimental diets: CON, control group with a plain diet without additives; PRO, a plain diet supplemented with 1 kg/t of Proporc (equivalent to 10^9 cfu of *Bacillus licheniformis* CECT 4536/kg of feed); and BUT, a plain diet supplemented with 3 kg/t of Gustor BP70 (equivalent to 2.1 g of partially protected SB salt/kg of feed).

Table 7.1. Ingredient and nutrient composition of the experimental diets as-fed basis, g/kg.

Ingredients	
Maize	280.8
Wheat	170.0
Barley 2 row	150.0
Extruded soybean	122.4
Sweet wheypowder (cattle)	100.0
Fishmeal	50.0
Soybean meal 44	50.0
Wheypowder 50% fat	30.3
Monocalcium phosphate	21.3
Calcium carbonate (CaCO3)	8.2
L-Lysine HCL	4.5
Vitamin-Mineral Premix [A]	4.0
Sodium chloride (marine salt)	3.0
DL-Methionine 99	2.4
L-Threonine	2.3
L-Triptophane	0.9
Analyzed composition	
DM	898.5
CP	170.5
CF	51.1
NDF	100.5
ADF	35.8
Ash	64.2

[A] Provided per kilogram of complete diet: 10,200 IU vitamin A, 2,100 IU vitamin D_3, 39.9 mg vitamin E, 3 mg vitamin K_3, 2 mg vitamin B_1, 2.3 mg vitamin B_2, 3 mg vitamin B_6, 0.025 mg vitamin B_{12}, 20 mg calcium panthotenate, 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.

7.2.3. Bacterial Strain

The bacterial strain used in the present study was a *Salmonella* Typhimurium *var. Monophasic* (formula: 4,5,12:i:-, resistance profile: ACSSuT-Ge, Fagotype: U302) that was isolated from a salmonellosis outbreak (mainly enteric and with sporadic septicemia) of fattening pigs in Spain, and was provided by the *Infectious Diseases Laboratory* (*Ref.*

301/99) of the UAB. The oral inoculum was prepared by 24 h incubation at 37ºC in buffered peptone water (BPW) (Oxoid, Hampshire, UK) and diluted (1:20) with sterile phosphate buffered saline (PBS) (Sigma-Aldrich, Madrid, Spain) to reach a final concentration of 2.5 x 10^8 cfu/ml.

7.2.4. Experimental Procedure

The duration of the study was 16 days, in which performance and clinical data were evaluated. BW was recorded on Days 1, 8, 12 and 16, while feed consumption was recorded at Days 1, 8, 11, 13 and 16. The ADG, ADFI and G:F were calculated by pen.

After one week of adaptation to the diets (Day 8), a single 2-ml dose ($5x10^8$ cfu) of *Salmonella* Typhimurium was administered to the challenged animals by oral gavage and a single 2-ml dose of sterile BPW to the non-challenged animals (challenge control group). Animals were checked daily for clinical signs to evaluate their status (i.e., dehydration, apathy and fecal score) after the *Salmonella* challenge, always by the same person. Fecal 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 (Thermoval Rapid, Hartmann, Spain) on Days 9 and 10 (1 and 2 post-inoculation [PI]). Mortality rate was also recorded and no antibiotic treatment was administered to any of the animals of the experiment.

For microbiological analysis, on Day 1 fecal samples were taken aseptically from 24 animals that were randomly selected from the total before distribution. Samples were taken after spontaneous defecation associated with the manipulation of the animal or by digital stimulation. On Days 8, 9, 11, 13 and 15 (Days 0, 1, 3, 5 and 7 PI), fecal samples were taken from the animal with the highest initial BW of each pen (N = 24).

At Days 4 and 8 PI (Experimental Days 12 and 16, respectively), one pig per pen was euthanized. On Day 4 PI, the animal selected was the one with the intermediate initial BW, while on Day 8 PI, the heaviest was selected. All NC animals were also euthanized at Day 4 PI.

Animals were euthanized and sequentially sampled during the morning (between 09:00 and 12:00 h). Prior to euthanasia, a 10-ml sample of blood was obtained by venipuncture of the cranial vena cava using 10-ml tubes without anticoagulant (Aquisel, Madrid, Spain). Immediately after blood sampling, selected piglets received an intravenous lethal injection

of sodium pentobarbital (200 mg/kg body weight of Dolethal; Vetoquinol S.A., Madrid, Spain). Once dead, animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract was excised.

Color range and consistency (on a scale ranging from 1 = liquid to 4 = semisolid) of intestinal contents, as well as the possible presence of fibrin, were recorded. Digesta (approximately 50 ml) from the ileum and proximal colon (considered to be 0.75 m from the ileocecal junction) was collected and homogenized. The pH of the contents was determined with a pH-meter calibrated on each day of use (Crison 52-32 electrode, Net Interlab, Barcelona, Spain) immediately after homogenization of the samples. Without delay, contents collected were sub-sampled and kept on ice all of the time. Colonic samples (1 g) were plated for *Salmonella* quantification the same day while samples for microbial counts of *Bacillus licheniformis* were kept refrigerated (4ºC) and plated the following day. A set of ileal and colonic content samples were preserved in a H_2SO_4 solution (3 ml of content plus 3 ml of 0.2 N H_2SO_4) for ammonia (NH_3) determination and were kept frozen at -20°C. An additional ileal and colonic sample set (approximately 20 g) was also frozen until analyzed for short-chain fatty acids (SCFA) and lactic acid.

For the histological study, 3-cm sections of the ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a 4% formaldehyde solution (Carlo-Erba Reagents, Sabadell, Spain).

Blood samples were centrifuged (3,000 x *g* for 15 min at 4ºC) after 4 h refrigeration, and the serum obtained was divided into different aliquots and stored at -20ºC to evaluate immune response.

7.2.5. Analytical Procedures

Chemical analyses of the diets including DM, ash, CP and diethyl ether extract, were performed according to the Association of Official Agricultural Chemists standard procedures (AOAC International, 1995). NDF and ADF were determined according to the method of Van Soest et al. (1991).

Bacillus licheniformis was analyzed by traditional microbiology to determine probiotic colonization. Three grams of sample were diluted into 300 ml of sterile saline solution (0.9%) + Tween 80 (0.4%) and homogenized in a sterile mincer, resulting in a starting dilution 10^{-2} from the original sample. The diluted sample was homogenized by stirring at

10,000 rpm for 1 minute, and afterwards treated at 80ºC for 1 minute in order to keep only the spore forms. Further decimal dilutions were done (up to 10^{-6}). Dilutions were plated in Triptic Soy Agar (Biokar Diagnostics, France), incubated for 48 h at 30ºC and manual cell counting was performed.

In digesta, NH_3 concentrations were determined with the aid of a gas-sensitive electrode (Hatch Co., Colorado, USA) combined with a digital voltmeter (Crison GLP 22, Crison Instruments, S.A., Barcelona, Spain). Three grams of acidified content were diluted (1:2) with 0.16M NaOH, after homogenization samples were centrifuged (1500 x *g*) for 10 min. The ammonia released was measured in the supernatants as different voltages in mV according to a procedure previously described in Hermes et al. (2009) that was adapted from Diebold et al. (2004). The SCFA and lactic acid analyses were performed by gas chromatography. The samples were submitted to an acid-base treatment followed by an ether extraction and derivatization with *N*-(*tert*butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MBTSTFA) plus 1% *tert*-butyldimethylchlorosilane (TBDMCS) agent, using the method of Richardson et al. (1989), modified by Jensen et al. (1995). For *Salmonella* bacteria counts, all samples were transferred (1:10) to BPW. Quantitative assessment was made by seeding the serial dilutions 10^{-2}, 10^{-4} and 10^{-6} of the samples in Xylose-Lactose-Tergitol-4 (XLT4) plates (Merck, Madrid, Spain). The qualitative assessment was made by incubating samples in BPW (37ºC, 24 h), transferring them to Rappaport-Vassiliadis enrichment broth (Oxoid, Hampshire, UK) for a second incubation (42ºC, 48 h) and seeding them in XLT4 plates in order to observe H_2S positive colonies.

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned to a 4-µm thickness and stained with hematoxylin and eosin. Measurements of 10 different villous-crypt complexes per sample were performed with a light microscope (BHS, Olympus, Barcelona Spain) using the technique described in Nofrarias et al. (2006). Serum concentrations of TNFα were determined by Quantikine Porcine TNF-α kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Pig major acute-phase protein concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain) as described in Saco et al. (2011). Serological antibodies of *Salmonella* were tested by ELISA *Salmonella* Herdcheck (Idexx, Hoofddorp, Netherlands), and the cut-off for positivity was established at optic density ≥40%.

7.2.6. Behavior Analysis

Behavioral measures were assessed applying the scan-sampling methodology found in the Welfare Quality Assessment protocol for pigs (2009) complemented with parameters observed in Escobar et al. (2007) and Temple et al. (2011). Active (positive + negative + exploration + feeding + drinking + walking + others) and inactive (lying laterally or ventrally and with or without contact with littermates) behaviors were recorded. An explanation of the recorded behaviors during the scan sampling is given in Table 7.2.

Table 7.2. Summary of recorded behaviors during the scan-sampling.

