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Modulation of gut health in monogastric animals through nutritional additives

Luca Lo Verso

Tutor:

Prof. Valentino Bontempo

Index

CHAPTER 1

Foreword

1. Foreword

Formulating diet for its effects on gut health is becoming a reality in the monogastric animal industries. This is because maintenance or enhancement of gut health is essential for the welfare and productivity of piglets and poultry when antibiotics are not allowed in feed: hence, producing animals without using antibiotic growth promoters represents a challenge. Disease problems often are elevated and general performance is compromised on farms practicing non-medicated production. That is true in particular in poultry industry, close to and shortly after hatch (when animals selected for an intensive growth rate can increase their bodyweight by 25% overnight), and in the pig industry, first of all during the immediate post-weaning period, that is critical in a piglet's life and is accompanied by nutritional, social and environmental stress: in fact, at weaning the diet is changed from a highly digestible and liquid diet (the milk) to a solid, more complex and less digestible diet, and post-weaning gut disorders cause important economic losses (Lallès et al., 2009).

Nowadays, these problems are linked to the astonishing performances that are requested to modern farm animals, coming from an intensive selection for growth rate, a meticulous attention to health and husbandry, and the advances in feed formulation, matching the nutrient contents of the feed with the nutrient requirements of the animals. As the growth period is progressively shortened and feed efficiency continuously improved, the health care and nutrition of the animals are becoming more demanding.

In this context and, most of all, with increasing concerns about antibiotic resistance and the ban on sub-therapeutic antibiotic usage in Europe, there is increasing interest in finding alternatives to antibiotics for farm production. Consequently, the need of more economic and safety substances as replacers of antibiotic growth promoters for livestock animals as probiotics, prebiotics, plant extract and other feed additives of natural origin, focused the attention of the research of the last years; however, questions, such as the primary mode of action, the metabolic pathways and optimal dosage of the additives, are still unanswered.

Therefore, the aims of the following trials were to study the effects of probiotics, prebiotics and natural substances on health and performance of poultry and post-weaned piglets.

1.1 The gastrointestinal environment

The gastrointestinal tract of a pig is a complex environment, with an enormous surface continuously exposed to a myriad of antigens, toxic compounds and bacteria, and thus is more susceptible to inflammatory response. A continuous and fine interaction between the enterocytes, microflora and gut immune system is fundamental to maintain correct cell functioning and to protect the cells against aggression from the external environment (Uzzau and Fasano, 2000; Xavier and Podolsky, 2000). Dysfunction of any component of this highly integrated mucosal system may lead to a disruption in communication and result in pathological inflammation (Roselli et al., 2005).

1.1.1. The intestinal epithelium

The intestinal epithelium constitutes the major barrier that separates the external from the internal environment and represents the first line of defense against pathogens and dangerous environmental agents (Roselli et al., 2005). Here, the tight junctions play an important role: they encircle the cells at the apical end of the lateral membrane and are composed of an array of proteins, including the occludin (an integral membrane protein), and the zonula occludens proteins (cytosolic peripheral membrane proteins). These proteins are in close apposition to the actin and myosin ring, in a dynamic adaptation to a variety of developmental, physiological and pathological circumstances.
The tight junctions have two functions, the barrier function and the fence function. The barrier function regulates the passage of ions, water, and various macromolecules through paracellular spaces. On the other hand, the fence function maintains cell polarity. In other words, tight junctions work as a fence to prevent intermixing of molecules in the apical membrane with those in the lateral membrane (Sawada et al., 2003).
Because of this functions, when tight junctions of epithelial cells that cover gastrointestinal tract become disordered, diarrhea occurs (Sawada et al., 2003).
Effects of cytokines and growth factors that serve as extracellular stimuli on tight junctions are listed in Table 1.1: transforming growth factor-beta (TGF-β) and II-10 prevent cytokine-induced decrease in the barrier function, although many factors cause the barrier function to deteriorate. Tumor necrosis factor-alpha (TNF-α) and interferon-γ downregulate occludin expression at the transcriptional level. Other factors may regulate tight-junction barrier function via various signal transduction systems such as the mitogen-activated protein kinase (MAPK) pathway (Sawada et al., 2003).

Table 1.1 - Cytokines, growth factors, and tight-junction function (Sawada et al., 2003)

Decrease the barrier function:	IFN-γ, TNF-α, HGF, TGF-α, IGF-I and IGF-II, VEGF, IL-1, IL-4, IL-13
Increase or protect the barrier function:	EGF, TGF-β, GDNF, neurturin, IL-10, IL-17

Gut epithelial cells are recognized to play an important role in innate immunity, forming a highly specialized physical and functional barrier to dietary and microbial antigens. These cells respond directly to colonizing bacteria using specific cell-surface pattern recognition receptors to detect and respond to the presence of bacteria and specific bacterial moeities. A number of diverse receptor systems are expressed on epithelial cell surfaces that recognize bacteria and communicate signals to underlying lymphoid cell populations. These receptor systems comprise glycan receptors, which recognize fimbrial lectins found on many commensal and pathogenic strains of bacteria and viruses, and toll-like receptors that recognize microbial molecular patterns (Stokes et al., 2004).
The mucosal barrier can be destroyed in some diseases and by some pathogens, allowing the indiscriminate passage of luminal antigens across the epithelial junctions. Because of that, some defense mechanisms are present in the gut environment: the mucus layer, composed of glycoconjugates and intestinal mucins: it is localized on the cellular surface of the intestinal epithelium and creates a physical barrier, greatly contributing to the health of the gut through lubrication, physico-chemical protection and prevention of bacterial adhesion (Forstner and Forstner, 1994); proteins produced by mucosal intestinal cells, such as defensins: upon microbial invasion, they are released quickly by proteolytic processing from precursor peptides antibiotics, and they have various activities: they have a broad-spectrum activity against various bacteria, fungi, and enveloped viruses, are chemotactic for monocytes, T lymphocytes and dendritic cells, inhibit the binding of ACTH to its receptors, suppress the activation of the classical pathway of complement, induce histamine release from mast cells and promote the binding of lipoprotein to the vascular matrix (Zhang et al., 2000).

