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Prebiotic and probiotic strategies in the prevention and control of post-weaning colibacillosis in piglets

Ph. D, Thesis

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A mis padres

Ten siempre a Ítaca en tu mente.
Llegar allí es tu destino…

Konstantinos Kavafis

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La realización de esta Tesis Doctoral no ha sido sólo el resultado del esfuerzo personal. He contado para ello con el apoyo incondicional de familiares, amigos y compañeros. Hecha esta aclaración, se impone como presupuesto dedicar un espacio a reconocer y agradecer la inestimable contribución de todas estas personas.

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Summary

This Thesis was designed with the main objective of evaluating the potential of some prebiotic, probiotic and synbiotic strategies to control digestive disorders of piglets around weaning. To achieve this purpose, three experiments were designed.

In Trial 1 we first investigated the potential of lactulose, a probiotic strain of *Lactobacillus plantarum*, or its synbiotic combination on the gastrointestinal environment and the performance of piglets after weaning. Animals were randomly divided into four groups and fed either: a control diet, the same diet supplemented with a daily culture of *L. plantarum* (10^9 CFU/ml sprayed on top; 20 ml/pig); a diet with 1% w/v lactulose or its synbiotic combination. The experiment lasted 14 days after which 8 piglets from each group were euthanized. Digesta from proximal colon was collected and pH, short-chain fatty acids (SCFA), microbiota composition were analyzed. Furthermore, blood urea nitrogen (BUN) and acute-phase proteins (Pig-MAP) were measured.

The trial 2 with a similar design to trial 1 attempted to evaluate whether lactulose, *Lactobacillus plantarum*, or its synbiotic combination could reduce the diarrhoea incidence and modulate the intestinal microbiota after an experimental challenge with enterotoxigenic *Escherichia coli* (ETEC) K88. For this, 72 weaning piglets were randomly assigned to one of the 4 treatments in the same way as trial 1. The pigs were orally challenged after receiving the supplemented diets for 7 days. Body weight, feed intake and diarrhoea incidence were measured. Six pigs per treatment were euthanized on days 6 and 10 post challenge (PC) and samples digesta of ileum and colon were collected for the analysis of lactic and SCFA and the quantification of different microbial groups. Ileal mucosa was also sampled for the analysis of adhered enterobacteria and *E*. *coli*. Ileal tissue and serum samples were also taken for histological studies and TNF-α and Pig-MAP determination.

Finally in **the Trial 3 it** was evaluated the prebiotic potential of a carob seed product to enhance the piglet gut health and performance after an experimental infection with ETEC

K88. A total of seventy two 28 day-old commercial piglets were used in a 15 days experiment. The treatments consisted of a control diet, the same diet to which 5 g/Kg of a product from carob seed was added, and a third diet with 3 g/Kg of ZnO as positive control. Body weight, feed intake and diarrhoea incidence were measured. Four and 8 days PC 8 pigs per treatment were euthanized and different samples taken similarly to trial 2.

Lactulose as prebiotic for weaned piglet

Results showed that addition of lactulose improved the feed intake, increased the lactic acid bacteria in ileum and at the same time reduced enterobacteria:lactobacilli ratio in colon. The colonic fermentation turned on more butyrogenic and less proteolytic with reductions on the BCFA and blood urea nitrogen**.** All these changes were also observed under challenging conditions. Moreover we detected other benefits on intestinal health indicators including: increases of ileum villous height or reductions on Pig-MAP concentration. All these benefits could be behind the improved weigh gain observed in the piglets during the post-challenge period.

Role of *Lactobacillus plantarum* on preventing and/or controlling of ETEC K88 infection.

Regarding supplementation of *Lactobacillus plantarum* the results demonstrated its ability to survive and colonize the distal part of gastrointestinal tract of weaned piglet even under ETEC K88 infection. The probiotic promoted a shift in the number and diversity of lactobacilli, with a decrease on the enterobacteria:lactobacilli ratio and on the percentage of branched fatty acids suggesting a reduction in the proteolytic microbial activity. The inclusion of the probiotic showed to diminish the incidence of diarrhoea ($P<0.05$), although we were not able to detect reductions in the *E. coli* K88 counts. Together to a possible control of enterobacteria, beneficial effects of *L*. *plantarum* could also be due to others mechanisms such as: (i) a promotion of a more healthy fermentation profile (mainly observed in colon with a lower ammonia concentration and increases in total SCFA and in the percentages of

butyrate), (ii) an improved barrier function (increased villous height and goblet cells), or (iii) a modulation in the inflammatory response, (reduction in plasmatic TNF and in IEL in ileum).

Synbiotic combination

Positive results obtained with both lactulose and *L*. *plantarum* were added in the synbiotic combination; despite that lactulose was not able to promote any additional increase in the numbers of *L. plantarum*. Therefore it was concluded that this combination could act as complementary synbiotic, adding the beneficial effects of each additive, but not as a synergic combination.

Efficacy of carob seed product on improving the health status of piglets after weaning.

The supplementation with the carob seed product reduced the diarrhoea incidence and the numbers of enterobacteria and *E. coli* in the hindgut and attached to ileal mucus. These results were associated: with increases in total SCFA and lactic acid in colon and a faster recovery of acute inflammatory response in similar manner to ZnO treatment (reducing IEL and serum Pig-MAP concentrations). These effects could be due to an indirect effect through reinforcement of the indigenous microbiota of piglets and also to a possible direct action interfering with the pathogen adhesion mechanisms to the intestinal epithelium, considering the high content of galactomannans of carob seed.

The results exposed in this thesis have highlighted that the use of prebiotic and probiotic may be a way to improve the adaptation of piglets around early weaning. These benefits are associated with the ability showed by *L*. *plantarum* to increase the population and activity of lactobacilli in order to prevent and/or controlling the incidence of digestive disorders. Lactulose demonstrated prebiotic effects with increases on lactobacilli, and promotion of a fermentation profile more butyrogenic and less proteolitic, increasing the ileal villous height. Dietary intervention with the carob seed product reduce the enterobacteria

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CHAPTER 1

General Introduction

1. General Introduction.

Weaning at early ages (21-28 days) is one of the most critical moments for the intensive pig production systems, because piglet suffers the stress of being abruptly separated from the sow and the transition to cereal based feed, which causes significantly reduced energy intake for maintenance of epithelial structure (Lallès *et al*., 2007). Damage to the epithelial layers also decreases nutrient digestibility which provides more substrates for pathogen proliferation (Pluske *et al*., 2002) and increases production of epithelial irritants such as ammonia (Heo *et al*., 2009). Furthermore the sudden removal of the maternal milk supply leads to the progressive withdrawal of the maternal protective IgA that acts locally in suckling piglet intestine (Gallois *et al.,* 2009). All these factors, when combined, can affect piglet immunity function, and negatively disturb the intestinal microbiota equilibrium.

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. Weaning of piglets, however, can lead to a disturbance of the ecological balance in the intestinal microbial community (Kostantinov *et al*., 2006). Indeed, changes in gut microbiota composition and activity often result in the lowering of resistance to endogenous and exogenous opportunistic pathogens, the weakening of digestion capability and poorer nutrient adsorption (Pluske *et al.*, 2002). Thereby increasing the susceptibility to gut disorders, infections and diarrhoea (Lallès *et al*., 2009)

Until few years ago, the efforts to prevent these intestinal diseases in piglets have been largely through the widespread use of antibiotics at sub-therapeutic doses or high dosis of minerals (pharmacological doses), especially ZnO and CuSO4 (Bomba *et al*., 2006; Heo *et al*., 2012). However, antibiotics can depress beneficial organisms in the gut, and can lead to the development of antibiotic-resistant strains of animal and human pathogens (Dibner and Richards, 2005). For this reason the use as antibiotic growth promoters has been completely banned throughout Europe since 01 January 2006 (Regulation (EC) No

1831/2003). Regarding the use of pharmacological doses of Zn or Cu, there are also concerns about their environmental accumulation throughout pig manure. Therefore, researchers in animal nutrition have as challenge the finding of alternatives that replaces to in-feed antibiotics as mean to prevent these digestive disorders.

As has been suggested, the dietary manipulation of the intestinal microflora could be a potential alternative to prevent the post-weaning disorders (Heo *et al*., 2012). One possible approach to obtaining desirable microflora and improved gut balance is to optimize the potentially beneficial components of weaner diets through the consumption of micro-ingredients such as probiotics, prebiotics and/or synbiotics (Gaggia *et al*., 2010). The main effects attributed to these feed additives are the improved resistance to pathogenic bacteria colonization and an enhanced host mucosa immunity; thus resulting in a reduced pathogen load and an improved health status of the animals. However, the mechanisms involving these possible effects are not completely described in weaned piglet, and is real potentiality to improve growth and increase health in challenge commercial conditions needs to be studied more in deep (Gaggia *et al*., 2010).

In the following chapters we will review the patogenia of post-weaning diarrhoea and the most recent evidences on the use of probiotics, prebiotics and synbiotic to prevent this problem.

CHAPTER 2

Literature Review

2.1. Post-weaning diarrhoea (PWD).

As we have seen, post-weaning diarrhoea and growth check are widespread problems in swine production. Generally, it is seen as yellowish or grey fluid and most commonly starts 3 to 5 days after weaning, lasting up to a week and causing emaciation, reduced feed intake, reduced nutrient digestibility, which may lead to dehydration, metabolic acidosis, and death (Gyles and Fairbrother, 2010). Many theories have been proposed as to why disease occurs at weaning. One hypothesis is the sudden deprivation of maternal antibodies and other protective factors in the sow's milk. Another possibility is sudden changes in diet and/or a compromised metabolism (Gyles and Fairbrother, 2010). The social stresses from mixing and fighting and crowding could also have a role, triggering cortisol release, most likely increasing transit time and depressing the immune response to bacterial infection. Also other factors to be considered could include a poor management with poor hygiene conditions, environmental temperature of less than 25 ° C, or excessive air currents.

This disease is usually caused by opportunistic pathogens, particularly pathogenic enterotoxigenic *Escherichia coli* ETEC (Fairbrother *et al.,* 2005) although the presence of other pathogens such as rotavirus in the environment increases the likelihood and severity of disease occurring (Pluske *et al*., 2002).

2.2. Enterotoxigenic *Escherichia coli* (ETEC) as main agent.

Various infectious agents cause diarrhoea in piglets and one of the most common of these agents is *Escherichia coli* (Fairbrother *et al*., 2005). *E. coli* is the type species of the genus *Escherichia*, which contains mostly motile gram-negative bacilli within the family *Enterobacteriaceae* and the tribe *Escherichia.* Pathogenic *E. coli* strains have been divided into different pathotypes based upon the diseases that they cause, the virulence factors that they possess, and their host of isolation (Karper *et al*., 2004) being the enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin (Stx) – producing *E. coli* (STEC),

and extraintestinal pathogenic *E. coli* (ExPEC), the most important pathotype affecting to piglets (Table 2.1).

Table 2.1. Major types of *E. coli* Implicated in piglet disease (Gyles and Fairbrother, 2010).

Disease	Pathotype	Virotypes	O serogroups
Neonatal diarrhoea	ETEC	STa:K99:F41,STa:F41, STa:987P, LT:STb:EAST1:K88ac, LT:STb:STa:EAST1:K88ac, STb:EAST1:AIDA	8,9,20,45,64,101,138,141, 147,149,157
Postweaning diarrhoea	ETEC	LT:STb:EAST1:K88ac, LT:STb:STa:EAST1:K88ac, STa:STb, STa:STb:F18ac, STa:F18ac	8,138,139,141,147,149, 157
	EPEC	Eae, Tir, EspA, EspB, EspD, EspC (enterotoxin)	45,103,123
Edema disease	STEC	Stx2e:F18ab:(AIDA), _Hly + 138,139,141	138,139,141
Extraintestinal colisepticemia	SEPEC	Aerobactin, F165 - 1 (P fimbrial family), F165 - 2 (S fimbrial family), CNF1 or CNF2, CDT	6,8,9,11,15,17,18,20,4 60,78,83,93,101,112, 115,116
Urogenital tract Infection	UPEC	P,S, aerobactin, CNF1	1,4,6,18

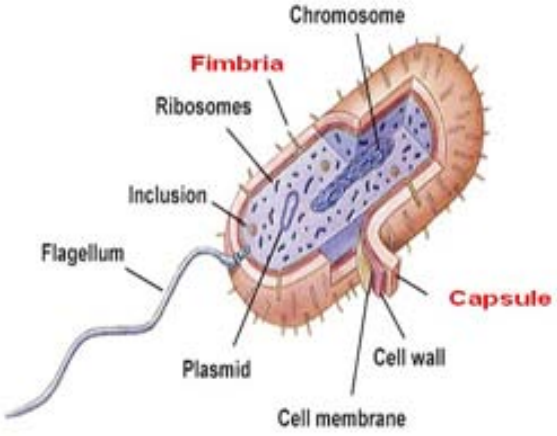
It also known that in most *E. coli* diseases, pathogenicity is associated with virulence genes encoded by plasmids, bacteriophages, or pathogenicity islands (PAIs). These genes include the plasmid-encoded genes for enterotoxins and fimbriae or pili; the phage– encoded genes for Stx, and the PAI-encoded genes for the attaching and effacing lesion (AE) in EPEC and enterohemorrhagic *E. coli* (EHEC). Other genes include the *pap*, *hly,* and *cnf1* in uropathogenic *E. coli* (UPEC), which is subsets of the STEC and ExPEC pathotypes, respectively (Gyles and Fairbrother, 2010).

E. coli are serotyped based on differences in the O, K, and H antigens determined by the polysaccharide portion of lipopolysaccharide (LPS), capsular polysaccharide, and

flagellar proteins, respectively. The K antigens are no longer routinely determined but more usually O and H antigens. At present there are 174 O antigens (O1 – O181, with O groups 31, 47, 67, 72, 93, 94, and 122 removed) and 53 H antigens (H1 – H56, with 13, 22, and 50 unassigned) in the international typing scheme. In addition another nomenclature is paralelally used including the classification of strains from the identification of F or fimbrial surface antigens (Gyles and Fairbrother, 2010). The F antigen is therefore often added to the serological formula for a strain, especially for ETEC of animal origin.

Porcine ETEC (Figure 2.1) involved in PWD typically belong to serogroups O138, O141, O147, O149 and O157 being the most commonly found in several countries (Francis, 2002; Vu Khac *et al*., 2006) of which O138, O141 O149 carrying the F4 (K88) adhesin seems to be the predominant serogroup in Spain ETEC (Garabal *et al*., 1996; Garabal *et al*., 1997).

Figure 2.1. Schematic structure of *E. coli* ETEC



2.2.1. Pathogenesis of *E. coli* post weaning diarrhoea

Newborn piglets ingest ETEC found in their environment; especially the mammary glands of the mother and the farrowing crate or pen with ETEC diarrhoea, asymptomatic carrier piglets, or sows (Fairbrother and Gyles 2006). Whereas in adult pigs, the stomach

can turn in to an efficient barrier against the entrance of *E. coli* to the gut, in weaned pigs, the pH of the stomach and duodenum is less acidic and the production of digestive enzymes lower, providing a favourable environment for the rapid multiplication of ETEC (Jensen, 1998; Franklin *et al*., 2002).

Once the ETEC pass throughout the stomach and reach the intestine (Figure 2.2), if present in enough numbers, colonize the small intestine (Gyles and Fairbrother, 2010). For that *E*. *coli* attach to receptors on the small intestinal epithelium or in the mucus coating the epithelium, by means of specific fimbrial adhesines. These bacteria then proliferate rapidly to attain massive numbers in the order of 10^9 CFU/g of tissue in the mid jejunum to the ileum. The degree of colonization determines whether or not disease results from infection. Colonizing ETEC produces enterotoxins which stimulate the secretion of water and electrolytes. This leads to diarrhoea if the excess fluid from the small intestine is not absorbed in the large intestine.

Moreover, ETEC infection can lead to increased paracellular permeability of the small intestine (Berkers *et al*., 2003). The loosening of the tight junction due to ETEC infection, therefore, further increases invasion of antigens, toxins and pathogens into the circulatory system and can trigger inflammatory cascades that results in the production of cytokines and chemokins) and recruitment of inflammatory cells (Berkers *et al*., 2003). Infection therefore can cause hemorrhagic gastroenteritis, congestion, and microvascular fibrinous thrombi and villous necrosis into the intestinal lumen (Fairbrother *et al*., 2005).

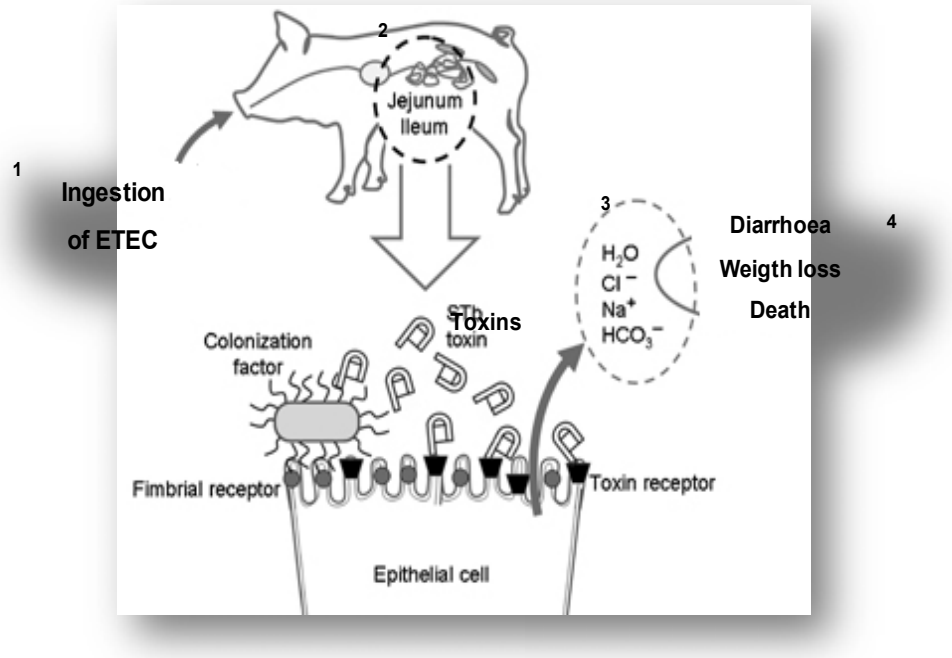


Figure 2.2. Schematic representation of the steps involved in the pathogenesis of ETEC infection. ETEC in the piglets environment are ingested (1), pass through the stomach, adhering to the small intestinal epithelium where they produce enterotoxins (2) that stimulate the secretion of water and electrolytes into the intestinal lumen (3). Loss of water and electrolytes leads to diarrhea, weight loss, and possibly death (4). Adapted to, Gyles and Fairbrother (2010).

2.2.2. Virulence Factors of ETEC

2.2.2.1. Fimbriae associated with ETEC in PWD

The fimbriae are important virulence factors that allow bacteria such as ETEC to adhere to specific receptors on mucosal surfaces preventing their removal by normal intestinal processes such as peristalsis. Fimbriae are 0.5-1.5 microns long proteinaceous appendages peritrichously distributed at the surface of the bacterium that allow adherence of the bacteria to fimbriae-specific receptors. There are between, 100-300 fimbriae per bacterium or even to 1000, and differ from pili which are rigid hollow structures with a diameter of 7-8 nm. Specifically F4 fimbriae have a variable appearance from a thin, flexible and extended structure to a wider, rigid and more condensed form (reviewed by Van den Broeck *et al.*, 2000). Besides of F4, ETEC possess four different fimbrial subtypes, F18, F5,

F6, and F41. The F5, F6, and F41 fimbriae are more often associated with *E. coli* causing neonatal diarrhoea, whereas the F4 and F18 adhesins are found on ETEC that predominantly causing post-weaning colibacillosis (Nagy and Fekete, 2005). These fimbriae have been named based on such criteria as strain of origin (F41, 987P, F107) or assumed structure (K88, K99). As explained above the system of F numbers was devised to designate fimbrial adhesins in the same way as the K or O antigens, and both, this nomenclature and the original designations, are used in the literature. Hence, the fimbriae originally named K88, K99, 987P, Fy, and F107, are synonymous with F4, F5, F6, F17, and F18.

The classification of fimbriae is generally performed according the presence and position of various amino acids in the primary sequence of their major fimbrial subunit. Another system for fimbriae classification is based to the ability of fimbriae to agglutinate red blood cells of different species in the absence or presence of 0.5% d-mannose, which allows them to be, classified as mannose sensitive and mannose resistant, respectively (Nagy and Fekete, 2005). Particularly, the F4 biogenesis and production can give rise to three fimbrial variants ‘ab’, ‘ac’, and ‘ad’ of varying lengths: F4ab and F4ad that are 264 aminoacids long, whereas F4ac is 262 aminoacids long (Van der Broeck *et al*., 2000). Between them, F4ac or the equivalent K88ac is the most common type expressed on *E. coli* strains isolated from diarrhoeic pigs (Fairbrother *et al*., 2005).

The antigenic differences among the three K88 variants can be ascribed exclusively to a small number of nucleotide changes in the major fimbrial subunit gene, which results in variants that all share common ‘a’ epitopes but contain unique ‘b’, ‘c’, or ‘d’ that distinguish the three K88 variants (Grange *et al*., 2002). Aside from their unique antigenic differences, all three F4 variants have their receptor binding sites defined by interactions between their variable and conserved regions. Interestingly all F4 fimbrial variants bind the F4 receptor (F4R) with equal avidity (Hacker, 2002; Nagy and Fekete, 2005).

Multiple studies have demonstrated that F4 fimbria (K88) is important for adherence of enterotoxigenic *E. coli* (ETEC) to the pig intestine. Both ETEC expressing F4 fimbriae, as well as purified fimbriae, were shown to be capable of binding IPEC-J2 cells (Koh *et al*., 2008; Johnson *et al*., 2009). The IPEC-J2 cell line, isolated from the jejunum of a neonatal pig, is a reliable cell line for the *in vitro* study of swine intestinal diseases and these cells specifically adhered to K88 ETEC (Koh *et al*., 2008). Additional support for the importance of F4 fimbriae in ETEC infection comes from Geens and Niewold, who used isogenic strains to show that only the strain possessing the F4 fimbriae was able to efficiently bind to *in vitro* IPEC-J2 cells (Geens and Niewold, 2010). Finally a novel study revealed that purified F4 fimbriae were able to be internalized via a clathrin-mediated endocytosis pathway, resulting in transcytosis of the fimbriae (Rasschaert *et al*., 2010). Results from these studies aid to clarify the underlying mechanisms by which the F4 cause damage on tight junction.

2.2.2.2 Enterotoxins of ETEC F4 responsible for post-weaning diarrhoea in pigs

The various pathogenic *E*. *coli* subtypes possess additional genetic material that encodes specific enterotoxins responsible for fluid homeostasis perturbation including secretion. Two major classes of enterotoxins are produced by porcine ETEC: heat labile (LT) toxins and heat-stable toxins (ST) which promotes chloride ions secretion from the small intestinal epithelial cells resulting in diarrhoea (Nagy and Fekete *et al*., 2005).

2.2.2.2.1 Heat-labile enterotoxin

There are two major serogroups of LT among *E. coli* strains: LT-I and LT-II. LT - I can be divided into LT - Ih, produced by human ETEC, and LT - Ip, produced by porcine and human ETEC, while LT-II is typically associated with diarrhoeal disease in several animal species (Zhang *et al*., 2006). However a direct role for LT-II in pathogenicity and virulence of porcine ETEC has not been fully demonstrated (Casey *et al*., 2012). Therefore, this section only focuses on the discussion regarding porcine LT-I.

The *E. coli* LT-I is an 86 kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors (i.e., GM1 that are recognized by the cholera toxin and its enzymatic activity (Tauschek *et al*., 2002). The B subunits bind predominantly to the GM1 ganglioside receptor acting as receptors on the cell surfaces (O'Brien and Holmes, 1996). Once the B subunits have fixed the toxin molecule to the cell surface, a fragment of A domain will translocate into the cell (Fleckenstein *et al*., 2010 and Erume *et al*., 2013) and lead to irreversible activation of adenylate cyclase and increases in intracellular levels of cyclic AMP (cAMP). In intestinal epithelial cells, this induction of cAMP causes a disregulation of cAMP-sensitive ion transport mechanisms, inhibiting intracellular salt absorption, increasing electrolyte transport into the gut lumen, and creating an osmotic gradient favouring intestinal water secretion (Nagy and Fekete, 2005).

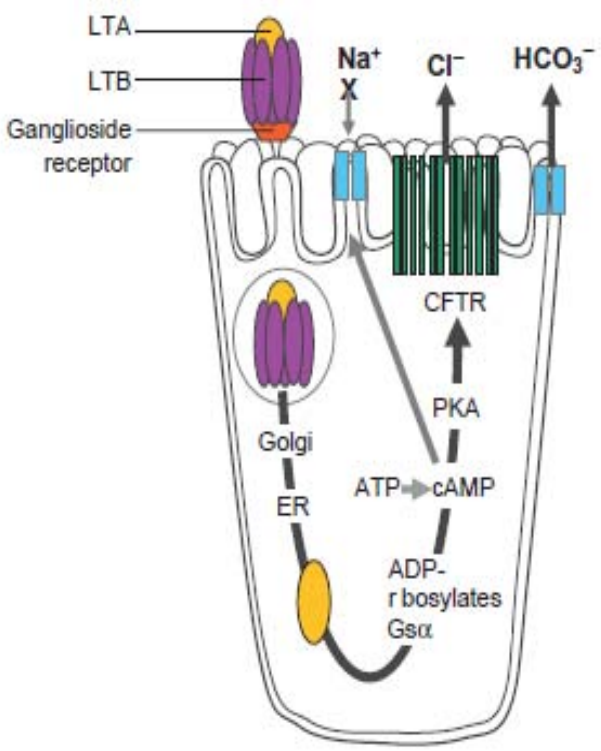


Figure 2.3. Major mechanism of action of LT. LT binds GM1 and other gangliosides, resulting in internalization by receptor-mediated endocytosis. Following transport to the Golgi and the ER, LTA1 is translocated to the cytosol where it ADP-ribosylates the regulatory protein Gsα which irreversibly stimulates production of cyclic AMP. Elevated levels of cAMP increase production of c-AMP-dependent protein kinase A, leading to phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR activation leads to secretion of Cl^- from secretory epithelial cells in the crypts, which stimulates $HCO3^-$ secretion from the $Cl^-/HCO3^-$ exchanger in villus epithelial cells. (reviewed by Gyles and Fairbrother, 2010)

Functions other than enterotoxicity including promotion of adherence, down-regulating innate host responses and inducing apoptosis capacity of lymphoid cells have been ascribed to LT (Fleckenstein *et al*., 2010). Recently, Santiago Mateo *et al.* (2012) showed that pre-inoculation with an avirulent strain expressing adhesive fimbriae and a non-toxic form of LT resulted in not only a reduction in severity of diarrhoea but also in colonization reduction of the intestine of weaned piglet. These findings strongly suggest that LT may also play a role in adhesion (Qi *et al*., 2011) by a mechanism that appears to require the ADP-ribosylation activity of this toxin (Jhonson *et al*., 2009).

To conclude, ETEC LT+ strains also produce STb as both genes are frequently on the same plasmid (Zhang *et al*., 2006). For example, of 111 LT+ ETEC examined by Frydendahl (2002), 110 also possessed the gene for STb. In addition, evidence suggested that LT is major contributor of virulence than STb in ETEC K88 infection (Casey *et al*., 2012).

2.2.2.2. Heat-stable enterotoxin

Regarding to STs they are small and monomeric molecules and may be associated with either human or animal disease. STa and STb are the two classes of STs first recognized and differ from each other in both, structure and enzyme activity. STa is produced by porcine and human ETEC as well as other bacteria, while STb is found only associated with porcine ETEC (Nagy and Fekete *et al*., 2005).

The STa **(**also called STI) is an 18 - or 19 – amino acid peptide of about 2-kDa which binds to a guanylyl cyclase C (GC - C) glycoprotein receptor on the brush border of villous and crypt intestinal epithelial cells and activates guanylate cyclase, which stimulates production of cyclic GMP (cGMP) (Giannella and Mann 2003 ; Turner *et al*., 2006).

Elevated levels of cGMP in the cell activate cGMP-dependent protein kinase IIresulting in phosphorylation of the chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR). Thereby, reducing the absorption of water and electrolytes

(Na^+) on villous tips and simultaneously increasing the secretion of Cl^- and H_2O in crypt cells.

Based on the concentration and affinity of the STa receptors, the posterior jejunum appears to be the major site of hypersecretion in response to STa. However, there is good evidence that STa binds to other receptors and that it has other activities but their relationship to diarrhea is not known (Sellers *et al*., 2008). We must remark that STa activity in newborn pigs is greater than in weaned piglets, consistent with decreasing affinity and density of STa receptors with increasing age. Not surprisingly, ETEC strains that produce STa as the only enterotoxin are associated with disease in neonatal pigs (Nagy and Fekete *et al*., 2005).

The STb is a 48-amino acid peptide of about 5 kDa, which has four cysteine residues involved in two disulfide bridges and is heat-stable but susceptible to degradation by proteolytic enzymes. The STb gene has been associated with of cases of neonatal diarrhoea and 81.3% of PWD (Chapman *et al*., 2006). The mechanism by which STb causes accumulation of fluid in the intestine is not known but it appears to involve an increased production of serotonin and prostaglandins E2 from intestinal cells (Figure 2.4) resulting in increased levels of cAMP leading to diarrhoea elevation ((Dubreuil, 2008).). Moreover, it has been suggested that fluid accumulation by the effects of STb, may lead to decreasing bacterial adherence (Eureme *et al*., 2012). Thereby, this and other recently results confirm a dominant role for STb on induced apoptosis (Jhonson *et al*., 2009; Zhu *et al*., 2011), as well as in the induced innate immune response through differential regulation of immune mediators like interleukin 1 and interleukin 17 (Loos *et al*., 2012)

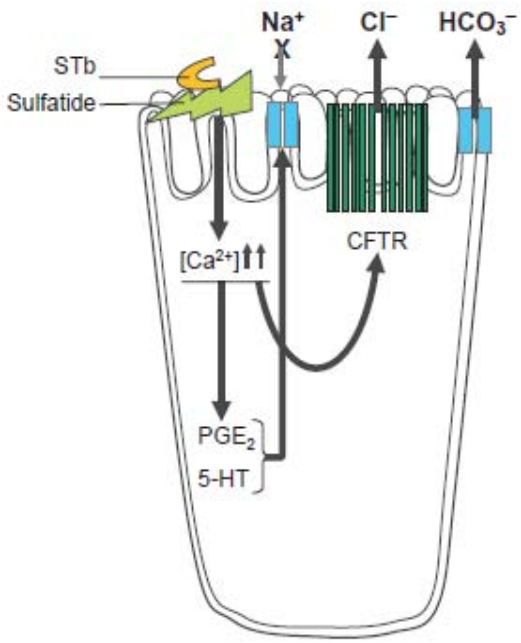


Figure 2.4. Mechanism of action of STb Binding of STb to its receptor sulfatide leads to elevated intracellular levels of calcium. The high calcium levels lead to activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and the secretagogues prostaglandin E2 (PGE2) and 5-hydroxytryptamine (5-HT). These lead to secretion of−Cland HCO3 and inhibition of absorption of Na+. (reviewed by Gyles and Fairbrother, 2010

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2.2.2.3. Others virulence factors.