Active behavior	
Negative social behavior	Aggressive behavior, including biting or any social behavior with a response from the disturbed animal.
Positive social behavior	Sniffing, nosing, licking and moving gently away from the animal without an aggressive or flight reaction from this individual.
Exploration of the pen	Sniffing, nosing, licking all features of the pen.
Feeding	Pig with the head in the feeder.
Drinking	Pig with the mouth at the nipple.
Walking	Two steps were minimum requirements to configure walking.
Other	Other active behaviors not cited (defecation, urination, air sniffing, etc.).
Inactive behavior	
Lying laterally without contact	Resting lying laterally without contact or with less than half of their body in contact with other pen mates.
Lying ventrally without contact	Resting lying ventrally without contact or with less than half of their body in contact with other pen mates.
Lying laterally with contact	Resting lying laterally with more than half of their body in contact with other pen mates.
Lying ventrally with contact	Resting lying ventrally with more than half of their body in contact with other pen mates.

Two observers, who were blind to treatments, carried out the direct visual observations using a focal sampling technique (Lehner, 1998). In order to minimize differences between observers and to standardize the behavioral observations, the observers followed an identical previous training. Behaviors were recorded during two periods (08:30 to 10:30 h and 14:00 to 16:00 h) in a 14 h light phase (7.30 h – 21.30 h) on Days 2 and 1 before the inoculation (Experimental Days 6 and 7) and Days 1, 2 and 3 PI (Experimental Days 9, 10 and 11). Behaviors recorded before and after the oral challenge were compared in order to

determine the challenge effect. Space data collection was performed together with behavior analysis using scan-sampling in order to evaluate the preference of pigs to use different areas of the pen: water nipple, heat-light and feeder area.

During the observation period, the frequency of times engaged in each behavior was registered at 2-min sampling intervals. Each pen received 20 scans every day in the morning and the afternoon period. Throughout the experimental period a total of 4,777 scans were made.

7.2.7. Statistical Analysis

The experiment was conceived as a complete randomized design that included three treatments (CON, PRO and BUT). Results are expressed as means with their standard errors unless otherwise stated. The general linear and mixed models of SAS (SAS Institute Inc., Cary, NC, USA) were used to analyze the effect of experimental treatments except on microbiological data, where frequencies of positive animals were analyzed as contingency tables with Fisher's exact test.

For behavioral records, no differences were found between Days -2 and -1 in relation to infection, so these data were pooled and named as -1. The time spent in different behaviors was expressed in proportion of the total number of observations (active + inactive animals) and transformed using a square-root transformation.

When treatment effects were established, treatment means were separated using the probability of differences function adjusted by Tukey–Kramer. The pen was considered as the experimental unit for analysis, and random effect was used to account for variation between pens. The α-level used for the determination of significance for all of the analysis was $P = 0.05$. The statistical trend was also considered for $P < 0.10$.

7.3. Results

7.3.1. Adaptation to Diets and Challenge Response

In general, the animals used in the study showed a good state of health at the beginning of the trial, none of the animals seeded *Salmonella spp.* on their arrival and no signs of diarrhea throughout the first week of adaptation to the diets were registered.

Bacillus licheniformis was only recovered in countable numbers from animals in the PRO group, with a mean concentration of 1.8±0.47 x 10^5 cfu/g for ileal content and 5.0±1.15 x 10^5 cfu/g for colonic content.

Regarding the experimental model of salmonellosis, the NC group was compared to the challenged CON group at Day 4 PI. After the challenge, animals showed a mild course of diarrhea, and the NC group showed numerically lower fecal scores, in comparison to the infected CON group (1.9 ± 0.44 vs. 2.3 ± 0.27, P = 0.225). Although none of the challenged groups reached fever levels, the NC group had a lower body temperature than did the challenged CON, specially manifested at 24 h PI (39.1ºC ± 0.11ºC vs 38.5ºC ± 0.17ºC, P = 0.037). As expected, none of the NC animals seeded *Salmonella* in feces. Although no significant differences were recorded in TNFα serological concentrations, Pig-Map tended to be significantly lower in the NC group, in comparison to the CON group (0.63 ± 0.158 mg/dl vs. 1.07 ± 0.137 mg/dl, P = 0.060).

Histological parameters did not differ except concerning a tendency for the NC group for higher villous height in the ileum, in comparison to the CON group (287 ± 16.7 µm vs 248 ± 14.5 µm; P = 0.100). Moreover, no significant differences were found between these two groups regarding microbial activity (pH, ammonia, SCFA and lactic acid concentration; data not shown).

7.3.2. Effects on Performance, Intestinal Environment and Immune Response

ADFI, ADG and G:F mean values for the different challenged groups are shown in Table 7.3. No significant differences were detected in animal performance parameters related to the use of the in-feed additives. No relevant changes were seen either in rectal temperature, fecal, ileal and colonic consistency or presence of fibrin (data not shown).

Table 7.3. Performance of piglets fed the experimental diets and orally challenged with *Salmonella* Typhimurium at Day 8.

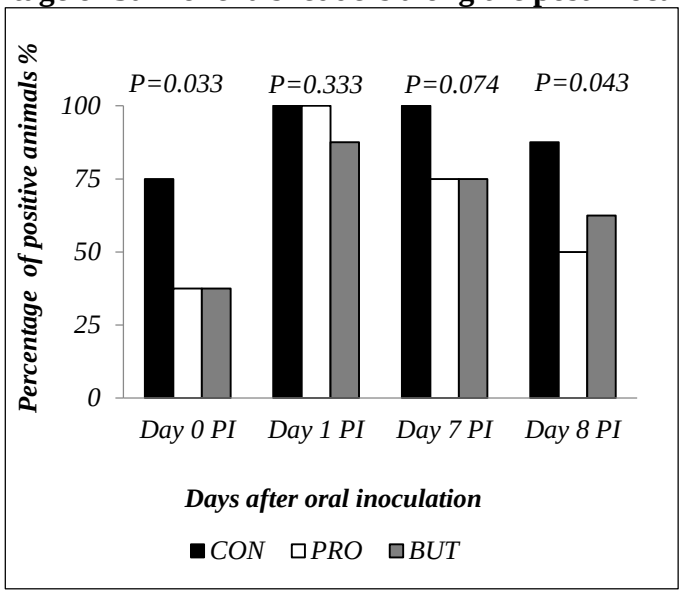
	Treatments [A]				
	CON	**PRO**	**BUT**	**SEM [B]**	***P*-value**
BW, kg					
Initial	8.3	8.2	8.3	*0.05*	*0.172*
Final	11.0	10.2	10.6	*0.69*	*0.725*
ADFI, g/d					
Pre-inoculation[C]	191	185	184	*16.2*	*0.941*
Post-inoculation[D]	360	336	385	*46.1*	*0.759*
ADG, g/d					
Pre-inoculation[C]	67.5	38.0	37.5	*30.28*	*0.734*
Post-inoculation[D]	243	198	208	*54.30*	*0.835*
G:F					
Overall[E]	0.62	0.48	0.46	*0.136*	*0.668*

[A] Treatments: CON, plain diet without additives; PRO, plain diet supplemented with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet supplemented with 3 kg/t of Gustor BP70 (2.1g of partially protected sodium butyrate salt/kg of feed). [B] Pooled SEM; n = 8/treatment. [C] Experimental Days 0 to 7. [D] Experimental Days 8 to 16 (0 to 8 PI). [E] Experimental Days 1 to 16.

Fecal prevalence of *Salmonella spp.* in feces (or colon digesta for Day 8 PI) after the oral inoculation is shown in Fig. 7.1. Significant differences among groups were seen on Days 0 (P = 0.033) and 8 PI (P = 0.043). When analyzing the *Salmonella* prevalence in the complete experimental period, both treatments succeeded in reducing the number of positive animals significantly (P = 0.016 for PRO and BUT) in relation to the control ones.

Despite the fact that most of the tested animals were positive in feces for *Salmonella* along the study, only 8% of the animals reached quantitative levels (>10^3cfu/g). No significant differences were observed among experimental groups in the frequency of quantifiable shedders or in the amount of *Salmonella* found in feces in the quantifiable animals (data not shown).

Figure 7.1. Percentage of Salmonella shedders along the post-inoculation (PI) period.



Percentage of animals (n=8) that showed *Salmonella* in feces at Days 0, 1, 7 post-inoculation (PI) or colon digesta at Day 8 PI. P-values obtained by Fisher's exact test. Treatments: CON, plain diet without additives; PRO, plain diet with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet with 3 kg/t of Gustor BP70 (2.1 g of partially protected sodium butyrate salt/kg of feed).

Table 7.4 shows pH values and fermentation products in the colon. No significant differences were found in pH values, ammonia, SCFA or lactic acid concentrations between treatments. Regarding the molar proportions of SCFA, no significant differences between treatments were found (data not shown), although at Day 4 PI the molar proportion of butyric acid was numerically higher in the BUT group (14.0% ± 1.69%, 10.8% ± 1.69%, and 16.0% ± 1.80% for CON, PRO and BUT, respectively; $P = 0.121$).

All of the animals euthanized remained serologically negative to *Salmonella* along the study. No change was detected in the mean levels of pro-inflammatory cytokine TNFα (91.1 ± 7.08 pg/ml, 98.2 ± 7.08 pg/ml and 97.6 ± 7.08 pg/ml for CON, PRO and BUT, respectively; $P = 0.532$) nor of acute phase protein Pig-MAP (1.81 ± 0.175 mg/dl, 2.09 ± 0.175 mg/dl and 1.92 ± 0.175 mg/dl for CON, PRO and BUT, respectively; $P = 0.737$).

The results of histological analysis revealed no significant differences among the experimental groups despite a trend for BUT treatment on Day 4 PI to increase crypt depth, when compared with the CON group ($P = 0.104$). Histological measurements at Day 4 PI are shown in Table 7.5.