1.1.2. The mucosal immunity

The intestinal mucosa is provided with one of the largest immunological organs of the body, able to remove pathogens, eliminate infected cells and develop an immunological memory to induce a more rapid response against a subsequent exposure to the same antigens (Roselli et al., 2005). The gut-associated lymphoid tissue (GALT) comprises follicles or groups of them defined as Peyer's patches, surrounded by specialized M cells, responsible for the transport in the patches of

antigens and bacteria coming from the intestinal lumen (Pabst et al., 1988; Stokes et al., 2004).
The GALT also includes diffuse lymphoid tissue in the lamina propria, intraepithelial lymphocytes and mesenteric lymph nodes. After antigen presentation by antigen presenting cells, lymphocytes leave the Peyer's patches (inductive sites of the intestinal mucosa), and migrate to effector sites, such as the spleen, lungs, respiratory and urogenital tracts. At intestinal sites, lymphocytes are recruited and spread in the lamina propria to produce antibodies (mainly IgA) or migrate into the epithelium.
The intestinal immune system must protect the mucosa against pathogens and noxious substances, but has to be able to avoid hypersensitivity reactions against food proteins, normal microflora and innocuous macromolecules present in the intestine.

1.1.3. Gut microflora

The intestinal microflora helps the host to fight the colonization of pathogenic bacteria and to protect against dangerous substances arriving in the colon.
It contains high numbers of various species of bacteria involved in the process of digestion: the total number of microbial cells within the gut of single stomached animals exceeds that of the host cells by at least one order of magnitude (Savage, 1977). Microbial composition is determined by mutual interactions between the host and the microorganisms, and also among different microorganisms. These factors are designated as "autogenic" (Fuller et al., 1978; Budiño et al., 2005). On the other hand, pH in the stomach, digestive enzymes, intestinal peristalsis, nutrients and immunity of the host are termed "allogenic" factors (Budiño et al., 2005; Roselli et al., 2005).
The colon contents support at least 400 different species, with numbers as high as 10^{10} and 10^{11} culturable bacteria/g digesta (Savage, 1977). In pigs, the majority of the large intestinal microbiota are obligate anaerobes, though some aerobic and facultative micro-organisms also exist (Varel and Yen, 1997). Ducluzeau (1983) reported that numbers of microorganisms can reach values of between 10^8 and 10^9 CFU/g of feces already 10 to 12 h after the birth of piglets and that their numbers stabilize within 24 to 48 hours after birth. However, the composition of microflora is not definitive. It develops gradually and numerous changes occur during weaning (Mikkelsen, 2003; Roselli et al., 2005).
The embryo intestine is sterile, and the gastrointestinal colonization starts after delivery: in one year, the microflora becomes similar to the adult one. Hence, the intestinal microbiota takes some time before developing a stable community: in fact, colonization is a complex process of natural selection and ecological

succession. It depends on various factors, some of which are of host origin, such as the genome and physiology of the animal, while others are of microbial origin, such as bacterial interactions (Bauer et al., 2006). During the first few weeks of life, microbial succession in the gut of monogastric animals is remarkably similar: after birth, the germfree gastrointestinal tract is rapidly colonized by anaerobic and facultative anaerobic bacteria.

The highest number of microorganisms is found in the caudal part of the intestines (Table 1.2), where around 500 different species of microorganisms have been described and identified (Budiño et al., 2005). In a well-balanced microbial environment, members of the following genera prevail: *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Peptostreptococcus*, *Enterobacter*, *Bacteroides*, *Porphyromona*, while the numbers of coliform bacteria *E. coli* and *Clostridium* sp. are lower (Fuller et al., 1978; Maxvell et al., 2004; Stokes et al., 2004; Budiño et al., 2005).

The predominance of beneficial species of microorganisms over pathogens is essential for stability of the immune system of the intestines and consequently of the entire body (Mikkelsen et al., 2003). In particular, the populations of *Lactobacilli* establish early in the intestine, and, although succession does occur throughout the pig's lifetime, they remain a predominant member of the intestinal microbiota (Bauer et al., 2006). On the other hand, fecal *Bifidobacteria* seems to be numerically lower (Mikkelsen et al., 2003; Konstantinov et al., 2004a).

Anaerobic conditions, favourable temperature, pH and slow passage of the digesta are the preconditions for the presence of large numbers of bacteria in the large intestine and caecum, up to 10^{10} CFU/g. In the caecum Gram-negative bacteria predominate, whilst these are outnumbered by Gram-positive bacteria in the colon (Mikkelsen et al., 2003).