Bacterial plasmids are self-replicating, extrachromosomal replicons that are key agents of change in microbial populations (Frost *et al*., 2005). Naturally occurring plasmids are able to promote the dissemination of a variety of traits including drug resistance, virulence, and the metabolism of rare substances (Table 2.2). Several types of *E. coli* virulence plasmids exist, including those essential for both, resistance and virulence of ETEC K88 (Gyles and Fairbrother, 2010). In the review conducted by Johnson and Nolan (2009) it was described that as earlier as 1966 Ørskov and Ørskov (1966) found that K88 antigen is encoded by transmissible plasmids that later were associated with the ability to utilize raffinose as a sole carbon source. Indeed, at 1978 Shipley *et al.* (1978) characterized a nonconjugative plasmid linked to genes encoding K88 production and raffinose fermentation. More recently in the genome of porcine ETEC K88 it was found the presence of 17 genes of interest in plasmid encoding to known and potential novel virulence factors, and antimicrobial resistance-associated elements (Shepard *et al*., 2012). In this study there were characterized three porcine ETEC K88 virulence-associated plasmids (pUMNK88_Ent,

pUMNK88_K88, and pUMNK88_Hly) encoding to K88ac fimbrial, LT and STb

Table 2.2. Sequenced virulence-associated plasmids of *E*. *coli* ETEC K88 from porcine origen (Shepard *et al*., 2012)

Plasmid	Virulence factor
pUMNK88_Ent	Heat-labile enterotoxin subunit LT-A and LT-B
	Heat-stable enterotoxin STb
pUMNK88_Hly	Putative invasin
	Hemolysin protein HlyA
	Hemolysin protein HlyD
pUMNK88_K88	Glycoporin RafY lacking transposase insertion
	Glycoporin RafY core region
	Putative malate dehydrogenase
	Putative choloylglycine hydrolase
	K88 minor fimbrial subunit FaeH
	K88 fimbrial chaperone FaeE
	Putative fimbrial protein
	Putative fimbrial subunit
pUMNK88_161	Multidrug resistance
pH10407_95	K88 fimbrial

Particularly, the pUMNK88_K88 is an 81-kb plasmid containing the K88ac fimbrial operon, this portions of the K88 operon was also found in pSE11-3 from human commensal *E. coli* strain SE11 (Oshima *et al*., 2008). In addition in the pUMNK88 it was also described an IncA/C multidrug resistance associated plasmid encoding gene resistance to phenicols (*floR* and *cmlA*), sulfonamides (*sul1* and *sul2*), aminoglycosides (strAB, aadA and aadA2 and mercury (*mer*). IncA/C plasmids have been shown to be highly prevalent among disease-associated porcine ETEC, underscoring their importance in the dissemination of multidrug resistance among bacteria of production animals (Johnson *et al*., 2011).

2.2.2.4. Role of Quorum Sensing mechanisms on virulence of ETEC K88

It is a recent finding that bacteria have the ability of communicating through the production of diffusible signal molecules termed autoinducers (AIs) (Fuqua *et al*., 1994). These molecules, accumulate during growth, and once reach a critical concentration can activate or repress a number of target genes. This is therefore, a phenomenon cell-density-dependent that has been called quorum sensing (QS). Different QS systems have been described during the last years, many of them able to control the expression of virulence genes in numerous micro-organisms (de Kievit and Iglewski, 2000).

Through the use of AIs, bacteria can regulate their behaviour according to population density. This capacity to behave collectively as a group has obvious advantages, 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 *Pseudomona aeruginosa* (Popat *et al.*, 2012)) and *E*. *coli* (Gonzalez-Barrios *et al*., 2006), which may afford protection from deleterious agent.

The LuxS/AI-2 is one of the major QS system found in a wide variety of bacteria, including both gram-negative and gram-positive species (Antunes and Ferreira, 2009). The system produces the universal signalling molecule called autoinducer-2 (AI-2) as a result of the activity of the *LuxS* protein, the product of the *luxS* gene. The *luxS* gene plays a role in pathogenesis in a number of bacteria, including *E. coli* (Caetano *et al*., 2010). Genetic studies in EHEC and EPEC from human origin revealed that *Lux*S controls expression of the type-3 secretion system encoded by the locus of enterocyte effacement (LEE) Pathogenicity Island (Sperandio *et al.*, 1999). This important virulence determinant is required for cause a characteristic histopathology in intestinal cells known as attaching and effacing. Subsequently it was performed a gene array analysis in order to elucidate the role that QS plays in the regulation of EHEC virulence and physiology by comparing a *luxS* mutant strain

of EHEC to wild-type EHEC (Sperandio *et al.*, 2001). The results from this analysis revealed that *LuxS* is a global regulator in EHEC, controlling the expression of over 400 genes. Many of these genes have functions related to bacterial virulence such as cell division, flagellar motility, surface adhesion and Shiga toxin production. However, subsequent research using purified and in vitro-synthesized AI-2 demonstrated that the signalling molecule affecting the LEE and motility was not AI-2 but was a distinct compound designated AI-3 (Sperandio *et al.*, 2003). The AI-3 is produced by the resident intestinal microbiota and this synthesis is not dependent on *Lux*S system (Sperandio *et al.*, 2003). Additional this molecule may be involved in EHEC cross talk with the epinephrine-norepinephrine host signaling through activate a two-component signalling regulator, renamed quorum sensing *E. coli* (QseBC) responsible for the regulation of flagella and motility (Sperandio *et al.*, 2003). Moreover, another two-component regulatory system may control the formation of AE lesions (Reading *et al.*, 2009). This system is composed of the histidine kinase QseE and the response regulator QseF. These two proteins are also required for the translocation of type III secretion system effectors into host cells (Reading *et al.*, 2009). Besides these two-component systems, two LysR-type regulators, QseA and QseD, are also required for the control of LEE expression, revealing that a complex regulatory cascade links quorum sensing and virulence gene expression in *E. coli* (Walters and Sperandio, 2006).

As far as we know the role of QS in ETEC K88 pathogenesis only has been evaluated in the study conducted by Zhu *et al*. (2011). Theses authors demonstrated the role of overexpressing *LuxS* by *E.coli* K88, in causing the cell damage and regulating the expression of enterotoxin geneS. However to reach a clear conclusion on the function of AI-2 in regulating the ETEC pathogenesis, further studies are needed.

2.2.2.5. Expression of receptors on porcine enterocytes.

It is a curious observation that *E. coli* are normally present in the digestive tract of pigs, and even pathogenic serotypes may be present without causing disease. This is because the presence of a pathogen is one of several (but not an exclusive) prerequisites for pathogenesis. Between the diverse pre-requisites that could make of not possible the course of the disease it should be mentioned the presence/absence of certain receptors in the intestinal epithelial cells. For instance it has been described that in the villous brush border of susceptible pigs there are three types of proteins, i.e. mucin-type sialoglycoproteins (IMTGP), an intestinal neutral glycosphingolipid and a transferrin glycoprotein, that facilitates binding of ETEC K88 to intestinal villi leading to colonization and diarrhoeal disease (Van den Broeck *et al*. 2000). These K88 receptors have different specificities that allow them to bind one, two, or all of the ETEC K88 variants. Data from glycoprotein blocking studies indicate that K88ad adhesin recognizes the terminal *N*-acetylglucosamin while the K88ac adhesin recognizes *N*-acetylgalactosamine and galactose (Grange *et al*., 2002).

However not all pigs express in a same way these brush border proteins. As early as 1977, Gibbons *et al*. (1977) showed that the adherence to ETEC K88 was inherited as an autosomal dominant Mendelian trait with the two alleles: *S* (adhesion, dominant) and *s* (non-adhesion, recessive). Hence, there are three genotypes: ss (resistant), SS and Ss (sensitive), being linked to a polymorphism on interesting candidate genes of F4 receptor (F4R) including MUC4 (Pen *et al*., 2007) MUC13 (Zhang *et al*., 2008), MUC20 (Ren *et al*., 2012). Actually, identification of these mutation(s) could be a promising tool, to identification the pigs as F4R positive (F4Rpos) or F4R negative (F4Rneg) and thus predicts the ETEC F4-susceptibility (Fu *et al*., 2012).

As early as 1975 was development a *in vitro* villous adhesion assay as alternative phenotypic method to classify pigs as F4Rneg or F4Rpos (Bosi *et al*., 2004). Sellwood *et al.*

(1975), using this test with ETEC F4ab and F4ac observed two brush border adhesion phenotypes: F4ab$^+$/ F4ac$^+$ and F4ab$^-$ /F4ac$^-$. Subsequently there were described other phenotypes (Bijlsma *et al.,* 1982; Baker *et al.*, 1997) until confirmed at least six adhesion phenotypes (Python *et al.*, 2005). Additionally, more recently Li *et al.* (2007) reported two other adhesion phenotypes: F4ab$^-$, F4ac$^+$ and F4ad$^-$ and F4ab$^-$, F4ac$^+$ and F4ad$^+$. Therefore, these studies have proved that receptor classification is more complex than a simple positive or negative categorization, since the presence of as many as six patterns of K88$^+$ *E. coli* adhesion among brush borders from different pigs suggests the existence of several K88 receptors, expressed individually or in various combinations.

Regarding the F4R expression along the pig life, the enterocyte F4R has been described to be expressed throughout their entire life time. However, their expression decreases between 3 and 8 weeks of age being older pigs less susceptible to disease (Chandler *et al*., 1994). Interestingly the F4R is also found in pig intestinal mucus due to enterocyte turnover their function is to bind ETEC F4 and aid in their removal from the intestine (Conway *et al.,* 1990). Thus, F4R in the mucus could help to protect the neonatal pig from infection with *E.coli*. Similarly to the receptor in the brush border, the presence of F4R in the mucus declines significantly at the time of weaning and ceases by 6 months of age (Chandler *et al*., 1994).

2.2.2.6. Role of the defence systems of the animal.

From birth, the piglet begins to develop non-specific and specific immune mechanisms which allow the recognition and inhibition of potential pathogens in the gastrointestinal tract. This selectivity function of intestinal mucosal barrier is the result of a dynamic equilibrium between their four major components (Table 2.3). A better appreciation of this intricate balance is paramount to the understanding of intestinal immunophysiology. In this aim the following section focuses in a brief description of some of these components,

which could be divided in:

2.2.2.6.1 External barrier function in passive innate immunity.

The mucosal epithelium is the primary barrier between the internal milieu which consists of nutrients and harmful elements such as pathogens and antigens. This epithelial barrier is protected externally by gastric acid and digestive enzymes, nonspecific antimicrobial substances and IgA antibodies secreted into the lumen, and an intestinal mucus layer coating the mucosal surface.

Table 2.3. Main components of the intestinal mucosal barrier

Gastrointestinal Barrier
1. External barrier • Gastric acid • Digestive enzymes • Non specific antimicrobial factors (eg, lysozyme, defensins) • Secretory IgA • Intestinal mucus • Peristalsis
2. Epithelial layer • Tight junctions • Innate immune receptors • Intraepithelial immune cells
3. Indigenous intestinal microbiota • Inhibition of colonization and adherence of pathogens • Stimulation of the intestinal immune system
4. Intestinal lymphoid tissue • Uptake and presentation of antigens • Induction of secretory IgA production • Local and systemic inflammatory responses

The acidic environment of the stomach and upper duodenum is the first line of mucosal defense. Normal intestinal peristalsis is an important defence factor as small itestine obstruction is associated with bacterial translocation in both humans and animals. Once, the

pathogens have overcome this barrier, other component of secreted mucosal are the next in line for intestinal defence.

One of the first physical contacts between the host and the luminal bacteria is the intestinal mucus layer, which covers the mucosal surface. This mucus is a product of goblet cells that actively secrete the mucin glycoproteins. The entrapment of bacteria within the mucus which contains secretory immunoglobulins (sIgA), coupled with peristalsis, results in the rapid expulsion of bacteria from the intestine (Oswald, 2006). During suckling, piglets ingest sows' milk which contains immunoglobulin IgA, being absorbed into the mucus covering the villous surfaces and prevents microorganism from attaching to the villous. After weaning, however, the passive immunity conferred by colostrum and milk is then gradually replaced by the own producing sIgA (Stokes *et al*., 2004).

Moreover the gastrointestinal epithelium includes other specialized cell types, such as Paneth cells, whose activities influence the local microbiota communities. Paneth cells secrete a variety of antimicrobial peptides (also called defensins) and large antimicrobial proteins (e.g. lysozyme and lactoferrin). The antimicrobial peptides act by disrupting the integrity of the microbial membranes through their net-positive charge and their ability to fold into amphipathic structures (Bosi *et al*., 2003). Thus these molecules may prevent the direct access of ETEC K88 to the intestinal mucosa, thereby acting as regulators of microbial density and protectors of nearby stem cells proteolysis.

2.2.2.6.2. Epithelial layer limiting the space for bacterial growth

If these foremost barriers fail to prevent the bacteria translocation, an additional physically barrier may be achieved through tight junctions (TJs) joining epithelial cells. When intestinal junction is compromised, the barrier function can become "leaky" allowing pathogens and toxins to enter the body (Groschwitz and Hogan, 2009).Before in this Chapter, we mentioned that ETEC K88 can adhere to the brush border of intestinal cells,

which may damage the structure of the TJs or adherens junction (Roselli *et al*., 2003). In this sense, in vitro study have confirmed that, following exposure to ETEC K88, disruption of TJs occurs in model epithelial monolayers (Yu *et al*., 2012). Those authors reported that ETEC K88 was able to increased the dextran permeability and a decreased the transepithelial electrical resistance. Besides, they were affected the expression levels of claudin-1, ZO-1, E-cadherin as well as of two signalinng molecules related with the maintaining the integrity of the junctions. The alterations of these parameters clearly evidenced the important damages that ETEC may cause on cell junctions.

2.2.2.6.3. Contribution of indigenous intestinal microbiota to barrier function

The resident microbiota itself forms an integral part of the natural defence system of the host. One of its basic physiological functions is act as microbial barrier against microbial pathogens. This appears to be a multi-factorial process involving:

- Competition for nutrients
- Production of toxic conditions or compounds (low pH, fermentation acids, bacteriocins, etc.)
- Competition for binding sites on epithelial surfaces, or in the tightly adherent mucus layer
- Stimulation of the immune system

The findings obtained by Blomberg *et al*. (1993), give support to the above sentences. Those authors described that indigenous lactobacilli reduced adhesion of *E. coli K88* to piglet ileal mucus (Blomber *et al*., 1993). More recently Roselli *et al*. (2007) reported that porcine *L. sobrius* may reduce the ETEC adhesion to IPEC cells line. Indeed the lactic acid bacteria form a barrier against other microorganisms in the intestine by rapid proliferation (Konstantinov *et al.,* 2004) or by production of antimicrobial molecules including short-chain fatty acids (Jin *et al*., 2000) and bacteriocins (Dobson *et al*., 2012).

2.2.2.6.4. The intestinal epithelium and immunity response.

Despite the multiple components of the external barrier to prevent bacterial contact with the epithelial layer occasional breaches are inevitable. Thus a second layer of the intestinal immune protection is the rapid detection and clearance of bacteria that penetrate the epithelial layer or go beyond it and into the lamina propria. This function is orchestrated by network of lymphoid cells, which is collectively referred to as gut-associated lymphoid tissue (GALT). This includes: intraepithelial lymphocytes (T-cells and B-cells), lamina propria and those aggregated into lymphoid nodules, such as Peyer patches and mesenteric lymph nodes.

The immune system has both innate and adaptive arms (Gallois *et al*., 2009). The innate system includes cells (macrophages, dendritic cells) that mount a non-specific response to the presence of any foreign antigen. The intraepithelial lymphocytes along with dendritic cells in the lamina propria can act as antigen-present cells and do the phagocytosis, against potential pathogenic bacteria and serves to restrict their transfer across the epithelium. In addition, once exposed to a pathogen, the adaptive system produces antibodies that are protective against subsequent infections by the same organism. In essence, bacterial invasion of epithelial cells or breach of the epithelial barrier provides a signal to epithelial cells to initiate responses that are of inflammatory nature in the majority of the cases and whose final goal is the clearance of invading microorganisms (Gallois *et al*., 2009). The mucosal immune system can detect microorganisms by discriminating between mutualism and pathogenicity using a sophisticated system of receptors, including membrane-bound Toll-like receptors (TLRs) and cytoplasmic Nod-like receptors (NLRs). In general, signalling via TLRs or NLRs leads to the production of pro-inflammatory cytokines and chemokines, thereby contributing to host defense and inflammation (Goto y Kiono, 2012). Giving support to this concept both *in vitro* and *in vivo* studies reported the expression

of IL-8 and TNF-α in response to ETEC K88 challenge (Devriendt *et al*., 2010; Nyachoti *et al*., 2012).

2.3. Prevention strategies

As it was appointed in the General Introduction, the main strategy for preventing the occurrence of PWD in piglets has been based on the use of in-feed antibiotics. However, worldwide concern about development of antimicrobial resistance and about transference of antibiotic resistance genes from animal to human microbiota (Mathur and Singh, 2005) led to banning its use as growth promoters in the European Union since January 1, 2006. This has led to increasing interest in alternatives to antibiotics in swine production and to a number of initiatives to evaluate the effectiveness of dietary inclusion of herbal extracts, essential oil, feed enzymes as well as organic and inorganic acids (de Lange *et al*., 2010; Heo *et al*., 2012). Among all these alternatives to antibiotics this review will be focus on probiotics, prebiotic and their synbiotic combination as promising strategies to improve the health status of the piglets and its adaptation to the weaning process.

2.3.1. Probiotics.

As it has been mentioned above how, the gastrointestinal microbiota plays an important role in the health and wellbeing status of weaned piglets. Therefore, it is reasonable that supplying the animal with certain microorganisms in sufficient quantities could confer direct benefits to the host. This approach is defined by the term 'probiotic', however the term "probiotic" can be used in different contexts and slightly different meanings. In simple term, probiotic means "for life". Lilly and Stillwell (1965) were the first to define this term as those substances which are produced by one microorganism and stimulate the growth of another. Later, Parker (1974) described the term as organisms and substances which contribute to intestinal microflora balance. Fuller (1989) modified the definition as a live microbial feed supplement which beneficially affects the host animal by

improving its microbial balance and has been used to describe viable microbial cultures, culture extracts, enzyme preparations, or various combinations of the above (Yoon and Stern, 1995). For this reason, the U.S. Food Safety Administration (U.S. FDA) has required feed manufacturers to use the term “direct-fed microbial” (DFM) instead of “probiotic” and has narrowed the definition to “a source of live, naturally occurring microorganisms” (Yoon and Stern, 1995).

Actually, is widely used the probiotic term defined 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, 2002). Therefore, this definition implies that a health effect must be demonstrated for the probiotic.

2.3.1.1. Uses of probiotic in the pig industry

Microbes from many different genera are being used in animal feed in the European Union (EU) although the most commonly used are bacteria members of the genera *Lactobacillus* and *Bifidobacterium* (Table 2.4); some others probiotics are microscopic fungi such as strains of yeast belonging to the *Saccharomyces cerevisiae* species.

Their use, cover a wide spectrum of the swine production cycle and include administration to sows during pregnancy (antepartum) and post-partum until piglet weaning, to piglets (pre-weaning and post-weaning) and to growing pigs up to market weight. Feeding of probiotics to sows before farrowing and during lactation; improved the sow feed intake; caused a fall in numbers of pathogenic microorganisms present in faeces, and resulted in the reduced occurrence of digestive disturbances and mortality (Bohmer *et al*., 2006; Taras *et al*., 2005). Those authors also reported that sows fed probiotics, nursed numerically more piglets and supported a higher sum of total nursing days of all piglets within each litter and had a higher litter size (Taras *et al*., 2005).

In growing pigs probiotics also have been used with success. Some reports have indicated that feeding probiotics also improves the performance in growing pigs (Wang *et al*.,

2009) and finishing pigs (Chen *et al*., 2006; Meng *et al*., 2010). Multi-strain combination of probiotics, also have been used with promising results. Hossain *et al*. (2012) investigated the effect green tea plus multi-strain probiotics on performance of finishing pigs. They reported that supplementation of probiotic has a positive effect compared to control group. In addition Chen *et al*. (2005) also observed an improvement when growing pig fed diets supplemented complex probiotic (*L. acidophilus*, *S cerevisiae* and *Bacillus subtilis*).

Table 2.4 Summary list of probiotics athorized by the European Union for its use as additive in animal feed (Gaggia *et al*., 2010).

Genus	**Species**
Bifidobacterium	*B animalis subsp. animalis, B. lactis subsp. lactis* and *B. longum subsp. longum*
Enterococcus	*E. faecalis*
	E. faecium
Lactobacillus	*L. casei subsp. casei (L. casei), L. plantarum subsp. plantarum (L. plantarum), L. reuteri, L. rhamnosus, L. salivarius* and *L. amylovorus (L. sobrius)*
Streptococcus	*S. salivarius subsp. Salivarius,* *S. thermophilus*
Bacillus	*B. cereus* var*. toyoi, B. licheniformis and B. subtilis*
Saccharomyces	*S. cerevisiae (S. boulardii) and S. pastorianus (S. carlsbergensis)*
Aspergyllus	*A. orizae and A. niger*

Regarding nursery pigs many authors also have found positive effects when diets added probiotic preparations. Post-weaning period could be considered as target period for probiotic administration, because represents a critical period (Mountzouris, 2007). In this sense, apart from beneficial effects on piglets daily weight gains prior to weaning (Zeyner and Boldt 2006) and improvements in overall weight gain to feed ratios post-weaning (Taras *et al*. 2005), the administration of probiotics during lactation resulted in a significant reduction in diarrhoea frequency and score pre-weaning (Zeyner and Boldt 2006), and in the incidence of diarrhoea post-weaning (Taras *et al*. 2005). Successful applications of probiotics have been achieved to reduce diarrheoa in post-weaning following challenge with enterotoxigenic *E*. *coli* K88 or *Salmonella* spp. (Casey *et al*., 2007; Bhandari *et al*., 2008; Zhang *et al*., 2010).

Despite the evidence of the beneficial effects of probiotics in many published works, there are also others that could not demonstrate any benefit, or even negative or contradictory results. For example dietary supplementation of *E. faecium* it was ineffective to improve the growth performance of weaning piglets as well as intestinal microbiota (Broom *et al*., 2006), immune parameters (Scharek *et al*., 2005; Broom *et al*., 2006) and controlling the diarrhoea incidence (Vahjen *et al*., 2007). Likewise, inclusion of lactic acid bacteria (Walsh *et al*., 2007), *Bacillus cereus, Pediococcus acidilactic (Simon et al., 2003)* or yeast culture (van Heugten *et al*., 2003) in weanling pig diets had not positive responses.

Likely, inconsistencies found in the literature could be related with an important number of causes, some of them related to the probiotics, others to the animal. Among, the probiotics one may list the variations in the, strains, dose (Li *et al*., 2012), timing and duration of the administration (Ohashi *et al*., 2004, Pieper *et al*., 2009; Mori *et al*., 2011). Among the factors related with pigs are include; the growth phase of the animal, stress and /or disease; apart the hygienic condition of the housing (Martin *et al*., 2012) and the heterogeneity of the experimental protocol utilized (Gaggia *et al*., 2010), just to name a few.

2.3.1.2. Probiotics mode of action.

Several have been the mechanisms proposed for the probiotics to explain their significant benefits on prevention of post weaning diarrhoea and pathogen infection (Figure. 2.5). Different modes of actions for probiotics have been classified (Brown, 2011) according to different levels of host–microbe interaction as following:

- Microbe–microbe interaction including the exclusion and inhibition of pathogens by prevention of adhesion, inhibition of replication of pathogens mediated through secretion of antimicrobial substances, competition for nutrients necessary for pathogen survival and anti-toxin effects.
- Microbe–epithelium interface, including adhesion to mucosal and epithelial cells, stimulation of mucus secretion, production of defensive molecules resulting in reinforcing gut barrier function.
- Microbe–immune system interaction comprising of immune-modulation and regulation of immune responses beyond the gut.

2.3.1.2.1. Microbe–microbe interaction

Besides competition for nutrients and gut microbiota modulation, probiotic are known to produce a variety of compounds that exert a direct antimicrobial action toward competing microorganisms (Lebeer *et al*., 2008). Consumption of a probiotic for example may result in lowering the pH value and producing organic acids which could exert antibacterial effects on certain intestinal pathogens and particularly on the ETEC K88 growth (Jin *et al*., 2000). Furthermore, it has been suggested that lactic acid might act synergically with proteinaceous substances of *L. rhamnosus* and *B. lactis* against invasion of ETEC (Gopal *et al*., 2001).

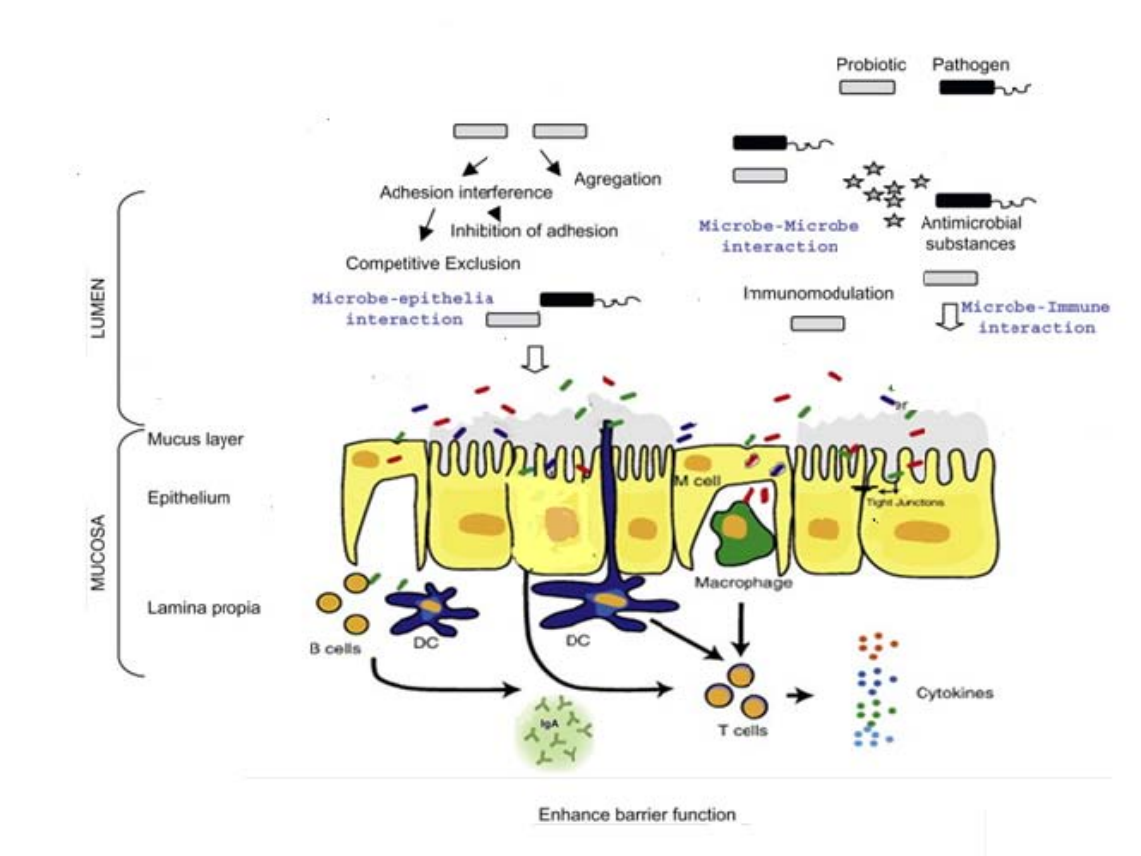

Figure. 2.5. Mechanisms of probiotics against pathogen infection: 1 Promotion of indigenous microbiota (indicated as different colored bacterial cells). 2. Antimicrobial substances against pathogens. 3. Immunomodulation. 4. Improvement of barrier function. 5. Adhesion: competitive inhibition with pathogenic bacteria, inhibition and displacement of pathogen's adhesion. 6. Aggregation and coaggregation with pathogens (adapted from Collado *et al*., 2010).

Together to lactic acid they have been described others antibacterial substances produced by probiotics such as: toxins, enzymes, bacteriocins and auto-inducer antagonist, which might directly interfere with the metabolism and the quorum sensing mechanisms of the pathogenic bacteria (Brown, 2011). These compounds may reduce not only the number of viable pathogenic organisms but may also affect bacterial virulence (Murali *et al*., 2010). On this regard, Schroeder *et al*. (2006) noted that the *E. coli* Nissle 1917 probiotic strain

could abolish the signs of secretory diarrhoea caused by porcine enterotoxigenic *E. coli*, trough the enterotoxins inhibition. Enterotoxins can disturb the structure of the tight junction and induce net secretion in intestinal segments being the main virulence factors responsible for diarrhoea in ETEC infections (Erume *et al*., 2012*;* Loos *et al*., 2012). In addition, it has been speculated on the potential of *L*. *sobrius* to produce bacteriocins against ETEC K88 (Konstatinov *et al*., 2008). Recently, Krause and Nyachoti (2011) patented a new colicin-producing *E. coli* targeting pathogenic *E. coli* K88. There are a number of other examples of the use of bacteriocin producing probiotics with respect to controlling pathogens in the gut. It has been established that production of enterotoncin-A by strain *E*. *faecium* EK13 protects newborn piglet against *E. coli* (Strompfová *et al*., 2006). Interestingly, Riboulet-Bisson *et al*. (2012) reported that administration of *L*. *salivarius* UCC118 bacteriocin Abp118-producing can induce the inhibition of certain gram negative microorganism despite the fact that this strain is normally not active *in vitro* against this group of microorganisms.