Table 7.4. Colonic pH values, ammonia concentration and fermentation products for Days 4 and 8 post-inoculation (PI).

		Treatment [A]				
	Days PI	**CON**	**PRO**	**BUT**	**SEM [B]**	***P*-value**
pH	4	5.89	6.13	6.09	*0.120*	*0.327*
	8	6.04	5.90	6.01	*0.086*	*0.478*
NH_3, *mM*	4	19.5	19.8	19.4	*1.99*	*0.990*
	8	55.2	46.2	50.9	*4.93*	*0.447*
Lactic Acid, mmol/kg	4	7.45	4.64	0.99	*2.458*	*0.225*
	8	1.75	2.49	0.94	*0.904*	*0.492*
Total VFA, mmol/kg	4	119.3	104.4	119.2	*8.71*	*0.405*
	8	129.6	115.8	129.4	*7.85*	*0.381*

[A] Treatments: CON, plain diet without additives; PRO, plain diet supplemented with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet supplemented with 3 kg/t of Gustor BP70 (2.1g of partially protected sodium butyrate salt/kg of feed). [B] Pooled SEM; n = 8/treatment.

Table 7.5. Histological determinations in ileum on Day 4 PI.

	Treatments [A]				
	CON	**PRO**	**BUT**	**SEM [B]**	***P*-value**
Villous height, µm	248	291	275	18.6	0.267
Crypt Depth, µm	203	238	251	15.9	0.107
Villus:Crypt Ratio	1.24	1.25	1.13	0.101	0.682
IEL[C], /100 µm	1.10	0.94	1.30	0.224	0.535
GC [D], /100 µm	1.19	0.92	1.23	0.170	0.384
Mitosis [E], /100 µm	0.33	0.30	0.29	0.053	0.832

[A] Treatments: CON, plain diet without additives; PRO, plain diet supplemented with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet supplemented with 3 kg/t of Gustor BP70 (2.1g of partially protected SB salt/kg of feed). [B] Pooled SEM; n = 8/treatment. [C] IEL= Villous intraepithelial lymphocytes. [D] GC= Villous goblet cells/100 µm. [E] Number of mitosis in crypts.

7.3.3. Effects on behavior analysis

Table 7.6 shows the mean frequencies of behaviors and use of spaces recorded during the scans in the morning and afternoon. Changes between these two periods were found, the sum of active behaviors being 51.5% in the morning vs 29.3% in the afternoon. Differences in pig expression of inactive behaviors between morning and afternoon were recorded too, with the lateral and ventral lying consuming about 70% of the time in the afternoon vs. 48% in the morning. Regarding the use of spaces, the use of the feeder area was higher in the morning (41.8% for the morning vs. 31.4% for the afternoon) and of the light area in the afternoon (53.8% for the morning vs 64.1% for the afternoon). The water nipples area use was similar in both periods. Due to the different pattern found in behaviors between morning and afternoon periods (8:30 to 10:30 h and 14:00 to 16:00 h), possible changes related to treatments were analyzed separately.

Table 7.6. General descriptive statistics of behaviors and use of the space.

	Morning	Afternoon
Behaviors, %		
Positive	4.2 ± 0.44	2.6 ± 0.32
Negative	2.0 ± 0.29	0.5 ± 0.18
Exploration	5.5 ± 0.55	2.2 ± 0.26
Feeding	30.7 ± 1.82	18.4 ± 1.79
Drinking	2.3 ± 0.22	1.7 ± 0.22
Walking	1.6 ± 0.18	1.2 ± 0.14
Others	5.2 ± 0.42	2.7 ± 0.31
Lying laterally without contact	2.3 ± 0.44	3.4 ± 0.63
Lying ventrally without contact	5.5 ± 0.60	7.0 ± 0.77
Lying laterally with contact	11.5 ± 1.32	20.6 ± 1.72
Lying ventrally with contact	28.7 ± 2.16	39.2 ± 2.39
Use of the space, %		
Feeder area	41.8 ± 2.16	31.4 ± 2.50
Light area	53.8 ± 2.29	64.1 ± 2.62
Drinker area	4.4 ± 0.38	4.8 ± 0.43

Pooled values of Days -2, -1, +1. +2 and +3 post-inoculation, recorded during morning (08:30 to 10:30 h) and afternoon (14:00 to 16:00 h). Means values ± SEM of untransformed data (n=120).

Behaviors in the morning: The general expression of pig behaviors in the morning period is summarized in Table 7.7.

Table 7.7. Pig behavior expressions in the morning (08:30 to 10:30h) according to dietary treatment and days in relation to challenge.

	Days in relation to challenge					*P*-value		
	-1[1]	**1**	**2**	**3**	**Total**	**TRT[2]**	**DAY[3]**	**TRT x DAY**
Positive contacts:								
CON	1.96 ^{c}	1.59 ^{c}	5.95 ^{a,b}	0.77 ^{c}	2.22			
PRO	8.29 ^{a}	2.02 ^{b,c}	3.88 ^{a,b,c}	2.13 ^{b,c}	3.72	0.142	0.038	0.025
BUT	2.28 ^{b,c}	1.82 ^{b,c}	1.42 ^{c}	2.34 ^{b,c}	1.96			
Total	3.72^{A}	1.82^{B}	3.50^{A,B}	1.66^{B}				
Negative contacts:								
CON	0.61	0.58	3.39	0.67	1.10			
PRO	0.86	0.06	2.04	1.02	0.81	0.571	0.080	0.563
BUT	1.04	0.30	0.61	0.52	0.59			
Total	0.83	0.27	1.82	0.72				
Exploration:								
CON	2.13	2.69	4.49	1.42	2.56 ^{B}			
PRO	8.94	2.56	4.93	5.71	5.29 ^{A}	0.050	0.347	0.265
BUT	4.41	3.65	1.72	2.19	2.89 ^{A,B}			
Total	4.80	2.96	3.53	2.86				
Feeding:								
CON	11.49	26.63	28.30	25.00	22.28^{B}			
PRO	29.05	33.87	55.06	34.81	37.58^{A}	0.003	0.030	0.850
BUT	19.45	20.52	30.47	19.18	22.18^{B}			
Total	19.27 ^{B}	26.73 ^{A,B}	37.09 ^{A}	25.91 ^{A,B}				
Others:								
CON	3.06	4.71	3.46	1.23	2.96 ^{B}			
PRO	5.95	5.90	6.60	3.72	5.48 ^{A}	0.039	0.192	0.966
BUT	3.84	2.86	4.24	2.28	3.28 ^{B}			
Total	4.20	4.41	4.67	2.28				

	Days in relation to challenge					*P*-value		
	-1[1]	**1**	**2**	**3**	**Total**	**TRT**[2]	**DAY**[3]	**TRT x DAY**
Lying laterally without contact:								
CON	0.86	1.00	0.81	1.06	0.92	0.821	0.448	0.967
PRO	1.44	0.41	0.46	1.77	0.92			
BUT	1.08	0.37	0.21	1.02	0.61			
Total	1.12	0.56	0.46	1.25				
Lying ventrally without contact:								
CON	1.80	3.96	4.41	6.66	4.00	0.937	0.001	0.887
PRO	2.02	1.99	2.92	9.80	3.69			
BUT	2.28	4.45	3.24	7.56	4.16			
Total	2.02 ^{B}	3.39 ^{B}	3.50 ^{B}	7.95 ^{A}				
Lying laterally with contact:								
CON	12.46	4.97	7.90	9.55	8.53 ^{A}	<0.001	0.258	0.946
PRO	3.88	2.37	0.64	1.00	1.77 ^{B}			
BUT	13.03	4.93	9.00	12.18	9.49 ^{A}			
Total	9.24	4.00	4.84	6.40				
Lying ventrally with contact:								
CON	31.47	38.81	25.30^{a}	24.21	29.70 ^{A}	0.008	0.249	0.683
PRO	10.11	21.44	5.52^{b}	21.90	13.76 ^{B}			
BUT	17.64	29.92	26.52^{a}	30.69	25.91 ^{A}			
Total	18.75	29.59	17.47	25.40				

[1] No differences were found between Days -2 and -1 before the inoculation, so these data were pooled and named as -1. [2] Treatment effect. Treatments: CON, plain diet without additives; PRO, plain diet supplemented with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet supplemented with 3 kg/t of Gustor BP70 (2.1g of partially protected sodium butyrate salt/kg of feed). [3] Inoculation effect (measured as difference between days before (-1) and after (1, 2, 3) the challenge). ^{A,B} LSmeans within rows without common letters differ by the Means-Tukey adjustment test ($P < 0.05$). ^{a,b,c} LSmeans within columns without common letters differ by the Means-Tukey adjustment test ($P < 0.05$). Values expressed are a proportion of total number of observations (active + inactive). For the statistical analysis, data were previously transformed using square root transformation. Drinking and walking behaviors are not shown, as they were not modified by any experimental treatment.