Table 1.2 - Comparison of microflora in the stomach, small and large intestines (CFU log10/g chime; Vondruskova et al., 2010)

Microrganisms	**Microflora in the stomach**	**Microflora in the small intestine**		**Microflora in the large intestine**	
		Proximal	**Caudal**	**Caecum**	**Colon**
Total count	5.5-9.5	5.5-8.5	5.5-9.5	8.5-9.5	8.0-10.0
Lactobacillus	5.0-9.0	5.5-8.5	6.0-9.5	8.0-9.5	7.5-9.5
Streptococcus	4.0-7.0	4.0-6.5	5.0-7.5	7.5	6.0-8.0
Bifidobacterium	4.5-6.5	4.0-5.5	5.5-7.5	5.0-8.0	5.5-8.5

Intestinal microorganisms participate in various physiological functions, by which they influence their hosts. Enteric pathogens may cause several damages to intestinal cells, including interference in the epithelial cell signalling that

controls both the transcellular and paracellular secretion pathways; consequently, protection against pathogenic and conditionally pathogenic microorganisms in the form of colonization resistance is most important (Roselli et al., 2005). Natural intestinal microflora adhere to intestinal mucosa and inhibit colonization by pathogens. This inhibition effect is caused by competition for nutrients and binding sites, bacteriocin production, lactobacilli fermentations and short chain fatty acid production (Teitelbaum and Walker, 2002; Roselli et al., 2005). Subsequently, immunological memory is created and intestinal immunity develops (Stokes et al., 2004).

1.1.4 Physiology, microbiology and immunology of the gastrointestinal tract at weaning

Physiology. The intestines display various functions including absorption of nutrients, absorption and secretion of electrolytes (and water), secretion of mucin and immunoglobulins and selective barrier protection against harmful antigens and pathogens (Lallès et al., 2004). Many changes in intestinal physiology occur during the 2 weeks post weaning (Boudry et al. 2004), related to changes in intestinal tissue (villus and crypt architecture) and depressed activities of many brush-border digestive enzymes, that have been well documented, in particular in the presence of pathogens, such as *Escherichia coli* and rotaviruses (Pluske et al., 1997).
Transient increases in net ion transport in the jejunum and colon and in glucose absorption capacity in the jejunum and decreased jejunal electric resistance have been documented in piglets fasted for 2 d after weaning (Boudry et al. 2004). Pre-weaning values are usually observed again at 5 d after weaning. However, long-lasting changes are also recorded up to 2 weeks after weaning. Jejunal glucose absorption and secretory responses to secretagogues decrease while ileal transmucosal electric resistance increases on day 5 post weaning and then stabilize. Permeability to macromolecules across the jejunum decreases on day 2 post weaning and remains low thereafter. Thus, weaning induces transient acute changes probably related to post-weaning fasting, followed by a period of intestinal maturation corresponding to voluntary feed intake resumption (Lallès et al., 2007).

Microbiology. As previously described, the gut is sterile at birth and is then colonised by microbes from the mother and the environment, starting with lactic acid bacteria, *Enterobacteria* and *Streptococci*. After the introduction of solid feed obligate anaerobes increase in number and diversity until an adult-type pattern is achieved (Inoue et al. 2005; Konstantinov et al. 2006*a*).

The commensal microbiota salvage energy from otherwise indigestible carbohydrates and protect the host from pathogens by forming a front line of mucosal defense (Zoetendal et al. 2004).
In contrast to adults the neonatal and weaning piglet is highly susceptible to enteric diseases: in the immediate post-weaning period the balance between the development of so-called healthy commensal microbiota and the establishment of a bacterial intestinal disease can be easily tipped towards disease expression. Piglets weaned within a 'production' environment experience major changes in intestinal microbiota composition that are influenced by diet, environmental factors and the host (Konstantinov et al. 2004*b*). In a short period of time the intestinal microbiota must ultimately develop from a simple unstable community into a complex and stable community, thus generating a tight 'colonization resistance' or 'competitive exclusion'.
Microflora composition can transiently change in response to various factors such as diet, health conditions and the environment: in particular, at weaning, which generally occurs early, the transition from milk to a solid diet leads to dramatic changes in the composition of the microbial population (Bauer et al., 2006). In this context, pathogenic strains of E. coli are the most frequent causes of post-weaning diarrhea in piglets, even though avirulent strains of the same bacteria are a common constituent of intestinal microflora. (Melin et al., 2004).
Hence, it is clear that microbial fermentation within the gastrointestinal tract is very important for the pig (Williams et al. 2001). The main products of fermentation include volatile fatty acids (VFA), which are known to play an important role in water (and Na^+) absorption, pH control and the inhibition of pathogens. In relation to host gut health, fermentation (i.e. microbial activity) is also important for gut motility, improvement of energy yield, vitamin production and the stimulation of gut immunity. It is also involved in the prevention of diarrhea and defense against pathogens (Lallès et al., 2007).

Immunology. At birth, the piglet is profoundly immunodeficient and highly dependent upon a supply of both specific and non-specific immune factors present in maternal colostrum and milk for immune protection, development and survival (Stokes et al., 2004). Directly after birth the piglet takes up macromolecules from the intestinal lumen in a non-selective way; a rapid "closure" of the gut for the macromolecular uptake occurs within 24 and 48 h after birth (Leece, 1973). Among them, an important role is played by immunoglobulins: IgG from the sow's colostrum is absorbed by the newborn via enterocytes; lymphocytes migrating into the lactating mammary gland provide the immunological information necessary for the production of secretory IgA that is released into the sow's milk for maintenance of humoral immunity in the offspring (Leece et al., 1973).

The functional immaturity of the neonatal cellular and secretory immune systems is such that newborn pigs are only able to generate limited T and B cell responses when challenged with pathogens, thus contributing to their immuno-compromised state (Butler et al., 2002).