Research conducted in recent years on bacteriocines secreted by probiotics demonstrated that the expression of these proteins could be interfering with the mechanism of quorum sensing. In cell free culture of *L*. *plantarum* there were identified and characterized several cycle dipeptides displaying antifungal activity (Ström *et al*., 2002), but also interfering with the QS mechanism of *Pseudomonas aeruginosa* (Hentzer *et al*., 2003). According to Medellin-Pena *et al*. (2007), *L*. *acidophilus* secretes a molecule that inhibits significantly the production of extracellular AI-2 and also the expression of important virulence genes throughout interfering with other QS signalling. Particularly they demonstrated the inhibition of the transcription of an *E. coli O157* gene, involved in colonization.

Probiotics may also competitively exclude *E*. *coli* K88 from binding to the intestinal epithelium, through mimicking the host cell receptor to which pathogenic bacteria adhere. Indeed, some probiotic such as *E. faecium* (Jin *et al*., 2000) *B. animalis* MB5,*L. rhamnosus*

GG (Roselli *et al*., 2006) and *L. fermentum* I5007 (Li *et al*., 2008), express binding cell receptors similar those of ETEC K88, inhibiting the adherence to this pathogen on intestinal epithelial cells. Additionally, it has been confirmed that *S. cerevisiae* var. *boulardii*, highly rich in galactomannan residues can bind to K88 adhesin, suggesting their role as a prophylactic agent against gastrointestinal infections in pigs (Badia *et al*., 2012).

2.3.1.2.2. Microbe-epithelium interaction

A loss of barrier function (increase of permeability) is involved in the pathogenesis of many gastrointestinal diseases such as intestinal infections with pathogenic *E. coli* (Gyles and Fairbrother, 2010). Therefore, the study of possible protective effects of probiotics on intestinal permeability takes on considerable relevance.**.**

Probiotics may reduce the intestinal permeability by its interaction with pathogenic bacteria in the gastrointestinal tract (explained above) or by interaction with host cells such as epithelial cells (Lodemman, 2010). In this context, shortly chain fatty acids produced by probiotics could act indirectly in prevention and prophylaxis of PWD; through stimulate the proliferation of intestinal epithelial cells, leading to an increase in the thickness of mucosa (Williams *et al*., 2001). An increase of villous length and number of goblet cells in the small intestine has been proposed as one of the mechanisms by which probiotics exert their action on maintenance of barrier function (Bontempo *et al*., 2006; Davis *et al*., 2008; Mair *et al*., 2010). It has been demonstrated that *L*. *plantarum* regulates the mucin gene expression (MUC-2 and MUC-3) by goblet cells enhancing the epithelial barrier against pathogenic *E. coli* (Mack *et al*., 1999). The number of goblet cells and the type of secreted mucins appeared in part modified in the large bowel of piglet dietary supplemented with *Bacillus cereus toyoi* (Lodemann *et al*., 2008).

Nevertheless, there may be other mechanisms by which probiotics can improve intestinal epithelium and villous. It has been established that the effects of probiotic bacteria

may also result from soluble factors that can protect against pathogen-induced membrane barrier disruption. *L. sobrius* (Roselli *et al*., 2007) and *L. amylophilus* D14 (Yu *et al*., 2012) might directly activate the host cell pathways that regulates the correct assembly of the tight junction and adherents junction, thus protecting the intestinal permeability from the perturbation induced by ETEC K88. Interesting dietary supplementation with the probiotic *L. fermentum* I5007, changed the expression of proteins that can inhibit cellular apoptosis, modulate stress response, and enhance detoxification capacity in piglet small intestine (Wang *et al*., 2012). Proteinaceous, factor released by *L. rhamnosus* GG (LGG), counteract the ETEC-induced alterations of TNF-α cytokine expression (Roselli *et al*., 2007) protecting intestinal cell against the inflammation-associated response caused by ETEC K88.

2.3.1.2.3. Microbe- immune system interaction

Ingested probiotic bacteria can interact with gut epithelial cells triggering a cascade of immunological defence mechanisms. Alternatively, probiotics may also be transported by cells localized in the follicle associated epithelium overlying Peyer's patches to the immune cells in the sub epithelial dome region, which can stimulate the innate or adaptive immune system (Corthésy *et al*., 2007). In this context evidence suggest that probiotic such as *B. cereus* var. toyoi could be internalized by epithelial cells, leading to an increase of intestinal IgA secretion in piglets (Schierack *et al*., 2007). This effect could be related to a more successful mucosal defence which in turn led to a lower level in systemic IgG production (Broom *et al*., 2006). It also has been shown that supplementation of probiotic *B. lactis* to piglet infected with *E.coli*, resulted in enhancing of cellular immunity as well as an increasing of titers of IgA, IgG, and IgM (Shu *et al.,* 2001).

On the other hand many probiotics have been demonstrated to promote changes in adaptive immunity. For example administration of *P. acidilactici* increased the $CD8^{+low}$ T lymphocytes in the ileum and $CD8^{+high}$ cells in the mesenteric lymph node (Lessard *et al*.,

2009). Similarly, increasing TNF-α in piglets supplemented with *L*. *rhamnosus* GG after a *E. coli* infection might have been beneficial (Zhang *et al*., 2010), Finally, *L. casei* supplementation has been also demonstrated to induce specific immune responses against ETEC K88 (Wen *et al*., 2012).

Probiotic strains could also regulate the degree of immune activation in response to pathogens or other harmful antigens, preventing excessive inflammation. On this regard, various *in vitro* and *in vivo* studies reveal positive effects of probiotics on production of both anti- and pro-inflammatory cytokines. The addition of *B. animalis* or LGG to Caco-2 cells infected with ETEC K88 not only regulated the production of pro-inflammatory cytokines IL-1β and TNF-α but also the anti-inflammatory cytokine TGF-β1 (Roselli *et al*., 2007).. Recently, Badia *et al*. (2012) showed that *S. cerevisiae* var. Boulardii decreased the mRNA ETEC K88-induced gene expression of TNF-α, IL-6, GM-CSF and chemokines CCL2, CCL20 and CXCL8 on intestinal IPI-2I cells. Administration of *P. acidilactici and S. cerevisiae var boulardii* significantly increased IL-6 and up regulated TNF-α gene expression, in the ileum of the ETEC F4-challenged piglets *(*Daudellin *et al., 2011).* Similarly*, L. rhamnosus* resulted in delayed increase of serum TNF-α concentration, while were up regulated TNF-α mRNA and decreased IL-8 expressions in jejunum (Li *et al*., 2012), indicating its important role in regulation of systemic immune responses.

In summary, scientific evidence support the role of probiotics inducing signals to the immune cells associated with the intestine by an increase in the cytokine production and an increase in the number of IgA-secreting cell as most important mechanisms involved in the gut immune stimulation by probiotics.

2.3.1.3. Criteria to select new probiotics.

Worldwide acceptable criteria for the selection of probiotic bacteria include isolation of strains capable of performing effectively in the gastrointestinal tract. To be effective, the

bacteria must possess a number of functional characteristics, including non pathogenic behaviour, resistance to technologic processes, resistance to gastric acidity and bile toxicity (viability), high properties to adhere to gut epithelium, ability to persist within the gastrointestinal tract, production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities. For the importance of these aspects we briefly discuss some of them.

- *Origen of strain. Should be of porcine origen?*

One philosophy for selection of a probioitc strains is whether they are naturally occurring in target site, and the predominant member of that species. Thereby guarantying that selected probiotic may be safer and also has a better ability to colonize the gut and may be more effective within the intestinal ecosystem. For example, supplementing the diet of weaning pigs with lactobacilli, especially of commensal origin, has been shown to improve the health status (Lalles *et al*., 2007; Hoang *et al*., 2010). Nevertheless, some strains that are not isolated from porcine have been shown to be effective probiotics (e.g., *Lactobacillus rhamnosus* GG isolated from human faeces), which negates this requirement (Li *et al*., 2012).

- *Resistance to gut barrier*

The viability of probiotic strains is considered crucial to ensure optimal functionality. This is explained by the fact that after ingestion these bacteria have to survive the inevitable three biological barriers such as: salivary lysozyme (Rada *et al*., 2010), acidic environment of the stomach and to the bile the acids in the duodenum (Saarela *et al.*, 2009). Therefore to ensure their survival during passage through the gastrointestinal tract, the probiotic strains are commonly tested in terms of resistance to pH and bile acids (De Angelis *et al*., 2007; Byeong *et al*., 2010). But not only can the viability of the probiotic be affected by low pH or

bile acids but also its functionality. In a recent experiment (Marcinakova *et al*., 2010) the abilityof several *E. faecium* bacteria to adhere to to IPEC-J2 cell line cells after their exposure to low pH and bile was tested. It was shown that the pretreatment of strains with HCl (pH 3.0) significantly reduced their adhesion, while the adhesion ability of *E. faecium* EE3 (isolate from canine feed) slightly increased after exposure to bile. Therefore, these results indicate that the adhesive properties of probiotics should be examined after passage through an intestinal model or an animal model.

- *Stability in the dry feed.*

The selection and design of probiotics for feed applications have also to take in to account the severe conditions of drying and storage that commonly probiotics has to withstands for its use in the feed industry, such as pelleting, in which a transient contact with high temperatures can occur (Simpson *et al*., 2005; Meng *et al*., 2008;). Careful screening of probiotic strains for their technological suitability should then be considered to be resistant to high temperatures, low water activity as well as the induction of known defense system at genetic level (e.g. heat shock proteins starvation stresses). This is probably one of the reasons that explain why *Bacillus* and *Enterococcus* are two of the most commonly probiotic genera used in the feed industry. The by far most stable probiotic strains are *Bacillus sps*., because their spores are heat resistant and stay viable during long term storage. For instance, the recovery of *Bacillus cereus toyoi* after pelleting at 87°C was 95 % and after 8 weeks in feed storage was 92 % (Simon *et al*., 2003). Vegetative, dehydrated cells like *Enterococcus faecium* are more sensitive to heat treatment but also quite resistant compared to other bacteria. *E. faecium* can resist approximately 50 % after pelleting and 8 weeks of storage (Simon *et al*., 2003). Nevertheless, tolerance to high temperature such as 50 °C or 55 °C is also well documented among lactic acid bacteria (Christiansen *et al.*, 2006; Santini *et al*., 2010), whereas bifidobacteria are known to be more sensitive (Schmidt and

Zink, 2000); however, it is known that thermal tolerance among bifidobacteria is subject to species and strain variation (Simpson *et al*., 2005).

- Adhesion to the ephitelium and proliferation in the gut

Adhesion to epithelial cells and mucus a well as the ability to colonize the GTI should be assessed during selection of probiotic bacterial strain. With this aim it has been used novel scientific tools such as in vitro cell and proteomic studies. In the last years it has been increased the use of IPEC-J2 cell line of to determine adhesion properties of probiotic bacteria for porcine applications. It have been demonstrated the adhesion rates of several *Enterococcus* strains from different origins (Marciňáková *et a*l., 2010)) or field isolates of *Lactobacillus plantarum* isolated from olives (Bevilacqua *et al*., 2010) as well as of 11 strains of *Lactobacillus* (Larsen *et al*., 2007). In this last study a competitive adhesion experiment on IPEC-J2 cells demonstrated that strongly adhesive strains, as *L. reuteri* DSM 12246 and *L. plantarum* Q47, significantly reduced the attachment of the less adhesive strains, such as *L. rhamnosus* GG and *L. johnsonii* NCC 533, indicating that bacteria may share the same binding sites for attachment to intestinal cells with different affinity for the receptors. This variability should be taking into account since poorly adhesive strains might be out-competed by strongly adhesive strains at natural conditions as well as in multi-strain complex probiotic formulations.

Advances in proteomics science have also contributed to identifiy the proteins and the proteomic patterns that may serve as bacterial biomarkers for the preliminary selection of strains with the best probiotic potential. In fact, these studies have allowed to indentify proteins involved in the adhesion of different probiotic to the gut mucosa like *L*. *plantarum* (Izquierdo *et al*., 2009; Hamon *et al*., 2012) or *L. reuteri* (Lee *et al*., 2008). Undoubtedly these advances will provide new insight to understanding the molecular mechanisms through which probiotic candidate may adapts to the intestinal tract.

As probiotic adhesion to epithelium can be influenced *in vivo* by multiples factors, , *in vitro* experiments may not be completely predictive. This is the reason why many published works studies the ability of probiotics to adhere the intestine using *in vivo* models.(Mare *et al*., 2006; Nencová *et al*,, 2012)

- *Safety*

Most of species and genera used as feed additive are apparently safe; however certain micro micro-organisms may be problematic, such as enterococci, which may harbour transmissible antibiotic resistance determinants and bacilli that are known to produce enterotoxins and an emetic toxin. Therefore to the use of novel microorganisms in animal nutrition the question of their safety and the risk to benefit ratio have to be assessed.

In this order the assessment of safety of probiotics comprises consideration of a variety of factor, such as: (i) record isolation history and taxonomic classification of the candidate probiotics (ii) absence of toxicity assessed at the strain level (e.g. through genotoxicity studies including mutagenicity) (iii) absence of virulence factors such as: hemolytic activity, production of enterotoxins or cytotoxins, or the capacity to invade host epithelial cells by comparing probiotic strains with isolates from pigs infection (iv) absence of antibiotic resistance and transferability of resistances (to prevent the undesirable transfer of antibiotic resistance or conferment of resistance to endogenous bacteria, probiotics should not carry resistance other than that required) and (v) absence of adverse immunological effects (Lara-Villoslada, *et al*., 2001., Anadon *et al*., 2006).

2.3.1.4 .Production of viable and stable probiotics

As the definition of probiotics indicates, viability is considered an important criterion for functionality. Therefore, means to improve stability of probiotics during food and feed processing and storage are of interest. Within this context, new technologies such as encapsulation of probiotic bacteria has proven effective in many applications for human

consumption (Anal and Singh, 2007 and Semyonov *et al*., 2010; Soccol *et al*., 2011). Based on this technology (Table 2.4), a wide range of microorganisms have been immobilized within semipermeable and biocompatible materials that modulate the delivery of cells. Moreover, according to Ding and Shah (2009), encapsulation may improve the survival of these microorganisms not only during processing and storage but also during passage through the gastrointestinal tract.

In this order, microencapsulation could represent a promising way of administering probiotics to piglets (Gaggia *et al*., 2010). In this regard micro encapsulating *Bifidobacterium* spp (Modesto *et al*., 2011), guaranteed the survival of probiotic bacteria during gastric transit and was effective to stimulate the development of the lactobacilli population in the hindgut.

Other strategy that also may guarantee the stability of probiotic added in feed includes is the growth of microorganisms on moist solid substrates in the absence of free flowing water. In that sense potential probiotic microbes, *Lactobacillus acidophilus*, *Bacillus subtilis Saccharomyces cerevisiae*, and *Aspergillus oryzae* were fermented by 7 days on corn-soybean meal mixture (Choi *et al*., 2011). After this, potential multimicrobe probiotics produced by solid substrate fermentation were dried at low (40°C) or high (70°C) temperature. Authors concluded that high drying temperature had no effect on the efficacy of potential multimicrobe probiotic product.

Summarizing, the administration of probiotic to weaned piglet seems to be a suitable strategy to prevent post-weaning diarrhea in piglets. The possible mechanisms of action could be multiple, being the most plausible the modulation of the gastrointestinal microbiota and the improvement in the immune response of the host. Selection of the most suitable strains deserve particular attention in their resistance to grastrointestinal conditions, their stability for applications in dry feed and its safety for the host and the final consumer.

Table 2.6. Techniques and processes used for encapsulating probiotic microorganisms. (reviewed by Anai and Singh, 2007)

Techniques	Types of materials for coating	Major steps in processes
Spray-drying	Water-soluble polymers	(i) Preparation of the solutions including microorganisms (ii) Atomization of the feed into spray (iii) Drying of spray (moisture evaporation) (iv) Separation of dried product form
Fluidized-bed coating/air-suspension	Water-insoluble and water-soluble polymers, lipids, waxes	(i) Preparation of coating solutions (ii) Fluidization of core particles
Coacervation/phase separation technique	Water-soluble polymers	(i) Core material is dispersed in a solution of coating polymer (ii) Deposition of the coating, accomplished by controlled, physical mixing of the coating and core materials in the vehicle phase (iii) Rigidifying the coating by thermal, cross-linking or desolvation techniques, to form self-sustaining microcapsules
Electrostatic method	Oppositely charged polymers/compounds	(i) Mixing of core and coating materials (ii) Extrusion of mixtures of core-coating materials in oppositely charged solutions (iii) Freeze-dry or oven-dry of microcapsules/microspheres/beads

2.3.2. Prebiotics

The prebiotics, are defined "as non-digestible food or feed ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson *et al*., 2005). However, the term prebiotic have been attributed to many feed components without consideration of the criteria required. In particular, almost every oligosaccharide and polysaccharide (including dietary fiber) has been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotic. There is, therefore, a need to establish clear derivatives for classifying a new feed ingredient as prebiotic (Figure 2.6). Such classification requires a scientific demonstration that feed ingredient: (i) resists the gastric acidity, hydrolysis by

mammalian enzymes and to GIT absorption; (ii) is fermented by intestinal microbiota; and (iii) selectively stimulate the growth and (or) the activity of intestinal bacteria such as *Lactobacillus* and *Bifidobacteria* associated with health and well-being (review by Heo *et al*., 2012).

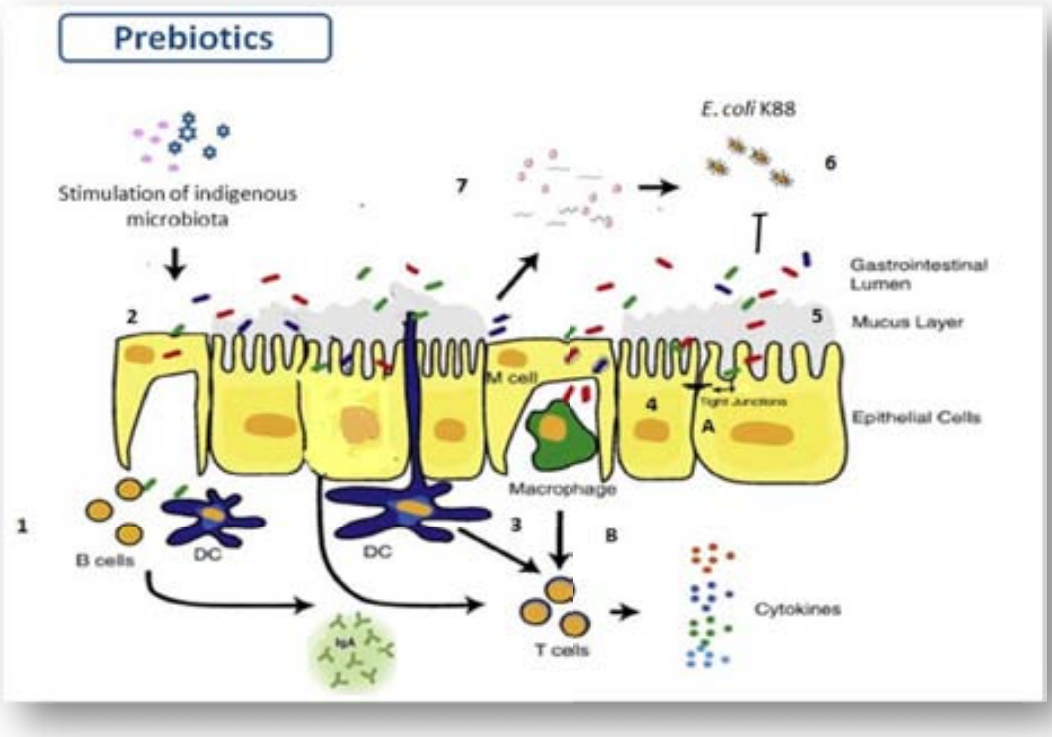

Figure 2.6. Prebiotic effects on intestinal epithelial cell of piglets (Heo *et al*., 2012). 1 Stimulation of the immune cell directly by prebiotics, 2 Irritation of ephitelial cell directly by prebiotics, 3 Stimulation of immune cell by the indigenous microbiota (indicated as different colored bacterial cells), 4 Stimulation of thigh junction genes/ proteins by the indigenous microbiota, 5 Stimulation of mucin production by the indigenous microbiota, 6 Production of antimicrobial substances by beneficial microbiota and stimulation of competitive exclusion against pathogens, 7 Stimulation of SCFA production by the indigenous microbiota leading to: A. Regulation of thigh junction function and stimulation of epithelial growth and differentiation. B. Regulation of pro-inflammatory cells.

2.3.2.1. Application of prebiotics in pig industry.

The most wildly prebiotics studied in piglet diets have been non digestible oligosaccharide (Table. 2.4) although, some types of protein, peptide and lipid have also be qualify as prebiotics (Berge and Wierup, 2012). It should be noted that dietary fibre not

digested enzymatically but fermented in the hindgut may also be a candidate (Bauer *et al*., 2006).

Table 2.4. Influence of some prebiotics in gut and host health of piglet

Prebiotic supplement	Effect on gut/microbiota	Effect on animal	Reference
Resistant starch 7%	Enhanced microbial diversity in colon	Reduced diarrhoea incidence	Bhandari *et al*., 2007
Mannan-oligosaccharides	Increased ileal and caecal microbiota diversity	Increased serum level of IgA, IgG and IgM	Whang *et al*.,2010
	Decreased enterobacteria in jejeunun	Promoted the intestinal morphology	Castillo *et al*., 2008
Trans-galactooligosaccharides	Not induced microflora changes in hindgut but promoted saccharolytic activities in colon		Mountzouris *et al*., 2006
Fructo-oligosaccharides	Increased Bifidobacteria in ileum	Improved the average daily gain	Shim *et al*., 2005
Inulin	Improved lactobacilli to coliforms ratio and decreased pH in proximal colon	Increased weight of caecum and colon but final body weight,	Wellock *et al*., 2007
	Increased the lactobacilli and bifidobacteria and decreased Enterobacteriaceae.	Higher blood hemoglobin concentration and down-regulated pro-inflammatory cytokine.	Patterson *et al*., 2010

Although in several studies, prebiotic inclusion in swine diets had no effect on growth performance parameters (Wellock *et al*., 2007; Modesto *et al*., 2009),t here are others where the determined effects ranged from an improvement in the the efficiency of gain, for example in mannan-oligosaccharides -supplemented diets (Castillo *et al*., 2008), to significant

improvements in daily weight gains, in diets supplemented with lactulose (Krueger *et al*., 2002), oligofructose (Shim *et al*., 2005) and lactose (Lynch *et al*., 2006) as some examples.

2.3.2.2. Mechanism of action of prebiotics.

2.3.2.2.1. Effects throughout changes in fermentation products and intestinal environment.

Different studies have shown the role of prebiotics preventing or reducing the growth and virulence of certain intestinal pathogens in pigs by modify the gastrointestinal microbiota. In this respect, batch culture studies with porcine faecal inocula showed that various carbohydrates including lactulose, increased numbers of *Bifidobacterium* group bacteria, while reduced *Salmonella* numbers (Martin-Pelaez *et al*., 2008). Others studies in pigs including inulin (Wellock *et al*., 2008), high level of lactose (Pierce *et al.,* 2006) and chito-oligosaccharide (Liu *et al*., 2008) in the diets showed favoured bifidobacteria and/or lactobacilli while decreasing *E. coli*.

Nevertheless, and despite the benefits against intestinal pathogens reported in many studies, other authors have also found contradictory effect with the use of prebiotics. In that sense Halas *et al*. (2009) including 8 % of inulin in the diets of pigs found a decrease in the total concentrations of short chain fatty acids and an increase in faecal ETEC shedding. Other negative effect was detected by Trevisi *et al*. (2008) those authors detected that supplementation with sugar beet fructo-oligosaccharides tended to increase the number of *E*. *coli* on cecum content.

Positive effects of prebiotics could be mediated by the stimulation of metabolic activities of indigenous microbiota. According to Pie *et al*. (2007), prebiotic supplementation of the diet influences short chain fatty acid content, branched-chain proportion, lactic acid concentrations and ammonia concentrations in the gut, which positively affects the secretion of mucins as well as the recovery of the epithelia. Produced SCFA could also have a protective effect regulating the tight junction function (Peng *et al*., 2009), the epithelial growth

and differentiation (William *et al*., 2001) and also modulating the pro-inflammatory response (Milo *et al*., 2002).

2.3.2.2.2. Effects on pathogen adhesion.

Prebiotics could also have a more direct effect on pathogens within intestine, through interference with their attachment and/or their toxins to the intestinal brush border (Badia *et al*., 2012). For example, it has been reported that prebiotic galacto-oligosaccharides (GOS) significantly inhibit the adherence of *E*. *coli* to both HEp-2 and Caco-2 cells, reducing the adherence until 65 and 70% (Shoaf *et al*., 2006). Beneficial role of glycoconjugates containing GOS (Tzortzis *et al*., 2005) and galactose residues in NSP hydrolysis products of soybean (Kiarie *et al*., 2008) have been also implicated in the interactions between epithelial and bacterial cells implying that these compounds also had the capacity to directly inhibit the attachment of *E. coli* and/or *Salmonella* to host cell surfaces by acting as competitive receptors. Other studies show that, in the presence of mannan products, the enteric pathogens attach to the mannan compounds in the gut lumen instead of the epithelia, which reduces their colonization (Kogan *et al*., 2007; Becker and Galletti, 2008).

2.3.2.2.3. Interaction with immune system

Prebiotics may act indirectly on the immune system (Lomax and Calder, 2009) by stimulating the growth of beneficial microbiota (reviewed by Licht *et al*., 2012) but also directly through the interaction with innate receptors on the plasma membrane of host cells, in particular in macrophages and dendritic cell (Wismar *et al*., 2011). As demonstrated the study conducted by Sheng *et al*. (2006) it was shown that mannan activate TLR4 signalling pathways in a dose dependent manner, inducing dendritic cell mature phenotype (CD40, CD80 and CD86) and up-regulating proinflammatory mRNA for IL1-β and TNFα cytokines as well as other Th1/Th2 cytokines.

Different studies can be found in the literature demonstrating the potential of prebiotics to improve the immune response in challenged piglets. Studies of Che *et al*. (2011) suggest that mannan-oligosaccharide (MOS) supplementation can enhance the shift from an innate to an adaptive immune response in piglets. They proved that MOS reduced the expression of IL-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, and TLR4 gene, while preventing over stimulation of the immune system. Supplementation with others oligosaccharides such as: arabinoxylan (Niewold *et al*., 2012) or chito-oligosaccharide (Liu *et al*., 2010) also have desmonstrated to modulate positively the small intestinal innate response of piglets challenged by ETEC K88.

Different prebiotics also have shown to increase the serum levels of inmunoglobulins. White *et al*. (2002) describe that the administration of MOS increase serum levels of IgG in piglets challenge with *E*. *coli* K88, associated with lower coliform counts. Similarly, dietary supplementation with galacto-mannan-oligosaccharides or chitosan enhanced serum levels of IgA, IgG and IgM, suggesting that both prebiotics may enhance the cell-mediated immune response in early weaned piglets by modulating the production of antibodies (Yin *et al*., 2008).

Therefore the use of prebiotics could be an effective alternative to improve enteric health of piglets around weaning. They can promote the beneficial bacteria populations enhance the intestinal defence systems (immunomodulatory action, pathogen displacement, bacteriocin production, etc.), increase the resistance to various health challenges and accelerate the recovery from digestive disturbances. However their, efficacy may be variable depending on the type of prebiotics, the dosis and the context of the administration.

2.3.3. Synbiotic

Synbiotic is a recent concept that was first conceived: as “mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live

microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare" (Gibson and Roberfroid 1995). Nowadays this concept has been not redefined, however as this definition is intrinsically bound to that of both prebiotics and probiotic, if it has evolved as a result of the changes in these definitions. In this sense Kolida and Gibson (2010) defined two types of synbiotic approaches: (i) Synergistic reserved for products in which the prebiotic compound is chose to selectively favours the chosen probiotic microorganism and (ii) Complementary, whereby the probiotic is chosen based on specific desired beneficial effects on the host, and the prebiotic is independently chosen to selectively increase concentrations of the beneficial microbiota components. Therefore both approaches may, directly or indirectly, comply with the synbiotic definition.

The beneficial effects of synbiotics combinations against pathogens have been reported mainly for humans with diverse synbiotic combinations being tested (reviewed by Kolida and Gibson, 2010). Synbiotic combinations for their application on animals, and particularly in pigs, have been however no so much explored, although several research works have been published in the last recent years (Mair *et al*., 2010a 2010b, Krause *et al*., 2010, Modesto *et al*., 2011, Chu *et al*., 2011, Bomba *et al*., 2012, Nemcová *et al*., 2012)

In most of them the combination of probiotics with fructo-oligosaccharide it has been studied. Nevertheless, other suitable components different to carbohydrates such as resistant starch, organics acids or polyunsaturated fatty acids (PUFA); may be used in synbiotic combinations.

2.3.3.1. Combinations including fructo-oligosaccharides

As explained in the section dedicated to prebiotic; the fructo-oligosaccharides (FOS) can significantly modulate the colonic microbiota by increasing the number of specific bacteria changing the composition of the microbiota and is one of the most studied

prebiotics. Because of this reason FOS has been one commonly prebioitc substrate used for synbiotic combination in pigs. Some of them have confirmed a significant synergistic stimulation on components of beneficial microflora (Bomba *et al*., 2002; Shim *et al*., 2005; Modesto *et al*., 2011) associated to a reduction of diarrhoea incidence. Furthermore, it has been observed that supplementation with the combination of *B*. *animalis* and fructooligosaccharide, resulted in increase of SCFA production (Bird *et al*., 2009) and modulation of inflammatory response by the expression of the TLR2 and –TNF-α gene expression (Trevesi *et al*., 2008). Furthermore the use of FOS in combination with *L. paracasei* has been demonstrated to have a stimulating effect on improves the morphology of intestinal villous (Shim *et al*., 2005). While with *L*. p*lantarum* it has been shown to reduce adherence of *E*. *coli* K88 to the intestinal mucosa of weaned pigs (Nemcova *et al*., 2007). Recently, it was also demonstrated that FOS supplementation was able to stimulate the endogenous bifidobacteria of the animal, but not had additional growth stimulus when it was combined with supplemented bifidobacteria (Modesto *et al*., 2009). Works of Mair *et al*. (2010a,b) using inulin and a multispecies probiotic also described additives distinct individual effects of inulin and the probiotic but not specific synergic effects. However, the impact of synbiotic combination including FOS on microbial population and particularly on opportunistic pathoges like ETEC is still far from being conclusive.