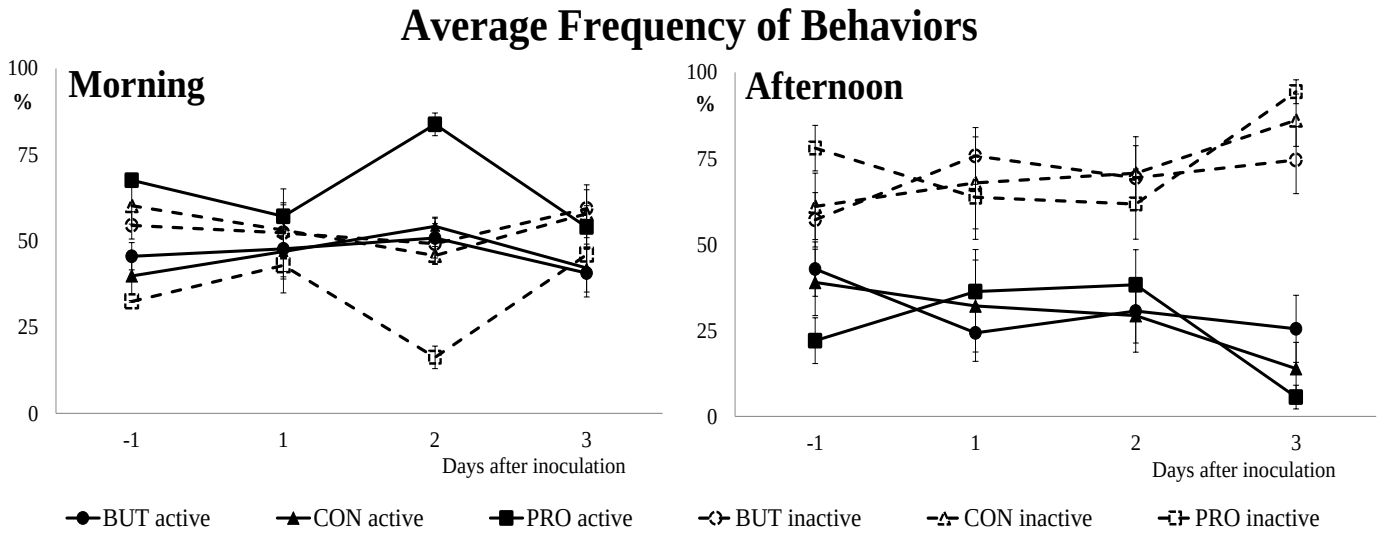
Challenge effects (evaluated as day effect) were less evident in the morning than in the afternoon. No interaction between day and treatment was found for any variable except for positive contacts ($P = 0.025$). Despite this, a general significant time effect could be seen during the morning on the time spent feeding, with values that increased on Day 2 PI ($P = 0.004$), and also in the time lying ventrally without contact, that also increased on Day 3 PI ($P < 0.001$).

Regarding the effect of the treatments on morning behaviors, pigs supplemented with PRO spent more time feeding ($P = 0.003$ for CON and $P = 0.002$ for BUT), exploring the pen ($P = 0.026$ for CON and $P = 0.054$ for BUT) and with other active behaviors ($P = 0.018$ for CON and $P = 0.040$ for BUT). Furthermore, the total time lying ventrally or laterally with contact was significantly lower in pigs fed the PRO diet, in comparison with others treatments ($P = 0.004$ and $P = 0.017$ vs. CON, $P < 0.001$ and $P < 0.001$ vs. BUT; for total time lying ventrally and laterally, respectively). These effects were seen not only after, but also during the days previous to the challenge. When the data for different lying behaviors were analyzed within the total resting time, there was no significant effect of treatments nor challenge or interaction between factors for time spent lying ventrally and lying laterally. Considering all the active or inactive behaviors together, the occurrences of active as well as inactive ones were significantly higher and lower in the PRO group's treatments ($P = 0.008$ and $P = 0.006$ vs. CON, $P < 0.001$ and $P < 0.001$ vs. BUT in active and inactive behaviors). A graphic display of the animals in active or inactive behaviors can be seen in Fig. 7.2.

In relation to the use of space (data not shown), pigs supplemented with PRO used the feeding area more frequently (30.7%, 36.1% and 50.7% for CON, BUT and PRO, respectively; $P < 0.001$ for PRO vs. CON and $P = 0.012$ for PRO vs. BUT) and the lying area less time (57.3%, 53.1% and 36.7% for CON, BUT and PRO, respectively; $P < 0.001$ for PRO vs. CON and BUT) than with the other treatments. No effect of treatments and challenge on the frequency of pigs in the drinker area was observed.

Behavior in the afternoon: In contrast to the morning, in the afternoon the factor with most effect on pig behavior was the challenge (days in relation to the challenge). As can be seen in Fig. 7.2, pigs were more active before the pathogen inoculation in comparison with Day 3 PI ($P < 0.001$).

Figure 7.2. Frequency of animals with active and inactive behaviors in the morning (08:30 to 10:30h) and afternoon (14:00 to 16:00 h), before and after the oral inoculation of the pathogen, for the different dietary treatments.



Each data point represents a mean value (n = 8). Treatments: CON, plain diet without additives; PRO, plain diet with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet with 3 kg/t of Gustor BP70 (2.1g of partially protected sodium butyrate salt/kg of feed). Active = positive + negative + exploration + feeding + drinking + walking + others. Inactive = lying laterally or ventrally, with or without contact with pen mates.

Table 7.8 summarizes the expression of behaviors of pigs in the afternoon. Because treatments had little effect on behavior at this time of the day, only the mean values for the different days are presented. The only difference found related to treatments was a less frequent behavior of lying laterally without contact for the animals on the PRO and BUT, in comparison to the CON groups (3.49%, 0.90% and 1.08% for CON, BUT and PRO, respectively; $P < 0.001$ for PRO vs. CON and BUT).

However, many significant differences were found related to the experimental days. Positive contacts, exploration and feeding behaviors ($P = 0.004$, $P < 0.001$ and $P < 0.001$, respectively) were less frequent on Day 3 PI, in comparison to the period before the inoculation. Lying laterally without contact and lying ventrally without contact increased on Days 2 and 3 PI, in comparison with before inoculation ($P < 0.050$). Lying ventrally with contact also increased after inoculation but only on Day 3 PI ($P = 0.016$).

Table 7.8. Expression of behaviors of pigs in the afternoon (14:00 to 16:00 h) according to days in relation to challenge.

Observations	Days in relation to challenge				*P*-value		
	-1[1]	+1	+2	+3	TRT[2]	DAY[3]	TRT x DAY
Positive contacts	1.61^{A}	1.25^{A}	1.90^{A}	0.23^{B}	0.632	0.015	0.894
Exploration	1.99^{A}	0.85^{B,C}	1.06^{A,B}	0.18^{C}	0.681	0.002	0.740
Feeding	15.60^{A}	11.29^{A}	13.18^{A}	2.96^{B}	0.786	0.003	0.159
Others	1.74	1.12	2.22	0.45	0.648	0.063	0.817
Lying laterally without contact	0.62^{B}	0.74^{B}	3.35^{A}	2.76^{A}	0.040	0.002	0.497
Lying ventrally without contact	2.66^{B}	2.89^{B}	7.95^{A}	7.73^{A}	0.835	<0.001	0.875
Lying laterally with contact	21.16^{A}	8.70^{B}	13.84^{A}	13.69^{A}	0.528	0.038	0.731
Lying ventrally with contact	30.14^{B}	37.58^{AB}	27.35^{B}	45.02^{A}	0.399	0.035	0.541

[1] No differences were found between Days -2 and -1 before the inoculation, so these data were pooled and named as -1. [2] Treatment effect. Treatments: CON, plain diet without additives; PRO, plain diet supplemented with 1 kg/t of Proporc (10^9 cfu/kg of feed of *Bacillus licheniformis* CECT 4536); BUT, plain diet supplemented with 3 kg/t of Gustor BP70 (2.1g of partially protected sodium butyrate salt/kg of feed). [3] Inoculation effect (measured as difference in days before (-1) and after (1, 2, 3) the infection). ^{A,B,C} Means within rows without common letters differ by the Means-Tukey adjustment test ($P < 0.05$). Data are mean (n=8) expressed in proportion of total number of observations (active + inactive). For the statistical analysis, data were previously transformed using square root transformation. Drinking and walking behaviors are not shown, as they were not modified by any experimental treatment.

In relation to the use of different areas (data not shown), in the afternoon the use of the feeder space decreased significantly (33.4% on Day -1; 16.6% on Day 1 and 9.6% on Day 3 PI; $P = 0.016$ for Day -1 vs. 1 PI and $P < 0.001$ for Days -1 vs. 3 PI) and the use of the lying area with light increased significantly (54.6% on Day -1 and 71.7% on Day 3 PI; $P = 0.040$) after the challenge. No significant differences were found concerning the use of the drinker area related to the experimental treatments.

7.4. Discussion

The *Salmonella* Typhimurium oral challenge resulted in an effective infection, as nearly all challenged animals excreted *Salmonella* after the oral inoculation, with the NC animals remaining negative until the end of the trial.

Experimental models of salmonellosis in pigs found in the literature have very variable responses. Whereas some authors report models with mild infectuous outcomes (Fraser et al., 2007; Szabó et al., 2009; Walsh et al., 2012), others report acute clinical responses (Balaji et al., 2000; Spiehs et al., 2008), and even responses that surpassed treatment capacity (Casey et al., 2007). These differences may be primarily attributed to different challenge dosages and different virulence of the strains. In our case, a mild outcome was planned to evaluate a treatment administered as a feed additive, and we succeeded by achieving minor clinical signs. The animals never stopped eating, and some even became negative in feces at the end of the trial.

Unexpectedly, after the first adaptation week, some animals from the challenged groups started to excrete *Salmonella* in feces (Fig. 1) at low levels (below countable numbers $<10^3$ cfu/g). It should be considered that in the 3 rooms in which the challenged groups were allocated, there had previously been another trial with *Salmonella* (same strain) and although the facilities were cleaned and disinfected, it could be possible that an ambient load could have remained. Low concentrations of *Salmonella* in the environment (10^2 to 10^3 cfu) have been reported as being able to infect the exposed animals (Boughton et al., 2007; Hurd et al., 2001). The fact that all fecal samples were negative for *Salmonella* on their arrival, and that all euthanized piglets remained sero-negative at the end of the study, reaffirms that these animals were not previously exposed to the pathogen in the farm of origin (Nielsen et al., 1995). Moreover, the NC group allocated in a room not previously used for *Salmonella* challenges remained negative along all the study.