Clearly, development of immunocompetence is an absolute requirement for optimum growth and performance. However, in the context of exposure to a wide range of antigens associated with pathogens and with commensal bacteria and food, a definition of immunocompetence must consider the ability to mount appropriate responses to antigens. This will include the ability to generate tolerance to food and commensal bacterial antigens as well active immune responses to pathogens (Bailey et al., 1998).

The cells and structures involved in mucosal immune responses are initially absent at birth, and preliminary studies have indicated that they populate the intestine of the young pig in a highly-programmed sequence (Bailey et al., 2005); four phases to this process have been identified (Stokes et al., 2004):

I. The newborn piglet has very few lymphocytes in its intestinal epithelium or lamina propria. Clusters of lymphocytes are present in the mucosa, in the areas that will subsequently develop into Peyer's patches (Barman et al., 1997), but these clusters have no clear immunological structure.

II. In the first two weeks of life the intestine rapidly becomes colonized with lymphoid cells. The Peyer's patches begin to organize during this period, reaching a relatively "adult architecture" by 10-15 days.

III. In piglets 2-4 weeks old the intestinal mucosa becomes colonized by CD4+ T cells, primarily in the lamina propria. CD8+ cells are still largely absent. Small numbers of B cells appear, preferentially expressing IgM.

IV. From the age of five weeks onwards, CD8+ cells begin to appear in the intestinal epithelium and around the epithelial basement membrane. In the crypt areas many IgA+ B cells are appearing. By 7 weeks the architecture of the intestine is comparable to that of the mature animal.

These phenotypic studies strongly suggest that the mucosal immune system remains relatively immature throughout the 'normal commercial weaning' period. The young piglet is capable of active immune responses to live virus and to dietary components by 3 weeks old, but quantitatively and qualitatively these responses differ markedly from that in older animals (Bailey et al. 2004). For example, whereas injecting 9-week-old piglets with soya results in a vigorous IgG1 and IgG2 response, injection at 3 weeks stimulates only a small IgG1

response (Bailey et al., 2004). Early weaning at 3 weeks of age is associated with a transient reduction in the ability of intraepithelial lymphocytes to respond to mitogens and splenic T-cells to secrete IL-2 (Bailey et al. 2005). Furthermore, tolerance to fed proteins introduced at weaning is not fully achieved until 8 weeks of age (Miller et al. 1994).
The first hypothesis put forward to explain intestinal damage shortly after weaning is adverse immune responses to dietary antigens (Dréau and Lallès, 1999). A second hypothesis is that the lack of intestinal stimulation as a result of post-weaning anorexia is a primary factor in intestinal inflammation, with responses to dietary antigens being secondary (McCracken et al. 1999). Indeed, inflammatory cytokine gene expression is transiently up regulated soon after weaning (Pié et al. 2004).

1.2. Producing swine without antibiotics

Weaning is a critical period in a piglet's life: a transient anorexia, as usually observed, leads to gut dysfunction, increased sensitivity to enteric infections and diarrhea. The most consistent patho-physiological changes effect the anatomy and function of the small intestine (Pluske et al., 1997), and these include a 20-30% reduction in mucosal weight associated with villous atrophy (Lallès et al., 2004). Hence, intestinal barrier function is compromised, resulting in increased secretion of electrolytes and water and increased permeability to potentially toxic substances (Lallès et al., 2004). In this context, Post-Weaning Diarrhea (PWD) is one of the most important consequence and one of the most frequent causes of heavy economic losses in pig herds (Vondruskova et al., 2010). It's considered a multifactorial disease and can be caused by a number of causative agents. In fact, early-weaned piglets are exposed to several stress factors that may cause diarrhea, with nutrition, etiology and indoor environment of housing being particularly implicated (Laine et al., 2008). Non infectious stress factors which are involved in the development of gastroenteric disorders are (Laine et al., 2008; Vondruskova et al., 2010):

- Age of piglets when they are weaned from their dam
- Low weaning weight
- Sudden change of feed from sow milk that provides piglets with immunoglobulins
- Irregular feed intake during the first weeks after weaning
- Low number of meals
- Feed structure
- Moderate cold stress/draught

- Animal hygiene and housing conditions
- Inadequate feeder space per piglet in the pen
- Vaccinating gestating sows against PRRS (porcine reproductive and respiratory syndrome)

Besides these, numerous changes in the early-weaned piglet body can initiate diarrhea, such as morphological and functional alterations of the small intestine, changes in intestinal colonization with predominance of pathogens and weakening of the immune system (Vondruskova et al., 2010).
Among infectious agents, the major bacteria which cause diarrhea are *Escherichia coli* and members of the genera *Clostridium*, *Lawsonia* and *Brachyspira*. Regarding viral agents, rotaviruses (Thomsson et al., 2008), coronaviruses (Song et al., 2006), transmissive gastroenteritis virus (Melin et al., 2004; Song et al., 2006; Thomsson et al., 2008) and others have been most frequently diagnosed. Particularly, during the first two weeks after weaning, pathogenic *Escherichia coli* plays a significant role in the etiology of PWD (Madec et al., 2000), although infection with pathogenic *E. coli* does not unequivocally lead to the development of diarrhea in weaned pigs (Melin et al., 2000; Madec et al., 2000).
Consequently, gastrointestinal diseases of pigs are economically important for pig production worldwide; regarding the fact that weaning greatly affects general health condition of piglets, it is necessary to stimulate the indigenous intestinal microflora and keep it well-balanced, because of its protection to animals against invasion by pathogenic microorganisms. Before 2006, health strategies widely used Antibiotic Growth Promoters (AGP) to reduce enteric infections and the occurrence of pathogens able to adhere to intestinal mucosa (Budiño et al., 2005): these were added to feed for piglets from birth to weaning with the aim of improving the composition of intestinal microflora in piglets and thus ameliorate the potential consequences of PWD (Sørensen et al., 2009). But the increased use of antibiotics has given rise to a fear of the development of resistant pathogenic (Budiño et al., 2005) and residual contamination of the food chain with antibiotics (Chen et al., 2005; Roselli et al., 2005). This has led to the adoption of safety measures and a gradual withdrawal of antibiotic promoters from pig diets. In 2006, the use of antibiotics as growth promoters was definitely forbidden in the EU (Vondruskova et al., 2010).
Because of the difficulties associated with producing pigs without antibiotics, intensive research has focused on the development of alternative strategies with the aim of maintenance of animal health and performance. Among them, an important role is played by nutritional strategies and alternative dietary supplements. Many different approaches have been proposed, but the one thing these approaches have in common is that they all aim at improving the pigs' ability to prevent pathogenic bacteria from colonizing in the intestinal system.