2.3.3.2. Combinations including resistant starch

Other potential synbiotic combinations that have been frequently tested are those including resistant starch. Examples in humans include maize resistant starch (RS) with *B. lactiis* (Worthley *et al*., 2009) and lactobacilli (Oláh *et al*., 2007) induced positive changes in faecal microflora and colonic patterns of fermentation. In porcine nutrition, it can be found examples of applications of resistant starch with *Bifidobacterium longum* (Brown *et al*., 1997) and with FOS and *Bifidobacterium animalis* (Bird *et al*., 2009) increasing the levels of faecal bifidobacteria. In this last study was demonstrated that when RS and FOS were fed together

there was an increase of bifidobacteria that exceeded the individual increases, suggesting that they operate by different mechanisms. Conversely to the fact that FOS may act as a metabolic substrate for bifidobacteria, the RS could promote the probiotic in a different way. In this sense it have been suggested that RS may promote bifidobacteria, through the physical protection offered by the starch particules that could facilitate the passage of LAB through the upper GIT. Nevertheless it not may discard that other type of starch can act as selective substrate for other bacterial species. Indeed in the study conducted by Krause *et al*. (2010) the combination of potato resistant starch with probiotic *E. coli*; increased the lactobacilli prevalence in the colon.

2.3.3.3. Other synbiotic combination

Other synbiotic combinations have also been tested in pigs. Combinations of maltodextrins and *L. paracasei* (Bomba *et al*., 2002) or *L. plantarum* (Nemcova *et al*., 2007) have been proved to increase the performance parameters of piglets, decreasing pathogenic *E. coli* growth in the digestive tract and/or their adhesion to intestinal epitelium. Improved efficiency and reduction of ammonia in intestinal content was also observed after the feeding piglets with biological preparations containing*, L. brevis* and *L. salivarius* with lactitol (Piva *et al*., 2005). Additionally, a mixture of *Lactobacillus spp* and mannan oligosaccharide, lactose, sodium acetate and ammonium citrate, revealed the reduction of faecal noxious gas (Chu *et al*., 2011).

The incorporation of probiotics with unsaturated fatty acids also yielded a superior synbiotic effect than the probiotics alone. The administration of PUFA demonstrated to improve the adhesion of *Lactobacillus paracasei* to the jejunal mucosa of gnotobiotic piglets (Bomba *et al*., 2012). Those authors suggested that dietary PUFA affects the attachment sites for the gastrointestinal microbiota, possibly by modifying the fatty acid composition of the intestinal wall. More recently Nencová *et al*. (2012) demonstrated the positive effect of PUFAs on *L.*

plantarum adhesion, which resulted in enhancement of the inhibitory effect of lactobacilli on *E. coli* K88 in the digestive tract of piglets.

In summary, it has been evidenced in this literature review that post weaning diarrhoea caused by ETEC K88 I as multifactorial disease dependent on several factors. An important factor is the adhesion of the pathogen to the intestinal mucosa that is generally increased by the lack of active immunity and damage of the intestinal barrier function provoked by weaning stressors. In the prevention and control of this disorder the use of dietary interventions reinforcing the gut barrier functionality seems to be a good alternative. Focussing on available knowledge in the literature, the application of probiotics, prebiotic and their combinations (synbiotics) have evidenced potential to improve the gastrointestinal function and health of pigs. Studies indicate a generally positive effect on maintaining gut health and performance of piglets (body weight gain, feed efficiency), through diversity, stability, metabolites and crosstalk with the epithelium and the underlying immune system. However, the data about their efficacy are sometimes variable and still far from being conclusive. Further, many of the studies we reported here were conducted in *in vitro* models, or in very much controlled experimental conditions far from what could be the environment we find in the field. It is necessary to be cautious extrapolating conclusions within the context of piglets managed in commercial farms and hence exposed to various opportunistic pathogenic agents and more challenging conditions.

Taking this in considerations and in order to obtain further evidence of the efficacy of these additives, it would be recommendable to evaluate any possible new candidate within the context of an infectious agent and experimental conditions more close to the challenging environment of the farms. Definitive conclusions will come, in a final stage, from controlled field studies, including different farms with variable productive conditions from which we will be able to definitively recommend or not their use in the industry.

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CHAPTER 3

Objectives and Experimental Desing

Objectives

In the last years our research group (Animal Nutrition, Management and Welfare group) have been involved in several projects with the aim of studying different feeding strategies to prevent the establishment of pathogenic microorganisms in early weaned piglets (AGL2005-07428-C02-01/GAN; AGL2007-60851/GAN; AGL2009-07328/GAN). Particularly, in these previous projects, it have been demonstrated *in vitro* the potential of different prebiotic and probiotcs to control the overgrowth of pathogens like *Salmonella* in the gastrointestinal tract and also the ability of some natural ingredients to interfere with the adhesion mechanisms of *E. coli* K-88 to the intestinal epithelium. The current Thesis intended to continue this line of research and for this purpose had the following main objectives:

1. To evaluate *in vivo* the potential of a synbiotic combination of lactulose and *L. plantarum* to increase the resistance to post-weaning colibacillosis in piglets.
2. To confirm *in vivo* the effectiveness of a natural ingredient derived from locust seed to improve the response of the animals against an infection of ETEC-K-88.
3. And a secondary objective, this Thesis also pretended to develop, and implement, a reproducible model of porcine post-weaning colibacillosis, adequate to evaluate the effectiveness of feed strategies in controlling post-weaning colibacillosis.

To achieve these three objectives, the following experiments were done and included in Chapters 4 to 6.

In the Experiment 1, it was analyzed the effects of lactulosa, a probiotic *L. plantarum* or its combination on intestinal microbial population, fermentative activity and performance of healthy weaning piglets. The experiment also aimed to explore the potential of lactulose, to specifically stimulate the growth *L*. *plantarum* in the synbiotic combination.

In the Experiment 2, it was investigated the efficacy of the same treatments (lactulose, *L. plantarum* or its symbiotic combination) in a similar design to Exp. 1, but under

challenging conditions. For that it was implemented an *in vivo* experimental model of colibacillosis by enterotoxigenic *E*. *coli* (ETEC) K88. Effects on intestinal architecture and some plasmatic pro-inflammatory indexes were also included.

The Experiment 3 was designed to study the effect of dietary inclusion of a product derived from the carob seed on improving the intestinal natural defence mechanisms of weaned piglets challenged with enterotoxigenic *Escherichia coli* K88. The design included a treatment with pharmacological dosis of ZnO as a positive control. Parameters studied included performance of the animals, changes on fermentative activity, microbial groups, morphology of the intestinal epithelium and some pro-inflammatory plasmatic indicators.

CHAPTER 4

Evaluation of the effect of a synbiotic combination of lactulose and *Lactobacillus plantarum* on the intestinal environment and performance of piglets at weaning.

4.1. Introduction

Pre- and post-weaning gastro-intestinal diseases remain a significant health and welfare burden affecting piglets causing an imbalance of the gastrointestinal microbiota (Lallès *et al*., 2004). In order to prevent and control these disorders it has been suggested that different feed additives are used to prevent disease and improve performance. Probiotics, prebiotics and more recently the combinations of both, known as synbiotics, have been proposed as potential feed additives (Zimmermann *et al*., 2001).

Probiotics are considered living organisms that, when incorporated into the diet in sufficient quantities, exert a positive effect on health (Guarner and Schaafsma, 1998). Many species of bacteria have been proposed as probiotics for inclusion in pig feed including; *Enterococcus* spp., *Lactobacillus* spp.*, Bifidobacterium* spp., *Pediococcus* spp., *Bacillus* spp. and *Escherichia coli* (Gaggìa *et al*., 2010). Among them *Lactobacillus plantarum* has been described as one of the most promising probiotics with proven beneficial effects in weaning piglets (De Angelis *et al*., 2007; Pieper *et al*., 2009; Mizumachi *et al*., 2009).

In the same way, prebiotics have been proposed as a feed supplement for the modulation of the beneficial microflora of the GIT (Rastall and Maitin, 2002). Prebiotics enhance the selective growth of certain indigenous gut bacteria considered to be positive microorganisms for the host. Lactulose, a synthetic disaccharide (galacto-fructose) used as a laxative in humans, has recently been proposed as a potential prebiotic (Panersar and Kumari, 2011). It has been shown that when lactulose is administered at low doses it positively influences the numbers of bifidobacteria and lactobacilli, whilst reducing the numbers of *Clostridium* sp, *Salmonella* sp *or E. coli* in the GIT (Schumann, 2002; Krueger *et al*., 2002). In pigs, this prebiotic is usually incorporated into feed at 10 g/Kg (Kamphues *et al*., 2007), but with variable results. Some investigators have found changes in short chain fatty acids SCFA concentration in the caecum (Martín- Pelaez *et al*., 2010) and other

microbial indicators like densities of clostridia (Kien *et al*., 2007) whereas others have reported no positive effects (Kamphues *et al*., 2007).

A synbiotic is defined as a mixture of probiotics and prebiotics that has a beneficial effect on the host by improving the survival and persistence of live microbial dietary supplements in the GIT by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid, 1995).

Regarding the potential of lactulose in a synbiotic combination, previous *in vitro* trials conducted by our group have demonstrated that it can be efficiently used as a growth substrate by several beneficial lactic bacteria like *L. acidophilus*; *L. casei* or *L. plantarum,* but not by potential opportunistic pathogens such as *Salmonella enterica* serovar Typhimurium (Martin-Peláez *et al*., 2008). From these studies it would appear that the combination of lactulose and *L. plantarum* could be a promising synbiotic mixture.

The objective of this study was to evaluate the effect of a combination of lactulose and *L. plantarum* on the intestinal environment of weaning piglets and its potential to improve the adaptation and performance of piglets after weaning.

4.2. Materials and Methods

4.2.1. Animals and diets

Ninety six commercial breed ([Large White x Landrace] x Pietrain), weaning piglets of 25 ± 2 days old with an average body weight 6.4 ± 0.54 kg were purchased from a single commercial farm and then transported to the experimental farm facilities of the Universitat Autònoma de Barcelona (UAB).

All procedures involving the treatment, management, housing, husbandry and euthanasia of animals received prior approval from the Animal Protocol Review Committee of the UAB (Permit number CEAAH 746) and ethics committee of the Animal Health

Veterinary Laboratories Agency (United Kingdom), and were carried out conformed to European Union Guidelines (The Council of the European Communities, 1986).

Piglets were housed in 32 pens distributed into four rooms in a randomised block design (three pigs per pen) taking litter size and initial body weight BW into account. Animals in each of eight replicated pens received one of four dietary treatments as follows: a control diet (Table 4.1) was formulated (CTR) to which either 15 ml/kg of lactulose syrup (Duphalac, Solvay Pharma, S.A. Barcelona) was added (equivalent to 1% w/v of lactulose) (LAC) or a fresh culture of *L. plantarum* (2 x 10^{10} CFU/animal/day) (LPLA). The probiotic was administered daily by spraying the feed with 20 ml of a pure culture (prepared as described below) per pig. Diets not including the probiotic treatment were sprayed with sterile Man, Rogosa and Sharpe broth (MRS, Merck 1.10661). The combination of both additives corresponded to the synbiotic treatment (SYN). All diets were fed as meal. Diets and water were offered *ad libitum*.

4.2.2. Probiotic strain

L. plantarum strain (B2984) originally isolated from the faeces of a healthy commercial pig was obtained from the Animal Health and Veterinary Laboratories Agency bacterial culture collection. For convenience 1 ml of lyophilized *L. plantarum* cultures were prepared: *L. plantarum* was cultured at 37°C in sterile Man, Rogosa and Sharpe broth (MRS, Merck 1.10661) medium to an OD_{650} of 1.2. The bacterial cells were centrifuged (7,600 × g) at 5°C for 10 min (Beckman Coulter Avanti J20-XPI) and resuspended in 10 ml of cryoprotective medium (1.5 % w/v skim milk and 4% w/v sucrose). The cultures were then lyophylised in vials of 1 ml. Each day the inocula were prepared by resuspending a single vial in 15 ml of MRS which was added to fresh sterile pre-warmed 500 ml of MRS broth and cultured for 16hrs at 37°C in to reach a final concentration of 10^9 CFU/ml. Serial dilutions of the inocula were plated on MRS agar and incubating for 16hrs at 37°C after order to

determine the CFU/ml.

Table 4.1. Composition of the control diet.

Ingredients	(g/kg fresh matter)
Corn Flakes	360.0
Wheat Flakes	240.0
Full fat extruded soybeans	110.0
Egg meal	92.7
Sweet whey	150.0
Soybean oil	16.5
Calcium Carbonate	5.6
Dicalcium Phosphate	8.10
L-Lysine	4.6
DL-Methionine	0.5
L-Threonine	1.1
L-Tryptophan	0.9
Salt	4.0
Vitamin-Mineral Premix*	6.0
Chemical analysis	(g/kg dry matter)
Dry matter	905.6
Gross energy (MJ/kg)	18.1
Crude protein (CP; N x 6.25)	172.9
Neutral detergent fibre	77.0
Acid detergent fibre	32.0
Ether extract	47.0
Ash	42.0

(*)Supplied per kilogram feed: 10,200 IU of vitamin A, 2,100 IU of vitamin D3, 39,9 mg of vitamin E, 3 mg of vitamin K_3, 2 mg of vitamin B_1, 3 mg of vitamin B_2, 3 mg of vitamin B_6, 0.025 mg of vitamin B_{12}, 20 mg of Pantotenate calcic, 60 mg of Nicotinic acid, 0.1 mg of biotin, 0.5 mg of Folic acid, 150 mg of Fe, 156 mg of Cu, 0.5 mg of Co, 120 mg of Zn, 49.8 mg of Mn,2 mg of I, 0.3 mg of Se.

4.2.3. Experimental procedures

.Animals received the experimental diets for 2 weeks. Feed intake was recorded daily and body weight weekly. Average daily weight gain was calculated individually and average daily feed intake and gain:feed (G:F) by pen.

Piglets with clinical signs of diarrhoea (watery faeces) and simultaneous dehydration, fever, growth check, and apathy (7 in total) were treated for a 3-d period with 1 ml

(intramuscular injection) per animal of marbofloxacin (Marbocyl 2%, Vetoquinol S.A., Lure Cedex, France). The results of clinical signs of diarrhoea were recorded daily.

On days 0, 7 and 14, faecal samples for microbiological analysis were taken aseptically (about 5 g) from two animals per pen corresponding to the animals with the intermediate and lowest BW. Sampled piglets were the same for the three sampling days. At the end of the experiment, an 8 ml-sample of blood obtained by venipuncture of the cranial vena cava using a BD Vacutainer (Becton Drive, Franklin Lakes, NJ) 10-ml plasma tube with sodium heparin (17 IU/ml) was taken from the animals with the intermediate BW of each pen. The plasma collected was stored at –80°C until the analytical use. Immediately after blood sampling, piglets were euthanised with an intravenous injection of pentobarbital sodium (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200 mg/kg BW). Animals were bled, the abdomen immediately opened and the whole GIT was excised.

Digesta from ileum and proximal colon (was considered 25 cm from ileocecal junction) (50mlL) was homogenized and pH was determined with a pH-meter (electrode Crison 52-32, Net INTERLAB SAL). Samples from proximal colon and ileal digesta (approximately 5g) were kept frozen in small zip-lock bags (-20 ºC) until analysed for SCFA. Additional samples were preserved in ethanol for DNA extraction (1 ml of digesta + 3 ml of ethanol).

4.2.4. Analytical methods

Culture methods. For bacterial counts, fresh faecal samples were suspended (10% w/v) in a Phosphate buffered saline (PBS) and subsequently homogenised for 5 min. Thereafter, 10-fold dilutions were made in PBS. Lactic acid bacteria were enumerated by plating onto MRS Agar plates which were incubated microaerophilically at 37ºC for 48 h, *L. plantarum* was enumerated using the specific growth medium described by Bujalance *et al*. (2006). Confirmation of the identity of colonies as *L. plantarum*, was performed by random

amplified polymorphic DNA (RAPiD) analysis of a total of 30 colonies randomly selected using primer OPL-05 (ACGCAGGCAC) (Van Reenen *et al*., 1996). For the enumeration of *E. coli* and total coliforms, 1 ml of each dilution was pipetted onto an *E. coli*/coliform count plate (3M Petrifilm, Europe Laboratories 3M Santé, Cergy-Pontoise, France) with violet red bile gel and an indicator of glucuronidase activity. The plates were incubated for 48 hrs at 37° C, and *E. coli* colonies and total coliform colonies were counted following the manufacturer's instructions. *Clostridium perfringens* were enumerated using Perfringens agar (Oxoid) and incubated 48 hrs at 37°C. All microbial enumerations were expressed as log colony forming units (CFU) per gram of faeces.

DNA extraction. Colon digesta samples (400 mg) were precipitated by centrifugation (13,000 g x 5 min) and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90º C and a posterior incubation step with lysozyme was added (10 mg/ml, 37º C, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 ml of Qiagen Buffer AE (Qiagen, West Sussex, UK) and stored at -80º C until use.

Terminal Restriction Fragment Length Polymorphism (t-RFLP): T-RFLP analysis of bacterial community in proximal colon was performed following the procedure described by Castillo *et al*. (2007). Briefly, a 1,497 bp fragment of the 16S rRNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5' AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR analyses were performed for each sample. The fluorescently labelled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µL of milli-Q water. After that, the resultant PCR product was subjected to to a restriction with Hha I endonuclease restriction enzyme (20,000 U/µl) (Biolabs Inc. New England, USA) for 8 hrs at

37^{o} C. The fluorescently labelled terminal restriction fragments (TRFs) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with 25-U detection threshold. Determinations of the sizes of TRFs in the range of 50 to 700 base pairs were performed with the size standard GS-1000-ROX (PE Biosystems).

Sample data consists of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all the TRF peak areas in the pattern was used to normalize peak detection threshold in each sample following the method of Kitts (2001). New total area was obtained by the sum of all the remaining peak areas in each pattern. Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms constructed using Fingerprinting II (Informatix, Bio-Rad, C, USA) software and un-weighed pair group method with averaging algorithm (UPGMA).

In order to infer the bacterial composition of the samples, *in silico* restrictions for the major pig gut bacteria (described by Leser *et al*. (2002)) with the primers and the enzyme used was obtained using the analysis function of MiCA web page (http://mica.ibest.uidaho.edu/default.php).

Quantification of microbial groups by Real time PCR. (q-PCR): Lactobacilli and enterobacteria population in colonic samples were quantified by real time PCR (qPCR) using SyBR Green dye, following the protocol described by Castillo *et al*. (2006).

4.2.5. Chemical analysis.

Diet samples were analysed for gross energy, crude protein, ether extract, neutral detergent fibre and acid detergent fibre, according to the Association of Official Analytical Chemists (AOAC, 1995) methods.

Short-chain fatty acids and lactic acid analysis were performed by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatisation with MBTSTFA+ 1% TBDMCS agent, using the method of Richardson *et al*. (1989) modified by Jensen *et al*. (1995).

Blood urea nitrogen was measured by Glutamate Dehydrogenase (GLDH) reaction, using the Olympus System Reagent® (Olympus, Ireland) and using a Olympus AU400 (series 3112676, Germany) device. Pig Major Acute-phase Protein (Pig-MAP) concentration was determined by a sandwich type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain), according to the manufacturer's instructions

4.2.6. Statistical Analysis

The effect of diet on performance, microbial and slaughter measurements was tested with an ANOVA using the GLM procedure of SAS statistics package (SAS Institute, INC. 8.1, Cary, NC). Data was analyzed as a 2 x 2 factorial arrangement of treatments, following this model:

$$Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk,$$

where Yijk is the dependent variable, μ is the overall mean, αi is the effect of including lactulose (PRE), βj is the effect of including *L. plantarum (*PRO), (αβ)ij is the interaction between lactulose and *L. plantarum* and ε represents the unexplained random error.

For performance analyses and for the microbial and slaughter measurements, pig was used as the experimental unit; and pen for average daily feed intake (ADFI) and gain: feed ratio (G:F). Multiple comparisons of the means were performed using the PDIFF function of SAS adjusted by Tukey Kramer.

The number of animals with clinical antibiotic treatment, the data of prevalence of *L. plantarum* in faeces and also the frequency detection of TRFs were analyzed by a χ^2 test using the same statistical software. Statistical significance was accepted at $P < 0.05$ and statistical trend when $P \leq 0.10$.

4.3. Results

During the experimental period all animals remained healthy although mild diarrhoea was observed in three pens of the CTR group and in two pens of the LAC and LPLA groups. In total 14 animals were treated with antibiotics during the first three experimental days. Differences between treatments were not significant (data not shown).

4.3.1. Animal Performance.

The effects of experimental treatment on ADFI, average daily gain (ADG) and G:F ratio are presented in Table 4.2. The addition of *L. plantarum* in the diet had no significant effect on the BW, ADG or G:F ratio. But, the incorporation of lactulose in the diet significantly improved ADFI (*P*= 0.03), ADG *(P* =0.01) and G:F ratio of piglets at 14 days of experiment (*P* =0.04).

4.3.2 .Microbiota composition.

L. plantarum was isolated from the faeces of 3, 4, 6 and 4 piglets from CTR, LAC, LPLA and SYN groups at day 7 regardless of the experimental treatment (Table 4.3). However at day 14 the number of piglets with more than 2 log UFC/g FM of *L. plantarum* (minimum level of detection) was higher *(P* = 0.01) in those groups receiving the probiotic bacteria 4 for LPLA and SYN compared to 1 in LAC and neither in CTR treatment.

Table 4.2. Effect of the experimental diets on growth performance of early weaned pig: Body weight (BW) (kg), average daily feed intake (ADFI) (g/day), average daily gain (ADG) (g/day), gain:feed ratio (G:F).

Items	Period	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
BW	0	6.4	6.4	6.4	6.4	0.54	0.93	0.96	0.94
	7	6.3	6.6	6.4	6.6	0.65	0.22	0.76	0.77
	14	7.6	8.0	7.5	8.2	0.78	0.13	0.55	0.90
ADFI	0-7	95	134	114	123	37.7	0.07	0.80	0.26
	7-14	260	285	267	305	34.9	0.02	0.25	0.61
	0-14	143	175	154	179	34.0	0.03	0.51	0.78
ADG	0-7	-4	25	-5	28	17.7	0.11	0.27	0.50
	7-14	166	192	180	216	52.4	0.30	0.73	0.13
	0-14	81	124	91	129	42.0	0.01	0.88	0.59
G:F	7	-0.10	0.04	0.05	0.07	0.158	0.151	0.10	0.30
	7-14	0.61	0.63	0.60	0.66	0.131	0.13	0.77	0.25
	0-14	0.57	0.69	0.60	0.72	0.173	0.04	0.94	0.64

PRE; main effect of the inclusion of Lactulose in the diets.
PRO; main effect of the inclusion of *L. plantarum* in the diets.

Table 4.3. Prevalence (number of animals with positive recoveries) and plate counts (between brackets mean values for positive animals: Log CFU/g fresh matter) of *Lactobacillus plantarum* in the faeces of early weaned pigs.

Period	Frequency			
(days)	CTR	LAC	PLAN	SYN
7	3 (3.57)	4 (4.36)	6 (4.33)	4 (4.18)
14	0^{y}	1 (3.00)^{y}	4 (3.83)^{x}	4 (3.88)^{x}

Minimun level of detection 2 log CFU/gr faeces.
^{xy} Different superscripts in the same row denote a significant difference ($P \leqslant 0.05$).

Results for microbial counts of other groups in faeces are shown in Table 4.4 No significant differences were observed for either, total coliforms, *E. coli*, *Clostridium* spp. or lactic acid bacteria.

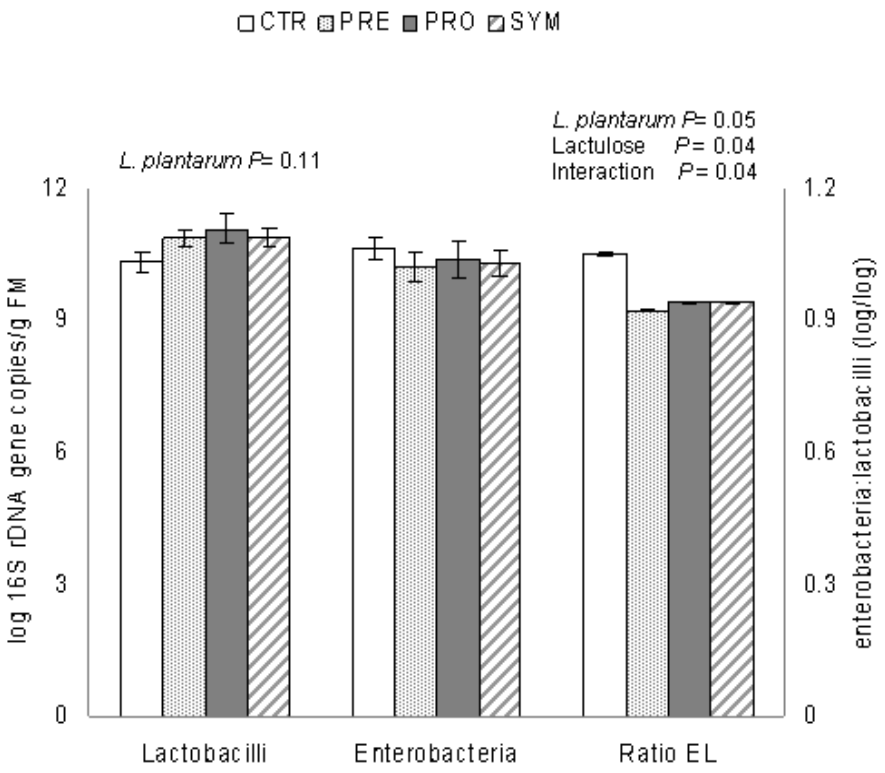
Table 4.4. Microbial counts *(E. coli*, coliforms, *Clostridium perfringens* and lactic acid bacteria) in faeces of early weaned pigs (Log CFU/ g FM faeces).

Items	Period	[a]Diets				SEM	*P*-value		
	(days)	CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
E. coli	7	8.0	7.7	7.3	7.6	0.78	0.89	0.11	0.28
	14	6.9	6.6	6.2	6.1	0.14	0.56	0.19	0.78
Coliforms	7	8.1	7.8	7.5	7.8	0.78	0.80	0.29	0.29
	14	7.2	6.8	6.9	6.7	0.10	0.49	0.62	0.72
C. perfringens	7	5.4	5.5	5.5	6.0	1.12	0.30	0.35	0.51
	14	5.0	5.2	5.4	4.8	1.48	0.70	0.87	0.48
Lactic acid bacteria	7	8.4	8.4	8.5	8.5	0.81	0.95	0.62	0.99
	14	8.1	7.6	7.9	7.6	1.22	0.23	0.72	0.73

PRE; main effect of the inclusion of Lactulose in the diets
PRO; main effect of the inclusion of *L. plantarum* in the diets

The total numbers of lactobacilli and enterobacteria were quantified in colon digesta at day 14 using qPCR (Figure 4.1). Results showed that enterobacteria counts were not significantly different between treatments. However, the lactobacilli population was numerically higher (P = 0.11) in the treatments including *L. plantarum*. On the other hand the ratio enterobacteria:lactobacilli was significantly (P = 0.05) higher in the CTR group than other groups (1.05 versus 0.92, 0.94 and 0.94 for CTR, LPLA, LAC and SYN groups, respectively).

Figure 4.1. Bacterial counts of lactobacilli and enterobacteria in colon digesta measured by quantitative PCR (log 16S rRNA gene copies/g FM)



The t-RFLP analysis of microbial community did not show differences in the species richness of the ecosystem defined as the total number of TRFs 20, 23, 26 and 24 for CTR, LAC, LPLA and SYN, respectively (P = 0.532) and the dendogram analysis (Figure 4.2) did not show any obvious grouping arrangement that was diet-related. However, compatibility analysis of TRFs showed some changes in particular microbial groups related to experimental treatments.

Figure 4.2. Dendogram illustrating the effect of experimental diets on microbial ecosystem.