7.4.1. In-feed additive effects in the animal´s response to Salmonella

The inclusion of the *Bacillus licheniformis* based probiotic in feed did not show significant effects on performance parameters. Other authors, however, have demonstrated positive effects of different *Bacillus spp.* based probiotics on performance (Alexopoulos et al., 2004; Link and Kováč, 2006). A possible explanation for these positive results could be the production of enzymes by the germinated spores in the gut, like proteases, lipases or amylases, that might help the digestion of nutrients from feed (Link and Kováč, 2006). Nevertheless, under challenge conditions, these positive effects are probably precluded by the limited number of replicates and the high individual variation in the clinical response. Similar results have been obtained by other authors testing different *Bacillus spp.* based

probiotics under challenge conditions who were not able to find differences in performance (Dänicke & Döll, 2010; Walsh et al., 2012a,b).

Our results demonstrate, as in Leser et al. (2008), the good viability of the *Bacillus* spp. bacteria in the gut. Actually, the cfu concentration found in the gut (between 2-5 10^5 cfu/g) would reflect that most of the administered spores were viable, considering that the in-feed doses were of 10^6 cfu/g and that dry-matter content of digesta usually vary by around 10% to 20%. Some authors have even demonstrated, in murine models, higher number of spores excreted in the feces than in the original inoculum, suggesting the ability of the administered spores to germinate and sporulate in the gut (Hoa et al., 2001).

The ability to stimulate the immune response has been largely attributed to *Bacillus* spp. probiotics (Duc et al., 2004), although very little information is available on the particular strain we tested. In our study, pro-inflammatory cytokine TNFα and acute-phase protein Pig-MAP were not affected by the probiotic treatment, similar to Walsh et al. (2012a), who did not find any differences in serum TNFα levels after the administration of a *Bacillus licheniformis* and *B. subtilis* combination. However, the absence of effects in these two broad indexes does not discard the possible effects of this probiotic on the immune response, as *Bacillus* spp. stimulation effects have been widely reported (Arena et al., 2006; Skjolaas et al., 2007; Walsh et al., 2012a).

In relation to *Salmonella* shedding, a significant reduction in the percentage of animals positive to *Salmonella* was seen when considering the overall PI period for the PRO group. Other authors have also described the potential of *Bacillus spp.* to reduce bacterial loads of *Salmonella* or *E. coli* after a challenge (Ahmed and Hoon, 2014). In contrast, Spiehs et al. (2008) and Walsh et al. (2012a) did not see any effective reduction in *Salmonella* shedding using a *B. licheniformis* and *B. subtilis* combination in piglets. Hence, it is important to bear in mind that the effect of probiotic bacteria can depend on the particular strain tested.

Regarding the inclusion of sodium butyrate in pig diets, the BUT treatment did not significantly influence performance. This lack of effects could also be due to the reasons stated above related to the limited number of replicates in this kind of controlled disease models. There are many authors who have found performance improvements in non-challenge trials with post-weaning piglets (Kotunia et al., 2004; Manzanilla et al., 2006; Lu

et al., 2008; Piva et al., 2010), although others reported no performance differences (Biagi and Piva, 2007; Mallo et al., 2012; Fang et al., 2014).

Inconsistencies found in the bibliography may be due to, among other factors, the form of presentations of the butyric acid. In some studies it is used as a free acid, but commonly it is used as its salt form (sodium or calcium salts with different solubility) to make handling easier. Also, these butyric salts can be protected with a fat coating or be combined with glycerol in esters of butyric acid, trying to delay its absorption in the gastrointestinal tract and its release along the intestine (Mallo et al., 2012). The butyrate source used in this experiment was a partially protected sodium butyrate salt that was expected to be released slowly in the gut and reach distal sections, but no significant differences in butyric acid concentrations were seen in the colon.

Different effects on the animals have been potentially attributed to butyrate. It is an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis. Among other functions, butyrate exerts potent effects throughout inhibiting inflammation and reinforcing various components of the defense barrier (Hamer et al., 2008). In response to a *Salmonella* challenge, it could be expected that the inclusion of butyrate in the diets would have better preserved the integrity of the intestinal architecture. In this regard, Jerzsele et al. (2012) and Chamba et al. (2014), who tested the same source of butyrate in challenged and non-challenged trials on broilers, found positive effects on the villus and crypt architecture (such as an increase in villus length, or villus:crypt ratio). In our study, although we were not able to demonstrate significant modifications, we found a trend in the ileal crypt depth to be increased at Day 4 PI.

Other positive effects of butyrate could be related to its ability to be transformed into butyric acid (combined with a hydrogen ion) and potentially influence the intestinal environment with antimicrobial activity. In our study, BUT treatment did improve *Salmonella* shedding, in consonance to Fernández-Rubio et al. (2009), who tested the same butyrate source and found a significant reduction of *Salmonella* Enteritidis in a broiler challenge model. These results agree with those of many other authors who demonstrated the benefits of sodium butyrate in challenges with *Salmonella in vitro* (Boyen et al., 2008; Gantois et al., 2006), in poultry (Van Immerseel et al., 2005) and in piglet models (Boyen et al., 2008). This bactericidal effect could be attributed to the undissociated form of newly formed butyric acid, which can penetrate a bacterial cell wall and dissociate to H^+ and

anions inside the cell, lowering intracellular pH and resulting in energy deficiency and osmotic problems in the microbial organism (Gálfi and Bokori, 1990; Warnecke and Gill, 2005).

In summary, both additives exerted a similar positive effect on the shedding of *Salmonella,* with a higher number of pigs that turned negative in feces for the pathogen at the end of the study, as compared with the control. It is also interesting to point out the differences seen for both experimental treatments after the first week of adaptation, when the animals were presumably exposed to a low environmental load of the pathogen (see comment above). In this case, the percentage of positive animals were reduced from 75% to 37.5%, suggesting that both additives would be specially effective when animals are exposed to low doses of *Salmonella* during prolonged times, the most frequent event in practical conditions.

7.4.2. Behavior analysis for in-feed additive evaluation

In this assay animal behavior was registered during the morning and afternoon. From the results shown, the best observational period to detect differences related to the diets resulted in being the morning, whereas the afternoon mostly showed effects due to the *Salmonella* challenge, assuming that changes observed between days are related to the infective process.

Sickness behavior, as reviewed by Weary et al., 2009, is an adaptive response of the animals to illness and some particular behaviors could be valid indicators to identify disease and discomfort of the animals. It is hypothesized that behaviors most likely to decline are those that provide long-term fitness benefits (such as playing), as animals divert resources to those functions of critical short-term survival such as maintaining body temperature. In our experiment, we were able to detect sickness behavior in the afternoon, when animals were more motivated to perform extra activities other than feeding, and these activities underwent a gradual reduction that reached statistical significance at Day 3 PI. Similar effects were also described by Rostagno et al. (2011), who hypothesized that infection would cause the pigs to feel sick, and thus be less willing to express investigative behavior. Moreover, Escobar et al. (2007) observed that resting animals may assume postures that conserve heat when sick; they found pigs infected with the porcine reproductive and respiratory syndrome (PRRS) virus spent more time in ventral decubitus. Even though their challenge differs from ours, this sickness behavior in ill pigs was also confirmed in our experiment by an increment of pigs lying ventrally on days post-

infection. Likewise, Rostagno et al. (2011) found that infected pigs with *Salmonella* spent more time in ventral recumbence to conserve body heat by lying on their feet and not exposing their underside.

Regarding the morning behaviors, feeding activity was prominent and, in accordance with Feddes et al. (1989), appeared to be driven by light changes. After the dark resting period, pigs were more motivated to feeding because they were hungry. In our study, the lights were turned on at 7:30 h and turned off at 21:30 h so in mornings the behavior of the pigs was recorded near the peak of feeding activity. In this regard, the use of the feeding area was also more frequent in the morning and the light-resting area in the afternoon. Food is an essential item and therefore the demand for it is inelastic, in other words, it would be the last behavior that the piglet would decrease when it gets sick (Matthews and Ladewig, 1994; Weary et al., 2009). We hypothesize that demand for food masked the effects in sickness behavior in the morning, and for this reason the effects of inoculation could not be perceived easily. We did not find any significant effects in the expression of active or inactive behaviors during this period.

Regarding the behaviors that were modified by the diets, pigs fed the probiotic diet showed positive, significant effects on exploring the pen, feeding and other active behaviors in the morning. Some authors have seen a correlation between feeding activity and food intake (Ahmed et al., 2015; Soltan, M. A., & Said, 2008) although in our experiment no increase in feed intake was seen in the PRO group. Our results also showed that lying with contact was less frequent in pigs fed with PRO. In accordance to Escobar et al. (2007), these results would suggest that supplementation brought some benefits for pigs because lying in contact with a pen mate is a strategy to achieve heat conservation for producing the beneficial febrile response in illness situations. These improvements were not only seen after the *Salmonella* challenge but also during the days previous to the inoculation (non-significant interaction of treatment*day). It must be remembered that weaning itself is a challenge for the piglets (Heo et al., 2013), and our results suggest that the probiotic diet may also have positive effects in the weaning response.