This can be accomplished via an improved immunological response to pathogens or via mechanisms that prevent the pathogens from adhering to intestinal tissue, and thus, reduce the damaging effects of the pathogens (Stein, 2007).

1.2.1. Nutritional strategies: management of the diet

In the past, diets for newly-weaned pigs have largely been formulated on the basis of overcoming the limitations or immaturity in digestive tract function (e.g., pancreatic and brush-border enzymes) in order to maximize growth of the whole animal, with due recognition to the cost of raw materials and processing. New researches (e.g. Wu et al., 2007), suggest that given the considerable advances made in the understanding of intestinal nutrient utilization and metabolism, an alternate or even complimentary goal in nutrition might be to formulate young pig diets with the specific task of optimizing the growth, function and health of the gut (de Lange et al., 2010).

1.2.1.1. Liquid feeding

Liquid feeding generally results in fewer intestinal upsets than dry feeding because liquid diets reduce gastric pH, which results in reduced or inhibited growth of pathogens in the intestinal tract; liquid feeding also prevents the atrophy of intestinal villi that often is observed during the post-weaning period in pigs provided a dry diet (Gu et al., 2002). With a healthier and more intact villi-structure in the small intestine, it is likely that pigs are less susceptible to *E.coli* infections, which in turn can explain why liquid feeding has a positive influence on overall pig health and pig performance (Stein and Kil, 2006). Fermentation of the liquid feed prior to feeding has been reported to reduce the concentration of pathogens in the diet and in the intestinal tract of the pigs; other benefits of fermented liquid feeding include improved protein digestion due to lower stomach pH and probiotic effects resulting from the supply of lactic acid bacteria via the fermented feed (Stein and Kil, 2006). Thus, fermented liquid feeding could have characteristics similar to probiotics and organic acidifiers.

1.2.1.2. Crude protein

The single most important nutritional factor for reducing scouring in pigs fed diets without antibiotic growth promoters is to reduce the dietary crude protein concentration (Stein, 2007). In fact, undigested crude protein entering the large intestine increases microbial fermentation in the hindgut and provide substrates for several potential pathogens, which are predominantly protein-fermenters and

would therefore grow more prolifically when proteins are freely available. For example, an imbalance between fermentable carbohydrates and potentially fermentable protein (i.e. undigested nitrogen entering the large intestine) has been proposed as a mitigating factor in the aetiology of PWD of newly-weaned pigs (Kim et al., 2008). It is also likely that the increased metabolic demand for deaminating excess aminoacids and excreting the extra nitrogen compromises the pigs' immune system. In addition, undigested feed protein may accelerate the production of toxic nitrogenous compounds such as ammonia which is harmful to intestinal health (Nyachoti et al., 2006); metabolism of proteinaceous materials by the microbiota in the large intestine may also increase levels of potentially toxic substances such as amines, indoles, phenols and branched-chain fatty acids, which have been implicated in the pathogenesis of PWD. These data are indicative of reduced protein fermentation by the microbiota, and indicate that feeding lower protein diets could be used to reduce PWD in piglets fed antibiotic-free diets.

For most groups of pigs, it is possible to reduce the dietary concentration of crude protein by 3-4% without compromising the pig's requirement for amino acids (Stein, 2007). Reynoso et al. (2004) demonstrated that by reducing the dietary crude protein concentration from 21.2% to 18.4%, a linear reduction in diarrhea was observed, but growth performance was not affected. The low protein diets that were used in this experiment were fortified with crystalline aminoacids, which is likely the reason performance could be maintained. Likewise, le Bellego and Noblet (2002) reduced dietary crude protein concentration from 22.4% to 16.9% with the addition of crystalline aminoacids without reducing pig performance.

It is usually possible to reduce the dietary concentration of crude protein in diets fed to weanling pigs to approximately 18% by including crystalline aminoacids (lysine, methionine, threonine, and tryptophan) in the diets without undersupplying any indispensable aminoacids. A study by Heo et al. (2008) was conducted to test the hypotheses that feeding a diet low in protein but supplemented with essential crystalline aminoacids to maintain an ideal pattern in the diet would reduce indices of protein fermentation in the gastrointestinal tract and reduce the incidence of PWD and not compromise growth to approximately 15 weeks of age: in this study the dietary protein level was reduced from 243 g/kg to 173 g/kg while maintaining diet digestible energy content. Reducing the dietary protein decreased the levels of fecal ammonia nitrogen, indicative of decreased protein fermentation; commensurately, this strategy decreased the number of antibiotic treatments, improved fecal consistency with and did not impact growth performance.