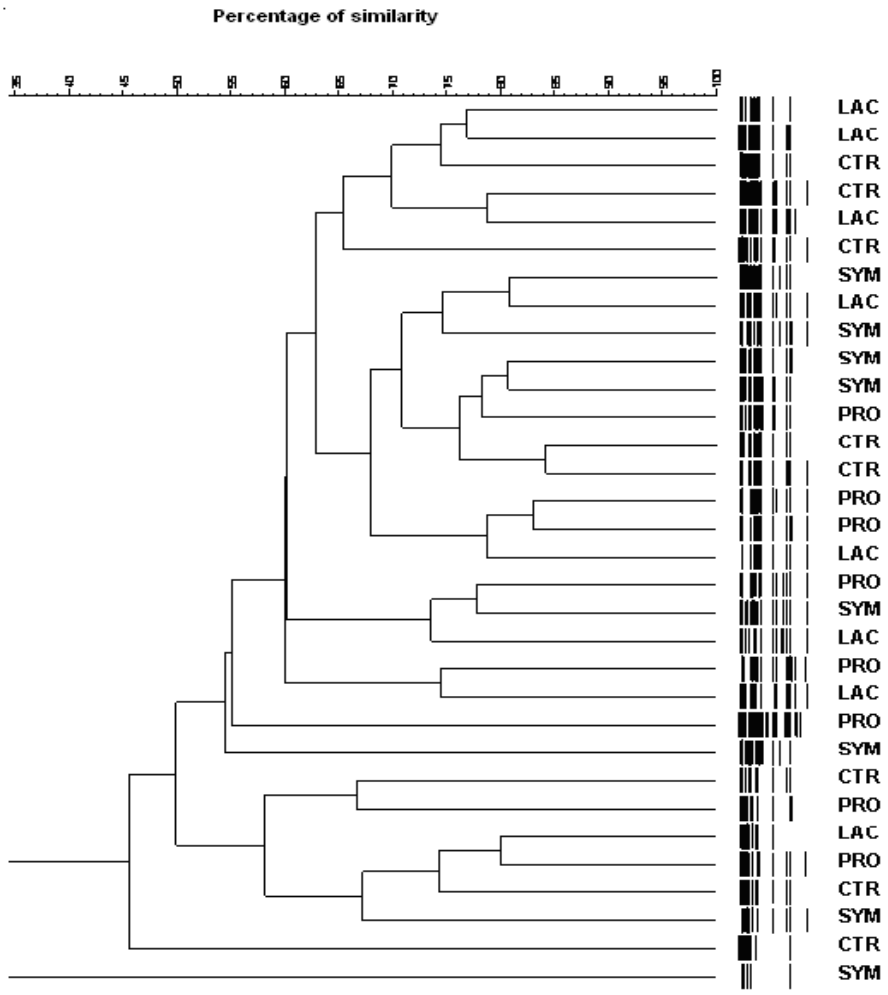


Table 4.5. Compatibility analysis of T-RFLP results for the major pig gut bacteria (n = 8 for CTR, LAC and PLAN; n = 7 for SYN)

Bacterial groups	Compatible bacteria [a]	*In silico* restriction[b]	Real restriction	Frecuency[d]			
				CTR	LAC	PLAN	SYN
Bacillus and Lactic acid bacteria	*L. delbruekii subsp. Lactis*	223	223	0y	5 (3.26)^{x}	2 (0.75)^{y}	1 (1.39)^{y}
	Bifidobacterium thermophilum	180	180	0^{y}	6 (1.59)^{x}	6 (1.46)^{x}	4 (1.36)^{x}
	Bacillus spp.	237-243	243-244	1 (0.68)	2 (0.97)	2 (0.97)	2 (0.97)
	L. plantarum	599	599	0	0	1 (0.99)	0
	Enterococcus spp.	217-218	214	2 (0.77)	2 (1.53)	1 (1.09)	0
Cytophaga	*Cytophaga*	90, 92, 94	82-94	6 (4.05)	8 (5.12)	8 (4.88)	4 (10)
Fibrobacter	*Fibrobater intestinales*	148	147-148	8 (9.38)	6 (13.8)	7 (14.0)	6 (13.8)
Clostridium	*Clostridium coccoides*	66	65-66	2 (0.91)	3 (0.86)	1 (0.64)	0
	Clostridium spp.	231-233	231-232	1 (1.92)	3 (1.09)	3 (1.62)	3 (0.81)
Proteobacteria	*Escherichia* spp.	371-373	371	8 (2.58)	8 (2.19)	8 (1.08)	7 (1.21)
	Other enteric bacteria (*Salmonella* spp.*, Citrobacter, Klebsiella*)	371-374	374-375	1(7.02)	5 (5.25)	4 (1.09)	2 (3.19)

[a] Major pig gut bacteria, based on the work of Leser *et al*. (2002), with a potential compatible fragment found in at least five animals.
[b] *In silico* restriction was performed using the analysis function of MiCA web page
[c] Terminal fragment length obtained after PCR product restriction with Hha I.
[d] Number of animals that showed the peak in each experimental group (in parenthesis mean peak area for positive animals).
[xy] Different superscripts in the same row denote a significant difference in frequency ($P \leqslant 0.05$).

4.3.3. Metabolic microbial activity

Table 4.6 presents pH values in proximal colon digesta and changes in the concentration and molar proportion of SCFA and in the concentration of lactic acid with the different experimental treatments. Colonic pH was reduced when lactulose was included alone into the diet, but not in the synbiotic combination (*P*-interaction = 0.03).

Table 4.6. Effect of the experimental diets on colonic fermentation of early weaned piglets. Table includes the pH values, the concentrations of short-chain fatty acids (SCFA) (mM, and molar proportions) and lactic acid (mM), and values of blood urea nitrogen (BUN) (mM).

Items	**Diets**				**SEM**	***P*-value**		
	CTR	**LAC**	**PLAN**	**SYN**		**PRE**	**PRO**	**Interaction**
pH	6.1^{y}	5.7^{x}	6.0^{y}	6.2^{y}	0.32	0.15	0.18	0.03
SCFA (mM)[a]	120.9	119.4	124.4	117.9	13.50	0.41	0.84	0.61
Acetic (%)	64.7^{y}	59.2^{x}	61.6^{X}	64^{y}	5.3	0.55	0.46	0.05
Propionic (%)	23.4	25.6	24.0	22.7	3.15	0.87	0.65	0.40
Butyric (%)	11.9^{Y}	15.2^{X}	14.4^{X}	13.3^{XY}	2.17	0.10	0.68	0.02
Valeric (%**)**	3.5	3	3.6	2.7	0.96	0.04	0.65	0.49
BCFA (%)[b]	3.3	2.9	2.4	2.1	0.77	0.12	0.002	0.77
Lactic (mM)	5.2	5.9	7.8	5.2	3.08	0.62	0.27	0.23

PRE; main effect of the inclusion of Lactulose in the diets.
PRO; main effect of the inclusion of *L. plantarum* in the diets.
[a] Total SFCA=acetic+propionic+butyric+valeric + branched chain fatty acids (BCFA)
[b] BCFA=isobutyric+isovaleric
[xy] Different superscripts in the same row denote a significant difference *(P* $\leqslant$ 0.05)**.**

Total SCFA and lactic acid concentrations were not modified by the experimental treatments. However, the experimental treatments were associated with modified molar proportions of SCFA in the proximal colon: The percentage of acetic acid was decreased with LAC and LPLA but not with SYN (*P*-interaction = 0.05). The percentage of butyric acid

increased with LAC and LPLA, but was not changed with SYN (*P*-interaction = 0.02). The percentage of valeric acid was decreased with the inclusion of lactulose (*P* = 0.04) and that of branched chain fatty acids decreased by the inclusion of *L. plantarum* (*P* = 0.002).

4.3.4. Serological results.

BUN results (1.1, 0.5, 0.9 and 0.6 mM for CTR, LAC, LPLA and SYM respectively) demonstrates a trend to decreased the concentration of urea in blood serum (*P* = 0.07) by the addition of lactulose. No significant effects were observed on PigMAP values (data not shown) associated with the experimental diets.

4.4. Discussion

In recent years a number of authors have reported the effect of lactulose on the metabolic activity and composition of the intestinal microbiota of weaning piglets (Kamphues *et al*., 2007; Martín-Pelaez *et al*., 2010). Furthermore, numerous studies have assessed the potential of *L. plantarum* as a feed additive promoting the establishment of a beneficial microbiota in the intestine (De Angelis *et al*., 2006; Pieper *et al*., 2009). However, no studies have evaluated the effect of a combination of both, lactulose and *L. plantarum,* as a growth promoter in weaned piglets. The aim of this study was to assess the impact of a synbiotic mixture of *L. plantarum* and lactulose on the growth performance, and digestive and gut metabolic parameters in piglets at weaning.

In this study, piglets that received lactulose in the diet showed a significantly higher feed intake (*P* =0.02) and weight gain (*P* = 0.01). Krueger *et al*. (2002) also reported increases in the weight of sows or weaned piglets that consumed lactulose. However other studies with weaning piglets receiving 1 % of lactulose in combination with other fermentable carbohydrates, did not find differences in growth (Konstantinov *et al*. 2004). The enhanced feed intake observed with the lactulose could be due to an improvement in gut health and a better adaptation to weaning but also to an increase in feed palatability. Although there is no

scientific evidence evaluating palatability of lactulose, piglets do have a preference for sweet flavours (Kennedy and Baldwin, 1972) and lactulose could have acted as a sweetener improving the acceptability of the diets.

The addition of lactulose to the diet did not significantly modify the concentration of total SCFA's or lactic acid. However, the relative amount of butyric acid tended to increase (P = 0.10) compared to other treatments. Similar results were obtained by Branner *et al.* (2004) feeding growing pigs with 1.5 % lactulose.. Interestingly, previous *in vitro* assays carried out by our group with batch culture systems using porcine inocula and 1 % lactulose, also showed significant increases in the percentage of butyric acid (Martín-Peláez *et al*., 2008) and in the total concentration of SCFA but not in lactic acid.

This scarce ability of lactulose to modify fermentation products in the pig hindgut compared to other prebiotics could be related to an insufficient dose, as suggested by Kamphues *et al*. (2007) or also to changes in the metabolism of different bacteria that could maintain a constant balance. Moreover it should be noted that *in vivo* conditions, concentration of SCFAs do not directly relate to their production, but to the balance between production and absorption, and therefore a lack of differences in concentration did not discard differences in production. In this regard our study, suggests an effect of lactulose on microbial activity by the changes observed in blood urea nitrogen (BUN) with lower concentrations when lactulose was included into the diet. This decrease could be related to an enhanced synthesis of microbial proteins in the hindgut. Interestingly, similar reductions in serum urea and intestinal ammonia with lactulose was described by Weber (1997)

Regarding the effect of lactulose on microbial composition, qPCR analysis showed a decrease in the enterobacteria/lactobacilli ratio in colon and also an increase in TRFs compatible with *Lactobacillus delbruekii* and *Bifidobacterium thermophilum* in the LAC diet. It has been previously described that lactulose can increase the population of lactobacilli (including *L. plantarum)* and bifidobacterium (Maxwell *et al*., 2004; Martín-Pelaez *et al*.,

2008). However, other *in vivo* assays could not demonstrate any effect of lactulose on lactic acid bacteria population in the hindgut (Kamphues *et al*., 2007; Martín-Peláez *et al*., 2010).

The ability of *L. plantarum* to survive the hostile conditions in the stomach and reach the distal parts of the GIT was evidenced by this study due to the higher faecal prevalence of this microorganism in the groups treated with PRO at day 14 of experiment. Similar results were obtained by Takahashi *et al.* (2008). It is not suppressive not finding TRFs compatible with *L. plantarum* in the T-RFLP analysis considering that this technique that do not target microbial groups under 1 % of the total population (Li *et al*., 2007).

In the studies presented here the administration of *L. plantarum* to the animals seemed to decrease the faecal *E. coli* numbers ($P = 0.11$) and the percentage of total peak area for the TRFs compatible with *E. coli.* Similarly results have been described by other authors (Takahashi *et al*., 2008). Reductions observed in the percentage of branched chain fatty acids with this probiotic could also be related to reductions in microbial populations with high proteolytic activity like *E. coli* (Bauer *et al*., 2006; De Lange *et al*., 2010). No differences were found in total lactic acid bacteria when quantified by qPCR but whether a decrease in the ratio enterobacteria/lactobacilli ($P = 0.05$) with the inclusion of *L. plantarum*. Also a higher frequency of detection for compatible TRFs with *Bifidobacterium thermophilus* was found. Positive effects of *L. plantarum* on the endogenous lactobacilli population have been described by other authors (De Angelis *et al*., 2007; Takahashi *et al*., 2008; Giang *et al*., 2010). However, cautions must be taken when comparing different studies, considering the reported variations in the particular strains, doses, way and method of administration (Gaggìa *et al*., 2010).

In a synbiotic combination it is assumed that the prebiotic provides the probiotic with an advantage to increase, not only its numbers, but also its metabolic activity through the supply of fermentable substrate. In this study, we chose the combinations of lactulosa and *L plantarum*, based on previous *in vitro* studies (Martin-Peláez *et al*., 2008). However, we did

not found any other publication that studied this synbiotic combination in vivo condition. Other investigators however, such as Nemcová *et al*. (2007) studied the use of othher synbiotic combinations of *L. plantarum* with maltodrextrins and/or fructoligosaccharides (FOS) in pigs and showed significant reductions in the numbers of *E. coli* K-88 adhering to the intestinal mucosa. In our study we were not able to demonstrate either a specific increase in the number of *L. plantarum* with the SYN or in total microbial activity compared to the *L. plantarum* alone. Regardless of this, the possibility of a specific improvement of *L. plantarum* activity by the prebiotic should not discard. It is important to remark that the increase in the metabolic activity of a probiotic (not only its numbers) could be fundamental for its health promotion properties.

4.5. Conclusions

The data presented here clearly demonstrates that the addition of lactulose to the diet of weaning piglet's increases feed intake and can affect colonic fermentation with increases in lactic acid bacteria and reductions on the blood urea nitrogen. On the other hand, *L. plantarum* seems to control the growth of *E. coli* shortly after weaning, increasing the total number of lactobacilli (P = 0.11) and reducing protein fermentation. Synbiotic combination adds all these positive effects although we were not able to demonstrate a specific growth of *L. plantarum* promoted by lactulose. The possible potential of lactulose to increase specifically the activity of *L. plantarum* in the pig intestine is worthy of further investigation.

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CHAPTER 5

Effect of a synbiotic combination of lactulose and *Lactobacillus plantarum* on the response of weaning pigs to an oral challenge with *Escherichia coli* K88

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of diarrhoea in weaned piglet (Holland, 1990). Several feeding strategies, alternative al use of antibiotics, have been suggested for the control and prevention of these disorders such as, supplementation of the feed with egg yolk antibodies, pharmacological doses of ZnO, spray-dried plasma, dietary acidification, phage therapy, or the use of prebiotic and probiotics (Fairbrother *et al*., 2005).

Probiotics, prebiotics and its synergist combination are considered functional components in swine diets that can have positive effects over one or several functions of the organism (Mountzouris, 2007). Probiotics, defined as life microbial feed supplements witch beneficially affects the host animal by improving its microbial balance (Fuller et al.,1989) could inhibit the growth of pathogenic microorganisms and prevent the diarrhoea by diverse modes of action including the enhancement of epithelial barrier integrity, competitive exclusion mechanisms, the secretion of bacteriocins, the interference with quorum sensing signalling, the attenuation of the inflammatory response and the modulation of the immune system against potentially harmful antigen (Brown, 2011). Prebiotics defined as "selectively fermented ingredients that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, which confer benefits on host well-being and health" (Gibson et al., 2004) could improve performance and prevent the post weaning diarrhoea, through the selective increase of beneficial bacteria, such as bifidobacteria and lactobacilli, the improvement of intestinal functions, the impairement of the epithelial binding of pathogenic bacteria,and the enhancement of the immune system of the host (Gaggìa *et al*., 2010, Robertfroid *et al.*, 2010).

In the last years the symbiotic concept as a combination of pre and probiotics has been explored by different researchers as a way to benefit the host by improving the survival

and persistence of live microbial dietary supplements in the GIT (Gibson and Roberfroid, 1995). Although some experimental trials can be found in the literature, nowadays the number of symbiotic combinations reported in piglets is scarce (Shin *et al*., 2005, Nemcová *et al*., 2007).

Regarding the potential of lactulose in synbiotic combinations, previous in vitro trials conducted by our group have demonstrated that lactulose selectively stimulate the growth of several beneficial lactic bacteria and particularly *Lactobacillus plantarum* (Martín-Pelaéz *et al*., 2008). Further *in vivo* trials also showed that both, lactulosa and *L. plantarum*, improved performance and colonic microbial activity of weaning piglets (Guerra *et al.,* 2012), however it kept to be studied their potential to improve the response of the animal in challenge conditions.

The aim of this study was therefore to evaluate the efficacy of lactulose, a probiotic strain of *Lactobacillus plantarum*, or its synbiotic combination against an oral enterotoxigenic *Escherichia coli* (ETEC) K88 challenge in weaning piglets.

5.2. Materials and Methods

5.2.1 Animals and diets

Seventy two commercial breed ([Large White x Landrace] x Pietrain) weaning piglets of 25 ± 2 days old with an average body weight 6.3 ± 0.75 kg were purchased from a commercial farm and were randomly allocated in 24 pens (three pigs per pen) at the experimental facilities of the Universitat Autònoma de Barcelona (UAB). All procedures involving the treatment, management, housing, husbandry and euthanasia of animals received prior approval from the Animal Protocol Review Committee of the UAB (Permit number CEAAH 746) and ethics committee of the Animal Health Veterinary Laboratories Agency (United Kingdom), and were carried out conformed to European Union Guidelines

(The Council of the European Communities, 1986). The experiment was conducted during the spring season (May) with an average inside room temperature of 30 ± 2 ºC.

Animals in each of six replicated pens received one of four dietary treatments as follows: a control diet (Table 5.1) (CTR) or the same diet to which 15 ml/kg of lactulose syrup (Duphalac, Solvay Pharma, S.A. Barcelona) was added (equivalent to 1 % of lactulose) (PRE) or a daily culture of *L. plantarum* (2 x 10^{10} CFU/animal/day) given (PRO). The probiotic was administered daily by spraying the feed with 20 ml of a pure culture (prepared as described below) per pig. The combination of both additives corresponded to the synbiotic treatment (SYN). All diets were fed as meal. Diets not including the probiotic treatment were also sprayed with sterile Man, Rogosa and Sharpe broth (MRS, Merck 1.10661). Diets and water were offered *ad libitum*.

5.2.2. Probiotic strain

L. plantarum strain (B2984) originally isolated from the faeces of a healthy commercial pig was obtained from the Animal Health and Veterinary Laboratories Agency bacterial culture collection. For convenience 1 ml of lyophilized *L. plantarum* cultures were prepared: *L. plantarum* was cultured at 37°C in sterile Man, Rogosa and Sharpe broth (MRS, Merck 1.10661) medium to an OD_{650} of 1.2. The bacterial cells were centrifuged (7,600 × g) at 5°C for 10 min (Beckman Coulter Avanti J20-XPI) and resuspended in 10 ml of cryoprotective medium (1.5 % w/v skim milk and 4% w/v sucrose). The cultures were then lyophylised in vials of 1 ml. Each day the inocula was prepared by resuspending a single vial in 15 ml of MRS which was added to fresh sterile pre-warmed 500 ml of MRS broth and cultured for 16hrs at 37°C in to reach a final concentration of 10^9 CFU/ml. Serial dilutions of the inocula were plated on MRS agar and incubating for 16 hat 37°C after order to determine the CFU/ml.

Table 5.1. Composition of the control diet.

Ingredients	(g/kg fresh matter)
Corn Flakes	360.0
Wheat Flakes	240.0
Full fat extruded soybeans	110.0
Egg meal	92.7
Sweet whey	150.0
Soybean oil	16.5
Calcium Carbonate	5.6
Dicalcium Phosphate	8.10
L-Lysine	4.6
DL-Methionine	0.5
L-Threonine	1.1
L-Tryptophan	0.9
Salt	4.0
Vitamin-Mineral Premix*	6.0
Chemical analysis	(g/kg dry matter)
Dry matter	901
Gross energy (MJ/kg)	18
Crude protein (CP; N x 6.25)	177
Neutral detergent fibre	74
Acid detergent fibre	28
Ether extract	46

(*)Supplied per kilogram feed: 10,200 IU of vitamin A, 2,100 IU of vitamin D3, 39,9 mg of vitamin E, 3 mg of vitamin K_3, 2 mg of vitamin B_1, 3 mg of vitamin B_2, 3 mg of vitamin B_6, 0.025 mg of vitamin B_{12}, 20 mg of Pantotenate calcic, 60 mg of Nicotinic acid, 0.1 mg of biotin, 0.5 mg of Folic acid, 150 mg of Fe, 156 mg of Cu, 0.5 mg of Co, 120 mg of Zn, 49.8 mg of Mn,2 mg of I, 0.3 mg of Se.

5.2.3. E. coli strain.

The bacteria strain used in this study was isolated from a colibacilosis outbreak in Spain (Blanco *et al*., 1997), serotype (O149:K91:H10 [K-88]/LT-I/STb), and was provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The infection inoculum was cultured by 16 hrs at 37ºC in Luria broth with slow agitation (1 x *g*) in an orbital incubator (WY-100, Comecta S.A., Barcelona, Spain), in to reach a final concentration of 10^9 CFU/ml.

5.2.4. Experimental procedures.

The experiment period was of 18 days. After one week of adaptation to the diets (on day 8), a single 2 ml oral dose (2 x 10^9 CFU/ml) of the ETEC (K88) strain was administered to the challenged animals. Feed intake and body weight were recorded on days 0, 7, 14 and 18. Average daily gain was calculated individually and average daily feed intake and gain:feed (G:F) by pen. Animals were checked daily to evaluate their status after the *E. coli* challenge. Briefly, clinical signs (i.e. dehydration, apathy and diarrhoea) were monitored daily. The diarrhoea incidence was measured as the percentage of animals in each pen that presented inconsistent to liquid faeces. The mortality rate was also recorded.

On day 0 faecal samples for microbiological analysis were taken aseptically in 24 animals randomly selected from total, whereas on days 7, 14 and 18, faecal samples were taken from two animals per pen corresponding to the animals with the highest and lowest BW. Sampled piglets were the same for the three sampling days of post challenge period.

At days 6 and 10 post challenge (PC) (experimental days 14 and 18 respectively) one pig per pen was euthanized. On day 6 PC it was selected the animal with the intermediate BW while on day 10 PC it was selected the heaviest of the two remaining piglets. Previous to euthanasia, an 8 mL-sample of blood was obtained by venipuncture of the cranial vena cava using a BD Vacutainer (Becton Drive, Franklin Lakes, NJ) 10-mL plasma tube with sodium heparin (17 IU/mL). The plasma collected was stored at –80°C until the analytical use. Immediately after blood sampling, selected piglets received an intravenous lethal injection of pentobarbital sodium (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200 mg/kg BW). Animals were bled, the abdomen immediately opened and the whole GIT was excised.

Content from ileum and proximal colon (was considered 25 cm from ileocecal junction were homogenized and pH determined with a pH-meter (electrode Crison 52-32, Net INTERLAB SAL). Fresh samples from ileum and proximal colon content were taken to microbial counts of *Lactobacillus* plantarum and to be preserved in HCl solution (3 mL of

content + 3 mL of HCl 0.2 N) for NH_3 determination. Additionally, two sub-samples (approximately 20 g) were kept frozen at -20 ºC until be analysed for SCFA and quantification of microbial groups by quantitative PCR (qPCR).

For the analyses of *Lactobacillus plantarum* attached to the ileum mucosa, 5-cm long sections of ileum were collected from each animal, washed thoroughly three times with sterile PBS, open longitudinally and scraped with a microscopy glass slide to obtain the mucosa scrapes content, that was placed in sterile container and immediately frozen at -20º C. Moreover for histological study, sections of 3 cm from the ileum were removed, opened longitudinally and fixed by immersion in Carnoy solution as described by Swidsinski *et al*. (2005). Tissue samples were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphological measurements were performed with a light microscope (BHS, Olympus, Spain), according to published parameters by Nofrarías *et al*. (2006).

5.2.5. Analytical methods

Culture methods. For bacterial counts, faecal, ileal and colon digesta and ileal mucosa scraping samples were suspended (10% wt/vol) in a buffered peptone solution (PBS) and subsequently homogenized for 5 min on a standard laboratory vortex. Thereafter, 10-fold dilutions were made in PBS. *L. plantarum* was enumerated using the specific growth medium described by Bujalance *et al*. (2006). Confirmation of the identity of colonies as *L. plantarum*, was performed by random amplified polymorphic DNA (RAPiD) analysis of a total of 30 colonies randomly selected using primer OPL-05 (ACGCAGGCAC) (Van Reenen *et al*., 1996). All microbial enumerations were expressed as log colony forming units (CFU) per gram of faeces.

DNA extraction. Colon digesta samples (400 mg) were precipitated by centrifugation (13,000 g x 5 min) and DNA from the precipitate was extracted and purified using the

commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90º C and a posterior incubation step with lysozyme was added (10 mg/ml, 37º C, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 ml of Qiagen Buffer AE (Qiagen, West Sussex, UK) and stored at -80º C until use.

Terminal Restriction Fragment Length Polymorphism (t-RFLP). T-RFLP analysis of bacterial community in proximal colon was performed following the procedure described by Castillo et al. (2007). Briefly, a 1,497 bp fragment of the 16S rRNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5' AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR analyses were performed for each sample. The fluorescently labelled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µL of milli-Q water. After that, the resultant PCR product was subjected to to a restriction with Hha I endonuclease restriction enzyme (20,000 U/µl) (Biolabs Inc. New England, USA) for 8 hrs at 37º C. The fluorescently labelled terminal restriction fragments (TRFs) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with 25-U detection threshold. Determinations of the sizes of TRFs in the range of 50 to 700 base pairs were performed with the size standard GS-1000-ROX (PE Biosystems).

Sample data consists of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all the TRF peak areas in the pattern was used to normalize peak detection threshold in each sample following the method of Kitts (2001). New total area was obtained by the sum of all the remaining peak areas in each pattern. Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated

and dendograms constructed using Fingerprinting II (Informatix, Bio-Rad, C, USA) software and un-weighed pair group method with averaging algorithm (UPGMA).

In order to infer the bacterial composition of the samples, *in silico* restrictions for the major pig gut bacteria (acording to Leser *et al*. (2002)) with the primers and the enzyme used was obtained using the analysis function of MiCA web page (http://mica.ibest.uidaho.edu/default.php).

Quantification of microbial groups by Real time PCR. (q-PCR) Lactobacilli and enterobacteria in colonic samples were quantified by real time PCR (qPCR) using SYBR Green dye following the protocols described by Castillo *et al*. (2006). To quantify *E*. *coli* K88 it was used an additional SYBR-Green PCR reaction according to procedure described by Hermes *et al*. (2012).

5.2.6. Chemical analysis.

Diet samples were analyzed, according to the Association of Official Analytical Chemists (AOAC, 1995) methods for dry matter (945.15), crude protein (979.09) and ether extract (920.39C). Neutral detergent fibre and acid detergent fibre were determined according to the method of Van Soest *et al*. (1991). The gross energy (GE) of was measured using an adiabatic bomb calorimeter (IKA 4000, Staufen, Germany).

Short-chain fatty acid and lactic acid analysis were performed by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatization with MBTSTFA+ 1% TBDMCS agent, using the method of Richardson *et al*. (1989) modified by Jensen *et al*. (1995).

Ammonia was determined with the aid of a gas sensitive electrode (Crison ISE- 9665, Crison Instruments, S.A., Barcelona, Spain) combined with a digital voltmeter (Crison GLP 22). Three grams of content were diluted (1:2) with 0.16 M NaOH, and after homogenization, samples were centrifuged (1500 x *g*) for 10 min. The ammonia released was measured in

the supernatants as different voltage in mV following the procedure described by Hermes *et al*. (2009).

Blood urea nitrogen was measured by Glutamate Dehydrogenase (GLDH) reaction, using the Olympus System Reagent® (Olympus, Ireland) and using a Olympus AU400 (series 3112676, Germany) device. The Tumor Necrosis Factor-α (TNF-α) concentrations were determined by Quantikine® Porcine TNF-α kit (R&D Systems, Abingdon, United Kingdom). Pig Major Acute-phase Protein (Pig-MAP) concentration was determined by a sandwich type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain), according to the manufacturer's instructions. Plasma was obtained by centrifugation of blood at 3,000 x *g*, 15 min at 4ºC.

5.2.7. Statistical Analysis

The effect of diet on performance, lactobacilli enterobacteria, *E*. *coli* K88 and slaughter measurements was tested with an ANOVA using the GLM procedure of SAS statistics package (SAS Institute, INC. 8.1, Cary, NC). Data was analyzed as a 2 x 2 factorial arrangement of treatments, following this model:

$Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk$, were Yijk is the dependent variable, μ is the overall mean, αi is the effect of including lactulose (PRE), βj is the effect of including *L. plantarum (*PRO), (αβ)ij is the interaction between lactulose and *L. plantarum* and ε represents the unexplained random error.

Pen was used as the experimental unit, and in the event that significant diet effects were established ($P < 0.05$), multiple comparisons of the means were performed using the PDIFF function of SAS adjusted by Tukey Kramer.

The data of prevalence of *L. plantarum* and the frequency detection of TRFs were analyzed by a χ^2 test using the same statistical software. Statistical significance was accepted at $P < 0.05$ and statistical trend when $P < 0.10$.

5.3. Results

5.3.1. Animal performance and health status.

The effect of the treatments on animal performance is shown in table 5.2. The addition of lactulose to the diet significantly increased the average daily gain (ADG) ($p<0.02$) between 14-18 days (PRO, $P=0.02$). Average daily feed intake (ADFI) did not show significant differences between treatments.

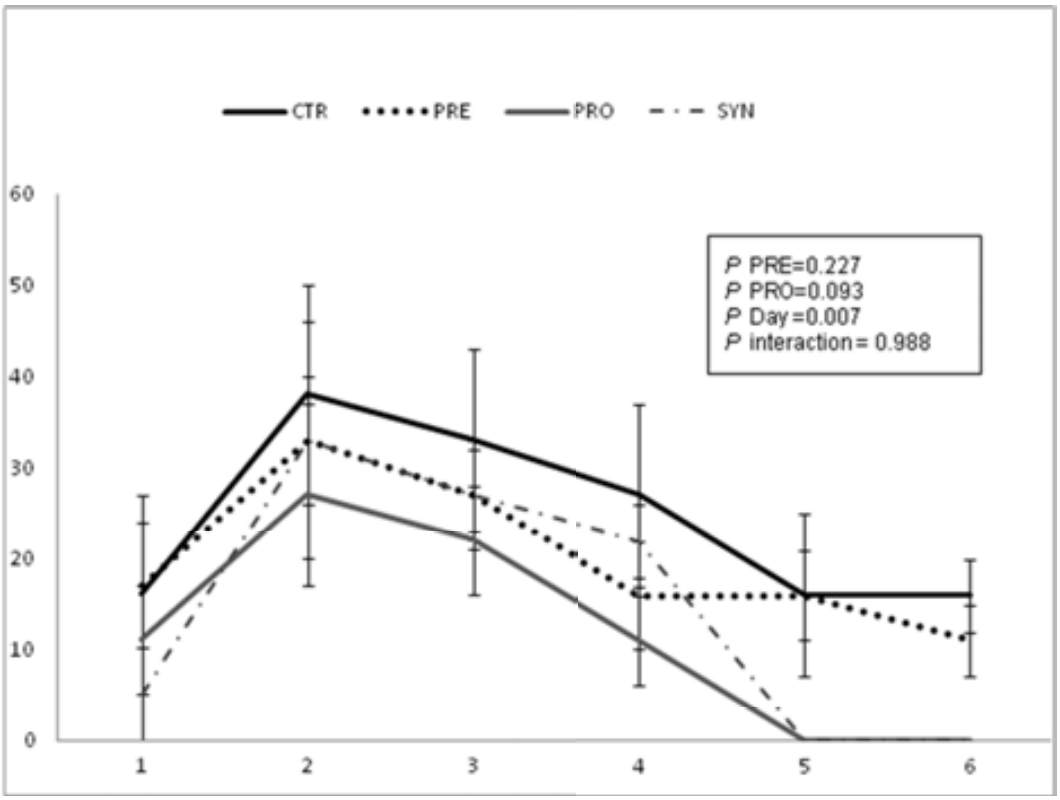
Table 5.2. Effect of the experimental diets on growth performance of early weaned pig: Body weight (BW) (kg), average daily feed intake (ADFI) (g/day), average daily gain (ADG) (g/day). gain:feed ratio (G:F).