Up until today, probiotics have been attributed several functions when fighting against pathogens, namely: reducing nutrients to pathogenic bacteria by competition in the gut; competitive exclusion in binding sites on the intestinal epithelium, and producing bacteriocines or stimulating the immune system (Cho et al., 2011). Nonetheless, our results

may suggest that other pathways could also be activated by probiotics. The gut-brain axis is emerging as an exciting concept where intestinal bacteria might influence nerve and brain function, and ultimately behavior (Cryan and O'Mahony, 2011). There is recent evidence that probiotics can reduce behaviors associated with stress, anxiety and depression by improving the role of GABA, a prevalent neurotransmitter in the brain. Stilling et al. (2014) reported some biochemical and behavioral parameters (including anxiety, sociability, hypothalamic-pituitary-adrenal axis, and tryptophan metabolism) that could be reversed in germ-free mice by re-colonization with a conventional microbiota or probiotic treatment. Moreover, other effects of probiotics on brain functions have been reported. Bravo et al. (2011), feeding mice with *Lactobacillus rhamnosus,* showed changed levels of signaling chemicals in the brain as well as increased numbers of receptors associated with learning, memory and emotional control. In humans, Messaoudi et al. (2011) showed how a combination of *Lactobacillus helveticus* and *Bifidobacterium longum* improved health scores designed to assess mental health, and chronically administered in rats, significantly reduced anxiety-like behaviors. In view of our data, we cannot determine the causes of the behavioral changes related to the probiotic treatment because other factors such as an up-regulation of the immune response or a decrease in pathogenic pressure could also contribute to diminish sickness stress. However, the lack of behavioral effects in the BUT group, which also reduced *Salmonella* shedding, indicate that probiotics may act by other mechanisms.

Nonetheless, the detection of so many effects was a surprising result and the concept of social facilitation should also be considered to interpret treatment effects in behavior, as the pig is a highly social animal and social facilitation is a common feature in its behavior (Hsia and Wood-Gush, 1984; Weary et al., 2008). According to Clayton (1976), social facilitation is an increase in the frequency or intensity of responses, or initiation of particular responses already in an animal's repertoire when shown in the presence of others engaged in the same behavior. Therefore, a slight difference in health in some pigs produced by treatments, even if not perceptible by clinical signs monitored, could be amplified in behavior expression by social facilitation. We suggest that this line of research is important to further characterize the animal response mechanism. Encountering sensible evaluation tools, especially in challenge trials, is a way of diminishing the cost of the experiment and improving welfare by applying one of the basic principles of the 3r's, reduction of the animals used.

On the other hand, to our knowledge, this is the first time that methodical observation has been performed to evaluate sodium butyrate in relation to animal behavior. In our study, although the treatment did exert a positive effect on the *Salmonella* shedding, we were not able to show behavioral changes promoted by this additive in a *Salmonella* challenge context.

To wrap up the behavioral analysis, the PRO treatment influenced the behavior and use of space positively. In the light of our results, behavior analysis may be a useful tool to appreciate significant changes in parameters studied by using a low number of animals.

7.5. Conclusions

Both products evaluated had a favorable effect against *Salmonella* Typhimurium, as they were equally able to reduce the colonization and shedding of *Salmonella* Typhimurium in piglets. In addition *Bacillus licheniformis* CECT 4536 had a positive significant effect on some behavioral displays, particularly those related to exploring the pen, feeding and other active behaviors in the morning period. Behavior analysis appears as a sensible tool to be considered in feed additive research, being able to detect slight improvements in the response of the animals and offering complementary information regarding other possible mechanisms of action of these additives.

Chapter 8. General Discussion

During the elaboration of the Thesis, few questions in relation to the use of probiotics were raised that in our opinion recall further consideration. Hence, the aim of this section is in first instance to address these loose ends. On a second instance, our experimental model and the new indicators will be briefly reviewed, to discuss their usefulness and whether they can be optimized.

8.1. Are we using probiotics correctly to control post-weaning diarrheas?

Whether probiotics are useful or not to control post-weaning diarrheas is an issue that has been addressed by many studies (including ours). However, up until today there is still no consensus in this question when considering the studies published. Many authors reported a positive effect when adding probiotics (Bhandari et al., 2008; Casey et al., 2007; Shu et al., 2001; Zhang et al., 2009) but there's also an important amount of research advising against their use (Broadway et al., 2016; Kreuzer et al., 2012; Szabó et al., 2009; Trevisi et al., 2011). Furthermore, some of the results of this Thesis (chapter 4), presented a different response on several parameters, depending on if the animals were exposed or not to a microbiological challenge. Overall, this uncertain background has lead us to question whether we are doing a correct use of probiotics at this productive stage.

As reviewed in the first chapter, parameters such as strain specificity, viability, dose, duration of the treatment, environment and delivery mode can greatly influence the treatment outcome. However, in our trials many of these variables were fixed or under control. This context is favorable to analyze the situation. Thus, some of the results obtained in this Thesis will be reviewed with the aim to discuss this lack of consistent results and, if possible, give suggestions on how to prevent it.

Firstly, results reported in Chapter 4, evaluating the single probiotic strain of B. *longum* subsp. *Infantis* CECT 7210 against bacterial pathogens, demonstrated the probiotic treatment was not able to prevent infection of the animals but was capable to reduce pathogen colonization or shedding to some extent. This should be regarded as a positive outcome, as it suggests that pathogen pressure was reduced in challenged animals receiving the probiotic. However, these positive results were not correlated with piglet global health status as interactions in the probiotic effects were observed depending on if

the animals were challenged or not, with more beneficial effects in non-challenged animals in comparison to the challenged ones (see Table 8.1).

Table 8.1. Effects of B.*longum* subsp. *Infantis* CECT 7210 reported in chapter 4.

B.*longum* subsp. *Infantis* CECT 7210	
Consistent effects	**Interactions challenge x probiotic**
Reduce Pathogens	Performance (ADFI)
Increase ileal IEL	SCFA concentrations
	Ileal Villus:crypt ratio
	Inflammatory response (Pig-MAP)

Probiotic effects observed with the probiotic strain of *B. longum* subsp. *Infantis* CECT 7210 in Chapter 4. Consistent effects were positive effects of the probiotic treatment but the interactions reflected negative effects in challenged animals.

This outcome was surprising, as we speculated that a reduction in pathogen pressure would be beneficial for the challenged animals (as reported in the other *Salmonella* challenges in Chapter 5 and 7). On the contrary, in general terms challenged animals receiving the probiotic did not present improvements in a response that was quite consistent in both challenges, including reduced ADFI, lower SCFA concentrations in the colon, increased inflammatory response and decreased villus:crypt ratio. These results suggest that the probiotic had a different effect depending on the gut health status in which it was applied, and a raised another question: are there potential risks of using probiotics in certain situations? In order to answer these questions we addressed scientific literature.

A baseline of bacterial translocation, possibly due to the increased para/trans cellular permeability in the enterocyte determined by the inflammatory stress, is normally associated with weaning (Lallès et al., 2004). This permeability has been reported to be affected by probiotic treatments such as in Trevisi et al. (2008), who reported an increase in translocation with a *B. animalis* and FOS treatment in post-weaning piglets. Consequently, elevated risk of sepsis could be speculated in post-weaning animals (Verna and Lucak, 2010). Moreover, it has also been reported that some probiotics may have immune-suppressive effects in the host (Siepert et al., 2014). This effect has no disadvantageous consequence in a healthy context. Nevertheless, it has been reported that in the need of a rapid humoral response, the immune activation is less efficient (Bosi and Trevisi, 2010) and therefore would also be deleterious in a disease situation.

This background could explain the results observed in our studies. It can be hypothesized the use of the probiotic when animals were exposed to a bacterial challenge led to a further epithelial stimulation. Thus, in a context of increased permeability, it could enhance immune response and even provoke sepsis in some animals, despite the observed reductions in pathogen loads in some cases. Besides, although probiotic mechanisms of action have already been described and will not be addressed in this section; a further remark should be done for the probiotic effects of increasing IEL. The IEL are a functionally heterogeneous population, which contains cells with antitumor activity, natural killer activity, allospecific cytotoxic T lymphocytes or their precursors and mast cells. It is well established that they have a relevant paper in gastrointestinal immune system (Ogra et al., 2012). However, regretfully the parameter IEL is too vague to further characterize the immune stimulation exercised by the probiotic, as we cannot determine which kind of response was exercised by each treatment (Hoang et al., 1997). Hence, the hypothesis of an immunosuppressed status of the animals that precluded an efficient immune response of the challenged animals cannot be ruled out.

On the whole, we can conclude that variability observed on probiotic outcomes can be due to the context where it is applied, being a potential risk the use of certain probiotics in animals with a damaged gut health or pathogen pressure.

In view of the situation, we speculate that setting up a strategy to minimize the risk of the probiotics at this stage would probably enhance positive outcomes of probiotic treatments and make them more consistent. From our point of view, the first step to address this issue would be to build clear differences whether probiotic usage is intended as a therapy or as prophylaxis. For instance, in human studies a clear distinction is done between research aimed to maintaining health and that aimed at treating a disease and this difference has important implications when designing trials and on regulatory affairs (Hill et al., 2014). Foods and supplements can be aimed to healthy population for disease prevention or quest for optimal health, but if used as a treatment they are considered drugs, the study has to be performed in population representative of that disorder and must conform to the standards of a pharmaceutical product (Kopp-Hoolihan, 2001; Sanders et al., 2013).