However, sometimes it may be necessary to formulate diets containing less than 18% crude protein during the immediate post-weaning period to avoid scouring

and intestinal malfunctions. In such diets, it may not be possible to include the indispensable aminoacids at recommended concentrations. Therefore, growth performance will be compromised (Stein, 2007). Stein and Kil (2006) indicated that pigs fed diets containing approximately 20% less aminoacids than recommended will have a reduced daily gain of 40-60 g per day (Table 1.3).

Table 1.3 - Effects of feeding low protein diets followed by either normal or high protein diets to weanling pigs (Stein and Kil, 2006).

Item	Days	Treatment			
		High/low	**High/high**	**Low/low**	**Low/high**
Crude protein/lysine (%)	0-14	20.8/1.35	20.8/1.35	15.7/1.15	15.7/1.15
	14-35	17.5/1.15	19.3/1.34	17.5/1.15	19.3/1.34
Average daily gain (g)	0-14	171^{xy}	180^{x}	148^{xy}	129^{y}
	14-35	516	529	499	535
	0-35	377	389	359	373
Average daily feed intake (g)	0-14	249	257	228	237
	14-35	790^{x}	756^{xy}	778^{xy}	735^{y}
	0-35	574	556	558	535
Average gain:feed ratio (g/g)	0-14	0.68	0.70	0.65	0.55
	14-35	0.65^{x}	0.70^{y}	0.64^{x}	0.73^{z}
	0-35	0.66^{x}	0.70^{y}	0.65^{x}	0.70^{y}

Values are means of six pens per treatment with 5 pigs per pen.
^{xyz} Values lacking a common superscript letter are different ($P < 0.05$).

If such diets are fed during the initial two weeks post weaning, then a total of 560-840 g of gain is sacrificed. However, if the protein concentration in the diet is returned to normal levels from day 15 post-weaning, then the pigs on the low-protein diets will compensate and by day 35 post weaning, there is no difference in the body weight of pigs regardless of the protein concentration they received during the initial 2 weeks post weaning.

1.2.1.3. Functional proteins

Certain proteins may have functions other than strictly providing amino acids and several proteins are believed to improve the immune status of weanling pigs. For example, it has been shown that the dietary inclusion of spray dried plasma may improve the immune status of weanling pigs (Bosi et al., 2004), which reduces the pigs' susceptibility to *E.coli* infections (Owusu-Asiedu et al., 2003). The immunoglobulins that are present in spray dried plasma are the functional units that provide the improved immunity (Pierce et al., 2005). Spray dried plasma also has been shown to down-regulate the inflammatory process in healthy pigs (Bosi et al., 2004), which in turn may contribute to increased feed intake and direction of nutrients towards gain of body weight.

If it is correct that it is the immunoglobulins in spray dried plasma that provide the improvement in pig immunity then it would be expected that other sources of immunoglobulins also can improve the immune status of pigs. The most abundant source of immunoglobulins is immunoglobulins from dairy cows that are present in the whey protein fraction of cows' milk (Stein, 2007): in fact, the cow transmits them and other physiologically important factors to the calf through milk, so it is reasonable to expect that milk would provide beneficial responses. Dried whey, the milk product most commonly used in diets
for young weaned pigs, contains several physiologically active proteins including immunoglobulins, lactoferrin, lactoperoxidase and lysozyme (Pettigrew, 2006). It has been assumed that significant bifidogenic activity may be associated with milk protein, either by direct stimulation of growth, or by antimicrobial effects: Liepke et al. (2002) showed that proteolytic fragments of major milk proteins are effective growth factors for bifidobacteria. Secretory immunoglobulin is a highly protective agent, which can prevent colonization and invasion by pathogens (Brandtzaeg, 2003).
Furthermore, egg proteins also contain immunoglobulins and other physiologically active agents, transmitted from the hen to her chicks through the egg, and the inclusion of dried whole eggs to diets fed to weanling pigs may also improve pig performance (Hong et al., 2004). Egg products, including dried whole eggs, are available as waste products from the food industry. If hens are immunized against certain pathogens, they will produce antibodies against these
pathogens and the antibodies will be present in the eggs from these hens. It is therefore possible to increase the concentrations of antibodies in eggs against certain pathogens and if these eggs are fed to weanling pigs, the pigs will gain an improvement in their immune status. It has been demonstrated that if eggs containing high concentrations of antibodies against *E.coli* are fed to weanling pigs, these pigs will experience fewer cases of *E.coli* associated diarrhea (Marquardt et al., 1999). There are, however, relatively large costs involved in the production of these eggs, and this practice is, therefore, not widely used in the industry.

1.2.1.4. Aminoacids

Several essential and non essential aminoacids are thought to play a role with regard to their metabolic, physiological, immunological and therapeutic effects on the gastrointestinal tract and on the whole organism (Kim et al., 2007). Apart from their role as building blocks for peptides and proteins (table 1.4), aminoacids can have bioactive properties. Particularly in certain disease conditions, specific aminoacids can promote health by improving tissue anabolism, by reducing stress and by modulating immunology. These aminoacids

can be supplemented to the diet in case of a relative deficiency during disease (de Lange et al., 2010).
Among aminoacids, glutamine and glutamate are important fuels for intestinal cells. They improve growth performance and feed efficiency post weaning and limit intestinal villus atrophy (Domeneghini et al. 2004, 2006): glutamine stimulates the division of enterocytes while decreasing apoptosis of enterocytes and lymphocytes; it also stimulates both innate and adaptative components of immunity, as shown by increased densities of macrophages and intra-epithelial lymphocytes. Alanine and glycine have been shown to stimulate the production of the so-called anti-secretory factor, improve growth performance and reduce the incidence of diarrhea: this anti-secretory factor provides protection against diarrheal diseases and intestinal inflammation, and it has been shown to be low immediately post weaning in pigs (Lallès et al., 2007). Arginine also prevents villus atrophy (Ewtushick et al. 2000). Finally, dietary supplementation with L-tryptophan can improve villus:crypt in the small intestine but it may depress feed intake and growth (Koopmans et al. 2006).