Items	Period	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
ADFI	0-7	47	58	56	53	25.70	0.44	0.84	0.49
	7-14	274	291	287	316	64.08	0.58	0.91	0.29
	14-18	309	373	333	379	90.43	0.79	0.87	0.34
ADG	0-7	-3.4	2.4	5.6	1.6	13.72	0.86	0.47	0.44
	7-14	141	163	161	187	55.13	0.72	0.76	0.66
	14-18	165	265	222	291	71.50	0.02	0.13	0.54

PRE; main effect of the inclusion of lactulose in the diets. PRO; main effect of the inclusion of *L*. *plantarum* in the diets.

Diarrhoea incidence was daily recorded after ETEC challenge. There was a significant effect of supplementation with *L*. *plantarum* on the reduction of the diarrhoea incidence ($P<0.09$). Mild diarrhoea was detected in all groups, with piglets in CTR group showing a much larger increase which lasted until five days (Figure 5.1).

Figure 5.1. Percentage of piglets that presented diarrhoea during the post-challenge period



PRE; main effect of the inclusion of lactulose in the diets.
PRO; main effect of the inclusion of *L*. *plantarum* in the diets.

5.3.2. Microbial activity and composition

Table 5.3 shows microbial counts for *L. plantarum* found in ileal scrapings, ileal and colon digesta and faeces. Results revealed no detection of *L*. *plantarum* in any of the samples of piglets fed CTR diet. However at day 6 PC, *L*. *plantarum* was detected in ileum scrapping, intestinal content and faeces of some of the piglets receiving PLAN and SYN diets, and four days after in most of the animals receiving these treatments. Regarding the animals fed the LAC diet *L. plantarum* bacteria was also detected in some piglets but ony at day 10 PC.

Table 5.3. Prevalence (number of animals with positive recoveries) and plate counts (Log CFU/g fresh matter) for positive animals (between brackets) of *Lactobacillus plantarum* in ileal scrapings, digesta, colon digesta and faeces of weaning piglets challenged with ETEC (K88) at 6 and 10 days post challenge (PC).

Localization	Period (days)	[a] Prevalence (counts)			
		CTR	LAC	PLAN	SYN
Ileal scrape	6	0	0	1(4.5)	1(3.3)
	10	0^{y}	$3(3.5)^{x}$	$4(3.6)^{x}$	$4(4.1)^{x}$
Ileum	6	0	0	1(2.3)	1(2.0)
	10	0^{y}	$2(2.0)^{xy}$	$4(2.7)^{x}$	$4(3.1)^{x}$
Colon	6	0	0	2(6.2)	1 (5.2)
	10	0^{y}	$1(3.1)^{y}$	$4(4.9)^{x}$	$4(5.3)^{x}$
Faeces	6	0	0	3 (5.2)	2(5.0)
	10	0^{y}	$3(6.0)^{x}$	$5(7.2)^{x}$	$5(7.7)^{x}$

Minimun level of detection 2 log CFU/gr faeces.
n = 6
^{xy} Different superscripts in the same row denote a significant difference ($P \leqslant 0.05$)

Lactobacilli, enterobacteria and *E*. *coli* k88 in colon content were enumerated by quantitative PCR (Table 5.4). On day 6 PC, there were no differences in the counts of lactobacilli or enterobacteria between treatments. However, on day 10 PC the supplementation of lactulose and *L*. *plantarum* increased the counts of lactobacilli (P=0.05), although increments were not additive in the SYN treatment (P interaction = 0.01). The administration of the probiotic strain trended to reduce enterobacteria population (P= 0.11) and to increase the ratio lactobacilli:enterobacteria (L:E) (P= 0.07) at day 10 PC.. *E. coli* K88 was always found at high counts in colon digesta without differences between diets.

Table 5.4. Bacterial counts of lactobacilli, enterobacteria, ETEC K8 in colon digesta measured by quantitative PCR (log 16S rRNA gene copies/g FM) in weaned piglets at days 6 and 10 post-challenge. Ratio of lactobacilli:enterobacteria (L:E) also is included as difference of logarithms.

Items	Period (days)	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
Lactobacilli	6	10.3	10.5	11.0	11.3	0.77	0.60	0.16	0.85
	10	10.9	12.0	12.1	11.9	0.61	0.05	0.05	0.01
Enterobacteria	6	9.7	9.9	9.3	9.2	2.03	0.56	0.48	0.43
	10	10.2	9.1	8.7	9.0	1.92	0.86	0.11	0.67
Ratio L:E	6	0.9	1.0	1.1	1.1	0.33	0.54	0.16	0.90
	10	1.1	1.3	1.5	1.5	0.27	0.17	0.07	0.50
***E. coli* K88**	6	8.5	8.4	8.3	8.1	1.89	0.68	0.50	0.28
	10	8.3	7.9	8.0	8.0	1.96	0.88	0.69	0.75

PRE; main effect of the inclusion of lactulose in the diets.
PRO; main effect of the inclusion of *L*. *plantarum* in the diet

Changes on microbial composition with the experimental treatments were also evaluated by T-RFLP in colon digesta at day 10 PC. Richness of the ecosystems was evaluated based on the total number of TRFs. There no were differences between treatments (18, 22, 21 and 20 for CTR, LAC, PLAN and SYN respectively *P* = 0.96). The compatibility analysis of TRFs represented in at least five animals is shown in Table 5.5. We could not find differences in the frequency of detection related to the diets, except for fragments compatible with *Escherichia* spp. and other enteric bacteria that were less frequently detected in piglets receiving the supplemented diets. Moreover in those animals receiving diets including lactulose the percentage of total peak area of positive animals was lower than for the rest of diets (5.26, 2.55, 3.07 and 2.02 % for CTR, PRE, PRO and SYN respectively *P* PRE = 0.04).

Table 5.5. Theorical restriction 5´-fragment length predicted for the major pig gut bacteria in colon digesta of weaned piglets at day10 post-challenge.

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Bacterial groups	Compatible bacteria [a]	*In silico* restriction [b]	Real restriction [c]	Diets			
				CTR	LAC	PLAN	SYN
Bacillus and lactic acid	*L. delbruekii sp. lactis*	223	223	1 (2.07)	2 (2.55)	2 (2.44)	3 (3.21)
bacteria	*L. acidophillus, L. brevis, L. bifermentum,*	597,598,599	597-599	0	1 (2.31)	2 (1.94)	2 (2.55)
	L. rhamnosus , L. Case, iL. plantarum						
	Bacillus sp	237-243	243-244	1 (0.68)	2 (0.97)	2 (0.97)	2 (0.97)
Cytophaga	*Cytophaga*	90, 92, 94	94	1 (5.03)	2 (1.83)	2 (1.03)	2 (2.45)
Fibrobacter	*Fibrobater intestinales*	148	147	1 (1.6)	2 (2.03)	2 (2.91)	2 (2.27)
Clostridium and relatives	*Clostridium coccoides*	66	67	3 (2.20)	4 (3.43)	5 (5.11)	5(2.07)
Proteobacteria	*Escherichia* spp	371-374	371-375	4^{y} (5.26)	2^{x} (2.55)	2^{x}(3.07)	2^{x}(2.02)
	Other enteric bacteria (*Salmonella* ssp*, Citrobacter, Klebsiella*)						

[a] Major pig gut bacteria, with a potential compatible fragment found in at least five animals.
[b] In silico restriction was performed using the analysis function of MiCA web page
[c] Terminal fragment length obtained after PCR product restriction with Hha I.
[d] Frequency of detection of each compatible fragment is given as number of animals that showed the peak in each experimental group. The mean peak area (%) value in these animals is also shown in brackets
[xy] Different superscripts in the same row denote a significant difference *(P* $\leqslant$ 0.05).

The effects of the diets on metabolic activity in ileum and colon are shown in table 5.6. Most of the significant changes registered in fermentation were related to the inclusion of *L. plantarum* in the diets. Consistently animals receiving the probiotic showed decreases in NH_3 concentration in ileum at day 10 (P = 0.03) and in colon at days 6 (P = 0.03) and 10 PC (P = 0.007). The pH values was not changed in ileum but showed a decrease in colon at day 6 PC (P= 0.05) and percentage of valeric and BCFA were lower at day 6 and 10 PC respectively (P = 0.03, P = 0.05). The rest of parameters were not significantly affected. The inclusion of lactulose only was associated to an increase in the percentage of butyric acid in colon at day 10 (P = 0.02).

5.3.3. Serological results

There were no significant differences, in the immunological factors in serum on day 6 PC due to the supplementation with lactulose and/or *L. plantarum* (data no shown). However on day 10 PC (Table 5.7) the consumption of *L. plantarum* reduced the value of TNF-α (P=0.01) and tended to reduce the Pig-MAP (P=0.08), whereas lactulose, reduced Pig-MAP value (P=0.01).

5.3.4. Changes in the intestinal morphology

The results of histological analysis of ileum are summarised in table 5.8. Most of the significant changes were registered with the diets including *L. plantarum*. The inclusion of the probiotic increased the villus height and the villus:crypt ratio at day 10 PC (P = 0.01, P =0.004 respectively). Also it was increased the intraepithelial lymphocytes at day 6 and 10 PC (P = 0.03, P = 0.01 respectively) and the number of goblet cells at day 10 (P = 0.02). No histological changes were registered with the inclusion of lactulose with the exception of an increase in villous height at day 10 PC (P = 0.05).

Table 5.6. Effect of the experimental diets on ileal and colonic fermentation of early weaned piglets at days 6 and 10 post-challenge. Table includes the pH values and concentrations of ammonia (mmol/L of FM), lactic acid, total short-chain fatty acids (SCFA) (mM) and the molar percentage of their different components.

Items	Period (days)	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
ILEUM									
pH	6	7.3	7.2	7.3	7.0	0.39	0.32	0.60	0.39
	10	7.2	7.5	7.2	7.3	0.35	0.73	0.68	0.63
NH_3	6	2.4	2.9	2.05	2.02	0.86	0.26	0.81	0.54
	10	1.01	0.92	0.59	0.61	0.36	0.98	0.03	0.63
SCFA (mM)[a]	6	16	21	27	23	9.49	0.41	0.06	0.81
	10	13	15	19	17	6.31	0.50	0.16	0.90
Lactic acid	6	8.1	7.8	9.4	9.0	4.12	0.92	0.60	0.85
	10	5.7	3.9	8.0	7.8	3.27	0.55	0.08	0.65
COLON									
pH	6	6.8	6.2	6.0	5.9	0.48	0.15	0.05	0.32
	10	6.1	6.0	6.0	6.0	0.23	0.73	0.68	0.63
NH_3	6	11.9	11.0	7.8	7.3	3.21	0.66	0.03	0.44
	10	12.5	7.4	5.1	5.3	3.85	0.17	0.007	0.63
SCFA (mM)	6	67	77	94	95	22.5	0.69	0.11	0.70
	10	83	96	99	116	20.2	0.15	0.08	0.84
Acetate (%)	6	64.8	64.9	63.6	65.6	4.43	0.37	0.60	0.80
	10	68.6	64.6	64.8	61.9	3.01	0.08	0.07	0.71
Propionate (%)	6	22.7	23.6	23.4	23.0	3.90	0.91	0.92	0.90
	10	20.3	22.3	21.1	23.1	2.10	0.08	0.70	0.67
Butyrate (%)	6	7.9	7.0	9.7	8.6	2.32	0.38	0.13	0.89
	10	7.1	11.2	10.5	12.0	2.51	0.02	0.07	0.61
Valerate (%)	6	1.6	2.2	1.8	1.7	0.90	0.60	0.63	0.39
	10	1.5	1.4	2.0	1.6	0.47	0.22	0.03	0.46
BCFA (%)[b]	6	2.9	3.0	2.2	1.0	1.01	0.30	0.05	0.14
	10	2.2	0.92	1.6	1.3	0.60	0.07	0.55	0.49

[a] Total SFCA=acet+propionic+butyric+branched chain fatty acids (BCFA)

[b] BCFA=isobutyric+isovaleric acids

[xy] Different superscripts in the same row denote a significant difference ($P \leqslant 0.05$).

Table 5.7. Effect of the diets on plasmatic levels of pro-inflammatory cytokine TNF-α and acute phase protein Pig-MAP in weaning piglets

Items	Period (days)	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
TNF-α (pg/mL)	6	58	53	52	55	21.2	0.85	0.88	0.71
	10	52	57	32	37	17.3	0.53	0.01	0.98
PigMAP (mg/dL)	6	1.7	1.9	1.6	1.3	0.87	0.96	0.38	0.43
	10	2.4	1.4	1.8	1.5	1.2	0.01	0.08	0.75

PRE; main effect of the inclusion of lactulose in the diets.
PRO; main effect of the inclusion of *L*. *plantarum* in the diets.

Table 5.8. Effect of the diets on intestinal morphology of ileum of weaning piglets at days 6 and 10 PC.

Items	Period (days)	Diets				SEM	*P*-value		
		CTR	LAC	PLAN	SYN		PRE	PRO	Interaction
Villous height (μm)	6	288	298	306	291	55.4	0.19	0.96	0.72
	10	280	301	311	320	52.5	0.05	0.01	0.43
Crypt Depth (μm)	6	199	197	197	202	22.4	0.81	0.78	0.61
	10	205	218	210	214	12.1	0.98	0.13	0.28
Villus:Crypt Ratio	6	1.4	1.5	1.5	1.4	0.21	0.56	0.37	0.51
	10	1.4	1.4	1.5	1.6	0.28	0.86	0.004	0.58
IEL (cells/100 μm).	6	11.7	9.2	7.0	8.3	2.17	0.30	0.03	0.40
	10	13.3	10.1	9.6	9.2	1.73	0.09	0.01	0.22
GC /100 enterocytes	6	7.0	8.4	7.8	7.4	0.84	0.50	0.72	0.37
	10	8.1	8.5	9.9	9.4	0.97	0.98	0.02	0.44

[a]GC Villus goblet cells /100 enterocytes
[xy] Different superscripts in the same row denote a significant difference ($P \leqslant 0.05$).
PRE; main effect of the inclusion of lactulose in the diets.
PRO; main effect of the inclusion of *L. plantarum* in the diets.

5.4. Discussion

Up to day it can be found in the literature different works aimed to evaluate the positive effects of prebiotics and probiotics in preventing the post weaning diarrhoea in piglets (De Lange *et al*., 2010). However, only a few of them have been carried out under ETEC challenge conditions and very few trying a potential synbiotic combination. In previous works of our group (Guerra *et al*., 2012) we studied the effects of lactulosa, *L. plantarum* and its combination on the intestinal environment and performance of weanling piglets. In this work we aimed to study the effectiveness of these treatments in weaned piglets challenged by an oral inoculation of ETEC K88.

We previously demonstrated that lactulose addition in prestarter diets improved performance at weaning mainly throughout increases in feed intake (Guerra *et al*., 2012). However, in this work under ETEC K88 challenge conditions, lactulose did not increase intake but enhanced ADG in the post-challenge period probably by other mechanisms. These tentative mechanisms could include the promotion of a more robust indigenous microbiota against E. coli, the improvement of the epithelium integrity, and a possible modulation of inflammatory response.

Regarding possible changes in microbiota composition and activity, lactulose increased the counts of colonic lactobacilli and a reduced frequency of detection TRFs compatible with *Escherichia* spp. The promoting of colonic lactobacilli, might have increased the production of lactic acid that used as a substrate for butyrate-producing bacteria (Tsukahara *et al*., 2002) could explain the significant increase reported in the butyric acid percentage in colon. Moreover this possible prebiotic effect of lactulosa could also have reduced the activity of proteolitic bacteria, as a reduction of BCFA percentage was also reported at day 10 ($P = 0.07$). All these results are in accordance with previous results in non-challenged piglets fed lactulose, which lead to an increase in the L:E ratio, in the butyric

percentage and a reduced proteolytic fermentation (Guerra *et al.,* 2012). However other authors did not reveal prebiotic effects for lactulose when including 1.5 % in the diet of growing pigs (Branner *et al*., 2004). The absences of effects were attributed by authors to the high pre-caecal digestion of lactulosa in these animals (79 %). In this regard Kamphues *et al*.(2007) comparing the effect of including lactulosa in piglet, growing pig (between 2.7-2.9 %) or sow diets (between 5.5-14 %) only were able to demonstrate prebiotic effects in piglets. Regarding these results prebiotics effects of lactulose could be restricted to very young animals in which intestinal digestive activity is not fully developed.

The higher butyrate production with diets including lactulose could have represented a major source of energy for colonocytes (Mountzouris, 2007). Butirate also has been reported to promote epithelial cell proliferation and to stimulate sodium and water absorption preventing the acute diarrhoea (Willians *et al.,* 2001). In that sense diets including lactulose showed an increased villous height despite no significant decrease in the diarrhoea frequency was observed and neither in the numbers of *E*. *coli* K88 in colon. However, the lower Pig-MAP values in animals fed lactulose, suggest an improvement in the animal response after the ETEC challenge. In swine, Pig-MAP is a major acute-phase protein and its higher serum concentration has been related to acute inflammatory processes and also with the extent of tissue injury, expressing strong and protracted responses to bacterial infections (Piñeiro *et al*., 2009).

Throughout the experimental, *L. plantarum* was never detected in the control group. However, in most of the animals receiving the probiotic, *L. plantarum* was detected in the ileal scrapes, ileal and colonic digesta and faeces at day 10 PC. These findings, confirm that the administered probiotic strain was able to survive in the gastrointestinal tract of the animals treated. Similar results were found in previous studies of our group with healthy piglets, where the recovery of *L*. *plantarum* in faeces was significant until 14 days of treatment (Guerra *et al*., 2012). Also it is interesting to remark that in some animals receiving

the LAC diet it was also possible to detect *L. plantarum* but only at the end of the experiment. These results could suggest a possible prebiotic effect of lactulose on indigenous strains of *L. plantarum*.

The inclusion of the probiotic in the diets was not associated with any improvement in performance indexes. However, other of the parameters studied were positively affected by the inclusion of *L. plantarum* in the diets. At day 10 PC, piglets fed *L. plantarum* showed a significant increase in the colonic lactobacilli population. Similarly other works have reported that administration on *L. plantarum* could stimulate the beneficial intestinal microbiota (Takahashi *et al*., 2007; Pieper *et al*., 2009, 2010).

Intestinal fermentation was significantly modified by the probiotic. It was relevant the reduction in ammonia concentration observed in ileum and colon that was simultaneous to a significant reduction of BCFA at day 6 PC. These results point out to a possible reduction in the proteolytic activit due to a suppression of putrefactive enterobacteria (Brown, 2011) suggested by the numerical decreased found on enterobacteria ($P< 0.11$) and in the frequency of compatible TRFs with *Escherichia* spp and enterobacteria. In previous studies with the same strain but in non-challenged pigs we also reported numerical inhibition of *E. coli* with significant decreases on BCFA (Guerra *et al*., 2012).

Although animals receiving the probiotic showed a diminishing in the incidence of diarrhoea ($P<0.05$) we could not detect any change in colonic *E. coli* K88 counts. Together to a possible control of enterobacteria and proteolytic activity, beneficial effects of *L. plantarum* could also be due to others mechanisms like the promotion of a more healthy fermentation profile, an improved barrier function, or a well balance immune response. Regarding a possible improvement on the fermentation profile, *L. plantarum* trended to increase lactic acid ($P = 0.08$) in ileum and total SCFA in ileum ($P = 0.06$) and colon ($P = 0.08$) with higher percentages of butirate ($P = 0.07$) at day 10 PC. Previous results with non challenged piglets also showed the ability of *L. plantarum* to increase the butyric production (Guerra *et al*.,

2012). In addition other authors reported the promotion of butyrate producing bacteria and reduction of diarrhoea incidence in piglet fed *L*. *plantarum* (Pieper *et al*., 2010).

Higher SCFA and butyric acid with the probiotic could have improved intestinal epithelia, exerting a positive effect on the maintenance of barrier function. The inclusion of *L. plantarun* in the diets modified positively most of the histological parameter included in this study, mostly at day 10 PC. The prevention of intestinal barrier disruption have been suggested by other as the mechanism involved in the improvement of villous height and crypt depth in ileum of weaned piglets fed *L. plantarum* (Suo *et al*., 2012). The significant increase we observed in villous height could be related to the reported reduction of ammonia concentration ($P<0.03$) since higher ammonia concentration has been related with a reducing of villous height (Nousiainen, 1991). Moreover an increased protection of the ileal epithelium by an improved mucus layer could have been promoted by the probiotic as it was shown an increase in the number of goblet cell at day 10 PC. Metabolites produced by probiotic bacterial fermentation have been described to improve the growth and maturation of goblet cell (Brown, 2011) and a higher number of goblet cell may increase mucus production (Pluske *et al*., 2003). Previous in vitro studies, with the same *L. plantarum* strain, also have demonstrated a protection against *Salmonella* Typhimurium, through an increase in the acidic mucin secretion (Collins *et al*., 2010).

Regarding a possible effect of the probiotic on the modulation of the inmmune response, we found lower IEL and a reduction in the plasmatic TNF-α level in the piglets fed the *L. plantarum* strain. Intraepithelial lymphocytes are primarily T-lymphocytes that participate in regulation of immune response and are influenced by the presence of microbial antigens within the gut *(Gaskins*, 1998). Moreover, pro-inflammatory cytokines as TNF-α contribute to host defence mechanisms in response to external invasion, but they have the potential to injure host tissues when over-induced. On this regard, Mizumachi *et al*. (2009) found that production of cytokines TNF-α and IL-8 were significantly decreased in *L.*

plantarum fed piglets. Moreover Willing and Van Kessel (2007) comparing germ-free pigs with piglets mono-associated with *E*. *coli* or *L. fermentum* demonstrated an increase in infiltration of IEL and in the expression of TNF-α that the authors associated to an induction of inflammation response promoted by *E. coli* but not by *L. fermentum*. Our data would point out to the potential of *L*. *plantarum* to modulate the immunity and inflammatory responses under ETEC K88 challenge.

A way of potentiating the efficacy of probiotic preparations may be the combination of both, probiotics and prebiotics as synbiotics (Gibson and Roberfroid, 1995). Despite, that previous *in vitro* assays had confirmed the ability of *L. plantarum* to growth efficiently on lactulose substrate (Martín-Pelaez *et al*., 2008), SYN dietary treatment did not resulted in promoting a specific growth of *L*. *plantarum* compared to PLAN diet, and neither in previous works with non-challenged animals (Guerra *et al*., 2012). Nevertheless, when combined both treatments, the beneficial effects of each were added, being the interaction effect not significant in most of the studied parameters. In this regard the SYN diet combined the beneficial effects of LAC and PLAN with decreases in ammonia in ileum and colon, increases the percentage of butirate in colon, decreases in plasmatic TNF-α and PigMAP and an improved membrane barrier function with higher villous height, number of goblet cells and reduced IEl in ileum. The synbiotic concept is relatively new and only a limited number of studies exploring potentially synbiotic combinations in pigs can be found (Gaggia *et al*., 2010) particularly in challenge conditions. In this regard works of Nemcová *et al*. (2007) using a combination *L. plantarum* with maltodextrinas and/or fructoligosacharides could demonstrate a beneficial effect of the synbiotics on reducing the adhesion of pathogenic *E. coli* K88 to the intestinal mucosa but, similarly they were unable to demonstrate any increase in the intestinal lactobacilli population. Seem clear that combining a probiotic with a prebiotic can be a way to potentiate the benefits of the probiotic, however this synergy could not came only from an increase in the number of the probiotic but also from an improvement

in its metabolisms or health promoting activities. Further research is required to fully understand the mechanisms involved.

5.5. Conclusions

In conclusion, the present study showed that lactulose increased the average daily gain in the post-challenge period likely due to a prebiotic effect on the growth of lactobacilli and butirate producing bacteria with improvements in epithelium architecture. A better response of the animals to the challenge was also confirmed by reductions on the plasmatic Pig-MAP concentration. *Lactobacillus plantarum* was also effective promoting the growth of lactobacilli and modulating the fermentative activity towards a less protetolytic one. Its inclusion in the diet increased villus height and globet cells with improvements in the immune and inflammatory response, suggested by reductions in IEL and plasmatic TNF-a. Positive effects of *L. plantarum* and lactulose were added in its symbiotic combination despite lactulose was not able to promote a specific growth of *the L. plantarum*.

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CHAPTER 6

Effects of a carob seed product on intestinal health of weaned piglets exposed to an Enterotoxigenic *E. coli* K88 challenge

6.1. Introduction

Early weaning is a critical period for piglets in which animals have to face to innumerable changes and stressors. After the sudden interruption on the maternal milk, weaners show an anorexia period of variable length that result in alterations on the morphology and functionality of the small intestine (Lallès, 2004). As a consequence, the early weaning is usually related to an increased risk of intestinal disorders. However gastrointestinal disorders results not only form alterations in the gastrointestinal architecture and function but also from major changes in the enteric microbiota (Konstantinow *et al*., 2004) and inmune system (Stokes *et al*., 2004). Between the most common pathogens enterotoxigenic *Escherichia coli* (ETEC) K88 is one of the most frequently implicated in the post-weaning diarrhoea (Fairbrother *et al*., 2005). ETEC K88 strains adhere to receptors on the small intestinal mucosa by fimbriae binding to K88-specific receptors present on brush borders of villous enterocytes (Nagy and Fekete 2005).

In the recent past, antimicrobial compounds have been used in weaning diets to control the incidence of post-weaning diarrhoea (PWD). However, the increasing concern in relation to the development of antibiotic resistant bacteria has resulted in restrictions in their use. At this respect the European Union (EU) banned the use of antibiotics as growth promoters since 2006. Furthermore, minerals such as zinc (Zn) and copper (Cu) are not feasible alternatives because their excretion is a possible threat to the environment. In the search of new alternatives in-feed additives like prebiotics, probiotics or organic acids has been proposed (Heo *et al*., 2012) and are nowadays being used in piglet diets.

The carob pod or “locust bean” is the fruit of carob tree (*Ceratonia siliqua)* and is commonly used as non-conventional feed for ruminant feeding (Vasta *et al*., 2008). The carob pulp comprises the 90% of the weight ripe fruit and is rich in sugars (48-56%). The other 10% correspond to the seeds, which are composed mainly by galactomannans. Galactomannans has been reported as a possible prebiotic in pigs (Wang *et al*., 2010) that

could prevent the adhesion of pathogens to the intestinal epithelium (Badia *et al*., 2012) and also positively modulate immune function (Badia *et al*., 2013). To date there is a limited number of studies regarding the use of carob seed products in weaned piglet diets. In some of then it have been evaluated the dietary inclusion of carob seed meal (Van Nevel *et al*., 2005) and carob gum (Van Nevel *et al*., 2005; Pellikaan *et al*., 2010; Badía *et al*., 2010) on bacteriological, morphological and fermentation characteristics along the gastrointestinal tract. However, up to our knowledge no work has been published evaluating its efficacy in the context of an enteric pathogen challenge model.

Therefore the aim of this study was to assess the effectiveness of a product derived from the carob seed and included in the diet, on growth performance, pro-inflammatory response and gastrointestinal health of weaned piglet exposed to an ETEC K88 challenge.

6.2. Materials and Methods

6.2.1. Animals and diets

This experiment was conducted at the experimental farm facilities of the Universitat Autònoma de Barcelona (UAB) and received prior approval from the Animal Protocol Review Committee of the UAB (Permit number: CEAAH 746). The treatment, management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines (The Council of the European Communities, 1986). The experiment was conducted during the spring season (May) with an average inside room temperature of 30 ± 2 ºC.

Eighty commercial breed [(Large White x Landrace) x Pietrain] weaning piglets of 25 ± 3 days old with an average body weight of 5.3 ± 0.75 kg were purchased from a commercial farm (Vic, Spain) in which mothers did not receive historic vaccination for *E. coli*. From these animals, 72 were randomly housed in three rooms of 8 pens each (24 pens, 3 pigs per pen) taking litter size and initial BW into account. Animals in each of eight replicated pens received one of three dietary treatments as follows: a control diet (Table 6.1) (CTR), the

same diet to which 3 g/Kg of ZnO (ZnO) was added and used as positive control or the CTR with 5 g/Kg of a product derived from carob seed (CRB) kindly provided by ARMENGOL HERMANOS, SA. Spain. The carob product used in this experiment was a meal obtained from the carob seed partially de-hulled, de-germened and micronized. It contained 142 g/kg of CP, 73 FND, 17 FAD, 16 EE, and 15 MJ/Kg of gross energy. Diets and water were offered *ad libitum*. The remaining 8 piglets were allocated in 4 pens in a separate room (two pigs per pen) and were fed with CTR diet. These animals were used as a negative control (NC) for the challenge model.

Table 6.1. Experimental diets

Ingredients	(g/kg fresh matter)
Corn Flakes	360.0
Wheat Flakes	240.0
Full fat extruded soybeans	110.0
Egg meal	92.7
Sweet whey	150.0
Soybean oil	16.5
Calcium Carbonate	5.6
Dicalcium Phosphate	8.1
L-Lysine	4.6
DL-Methionine	0.5
L-Threonine	1.1
L-Tryptophan	0.9
Salt	4.0
Vitamin-Mineral Premix*	6.0
Chemical analysis	(g kg^{-1} dry matter)
Dry matter	904
Gross energy (MJ/Kg)	19
Crude protein (CP; N x 6.25)	189
Neutral detergent fibre	85
Acid detergent fibre	29
Ether Extract	61

* Supplied per kilogram of feed: 10,200 IU of vitamin A, 2,100 IU of vitamin D as cholecalciferol, 39,9 mg of vitamin E, 3 mg of vitamin K_3, 2 mg of vitamin B_1, 3 mg of vitamin B_2, 3 mg of vitamin B_6, 0.025 mg of vitamin B_{12}, 20 mg of calcium pantothenate, 60 mg of nicotinic acid, 0.1 mg of biotin, 0.5 mg of folic acid, 150 mg of Fe as iron sulphate, 156 mg of Cu as copper sulphate, 0.5 mg of Co, 120 mg of Zn, 49.8 mg of Mn as manganese oxide, 2 mg of I, 0.3 mg of Se, as sodium selenite.

6.2.2. E.coli strain.

The bacteria strain used in this study was isolated from a colibacilosis outbreak in Spain (Blanco *et al*., 1997), serotype (O149:K91:H10 [K-88]/LT-I/STb), and was provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The infection inoculum was cultured by 16 h at 37ºC in Luria broth with slow agitation (1 x *g*) in an orbital incubator (WY-100, Comecta S.A., Barcelona, Spain), to reach a final concentration of 10^9 CFU/mL.