Secondarily, strategies in order to reduce their detrimental effects or enhance their positive effects should be undertaken with probiotic treatments. The first step should be a careful selection of the probiotic strain based on the current knowledge. As mentioned, probiotic

effects are strain dependent and there are some strains that have not been reported to be detrimental in any case, such as in Chapter 7. Another possibility would be to combine probiotics with complementary actions. For instance, as observed in Chapter 5, in a similar challenge situation the combination of bifidobacterial probiotics (including the strain discussed before) presented a positive outcome in both, challenged and non-challenged animals. Finally, other alternatives to potentiate effects could be the addition of specific prebiotic substrates or maybe in a near future genetic manipulation of the strain.

To sum up, we conclude a systematic approach should be taken when designing the probiotic intervention to characterize potential risks factors of the target animals and the suitability of a particular probiotic strain. However, we recon this process is difficult in pig production were a collectivity is being treated. From our point of view, more basic research is needed to further characterize the mechanisms of action of probiotics and their interaction in different gut health situations. Eventually, we are confident that when sufficient evidence is build up, we will be able to make reliable recommendations for every particular situation and hopefully, some common mechanisms of action will be described as useful in a wide range of situations as well. When we get to this point, we will be using probiotics correctly to control post-weaning diarrheas.

8.2. Are probiotics useful to increase performance of weanling animals?

Increased animal performance with a probiotic treatment is desirable because the main goal of porcine production is to obtain healthy adult slaughter-weight pigs with the minimum expense of feed and growth days. However, probiotics are zootechnical additives, in the category of digestibility enhancers or gut flora stabilizers for healthy animals to help weaning piglets and are not intended to increase body weight gain (Bernardeau and Vernoux, 2013). Still, some probiotic treatments have reported increased performance in weanling animals (Ahmed et al., 2014; Bhandari et al., 2010; Casey et al., 2007; Mallo et al., 2010; Yu et al., 2008). Should we expect increased performances with probiotic treatments? Focusing on our own data obtained on a regular, non-challenged situation, we will analyze whether the probiotic is directly capable to increase the animal performance of weanling animals and what mechanisms could be presumably involved. Mean values of the

parameters will be presented to illustrate the discussion, however, no statistical analysis will be presented as the low replicates of these groups (n=4) preclude the potency of the test to achieve meaningful comparisons.

To start with, Table 8.2 recalls performance data through the entire experiments (approximately 2 weeks post-weaning). Small consistent numerical improvements were observed in animal performance, especially in G:F ratio and when the ADFI data was analyzed as repeated measures (data not shown). It is worth to mention, that important (and significant) changes on performance due to feed additives in this type of trials are rarely seen because the number of animals or the replicates are normally insufficient, taking into account the variation coefficients of the piglet's weight (Aaron and Hays 2003). Moreover, the environment is not the adequate to maximize the effects (Kenny et al., 2011).

Table 8.2. Performance data of experiments reported in chapter 4 and 5.

Chapter	***Trial***	***Probiotic Treatment***	***Control (NN groups)***	***Probiotic (NP groups)***
			ADFI (g/day)	
4	*Salmonella*	*B. infantis*	328	360
4	ETEC K88	*B. infantis*	222	233
5	*Salmonella*	B. combination	383	380
			ADG (g/day)	
4	*Salmonella*	*B. infantis*	198	220
4	ETEC K88	*B. infantis*	120	120
5	*Salmonella*	B. combination	185	188
			G:F	
4	*Salmonella*	*B. infantis*	0.60	0.62
4	ETEC K88	*B. infantis*	0.49	0.52
5	*Salmonella*	B. combination	0.47	0.50

Pooled performance data through the entire experimental periods (approximately 2 weeks post-weaning) of the non-challenged groups (n=4).

These increases could be due to a direct probiotic effect. A higher nutrient availability for the animals treated with the probiotics cannot be discarded; however, the short-time treatment and the limited probiotic colonization observed would not support this hypothesis. Alternatively, probiotics could play a role in energetic costs of immune response, as it will be discussed further on. Furthermore, it is generally accepted that health effects and zootechnical effects are closely related (Bernardeau et al., 2006). Hence, considering the post-weaning status of the animals, an indirect consequence of the

alleviation of post-weaning stresses and ameliorated health status could reasonably be responsible for such improvements.

For instance, Table 8.3 summarizes the total concentrations of SCFA on the previous groups. Animals receiving probiotic generally had higher SCFA concentrations. This effect is commonly reported with probiotic treatments and has been attributed to an increase in the fermentation due to a healthier microbial profile and alleviation of weaning stresses (Nagpal et al., 2012; Patil et al., 2015). Furthermore, increased SCFA have been reported to higher nutrient availability for pig gut (Scheppach, 1994). The only exception would be the Day 8 PI when assessing the Bifidobacterial combination. However, in this case, as already discussed in Chapter 5, a lack of fermentative substrate due to increased fermentation levels in the ileum can explain the reduction. As for the SCFA profiles (data not shown), significant differences were rarely seen on the proportions of the different SCFA. From our point of view, this fact is mainly attributable to the high variability observed in the results, which in turn, is probably related to a different microbial colonization pattern on the animals. In addition, a better gut health status was also suggested by the mean fecal scores, that were significantly lower with the probiotic combination and numerical differences were reported with the single strain (data not shown).

Table 8.3. Total concentrations of SCFA reported in chapter 4 and 5.

SCFA *(mmol/kg)*	**Chapter**	**Trial**	***NN***	***NP***
			B. infantis	
	4	*Salmonella*	127.4	141.7
	4	ETEC K88	118.7	133.2
			***B.* combination**	
	5	*Salmonella*	138.3	130.8

Pooled total concentrations of total SCFA (mmol/kg) of Days 4 and 8/9 PI. Non-challenged groups (n=4).

Secondly, Table 8.4 summarizes the concentrations of Pig-MAP and TNFα recorded in these groups. It can be observed that the probiotic treatments provoked an immune-suppressive response by decreasing concentrations of both pro-inflammatory products. Immune stimulation represents a loss of energy in terms of mounting an immune response. Hence, the probiotic anti-inflammatory properties reported could contribute to a higher energy availability. However, although these responses were quite consistent, significant

differences detected were scarce. Probably the reason is the high residual variability observed in these immune response parameters.

Table 8.4. Total Pig-MAP and TNFα concentrations reported in chapter 4 and 5.

Chapter	Trial	*NN*	*NP*	*NN*	*NP*
		Pig-MAP (mg/ml)		**TNFα (pg/ml)**	
			B. infantis		
4	*Salmonella*	0.84	0.69	91.5	83.6
4	ETEC K88	0.82	0.71	77.05	63.55
			***B.* combination**		
5	*Salmonella*	1.24	1.08	75.8	69.6

Pooled total Pig-MAP and TNFα concentrations of Days 4 and 8/9 PI. Non-challenged groups (n=4).

Finally, another explanation could be found on the epithelial integrity of the gut, as it is the first line of defense of the gut and can also influence greatly on immune status and nutrient absorption. We studied histomorphological parameters in order to evaluate indirectly barrier function, and interesting information can be seed with the response observed on the GC. Consistent reductions of GC were observed with the probiotic treatments in the different trials although no significant changes were detected, once again, probably due to the limited amount of animals and high residual variability. GC produce mucin forming a protective layer on the epithelium preventing direct epithelial contact with luminal microorganisms (Specian and Oliver, 1991). Some probiotics have been described to enhance mucin production (Caballero-Franco et al., 2007; McCracken and Lorenz, 2001), yet in our non-challenged animals, the decrease in GC could be related to a decreased mucin production. Hence, on these animals it could be speculated that there was a healthier barrier with less need for protection or perhaps it was a consequence of the immune suppression previously reported.

Table 8.5. Total goblet cell (GC) counts reported in chapter 4 and 5.

	Chapter	Trial	*NN*	*NP*
GC *(Nº cel/ 100 µm)*			***B. infantis***	
	4	*Salmonella*	1.23	0.97
	4	ETEC K88	1.33	1.16
			***B.* combination**	
	5	*Salmonella*	0.69	0.55

Pooled total goblet cells (GC) numbers of Days 4 and 8/9 PI. Non-challenged groups (n=4).

Altogether, our results suggest that even if we observed increases in animal performance, an important part of it could probably be attributable to probiotics effects downregulating immune response and mitigating the post-weaning syndrome. This effects would be preventing energy loses and eventually helping animals to better express their growth genetic potential.

This observation is not only relevant in pig production, but also for human related studies. In humans, obesity is becoming one of the most serious health problems in modern societies (Marie et al., 2014). Thus, the use of probiotics with growth promoting effects in the farming industry in addition to scientific evidence that obesity may be related to a specific microbiota profile (Delzenne et al., 2011; Ley et al., 2006) has led some scientist to question whether probiotics can be regarded as safe for humans (Raoult, 2009). Interestingly, the use of probiotics in humans has been in many cases intended to fight against obesity and reduce the metabolic syndromes associated. Can probiotics be appropriate for both objectives?

Our experimental results suggest that even if detecting increased performance, it would probably be related to a healthier status achieving optimum growth rate rather than fat deposition leading to obesity. Therefore, paradoxically, probiotics can be used in pig production with the aim to increase body weight and in obese humans to relieve their situation. This is plausible because the target for the use of probiotics is in both cases to ameliorate health status and stress response, in pigs associated to weaning and in humans associated to metabolic syndrome.