Table 1.4 - Nutritionally essential and non essential amino acids in monogastric animals (Kim et al., 2007)

Essential Aminoacids	**Non Essential Aminoacids**
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Cysteine
Lysine	Glutamate
Methionine	Glutamine
Phenylalanine	Glycine
Threonine	Proline
Tryptophan	Serine
Valine	Tyrosine

Amino acids may be substrates for the synthesis of many biologically active substances (including NO, polyamines, glutathione, nucleic acids, hormones, and neurotransmitters) that are essential to the life and productivity of animals (Table 1.5). Their abnormal metabolism negatively alters feed intake, disturbs whole body homeostasis, impairs animal growth and development, and may even cause death (Kim et al., 2007).

Table 1.5 - Important nitrogenous products of amino acid metabolism in animals (Kim et al., 2007).

Precursors	Products	Functions
Arginine	NO	Vasodilator; neurotransmitter, signaling molecule; angiogenesis; cell metabolism; apoptosis (programmed cell death); immune response
	Agmatine	Signaling molecule; inhibitor of NO synthase and ornithine decarboxylase; brain and renal functions
Cysteine	Taurine	Antioxidant; muscle contraction; bile acid conjugates; retinal function
Glutamate	γ-Aminobutyrate	Neurotransmitter; inhibitor of glutamatergic, serotonin and NEPN activities
Glutamine	Glucosamine	Glycoprotein and ganglioside formation; inhibitor of NO synthesis
	Ammonia	Renal regulation of acid-base balance; synthesis of carbamoylphosphate glutamate and glutamine
	Serine	One-carbon unit metabolism; ceramide and phosphatidylserin formation
Glycine	Heme	Hemoproteins (e.g., hemoglobin, myoglobin, catalase, and cytochrome C); reduction of carbon monoxide (CO, a signaling molecule)
	Histamine	Allergic reaction; vasodilator; gastric acid and central acetylcholine secretion
Histidine	Homocysteine	Oxidant; inhibitor of NO synthesis; risk factor for cardiovascular disease
Methionine	Betaine	Methylation of homocysteine to methionine; one-carbon unit metabolism
	Choline	Synthesis of betaine, acetylcholine (neurotransmitter and vasodilator) and phosphatidylcholine
	Cysteine	An important sulfur-containing amino acid; formation of disulfide bonds
	DCSAM	Methylation of proteins and DNA; polyamine synthesis; gene expression
	Tyrosine	A versatile aromatic amino acid containing a hydroxyl group
Phenylalanine	H_2O_2	Killing pathogens; intestinal integrity; a signaling molecule; immunity
Proline	P5C	Cellular redox state; DNA synthesis; cell proliferation; ornithine formation; bridging the urea cycle with Krebs cycle; gene expression; tumor growth
	Glicine	Antioxidant; bile acid conjugates; neurotransmitter; immunomodulator; one-carbon unit metabolism
Serine	Serotonine	Neurotransmitter; smooth muscle contraction; hemostasis; immunity
Tryptophan	N-acetylserotonin	Inhibitor of sepiapterin reductase and thus tetrahydrobiopterin synthesis
	Melatonin	Circadian and circannual rhythms; free radical scavenger; antioxidant
	Anthranilic acid	Inhibiting production of proinflammatory T-helper-1 cytokines; preventing autoimmune neuroinflammation; enhancing immunity
	Dopamine	Neurotransmitter; apoptosis; lymphatic constriction; control of behavior
Tyrosine	EPN - NEPN	Neurotransmitters; smooth muscle contraction; cAMP production; glycogen and energy metabolism
	Melanin	Dark-color pigment; free radical scavenger; chelator of metals
	T3 – T4	Gene expression; tissue differentiation & development; cell metabolism
Arg and Met	Polyamines	Gene expression; DNA and protein synthesis; ion channel function; apoptosis; signal transduction; antioxidants; cell function, proliferation and differentiation; spermatogenesis; viability of sperm cells
Gln, Asp and Gly	Nucleic acids	Coding for genetic information; gene expression; cell cycle and function; protein and uric acid synthesis
Gln and Trp	NAD(P)	Coenzymes for oxidoreductases; substrate of poly(ADP-ribose) polymerase
Arg, Pro or Gln	Ornithine	Glutamate, glutamine and polyamine synthesis; mitochondrial integrity
Arg, Met and Gly	Creatine	Energy metabolism in muscle and nerve; antioxidant; antiviral; antitumor
Cys, Glu and Gly	Glutathione	Free radical scavenger; antioxidant; formation of leukotrienes, mercapturate, glutathionylspermidine, glutathione-NO adduct and glutathionylproteins; signal transduction; gene expression; apoptosis; spermatogenesis; sperm maturation; cellular redox state; immunity
Gln, Glu and Pro	Citrulline	Free radical scavenger; arginine synthesis; urea cycle
Lys, Met and Ser	Carnitine	Transport of long-chain fatty acids into mitochondria for oxidation; storage of energy as acetylcarnitine