6.2.3. Experimental procedures.

The experiment period was of 2 weeks. On day 7 of experiment, a single 6 ml oral dose (2 x 10^9 CFU/ml) of the ETEC (K88) strain was administered to the animals. Prior the challenge piglets were fasted during 10 hours, and fed again 30 minute before the inoculation. Feed intake and body weight were recorded on days 7, 11 and 15 of the experiment. Average daily gain was calculated individually and average daily feed intake and gain:feed ratio (G:F) by pen. Animals were checked daily to evaluate their clinical signs (i.e. dehydration, apathy and diarrhoea). The diarrhoea incidence was measured as the percentage of animals in each pen that presented inconsistent to liquid faeces. On days 7, 11 and 15 faecal samples (about 5 g) were taken for microbiological analysis from two animals per pen corresponding to the animals with the highest and lowest BW. Additionally on day 0, faecal samples were taken from 24 animals randomly chosen.

On days 4 and 8 post challenge (PC) (experimental days 11 and 15 respectively) some animals were euthanized with an intravenous injection of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200 mg/kg BW). On day 11 all the animals of NC were euthanized. For the rest of treatments, on day 11 it was taken the animal per pen with the intermediate BW and on day 15 the heaviest one. Before pentobarbital injection, blood samples were obtained by venipuncture from the cranial vena cava using a 10-mL plasma tube with sodium heparin (17 IU/mL) (BD Vacutainer; Becton Drive, Franklin Lakes, NJ). The

plasma collected was stored at -80°C until analysis. Immediately after blood sampling, piglets were died, bled, the abdomen immediately opened and the whole GIT tied and excised.

Digesta from ileum (was considered 25 cm from ileocecal junction) and proximal colon was collected, homogenized and pH determined with a pH-meter (electrode Crison 52-32, Net INTERLAB SAL). Samples from ileum and proximal colon digesta (approximately 20 g) were kept frozen in small zip-lock bags (-20 ºC) until analysed for short chain fatty acids (SCFA). Additional samples from colon were also maintained at -20ºC for quantification of microbial groups by quantitative PCR (qPCR). For the analyses of the enterobacteria and *E.coli* attached to the ileum mucosa, 5-cm long sections of terminal ileum were collected from each animal, washed thoroughly three times with sterile PBS, open longitudinally and scraped with a microscopy glass slide to obtain the mucosa scrapes content. For histological study, sections of 3 cm from terminal ileum were removed, opened longitudinally and fixed by immersion in Carnoy solution as described by Swidsinski *et al*. (2005). Tissue samples were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphometric measurements were performed with a light microscope (BHS, Olympus, Spain). Villus height and crypt depth, and the goblet cell number in crypts were measured. Measurements were taken in ten well-oriented villi and crypts from each intestinal section of each animal. The villus height and crypt depth were measured using a linear ocular micrometer (Olympus, Ref. 209-35 040; Microplanet, Barcelona, Spain). On the basis of the cellular morphology, differences between goblet cells and lymphocytes were clearly distinguishable at 400 × magnification. Cell density was expressed as the number of lymphocytes per 1000 μm^2. All morphometric analyses were done by the same person, who was blind to the treatments.

6.2.4. Culture methods.

For bacterial counts, fresh faecal samples and ileum mucosa scraping samples were suspended (10% w/vl) in a buffered peptone solution (PBS) and subsequently homogenized for 5 min. Thereafter, 10-fold dilutions were made in PBS and plated in agar MacConkey (Oxoid, Ref. CM 115, Oxoid S.A., Madrid, Spain) for enterobacteria counts (dilutions 10^{-c} to 10^{-t}) and in chromogenic *E. coli* media without antibiotic (Oxoid, Ref. CM0956) for count the bacterial *E. coli* population (dilutions 10^{-p} to 10^{-t}). Enterobacteria and *E. coli* were enumerated after 24 h incubation (37°C) and were expressed as log colony forming units (CFU) per gram of fresh matter.

6.2.5. DNA extraction and quantification of microbial groups by Real time PCR. (q-PCR)

Colon digesta samples (400 mg) were precipitated by centrifugation (13000g x 5 min) and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90ºC and a posterior incubation step with lysozyme was added (10 mg/mL, 37ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 mL of Qiagen Buffer AE (Qiagen, West Sussex, UK) and stored at -80º C until use. Enterobacteria and *E. coli* K88 population in colonic samples were quantified by real time PCR (qPCR) using SyBR Green dye, following the protocol described by Castillo *et al*. (2006) and Hermes *et al*. (2012).

6.2.6. Physic-Chemical analysis.

The viscosity of ileal digesta was measured in a Brookfield DV-E I viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA) using a SC4-18 spindle and a 13 R adapter suitable for small samples at a shear rate of 7.92 s^{-1}. Values were converted to a log scale to reach a normal distribution.

Diet samples were submitted to chemical analyses, following AOAC (1995) methods, for dry matter (945.15), crude protein (979.09) and ether extract (920.39C). Neutral detergent fibre and acid detergent fibre were determined according to the method of Van Soest *et al*. (1991). The gross energy (GE) of was measured using an adiabatic bomb calorimeter (IKA 4000, Staufen, Germany).

Short-chain fatty acid and lactic acid analysis were performed by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatization with MBTSTFA+ 1% TBDMCS agent, using the method of Richardson *et al*. (1989) modified by Jensen *et al*. (1995).

The Tumor Necrosis Factor-α (TNF-α) concentrations were determined by Quantikine® Porcine TNF-α kit (R&D Systems, Abingdon, United Kingdom). Pig Major Acute-phase Protein (Pig-MAP) concentration was determined by a sandwich type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain), according to the manufacturer's instructions. Serum was obtained by centrifugation of blood at 3,000 x *g*, 15 min at 4ºC.

6.2.7. Statistical Analysis

Data on performance, microbial and slaughter measurements were tested with a one way ANOVA with dietary treatment as the classification factor, using the GLM procedure of SAS statistics package (SAS Institute, INC. 9.2, Cary, NC).

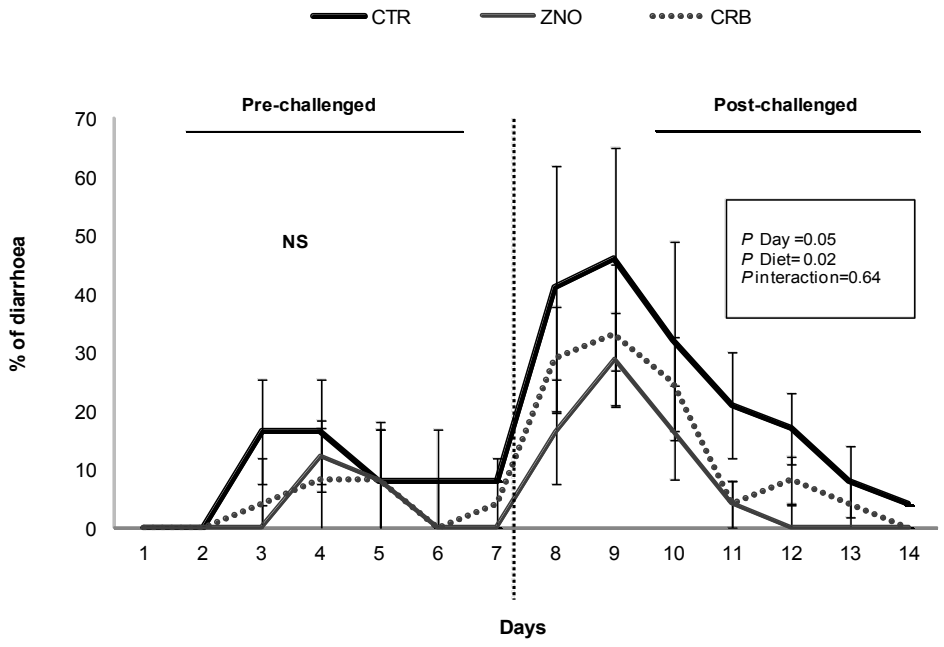
For all analyses the pen was used as the experimental unit. In the event that significant diet effects were established ($P \leq 0.05$), multiple comparisons of the means were performed using the Student Newman Keuls (SNK function) of SAS. Statistical significance was accepted at $P \leq 0.05$ and statistical trend at $P \leq 0.10$.

6.3. Results

The animals used in this study showed a good health status at the beginning of the trial with no signs of diarrhoea throughout the first week of adaptation to the diets. Regarding the experimental model of colibacillosis, it showed a mild course of diarrhoea with no mortalities during this study. At this respect the group NC, that received the CTR diet but was not orally challenged, did not show any sign of diarrhoea during the whole study with reduced numbers on faecal populations of enterobacteria (7.3 vs. 9.5 Log CFU/ g FM; *P* = 0.02) and *E. coli* (7.0 vs. 9.0 Log CFU/ g FM; *P* = 0.01) compared to CTR and with *E. coli* K88 counts below the minimun level of detection of the qPCR method (below 3.32 Log of 16S rRNA gene copies/g of FM sample). These animals also showed lower Pig–MAP values than CRT group (0.67 vs 1.20 mg/dL; *P* = 0.02).

The incidence of diarrhoea after the oral challenge is shown in Figure 6.1. Animal receiving the CTR diet showed a higher incidence (*P* = 0.02) than animals in groups ZnO or CRB. There were no differences between piglets receiving the ZnO and CRB diets.

.**Figure. 6.1.** Percentage of piglets that presented diarrhoea during the experimental period



[a] NS: Non-significant differences

Table 6.2 showed performance results for the different experimental groups. During the whole experimental period piglets from ZnO group had higher ADFI than those in CRB and CTR groups, with an increase of average daily gain (ADG) between days 11-15 days (*P*=0.01) and higher body weight at the end of the experiment (7.4, 6.8 and 6.6 kg BW for ZnO, CTR and CRB respectively; *P*=0.03). No significant differences were reported in the G:F ratio.

Table 6.2. Effect of the experimental diets on growth performance of early weaned pigs at days 7, 11 and 15 of experiment: average daily feed intake (ADFI) (g/day), average daily gain (ADG) (g/day) and gain:feed ratio (G:F). Animals were orally challenged with ETEC K88 at day 7.

Items	Period (Days)	[a]Diets			SEM	P-value
		CTR	ZnO	CRB		
ADFI	0-7	87^{x}	80^{x}	64^{y}	25.8	0.04
	7-11	251^{xY}	262^{X}	211^{y}	49.03	0.06
	11-15	270^{xy}	306^{X}	243^{y}	37.9	0.03
ADG	0-7	14	13	10	4.5	0.37
	8-11	91^{y}	121^{x}	79^{y}	28.9	0.08
	11-15	141^{y}	208^{x}	130^{y}	46.0	0.01
G:F	0-7	0.14	0.17	0.16	0.067	0.49
	8-11	0.38	0.47	0.36	0.151	0.21
	11-15	0.56	0.66	0.53	0.180	0.53

[a] Diets: CTR (control diet). ZnO (zinc oxide diet). CRB (diet with carob seed meal).
[xy] Different superscripts in the same row denote a significant difference *(P* ≤ 0.05) or an statistical trends *(P* ≤ 0.10).

Results of different fermentation parameters are shown in Table 6.3. Lactic acid was decreased in ileon at day 4 PC with ZnO and increased in colon at day 4 PC with CRB diet

(*P*=0.03). Total SCFA in ileon were not significantly affected by the experimental diets but were increased in colon with ZnO and particularly with CRB diet at day 4 PC (*P* = 0.01). Molar proportions in colon were scarcely modified by ZnO and CRB diets although there was seen a trend to increase in the proportion of butyric acid (*P*=0.09) and to decrease in BCFA (*P*=0.10) at day 4 PC. PH values in colon were not significantly modified by the diets (data no shown), however at day 4 PC there was an increase in the ileum pH of piglets fed the ZnO diet (6.9, 7.2 and 6.7 for CTR, ZnO and CRB diets respectively; *P* = 0.04). Furthermore, we must remark that CRB diet significantly increased the viscosity of ileal digesta at both, day 4 (*P* = 0.05) and 8 PC (*P* = 0.03).

Changes in intestinal microbial groups with the experimental diets are shown in Table 6.4. At the beginning of the experimental period (day 0) there were no significant differences in counts of enterobacteria or *E*. *coli* between diets. However at day 4 PC, challenged piglets fed the CRB and ZnO diets had a lower faecal population of enterobacteria and *E. coli* (*P* = 0.05 and *P* = 0.03) than piglet fed CTR diet. A similar trend was also observed for ileal mucosa scrapings (*P* = 0.09 and *P* = 0.07). On the other hand quantitative real-time PCR-based methods were developed to specifically measure colonic enterobacteria and *E*. *coli* K88 populations. Results revealed that on day 4 PC administration of CRB and ZnO diet also reduced the enterobacteria in colon on day 4 after ETEC challenge (*P* = 0.03), being also numerically decreased until day 8 PC (*P* = 0.10). Moreover piglets fed ZnO had lower counts of *E*.*coli* K88 (*P* = 0.05) than those fed CRB and CTR diet.

Table 6.3. Effect of the experimental diets on intestinal fermentation and ileal viscosity. Fermentation products include lactic acid (mM) and total short-chain fatty acid (SCFA) (mM) concentrations and their molar proportions, in ileal and colonic digesta of weaned piglets, 4 and 8 days after an oral challenge (PC) with ETEC K88.

Items	Period	[a]Diets			SEM	P-value
	Days PC	CTR	ZnO	CRB		
Fermentation products.						
Ileum						
Lactic acid	4	23.9 ^{x}	8.4^{y}	18.1^{x}	9.21	0.10
	8	25.9	13.3	19.7	13.35	0.38
SCFA (mM)[b]	4	8.0	5.5	7.8	3.23	0.46
	8	11.7	6.8	9.7	5.95	0.41
Colon						
Lactic acid (mM)	4	6.6 ^{y}	9.6 ^{xy}	14.4 ^{x}	5.17	0.03
	8	7.1	6.4	8.2	3.22	0.52
SCFA (mM)	4	113	134	159	32.4	0.10
	8	102 ^{y}	141 ^{xy}	166 ^{x}	34.9	0.01
Acetic (%)	4	57	60	62	4.29	0.28
	8	59	59	61	4.58	0.76
Propionic (%)	4	26	23	22	3.46	0.43
	8	28	27	25	3.92	0.69
Butyric (%)	4	9.1^{y}	11.2^{x}	11.6 ^{x}	2.15	0.09
	8	8.6	10.0	10.2	1.98	0.32
BCFA (%)[c]	4	3.0^{y}	1.9^{x}	1.7^{x}	1.04	0.10
	8	1.9	1.4	1.8	0.82	0.49
Ileal Viscosity (Log of cP)	4	1.97^{x}	1.93^{x}	2.33^{y}	0.424	0.03
	8	1.91 ^{x}	1.96 ^{x}	2.36 ^{y}	0.251	0.05

[a] Diets: CTR (control diet). ZnO (zinc oxide diet). CRB (dieta with carob seed meal).
[b] Total ileal SFCA include only acetic and butyric acids. Propionic and BCFA were below level of detection.
[c] BCFA=isobutyric+isovaleric acids.
[xy] Different superscripts in the same row denote a significant difference (*P* ⩽ifferent superscripts in the same(*P* ⩽ 0.10).

Table 6.4. Microbial counts of enterobacteria and *E. coli* in feces, ileal scrapings and colon digesta, determined by culture methods (Log CFU/g of fresh matter) or by qPCR (log gene copy number/g of fresh matter), in weaning piglets at 0, 4 and 8 days post-challenge (PC) with ETEC K88.

Items	Period Days PC	Diets			SEM	P-value
		CT	ZnO	CARB		
Faeces (CFU/g MF)						
Enteterobacteria	0	9.5	9.3	9.7	1.49	0.56
	4	10.9^{y}	9.3^{x}	8.9^{x}	1.86	0.05
	8	10.3	8.9	8.7	1.98	0.34
E. coli	0	9.0	9.2	9.0	1.45	0.65
	4	10.4^{y}	9.0^{x}	8.7^{x}	1.56	0.03
	8	9.6	8.6	8.2	2.01	0.26
Ileal scrapings (CFU/gr MF)						
Enterobacteria	4	6.3^{y}	5.8^{y}	4.2^{x}	1.80	0.09
	8	5.4	5.5	5.0	1.48	0.99
E. coli	4	6.1^{y}	4.6^{x}	4.1^{x}	1.78	0.07
	8	5.9	5.3	5.0	1.41	0.96
Colon digesta (log gene copy number/gr MF)						
Enterobacteria	4	11.9^{y}	9.1^{x}	9.7^{x}	0.64	0.03
	8	10.2^{y}	8.0^{x}	8.3^{x}	1.45	0.10
E.coli K88	4	10.3^{y}	7.3^{x}	8.9^{y}	0.83	0.05
	8	8.3	7.1	7.4	1.95	0.52

[a] Diets: CTR (control diet). ZnO (zinc oxide diet). CRB (carob seed meal diet).
[xy] Different superscripts in the same row denote a significant difference *(P* ⩽ifferent superscripts in the same*(P* ⩽ 0.10).

Regarding the effect of dietary supplementation on immune response (Table 6.5) CRB and ZnO diets piglets were able to reduced Pig-MAP plasmatic levels, in comparison to piglets fed CTR diet (*P* = 0.03) both at 4 and 8 days PC. No differences were found in tumor necrosis factor-*α* (TNF-*α*) with regard the treatments.

Table 6.5. Effect of the diets on plasmatic levels of cytokine TNF-α and acute phase protein PigMAP in weaning piglets at 4 and 8 days after an oral challenge (PC) with ETEC K88.

Items	Period	[a]Diets			SEM	P-value
	Days PC	CTR	ZnO	CAR		
TNF-α (pg/mL)	4	68.4	67.6	66.2	19.04	0.99
	8	76.5	64.3	72.0	17.13	0.38
PigMAP (mg/dL)	4	1.20^{y}	0.70^{x}	0.74^{x}	0.341	0.03
	8	1.37^{y}	0.53^{x}	0.83^{xy}	0.575	0.03

[a] Diets: CTR (control diet). ZnO (zinc oxide diet). CARB (carob seed meal diet).
[xy] Different superscripts in the same row denote a significant difference *(P* ⩽ifferent superscripts in the same*(P* ⩽ 0.10).

Table 6.6 shows the results for the ileal histological measurements. Villous height and therefore villous height/crypt depth ratio were higher for ZNO diet compared to CTR and CRB groups 8 days PC In addition, it was observed a higher villous goblet cell (*P* = 0.04) and reduced intraepithelial lymphocytes (IEL; *P* = 0.05) in piglet fed ZnO and CARB diets at 4 days PC**.**

Table 6 6. Effect of the diets on ileal histological measurements in weaning piglets at 4 and 8 days after an oral challenge (PC) with ETEC K88.

Items	Period (days)	[a]Diets			SEM	*P*-value
		CTR	ZnO	CAR		
Villous height (µm)	4	308	323	321	14.4	0.20
	8	322^{xy}	349^{x}	317^{y}	20.5	0.01
Crypt Depth (µm)	4	211	215	201	12.5	0.81
	8	207	197	206	18.4	0.57
Villus:Crypt Ratio	4	1.5	1.5	1.6	0.21	0.56
	8	1.6^{y}	1.8^{x}	1.5^{y}	0.66	0.01
IEL (cells/100 µm).	4	9.2^{y}	7.0^{x}	7.9^{x}	1.29	0.05
	8	10.0	9.0	8.7	1.17	0.18
GC /100 enterocytes	4	6.8^{y}	8.2^{x}	8.9^{x}	0.91	0.04
	8	8.8	9.6	9.1	1.17	0.15

[a] Diets: CTR (control diet). ZnO (zinc oxide diet). CRB (carob seed meal diet).
[b]GC Villous goblet cells
[xy] Different superscripts in the same row denote a significant difference *(P* ⩽ifferent superscripts in the same*(P* ⩽ 0.10).

6. 4. Discussion

The inclusion of ingredients rich in non starch polysaccharides (NSP) in weaning piglet diets has been reported by some authors as a way to reduce the occurrence of PWD, having a beneficial effect on gut health (Wellock *et al*., 2008). The carob seed is a vegetable ingredient with an endosperm rich in galactomannans (more than 30-40% % of the seed, Karababa and Coşkuner, 2013) that traditionally have been used in the food industry as a source of thickener agent. Specifically, the product used in this study was mostly obtained from the carob seed partially dehulled, degermed and micronized.

In the current study it was performed a challenge model with ETEC K88, representative of typical commercial rearing conditions, which allowed testing the proposal

dietary treatment in a real pathogenic environment. ETEC challenge resulted in mild diarrhoea that resolved spontaneously. These effects of the experimental *E. coli* challenge are in accordance with a previous report using the same model (Hermes *et al*., 2012). The effects of the challenge were evaluation in comparison with the NC group. These non challenge animals showed absence of diarrhoea, a significantly lower serum Pig-MAP and faecal enterobacteria and *E. coli* population as well as no detectable levels of *E. coli* K88 in colon.

6.4.1. Effects on performance parameters

Diarrhoea incidence was reduced in piglets fed CRB and ZnO diets. This result was coupled of reduced faecal count of enterobacteria and *E. coli*, indicating inhibition of colonization these pathogens in gastrointestinal tract. Similar effects with pharmacological doses of ZnO were reported by other authors (Owusu-Asiedu *et al*., 2003, Mollist *et al*., 2011).

During the whole experimental period, as expected, piglets fed ZnO shown a better ADFI and final BW than those fed CTR and CRB treatments. Increases in feed intake and gains, with high dosis of ZnO has been reported by other authors (Broom *et al*., 2006; Vilà *et al*., 2010). Regarding CRB diet, it was not able to improve the performance of the animals in terms of intake and weight gain. It was seen however an increase in the viscosity of ileal digesta. This latter effect might have had a negative impact on mucosal enzymatic activity and feed intake (Owusu-Asiedu *et al*., 2006; Hooda *et al*., 2010). However, CRB diet did not decrease significantly any performance parameter. Therefore it is possible that, under challenge conditions, the potentially detrimental effects of an increased viscosity had been compensated by other benefits like the promotion of a healthier microbiota or the improvement of the barrier function or the immune response. In that sense previous works with an inclusion up to 10% of carob seed also reported increases in the digesta viscosity but without compromising gain/feed ratio (Van Nevel *et al*., 2005).

6.4.2. Effects on intestinal microbiota

In the current experiment changes on pattern of fermentation were mainly detected in colon. Although both, ZnO and CRB diets resulted in increasing of SCFA and lactic acid concentration, the CRB diet showed higher numerical increases. The inclusion of the carob seed producto could have increased the concentration of total SCFA throughout an increase in the amount of fermentable material and particularly non-digestible oligosaccharides arriving to the colon. In this sense CRB diet could had acted as a prebiotic improving the composition and functionality of health-promoting bacteria enhancing colonization resistance against enterobacteria. Previously, Van Nevel *et al*. (2005) suggests that dietary administration of 10% of carob seed to weaned piglet, tended to increase the total SCFA in cecum. Administration of diets rich in fermentable carbohydrates to weaned piglets, has shown a promotion in the composition and activity of microbial communities in colon, especially lactobacilli (Kosntatinov *et al*., 2006; Wellock *et al*., 2007), reducing BCFA concentration and enhancing the barrier function.

Regarding the use of carob seed products, *in vitro* studies also have reported reductions in *E*. *coli* K88 (Hermes *et al*., 2011; Badía *et al.,* 2012). The effects on enterobacteria could be due to a prebiotic effect of the carob product but also to other mechanisms. Some authors have reported a possible interference of this ingredient in the mechanisms of adhesion of ETEC K88 to the intestine. Inhibition of the adhesion of ETEC by carob seed gum (Badia *et al*., 2012) or carob seed extract (Hermes *et al*., 2011) has been recently reported. These findings could explain the trend to reduce the enterobacteria and *E. coli* mucosa seen with CRB.

The inclusion of ZnO in the diet also promoted changes in the fermentation patter, especially with increases in the molar proportion of butyric acid in colon and decreases in BCFA. These effects would be difficulty attributed to increases in the amount of fermentable

material arriving to the hindgut but more probably to changes in the fermentation through selective changes in microbial populations. Particularly ZnO could improve the fermentation, by antimicrobial effects on proteolytic bacteria, including *E*. *coli* K88. The reduction of *E*. *coli* K88 by ZnO, has been observed in both *in vitro* and *in vivo* studies (Owusu-Asiedu *et al.,* 2003, Roselli *et al*., 2003;; Slade *et al*., 2011). Furthermore, is likely that ZnO did not affect the *Lactobacilli*, and thus favoured saccharolytic fermentation. A previous study, showed that ZnO, was able to reduce the lactic acid bacterial activity (Højberg *et al*., 2005), but more recently other authors have detected very high zinc resistance of lactic acid bacteria (Slade *et al*., 2011; Liedtke and Vahjen, 2012). Therefore ZnO could have modified fermentation throughout changes in microbial ecosystem.

6.4.3. Intestinal epithelial architecture and inmmune response

We have seen that both dietary treatments CRB and ZnO, had beneficial effects on intestinal environment, particularly with reductions in the number of enterobacteria and *E. coli*. Together with the possible reasons mentioned above, the reduction in this bacterial group could be also explained by an enhancement of the barrier function and immune response. In that sense both treatments promoted an increase in the goblet cells numbers responsible of the production of mucus. It is accepted that mucus contains similar ETEC K88 receptors than those found on enterocytes (Blomberg *et al*., 1993). Hence, increases of mucus layer secreted by goblet cell might have prevented the ETEC K88 adherence to epithelial small intestine.

Regarding changes in the intestinal architecture, we did not find either change in the villous/cript ratio, but a reduction in the IEL and also in the acute phase proteins (APP). In that sense it has been reported that mannans via its uptake into Peyer's patches could improve the immune response associated with lowered intraepithelial lymphocytes (Davis *et al*., 2004). Administration, of carob seed gum also has been reported to reduce C-reactive

protein CRP in piglets challenged with ETEC K88 (Badia *et al*., 2010). The Pig-MAP and CRP belong to the major positive acute phase proteins (APP) in swine. According to Pertensen *et al*. (2004) and Piñeiro *et al*. (2009) an increase in concentration of these APPs can indicate the extent of inflammation and tissue damage caused by bacterial infections such as ETEC. Therefore, under ETEC K88 challenge conditions, the inclusion of the carob seed product in the diet might have a favourable influence on the inflammatory response

The improvements seen on small intestine morphological in piglets fed ZnO, and particularly in the increased villous height and villous/crypt ratio could be due to direct antimicrobial effect on ETEC K88 but also by reducing the pathogen binding sites. Roselli *et al*. (2003) reported that ZnO might protect intestinal cells from *E. coli* K88 infection by inhibiting the adhesion and internalization of bacteria. In addition, ZnO may increase ileal villous height, goblet cells, mucus thickness (Li *et al*., 2001; Slade *et al*., 2011) and down-regulate the expression of gene associated with inflammation caused by ETEC K88 (Sargeant *et al*., 2010). So, evidence suggests that ZnO could also potentiate the non-immunological defence mechanisms, reducing the ETEC-associated damage on small intestine morphology

Regarding possible changes on pro-inflamatory cytokines like TNF-α, previous in vitro reports with carob gum (Badía *et al*., 2012) and ZnO (Sargeant *et al*., 2011) demonstrated a decrease in the expression of TNF-α on porcine intestinal epithelial cells (IPEC-J2) infected with ETEC K88. However in the current study we were not able to detect changes in the concentration of plasmatic TNF-α, suggesting that its role might be restricted to a local response in the intestine.

6.5. Conclusion

In conclusion, the current study demonstrated that the dietary inclusion of 0.5% of a carob seed product in weaning piglets can reduce the diarrhoea incidence and the overgrowth of enterobacteria and its attachment to the ileal epithelium after an ETEC-K88 challenge. These effects are correlated to increases in SCFA and lactic acid concentration in colon and improvements in the intestinal barrier function with increased goblet cell numbers and decreases in the ileal IEL and plasmatic Pig-MAP.

6.6. References

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CHAPTER 7

General Discussion

7. GENERAL DISCUSSION

Weaning constitutes one of the most challenging situations in the life of a pig. It introduces a number of stress factors, which may affect the immune functions and the intestinal microflora of the piglets negatively. These factors together with the presence of potentially pathogenic microorganisms such as *Escherichia coli* K88, may lead to outbreaks of diarrhoea following weaning. Antimicrobials compounds such as: antibiotics and ZnO have been effectively used as in-feed growth promoters to prevent the detrimental effects of weaning. However, from 2006 the use of antibiotic growth promoters for prevention of diarrhoeal diseases in piglets has been banned in the EU. This measure led to the investigation of dietary alternatives to in-feed antibiotics, including prebiotics and probiotics. There is evidence to suggest that probiotics, prebiotics and their combinations (synbiotics) could be functional components in piglet's diet, able to improve growth performance, gastrointestinal function and health. Several studies have been conducted with unchallenged piglets and different pre and probiotics, however, their impact on control and prevention of post-weaning diarrhoea caused by ETEC K88 is still far from being conclusive. It is necessary to know not only the effect of a probiotic on a normal healthy microbiota but particularly its effect when the composition of microbiota is unbalance by the overgrowth of a pathogen. This knowledge could be a requisite to select the right probiotic, prebiotic or symbiotic combination for a particular disease.

In this chapter, the main findings of this thesis are discussed in relation to the effects of prebiotics, probiotics and synbiotics in swine feeds on the gastrointestinal ecosystem, health and performance effects of weaned piglets exposed to an enterotoxigenic *Escherichia coli* K88 challenge.

7.1. Probiotic and prebiotic strategies to prevent the post weaning diarrhoea

Based in the results obtained by Martin-Pelaéz *et al*. (2008) and Collins *et al.* (2011) we aimed to evaluate, in the current thesis, the effect of a porcine *Lactobacillus plantarum* strain combined or not with lactulose, in the control and prevention of ETEC K88 infection. The experimental sequence followed in this thesis was appropriate to achieve this goal. Firstly, feed additives were tested in absence of pathogenic infection for subsequently to be studied in Study 2, under ETEC K88 challenge conditions. Both experiments provided valuable information in relation with the effects of these additives on a wide range of parameters including: growth performance, gastro intestinal microflora composition and metabolic activities, gut morphology and inflammatory responses. A better explanation of mechanisms of action involving these effects will be discussed bellow.