8.3. Experimental models of disease: need of sensible indicators

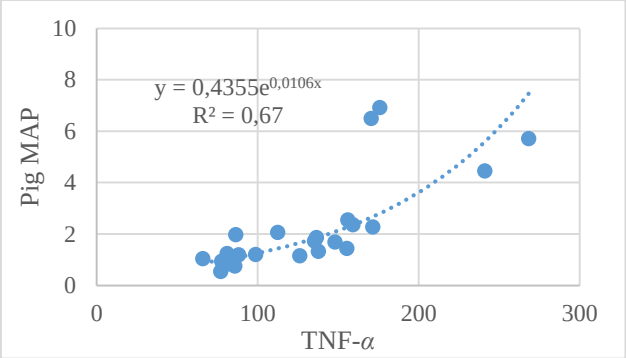
Experimental models aim to resemble in a repeatable way what occurs in natural conditions, in our case, early gut development stresses caused by weaning and potential opportunistic pathogen pressure. From our point of view, an appropriate disease model to study a probiotic strategy is one that promotes moderate diarrhea without precluding treatments to colonize and act in the gut, as it has been described as a requirement for many probiotics (Gareau et al., 2010; Oelschlaeger, 2010). Moreover, for ethical and economic reasons, models should use the minimum amount of animals possible, a characteristic that is especially important when animals are induced to a disease state (such as in our trials).

In this context, we need highly sensible indicators in order to assess animal status and extract useful information. Classic indicators assessed in these types of trials are performance, which as commented before can give a broad idea of the health status of the animals; SCFA, to assess fermentation profiles; histomorphology, to assess gut function and epithelial integrity and pathogen recounts in the case of experimental infections. However, many times these indicators fail to present significant differences probably to the high residual variability they present and the low number of animals used. For this reason, a secondary objective of this Thesis was to develop new sensible tools to evaluate the response of the animals to probiotics under experimental models.

On a first instance, a set of blood parameters was evaluated with a portable blood analyzer. As already reported in Chapter 6, good correlations were obtained with the challenge and the probiotic treatment, turning out to be sensitive methods to evaluate the probiotic treatment effects. Moreover, when the parameters were correlated with the body weight interesting correlations were seen, indicating that they were good descriptors of piglet productive status. Furthermore, we would like to discuss whether they were good indicators of the immune response and clinical status of the animals. Systemic immune parameters assessed in our studies were TNFα, a pro-inflammatory cytokine produced predominantly by activated macrophages and involved in the up-regulation of inflammatory response (Zhang and An, 2007); and Pig-MAP, a major acute-phase protein in swine with a synthesis regulated by pro-inflammatory cytokines that is normally increased by tissue injury or stress (Piñeiro et al., 2009).

Naturally, both of these parameters correlated together, and present a normal distribution in the physiological range with exponential increases in the most seriously compromised animals (see Figure 8.1).

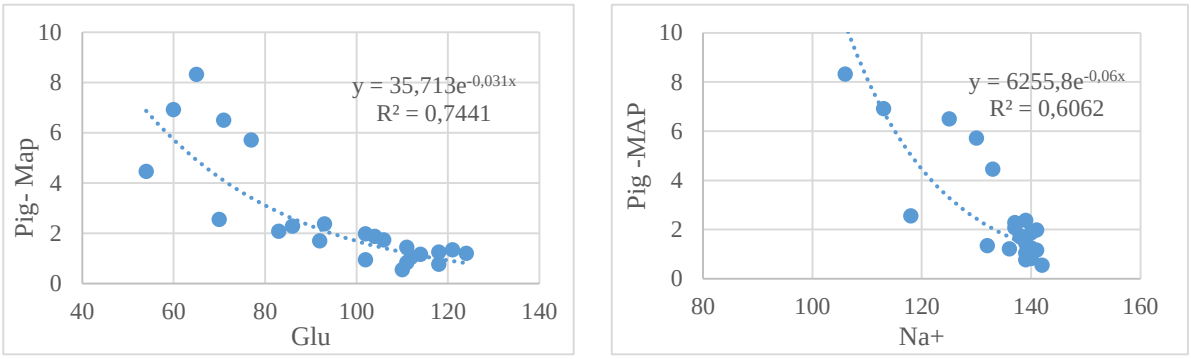
Figure 8.1. Correlation between pro-inflammatory products Pig-MAP and TNFα concentrations reported in chapter 5.



Similarly, electrolytic parameters (Na^+), and chemical biochemistry (Glu) showed a high correlation with inflammatory parameters and succeeded on the identification of animals with a more severe dysregulation. In particular, animals that presented a high inflammatory response had the lowest levels of sodium and glucose in blood (see Figure 8.2). As reported when electrolyte levels were analyzed by body weight gain in Chapter 6, the results suggest the existence of a breaking point and the loose of homeostasis reached only by those animals seriously compromised. Probably, these low concentrations could be explained due to the loss of electrolytes through acute diarrhea or a reduced feed intake if the animals were feeling ill (Nyachoti et al., 2004). In addition, a higher blood glucose uptake of the immune system has also been described when the metabolism is responding against the *Salmonella* infection (Correa-Matos et al., 2003; Sakaguchi et al., 1979), indicating those animals would be more seriously compromised. In any case, these more severely affected animals are the ones with worse prognostic; hence, it is interesting to further characterize them because it permits not only to assess the treatment mechanisms of action, but also could be used to build therapeutic strategies that potentiate the effect of the treatment if needed.

Alternatively, behavior analysis has also demonstrated to be a sensible tool to consider in this type of trials. It is particularly interesting when studying probiotics, to evaluate probiotics modulation on gut-brain axis. Still, the specificity of this parameter should be further assessed as differences in our trials could also be related to the up-regulation of the immune response and/or variability in the illness stress.

Figure 8.2. Correlation of Na^+ and Glu with pig major acute phase protein (Pig-MAP).



To improve this aspect, behavior data should be correlated with other analysis assessing the gut-brain axis such as gene expression or endocrine pathways (Montiel-Castro et al., 2013). Furthermore, one of the cons related to the behavior analysis was that it was a largely time consuming task, which is undesirable in these type of trials that require a huge workload in a short period. Nevertheless, this situation could be solved and refined by the installation of cameras and processing the images with video tracking systems, which would ease data acquisition and would enable to assess animals through larger time periods (Moll et al., 2007; Noldus et al., 2001).

We speculate this indicator will probably gain a commercial and legislative value in the near future (other than being a good descriptor of animal health status). Governments and customers are everyday gaining more sensibility with the wellbeing of farm animals, and as behavior is intimately related to animal welfare (Temple et al., 2011), the opportunity to assess not only health status, but also welfare status with one indicator, will probably make it a desirable parameter to evaluate in the near future on experimental models of disease.

To conclude, our results suggest that when probiotics increase body weight in weanling animals, it is probably due to a healthier status achieving optimum growth rate rather than fat deposition leading to obesity. In addition, although probiotics are potential alternatives to antimicrobials, more research should be performed in order to build up reliable treatment strategies useful in the wide range of situations present in pig production. To obtain more information related to their mechanisms of action, new indicators used in this Thesis are potentially useful parameters when evaluating feed additives in experimental models of disease.

Chapter 9. Conclusions

1. A daily dosage of 10^9 colony-forming units of the probiotic strain *Bifidobacterium longum* subsp. *infantis* CECT 7210 was able to stimulate the intestinal immune response by increasing ileal IEL and to reduce pathogen loads. Concretely, fecal excretion of *Salmonella* and the mucosal colonization of coliforms were reduced after an oral challenge with *Salmonella* Typhimurium and ETEC K88 respectively.

2. A daily dosage of 10^9 colony-forming units of the probiotic *Bifidobacterium longum* subsp. *infantis* CECT 7210 promoted a different response in feed intake, SCFA concentrations and villous:crypt ratio depending on the microbiological challenge to which the animal were exposed. Whereas feed intake, SCFA concentrations and villous:crypt ratio were increased by the probiotic in the non-challenged animals, if animals were orally inoculated with the pathogens ETEC K88 or *Salmonella* Typhimurium concentrations decreased.

3. When *Bifidobacterium longum* subsp. *infantis* CECT 7210 was combined with *Bifidobacterium animalis* subsp. *lactis* BPL6 in a daily dosage of 10^9 colony-forming units, it improved the beneficial effects of the single strain in front of a *Salmonella* Typhimurium challenge. It not only stimulated ileal IEL and reduced the fecal excretion of *Salmonella*, but also decreased rectal temperature to similar levels to non-challenged animals and improved the villus:crypt ratio. In addition, this combination improved voluntary feed-intake, decreased diarrhea scores and promoted healthier fermentation profiles in weaning piglets, regardless of the microbial challenge to which the animals were exposed.

4. The probiotic strain *Bacillus licheniformis* CECT 4536 in a dosage of 10^9 colony-forming units/kg of feed was able to modify positively the behavior of piglets at weaning with an increase in active behaviors, such as exploring the pen or feeding behaviors particularly in the morning period. The strain was also able to reduce intestinal colonization and fecal shedding of *Salmonella* after an oral challenge with *Salmonella* Typhimurium.

5. Behavior analysis was a sensible tool to be considered in feed additive research, being able to detect slight improvements in the response of the animals and offering complementary information regarding other possible mechanisms of action of these additives.

6. Blood parameters have demonstrated to be good descriptors of pig status. Micro-minerals zinc and cupper were useful descriptors of pig performance. Blood electrolytes (sodium, chloride and potassium) and acid base indexes (bicarbonate, total carbon dioxide and base excess in the extracellular fluid compartment) may enable to detect the most distressed animals. Finally, biochemical parameters assessed (glucose, hematocrit and hemoglobin) are good descriptors of health status and have demonstrated to be sensible simple indexes to be included in experimental designs aimed to evaluate health status and feed strategies in weanlings. However, they may not be useful to identify most severely affected animals.

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