7.1.1. Lactulose for prevention of PWD

Lactulose (4-O-β_-D-galactopyranosyl-D-fructose) is a semi-synthetic disaccharide formed by isomerization of lactose by a chemical reaction (Clausen and Mortensen, 1997). It has been used since the 1950s as a prebiotic, referred to as bifidus factor, and is classified as a drug, although it is similar in composition to feed additives currently being used as prebiotics (Schumann, 2002). Prebiotic uses had included the prevention and treatment of enteric disorders in humans but also in some animal species (Vondruskova *et al*., 2010). Together with prebiotic applications, lactulose has been also used in higher doses for the treatment for constipation in humans. However there is scarce information regarding the effects of lactulose as a possible prebiotic in pigs. A study conducted by Krueger *et al.* (2002) revealed that lactulose supplementation to piglets led to a significant increases of daily weight gains. Furthermore, in vitro studies showed lactulose promotion of growth and/or activity of indigenous intestinal *Bifidobacterium* and *Lactobacillus,* with reductions of the activity of proteolytic bacteria (Maxwell *et al*., 2004; Martin-Pelaéz *et al*., 2008). However, *in*

vivo experiments have not been so obvious regarding these beneficial effects. For example, for weaning piglets the lactulose promoted the indigenous *Lactobacilli* communities in both ileum and colon (Konstantinov *et al*., 2004) but did not mofidied the specific *L. amylovorus* and *L. reuteri* population, while in infected pigs did not affect *Salmonella* population (Martin-Pelaéz *et al.,* 2010).

Results described in the current thesis, revealed the potential of lactulose to control the PWD caused by ETEC K88 and to improve the performance of piglets at weaning. It seems that lactulose improved the growth through a direct effect on intake or indirectly enhancing other health parameters like the modulation of microbial population or the increase of villous height in ileum. Beneficial effects on epithelial architecture could be more relevant under ETEC K88 challenge.

Additionally, it must be explained that syrup of lactulose used in these studies also contained galactose, tagatose and epilactose. All these compounds are known to be sweetness in humans, but also preferred in piglets (Glaser *et al*., 2000), then probably its inclusion could increase the preference of the diet, improving the consumption.

Supplementation of lactulose, also resulted in increasing population of lactic acid bacteria in ileum and lactobacilli in colon and, although did not reduce the colonic enterobacteria population, did improve the lactobacilli to enterobacteria ratio. A higher lactic acid bacteria population could had play an important role, in digestion of nutrients (Berg, 1996), besides increase the production of butyric acid in colon. In this regard the first two studies showed increased in the percentage of butyric acid in colon.

Moreover results of the second trial, showed increases in the villous height when lactulose was included in the diet. Therefore due villous atrophy is frequently described after an ETEC K88 infection (Heo *et al*., 2012) supplementation with lactulose could ameliorate these effects maintaining the gut absorption capacity and reducing the growth check. These could be one of the explanations for the increased ADG observed in the post-challenge

period in the animals fed lactulose and also the reduced concentration on plasmatic PigMAP.

In addition our results also suggest that lactulose could have reduced the proteolytic fermentation. On one hand BUN concentration was reduced in the Study 1 and in both trials there were a trend in colonic BCFA to decrease. In agreement with these finding Vondruskova *et al*., (2010) affirmed that lactulose reduces the activity of proteolytic bacteria.

Summarizing our results demonstrated the prebiotic activity of lactulose when included at 1 % in the weaning diets. Lactulose was able to promote the lactobacillli population, modifying positively the microbial fermentative activity and was also able to improve epithelium architecture with increases in the villous height. All these benefits could be behind the improved ADG observed in the post-challenge period.

7.1.2. Role of L. plantarum in control and prevention of PWD caused by ETEC K88

In the control and prevention of PWD, the most promising effects of the use of probiotics are related to competitive exclusion mechanisms against pathogenic bacteria. However other effects of probiotics could include improvements on gut microbiota balance, positive influences on intestinal epithelium integrity and a more appropriate maturation of the gut associated tissue and immunogenic activity (Metzler et al., 2005). The potential of lactobacilli probiotics (*L. sobrius*) to inhibit particularly ETEC K88 in pigs had been previously described by others using in vitro and in vivo approaches (Roselli *et al*., 2007; Konstantinov *et al*., 2008).

An appropriate selection criterion for the design and development of a new probiotic is undoubtedly a key point. Many authors have suggested that a good strategy would be to select favourable commensal strains from the pig gut that could be more successful colonizing the gut and excluding pathogens. In this regard Yun *et al*. (2009) selected four lactic acid bacteria strains (*L. fermentum, L. salivarius, L. plantarum and L. reuteri*) isolated

from weaning pigs based also on its survival under acidic, high concentration of bile salts and in its ability to inhibit *E*. *coli* K88 and *Salmonella typhimurium* (Yun *et al*., 2009). The probiotic *L. plantarum* strain used in this study was previously selected based on similar criteria.

The viability and actual level of probiotic bacteria in the intestinal tract is also a key point because the effect of a probiotic depends on its viable count in the gastrointestinal tract (Mountzouris *et al*., 2007). As we have seen, their resistance to gastric acid, bile acid, and digestive enzymes is important and related with this also its way of administration. Depending on the resistance of the probiotic to the gastric barrier and its ability to colonize the gastrointestinal tract it can be needed a different pattern of administration. This could be one explanation for different results reported in the literature. While in piglets supplemented with a single oral dose of *L. plantarum*, the presence of probiotic bacteria was not demonstrated (Pieper *et al.,* 2009; 2010), other results showed that daily administration of *L*. *plantarum* isolated from infant faeces could be detected although do not become established as a normal microbiota of piglets (Sou *et al*., 2012). This fail in permanent colonization could be evidencing that ability of an allochthonous strain to colonize the host gastrointestinal microbiota may be low (Dunne *et al*., 1999). Regarding our studies we tried to guarantee a stable and high level of *L*. *plantarum* in the gastrointestinal tract, based on two conditions: The first that probiotic was administered daily. The shorter interval may lead to an elevation of the mean number of probiotic bacteria in the colon, ensuring the colonization. Second, as described above, the criteria used to select the potential probiotic bacterial strain resulted in the identification of porcine *L. plantarum* capable of resisting the effects of bile and low pH, adhering to piglet epithelial cell lines, and exhibiting antimicrobial activity in vitro (Collins *et al*., 2007; Collins *et al*., 2011).

The ability of probiotics of adhering to the intestinal epithelial cells and mucus is considered important as this capacity, is expected to strongly interfere with the adhesion of

pathogenic bacteria (Fuller 1991). Furthermore, the adherence of probiotic bacteria is associated with their immunological effects (Moutzouris, 2007). Although the *L. plantarum* used in our study, possessed the ability to adhere to the porcine intestinal epithelia (Collins *et al*., 2007), our results showed not antagonist effect against luminal *E.coli* K88 population. It is truth that the number of adhered *E. coli K88* could had been different between treatments despite the similar numbers found in the digesta. However it is reasonable to expect correlation between both parameters at the ileal level (Chandler and Mynot, 1998). Regarding the potential of probiotic bacteria to competitively interfere with the adhesion of ETEC K-88 to the epithelium, *in vitro* studies of Roselli *et al*. (2007) reported that probiotic *L. sobrius* strongly reduced the *E. coli* K88 adhesion.. Authors explained these results by a competition between both bacteria for the same mucus receptor in which would be involved a similar glycoprotein. Although we cannot discard a possible effect of our strain reducing the attachment of ETEC to the intestinal epithelium, our results suggests that other mechanisms could have been also behind the observed effects, like: (i) modulation of intestinal microbiota, (ii) stimulation of epithelial barrier and non immunological defence function; (iii) stimulation of immune responses via increases of IEL and regulation of pro-inflammatory cytokines.

In Studies 1 and 2, we demonstrated that administration of *L. plantarum* strain modulated the intestinal microbiota, favoring, the ratio lactobacilli to enterobacteria (study 1) and increasing the percentage of lactobacilli (study 2). Increases of total lactobacilli could be explained by the observed increases in the total *L. plantarum* population and also by the increase in the abundance of other lactobacilli such as *L. delbrueki, L acidophilus or L. brevis.* Other authors using a probiotc *L. plantarum* strain also described increases in the intestinal microbial diversity specially lactobacilli population (Pieper *et al.,* 2009; Pieper *et al.,* 2010). Increases in the lactobacilli could have represented a benefit for the weaned animal and their ability to respond to ETEC K88 infection. However, since the count of *E. coli K88*

was not reduced with the administration of *L. plantarum*, it can only be speculated that other possitive effects could had be behind the improved response to the challenge. Taking into account that butyrate is a key to many aspects of health and integrity of intestinal mucosa (Hamer *et al*., 2009) its numerical increase could had contributed to amelioration the effects of mucosal inflammation. Secondly the decreasing in BCFA and NH_3, also was indicative of an improved colonic fermentation. As BCFA and NH_3, are produced exclusively from the fermentation of protein (Williams *et al*., 2001), the reduction of these by-products of microbial activity indicates a reduction in the proteolitic activity of microbiota. Curiously, evidences in the literature on the influence of probiotics on proteolytic fermentation and therefore in the production of toxic metabolites such as ammonia, amines, volatile phenols and indoles are scarce. Reductions of these metabolites could have a beneficial impact on the health of the gastrointestinal tract because they can negatively affect the growth and differentiation of intestinal epithelial cells (Suzuki et al., 2002), and indirectly initiate inflammatory reactions and increase the incidence of diarrhoea.

Positive effects of the probiotic on the intestinal architecture and in the inflammatory response were supported by observations showed in the Study 2. These data revealed that inclusion of L. plantarum in the diet increased villous height and goblet cells in ileum. Improvements in the immune response were also suggested by reductions in IEL and plasmatic TNF-α with this treatment. These positive effects were also confirmed by reductions on the Pig-MAP serum concentration. Other authors also have described increases of villus heigh with the use of probiotic *L. plantarum* strains (Thu *et al*., 2011; Sou *et al*., 2012). Relative to the increasing of goblet cells, this could lead to a higher production of mucin (Deplancke and Gaskins, 2001). Previously Collins *et al*. (2011) demonstrated that *L. plantarum* did induce mature acid mucin secretion. The quantity and maturity of mucins covering the epithelial surface are important factors for optimum pathogen resistance. Particularly, mucins more acidic and viscous are highly resistant to the bacterial proteases

(Montagne *et al*., 2003). Other authors, using the HT-29 cell-line secreting mucus infected with EPEC, found an inhibition in the attachment of bacteria to the cells by *L. plantarum*, which was associated with an induction of mucin gene expression (MUC-2 and MUC-3) (Mack *et al*., 2003; Caballero *et al*., 2007) Altogether these results might indicate that *L*. *plantarum* could had increased both the activity and numbers of goblet cells.

Disruption of intestinal morphology has been reported as one of the clinical signs of ETEC K88 enteric infections. The maintenance of epithelium integrity might be an important mechanism through which the *L*. *plantarum* benefited the piglets under disease conditions. As has been already suggested, the patterns on fermentation in piglet fed *L. plantarum* could have had a positive influence on the maintenance of villous height. However, there may be other molecular mechanisms behind this probiotic effect. For example *L. plantarum* could have triggered responses of the host epithelial cells throughout mannose-specific interactions. This is possible by the up-regulating of innate immune response factor PAP (Gross *et al*., 2008). When it is augmented it contributes to epithelial repair as well as it exerts an antimicrobial action to enterotoxigenic *E*. *coli* (Cash *et al*., 2006; Niewold *et al*., 2007). Other theories implicated the action of soluble proteins and metabolites produced by of *L*. *plantarum* that would diminish significantly oxidative-stress-mediated disruption of barrier integrity (Paszti-Gere *et al*., 2012). Moreover, *L. plantarum could had* transformed dietary polyphenols to bioactive compounds with potential healthy effects (Jakesevic *et al*., 2008) reducing the *o*xidative damage induced by ETEC K88. In this regard it has been described how *L. plantarum* possess a tannase and two inducible decarboxylases able to hydrolyse tannins and others phenolic compounds (Osawa *et al*., 2000; Barthelmebs *et al.*, 2000).

As it is know ETEC K88 infection is associated with inflammatory responses in piglets and porcine intestinal cell lines (Bosi *et al*., 2007; Roselli *et al*., 2007). An adequate modulation of the the immune system, including inflammatory responses, could be therefore

an important point in animals challenged by ETEC K88. Fimbrial colonization factor antigen and heat labile enterotoxin of *E. coli* K88 are potent stimulators of the immune system suggesting that it is the intestinal colonization by ETEC K88 that triggers immune responses (Gyles and Fairbrother, 2010). From that point of view, diets including the probiotic could had reduced inflammation by reducing ETEC proliferation and subsequent colonization of the gut (Heo *et al*., 2012). However in our work (Study 2) we were unable to detect decreases in the numbers of ETEC K88 in the gut. Despite of this, we found clear evidences of positive effects on immune and inflammatory responses.

IELs are considered to be a T-cell population playing the first line of host defense against a variety of antigens and pathogens, contributing to the maintenance of intestinal homeostasis at epithelial sites (Oswald *et al*., 2006). These cells accumulate at inflammation sites, where they have been shown to reduce the inflammatory reaction and tissue damage in ETEC infection (Gyles and Fairbrother, 2010). Thus in Study 2 a higher IELs in piglets fed *L. plantarum*, was one of possible protective mechanism involved in maintaining and recovering the ephitelial mucosa. Regarding the decrease we also observed in TNF-α, other authors also found similar decreases in non challenge (Mizumachi *et al*. 2009) and also ETEC challenged pigs (Lee *et al*., 2012). Likely, this could had been due to the presence of two bioactive peptides of 21 and 31 kDa molecular weight, described by Paszti-Gere *et al*. (2012) to down regulate the expression of TNF-α and IL-8 of IPEC-J2 cells.

In general, our data suggest that probiotic *L*. *plantarum* could had promoted intestinal health and improved the response against to an ETEC K88 by several mechanisms including a shift in the numbers and diversity of lactobacilli, improvements in the fermentation profile with decrases in the proteolitic activity and also a better modulation of the inflammatory response throughout reductions in the TNF-a response and in the IEL..

7.1.3. Lactulose and L. plantarum as synbiotic combination

In Studies 1 and 2, the synbiotic approach showed consistent additive effects on the intestinal microbiota and on animal response. However, contrary to expected, we did not find an specific growth of *L. plantarum* in the symbiotic combination. In previous in vitro studies (Martin-Pelaéz *et al*., 2008) it had been demonstrated that lactulose was more efficiently used by *L. plantarum* compared to other probiotic strains, and moreover, pathogens like *Salmonella* were not able to growth on lactulose. Various factors could be considered to explain the fail of lactulose to increase the growth of *L. plantarum* in our *in vivo* conditions. First, the effects of lactulose *in vitro* cannot be directly extrapolate to in vivo, considering the complexity of gut porcine environment where it is possible that the lactulose does not exert the same promoter effect. Second, the *L*. *plantarum* used in this study, initially isolated from pigs, could have a great capacity to survive and establish in porcine gastrointestinal tract. Therefore it is possible that lactulose only had effects on *L*. *plantarum* population when it is in low numbers (endogenous population), but not when it is already in high numbers due to its supplementation as a probiotic source.. In this regard the development of synbiotics might be more important for strains of probiotic with poorer survival properties. Thirdly, it is possible that the dose of the prebiotic used (1% w/w) was too low to ascertain a specific growth promotion effect on *L. palantarum.* Finally we not discard that on intestinal porcine environment lactulose could have promoted the growth of other microbial groups like bifidobacteria instead of *L. plantarum*. For examples, several studies showed the effectiveness of lactulose to stimulate the growth of bifidobacteria (Olano and Corzo, 2009; De Souza Oliveira *et al., 2011*).

Summarising, when combined, lactulosa and *L. plantarum* add their individual advantages but we could not find synergic effects. Regarding the definition of Kolida and Gibson (2010) we would be talking then of a “complementary” symbiotic and not at “synergistic” one. Nevertheless it cannot be discarded that although supplementation of

lactulose did not increase the numbers of *L. plantarum* it could had improved its metabolisms and its beneficial effects on the host. More studies would be needed to explore this possibility.

7.2. Carob seed product as functional ingredient

Another objective of the current Thesis was to better understand the potential health promoting activities of a derivative form carob seed to prevent and controlling the PWD caused by ETEC K88. With this aim it was designed the Study 3. The results showed that the carob seed diet have some positive effects similar to those obtained with pharmacological doses of ZnO like decreasing diarrhoea reductions in enterobacteria population, enhancement in the colonic fermentation and modulation of inflammatory response. Up to day it is very limited the number of published works using carob products in the piglets diets and particular a product like that used in this study that was mostly obtained from the carob seed partially dehulled, degermed and micronized. Other studies evaluated the inclusion of carob pods meal (Lizardo *et al.*, 2002; Andrés-Elias *et al*., 2007), whole carob seed (Van Nevel *et al.,* 2005) and carob gum (Van Nevel *et al.,* 2005; Pellikaan,*et al*., 2010).Carob pod is a very good source of sugars (48 to 56%) making them palatable besides being a high energy feed for animal nutrition. However, an increasing in the inclusion rate up to 12.5% results in a significant decrease in nutrient digestibility due to their relatively high content of tannins (Kotrotsios *et al*. 2010). On the other hand the constituents of the seed are (by weight): coat (30-33%), endosperm (42-46%) and embryo or germ (23-25%). The seed coat also contains tannins, while the endosperm is the galactomannan carob bean gum source (Dackia *et al*., 2007).

Galacto-mannan compounds, in which is rich the product studied in this Thesis, could had interfered with the attachment of ETEC K88 to the enterocytes. In this regard, previous *in vitro* studies of our group have demonstrated the ability of an ingredient including carob

seeds to reduce the adhesion of ETEC K88 to the IPEC-J2 cells (Hermes *et al*., 2011). Also other authors using a prebiotic derived from carob bean and also rich in β-galactomanans described similar effects (Badía *et al*., 2012).

In our study we did not determine the number of ETEC K88 adhered to the intestine, therefore we cannot conclude regarding a interference of the carob diet in the adhesion of this particular pathogen, however we did found significant reductions of more than 2 log units in the number of total enterobacteria an *E. coli* adhered to ileum, 4 days PC. These results would suggest that carob diet could have exerted some inhibitory effect on the microbial adhesion to the intestine, and particularly the *E. coli* population.

The increase in the gut viscosity in pigs fed the carob diet, was not surprising considering the properties of carob seed endosperm and that the dose used in the study was in the upper limit of the recommendations given by the manufacturer (between 3-5 g/kg). An increase in digesta viscosity has been generally seen as negative as it has been related to lower feed intakes and detrimental effects on digestibility (Hopwood *et al*., 2004). However in our study we could not find significant effects on the performance parameters. Despite of this, possible detrimental effects during longer period of administration should not be discarded and would need to be tested. On the other hand more studies evaluating a possible reduction in the doses also should be considered.

Our carob seed product also could have act as a prebiotic, promoting the growth of particularly health beneficial bacteria. Different plant polysaccharides are now recognized as having prebiotic effects (Gaggia *et al.,* 2010; Heo *et al*., 2012). In this context the carob diet could had modulated favourable microbial population, since it was found an increase the production of lactic acid and SCFA, especially butyrate. We had hypothesized that these effects could be due to provision of more galactomannans acting as substrate for gut microbiota. In this regard similar soluble NSP present in sugar beet pulp also increased the butyrate percentage associated with the promotion of a beneficial shift in the microbial

colonization of newly weaned pigs (Molist *et al*., 2009). In addition was previously showed that supplementation of the diets with galactomannans increased the diversity of both ileal and colonic microbiota of weaned piglet (Wang *et al*., 2010).

According to the fact that the microbiota can modulate the local immunity of the host, the locust bean induced shift observed in gut microbiota could also explain the decrease of intraepithelial lymphocytes observed (Gaskins *et al*., 2008). We do not discard however a possible direct effect of the mannan fraction on immune response throughout epithelial receptors, as it has been reported interactions effects of mannans on *Peyer's patch* activating the immune cell of intestinal mucosa (Davis *et al*., 2004). In addition, Nochta *et al*. (2009) found that mannans increased the responsiveness to lymphocyte stimulation test in weaned pigs.

Therefore our carob seed derivative, could had acted as a prebiotic substrate for beneficial microbiota facilitating the *competitive exclusion* against enterobacteria and *E. coli*,population, all together modulating the inflammatory response. Also a possible interference in the attachment of ETEC to the intestinal epithelium could be behind the positive effects observed for this ingredient.

7.3. Zinc oxide as a mean of controlling PWD in piglets

There are several reports suggesting that the beneficial effects of pharmacological dosis ZnO on the prevention of PWC is based on its antimicrobial activity (Højberg *et al*., 2005; Pluske *et al*, 2007). However the improvement in health and performance observed in piglets could also be due to other mechanisms, *Z*n is an essential element for pigs that is a component of many metalloenzymes, including DNA and RNA synthetases and transferases, many digestive enzymes, and is associated with insulin and as such, it plays a crucial role in lipid, protein and carbohydrate metabolism in the pig.

During the Study 3 of the current Thesis we included a treatment with ZnO as positive control for the carob diet, as expected we found positive results in the performance an in the response after the challenge. Some of the particular effects because of their relevance we think would deserve some particular discussion.

7.3.1 Effect of ZnO increased voluntary feed intake

The supplementation of ZnO at pharmacological level (up to 3000 mg/kg) did enhance the piglet growth performance. This effect was primarily a result of increased feed intake. Likely, due to a direct effect of ZnO, altering the expression of genes responsible for glutathione metabolism and apoptosis (Li *et al*., 2006; Wang *et al*., 2009), which enhance gastric ghrelin secretion and intestinal morphology (Yin e*t al*., 2009) or through increasing production of digestive enzymes (Hedemann *et al*., 2006). Moreover, we no discard that ZnO could modulate the gastrointestinal bacterial composition and their metabolic activity (Højberg *et al*., 2005; Broom *et al*., 2006) thus exerting an indirect effect on the increase of feed intake.

7.3.2. ZnO modifying the intestinal environment

Regarding the effect of ZnO on gut environment,it was observed a significantly increase of lactic acid production in ileum, likely due to a modulatory effect of ZnO on microbial population with increases in lactic acid bacteria. We did no determine lactic acid bacteria but we found also decreases in enterobacteria and *E. coli.* . Results found in the literature regarding the effect of ZnO on particular microbial groups are sometimes inconsistent and contradictory. The results obtained by Piepper *et al*. (2012) showed that increasing level of dietary ZnO, resulted in an increase of bacterial diversity in the ileum, which reflects ecosystem stability. The same authors describe that lactobacilli, as well as lactate, were not influenced. Additionally, recent data suggested, the high Zinc resistance within the *Lactobacillus* group at the species level (Vahjen *et al*., 2012, Liedtke and Vahjen,

2012). However, previous findings showed a decrease of lactic acid bacteria with ZnO, which resulted in significantly lower concentration of lactic acid, SCFA (Højberg et al., 2005) and no effect on *E*. *coli* population (Hojberg *et al*., 2005; Broom *et al*., 2006). Moreover, Piepper et al. (2012) also reported that high dietary zinc levels led to increasing numbers of enterobacter but also resulted in significantly ammonia reduction. Conversely, in both the Study 3 and the conducted by Slade *et al*. (2011) the supplementation of ZnO, resulted in reduction of *E*. *coli* K88 population. This inhibitory effect could have influenced the proteolytic fermentation, marked by the numerical reduction of BCFA. Although, it should not be discarded that ZnO might also inhibit bacterial metabolism (Choudhury and Srivastava, 2001). In summary the effect of ZnO on microbial groups and particularly in lactobacilli, enterobacteria and *E. coli* is not clear and inconsistent results are found in the literature. Probably variables including the microbial status of the animal, the hygienic conditions, the composition of the diets or the dosis administered could be behind this variability.

7.3.3. Zinc oxide modifying the immune and inflammatory response.

The reduction of Pig-MAP was other positive indexes obtained by ZnO supplementation, which indicated the faster recovery of acute inflammatory response to ETEC K88 challenge. Reduction of inflammatory response could be due to reductions in pathogen growth or adherence. Several studies have elegantly demonstrated the decreasing in expression of immune response genes concerned with inflammation, related to the stage of ETEC K88 infection (Sargeant *et al*., 2010; Sargeant *et al*., 2011). However reduction of Pig-MAP could also be results of a direct effect of Zn on inflammatory or inmunitary response. .Feeding piglets with ZnO, resulted in significantly increase of the serum concentrations of immunoglobulin (Wang *et al*., 2012). These finding might be explained by the fact that zinc can inhibit the induction of nuclear factor κ**B** possibly through heat shock proteins and thus the cytokine response,(Sargeant *et al*., 2011).

Summarizing the observed benefits by dietary ZnO could be mediated through a directly improve of feed intake, changes in microbial equilibrium with reductions on ETEC K88 populations, a better control of inflammatory response and improvements in the intestinal mucosal barrier.

7.4. Experimental model of porcine post-weaning colibacillosis to study efficacy of dietary strategies.

One of the secondary objectives of this Thesis was to develop a reproducible model of porcine post-weaning colibacillosis resembling what occur on commercial piggeries. Since our point of view, an appropriate disease model for the study of dietary strategies would be one that would promote mild diarrhoea that could be solved spontaneously. However there are still many criticisms related to the incidence and severity of diarrhoea in the experimental conditions. Natural conditions of the disease are difficult to reproduce since, the post-weaning digestive disorders are multifactorial and experimental infection with *E. coli* alone is not sufficient to reproduce the syndrome as observed in the field. Predisposing factors to post-weaning diarrhoea include infective dose to use, bacterial survival after gastric passage, rearing conditions, particularly environmental temperatures, hygiene and dietary composition (Madec *et al*., 1999, Wellock *et al*., 2008; Rossi *et al*., 2012). In this respect, it must be emphasised that the conditions under which the piglets were kept in infection model experiments were of a high standard, and the animals lacked most of the risk factors for PWD. Taking the above aspects in consideration, the infection model was focused in selecting the infective ETEC K88 dose to guarantees its survival in gastrointestinal tract and subsequently a sub-acute infection. A higher dose of ETEC to achieve the infection may conduce to acute disease, while an insufficient dose may not assure a disease state piglet. Both conditions could limit the study of mechanisms of action involved in control of disease by the proposed strategies.

In our experiments piglets were challenged with a similar dose of pathogenic *E. coli* that has been used widely in previously models of PWD (Molist *et al*., 2008; Hermes *et al*., 2012). As expected, the challenge with ETEC K88 resulted in a sub-acute incidence of diarrhoea. This started between 24 and 48 hours after the challenge and lasted for more 4 days post-challenge and was also associated with recovery of ETEC K88 in colon content.

To achieve the bacterial survival rate after gastric passage, piglets were fasted during 10 hours prior the inoculation, after which were fed during 30 minutes. In contrast in other studies it was used the bicarbonate to guarantee the safe transfer of the inoculum into the small intestine (Jensen *et al*., 2006; Rossi *et al*., 2012). However, there is discrepancy about the importance of neutralize gastric acid. This could be explained by the ability of some *E*. *coli* organisms to activate an acid resistance system (Foster, 2005). Thus, if the challenge strain possesses such a system, the use of gastric acid neutralization would be unnecessary.

Other important point that could make variable the response of animals to the challenge would be a possible acquired immunity against the pathogen throughout their mothers. For the experiments with ETEC K88 challenge, piglets were selected from mothers with no historic of vaccination for *E*. *coli.*

The genetic susceptibility of the animals to ETEC K-88 infection it is also a variable to take into account. It is known the genetic resistance of piglet to ETEC K88 colonisation (Fu *et al*., 2012). However although we did not selected the piglets in our study for K88 susceptibility is truth that our animals had not been genetically selected for this gene and the natural presence of susceptible animals is quite high. Moreover the bacteriological examination showed sufficient colonization of the intestines with ETEC in challenged piglets. This would support that most of our animals were susceptible and/or that probably the presence of the F4R is not so determinant (Madec *et al*., 2000).

In the Study 3 we also included a control group of animals in which there were no

challenge. At day 4 post- challenge, these animals showed lower values of Pig-MAP than in the challenged pigs. As Pig- MAP concentrations generally increase during the acute state of disease (Heegaard *et al*., 2011) suggesting that the aim of inducing sub-clinical PWD was successfully achieved. In addition the count of ETEC K88 in these animals was below the minimal level of detection of method (3.3 log of 16S rRNA gene copies/g of FM sample) whereas in challenged animals the counts were between 8.9 -10.3 log of 16S rRNA gene copies/g of FM sample

As it has been shown, the experimental conditions and the infection procedure described in the challenge studies were effective to inducing diarrhoea in weaned piglets, allowing evaluation of dietary effects during infection and recovery processes.

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CHAPTER 8

Conclusions

Based in the results obtained in the current Thesis it was concluded that under our experimental conditions:

1. The inclusion of lactulose at 1% in the diet of weaning piglets increase their ADG after an ECET-K88 challenge. Lactulose increase the growth of lactobacilli, promote changes in the fermentation profile through a more butyrogen and less proteolitic one and increase the ileal villous height.

2. The daily supplementation of weaning piglets with 2 x 10^{10} CFU/d of *L*. *plantarum* effectively increases the number of *L. plantarum* in the gut and improves the response against an ETEC K88 challenge, decreasing diarrhoea incidence. Several mechanisms could be behind considering the ability of this probiotic to shift the numbers and diversity of lactobacilli, to improve the fermentation profile, less proteolytic, and also to modulate the inflammatory response, reflected by reductions in the TNF-α response and in ileal IEL.

3. Despite, that lactulose administered alone is able to stimulate the population of endogenous *L*. *plantarum,* when administered in association with a probiotic *L. plantarum* strain, do not promote any additional increase in their numbers. Considering this fact, the combination of lactulose and *L. plantarum* act as a complementary symbiotic, adding the beneficial effects of each additive, but not as a synergic combination.

4. The dietary inclusion of 0.5% of a carob seed product in weaning piglets can reduce the diarrhoea incidence and the overgrowth of enterobacteria and its attachment to the ileal epithelium after an ETEC-K88 challenge. These effects are correlated to increases in lactic acid concentration in colon and improvements in the intestinal

barrier function with increased goblet cell numbers. Enhancement in the inflammatory response is suggested by decreases in the ileal IEL and plasmatic Pig-MAP.

5. Under an ETEC-K88 experimental challenge, the supplementation of diets with pharamacological dosis of ZnO (3000 kg/Tm) increase the feed intake of weaning piglets and alleviate the deterioration of intestinal morphology increasing ileal villus heights and goblet cell numbers. Reduction in inflammatory response is suggested by decreases in the ileal IEL and plasmatic Pig-MAP.

6. The experimental model of colibacillosis by ETEC-K88 implemented in this Thesis is a reproducible model that can be successfully used to test new feeding strategies aimed to control the post-weaning diarrhoea.