1.2.1.5. Fibre

The roles of dietary fibre in post-weaning diets for pigs and its relationship to post-weaning disturbances such as diarrhea have been of great interest for many years. Unlike nutrients and dietary components (e.g. energy) where there are firm dietary recommendations, there are no such recommendations for dietary fibre, with the dietary level used being a consequence of the ingredients used to make the diet at least-cost. Partly because of this, there has been considerable interest in the use of dietary fibre in ameliorating post-weaning disturbances of the gastrointestinal tract (de Lange et al., 2010). Reports dating back to the 1960s and 1970s showed that the addition of insoluble fibre sources such as the husks from cereals, e.g., barley, could reduce the excretion of haemolytic *E. coli* and the incidence of diarrhea after weaning (de Lange et al., 2010). Nevertheless and as evidenced by research in Australia and Spain using cooked/uncooked rice fed to newly-weaned pigs (e.g., Hopwood et al., 2005; Vicente et al., 2008), feeding diets lower in dietary fibre paradoxically may also have benefits in terms of reducing PWD and (or) enhancing growth in the post-weaning period. However, the question of the correct amount of dietary fibre to include in a weaner diet is still complex, as it most likely depends on what the nutritionist/producer is trying to achieve, and may be better answered by investigating dietary ingredients that deliver an appropriate combination of carbohydrates (e.g. differing in digestibility and fermentability), as well as proteins, to the gut to address the issue.

1.2.2. Feed additives

Feed additives are non-nutritive products used in swine diets to improve production efficiency and performance. If chosen carefully and used properly, they can be effective and can help increase the profitability of pig production. A large number of feed additives have been evaluated that are aimed at enhancing the pigs' immune response, reducing pathogen load in the pig's gut, stimulating establishment of beneficial gut microbes and digestive function. In this section some feed additives are discussed.

1.2.2.1 Acidifiers

Acidifiers are compounds that have acidic properties: they may be organic or inorganic acids. Organic acids play an important role in the preservation of foods, as they are very effective inhibitors of microbial growth (Brul and Coote, 1999). While some foods are naturally acidic, sometimes acids are directly added to the food or produced inside it by organisms such as lactic acid bacteria. Organic acids that have shown positive effects on growth performance in

weaned pigs include citric, formic, fumaric, lactic and propionic acids: adding these acids to the diet of pigs has been reported to be helpful in overcoming problems of the post-weaning period (Partanen and Mroz, 1999). Inorganic acids are usually less costly and are often used in commercially available acidifiers as a combination with organic acids: in fact, the response to mixed acids is generally better than to single acids, apparently due to dissociation properties of these acids at various locations in the pig's digestive tract.
Acidifiers can be administered via feeds or drinking water. The solid acidifiers are easier to handle, whereas the liquid forms may be volatile (up to 20%) during spraying, and their disadvantage could be the corrosiveness and unpleasant odour (Mroz, 2005).
Among supplemental acids and their salts for pigs, a particular practical interest is focused on those listed in Table 1.6.

Table 1.6 - Some physicochemical properties of most common acids and salts (Mroz, 2005, modified).

Organic acids	**Organic salts**
1. Formic	10. Ca-formate
2. Acetic	11. Ca-lactate
3. Propionic	12. Ca-propionate
4. Butyric	13. K-diformate
5. Lactic	14. Ca-butyrate
6. Sorbic	15. Mg-citrate
7. Fumaric	16. Na-lactate
8. Malic	
9. Citric	

The mode of action of particular organic acids and salts is not uniform, although a consensus seems to be achieved on the following items (Mroz, 2005):

- Undissociated forms diffuse across cell membranes of pathogens, destroying their cytoplasm or inhibiting growth (inactivation of bacterial decarboxylases and catalases);
- Intestinal dissociation liberates H+ ions serving as a pH barrier against pathogen colonization on the brush border;
- Reduced gastric pH in complementariness to endogenous HCl;
- Gastric hydrolysis liberates H+ ions activating pepsinogen and inhibiting bacterial growth (bactericidal/bacteriostatic effects);
- Energetic substrate or modulator/stimuli for mucosal development, epithelial cell growth and greater absorptive capacity;
- Precursors for synthesis of non-essential amino acids, DNA and higher lipids required for intestinal growth;
- Increased blood flow and hypocholesterolemic effect.

The effectiveness of feeding acids to pigs will vary with the types and combinations of acid, the animal's state and feed characteristics, in particular the diet's buffering capacity. In a porcine intestinal organ culture model, it was established that coliform bacteria were most effectively killed by benzoic> fumaric>lactic>butyric>formic>propionic acid. With respect to *Salmonella typhimurium* the order was benzoic>sorbic>lactic>propionic>formic>acetic acid. Overall, benzoic acid is most effective, lactic acid is intermediate and both formic and propionic acids are least effective at killing both coliforms and *S. typhimurium* (Lallès et al., 2009).
In vivo trials with acidified diets in weaned piglets show various results: the inclusion of a combination of 1% lactic acid and 1% formic acid in the diet reduced gastric pH and concentration of lactic acid bacteria and *Enterobacteria* (Hansen et al., 2007). Lactic acid bacteria were also reduced by 0.5% benzoic acid (Guggenbuhl et al., 2007) and by the addition of 200 mEq/kg of a 1:1 formic:fumaric acid mixture, but not by a 1:1 formic:lactic acid mixture (Franco et al., 2005). Benzoic acid at 0.5% or 1.0% reduced the number of total aerobic, total anaerobic, lactic acid forming and Gram-negative bacteria in the stomach (Kluge et al., 2006). In another study benzoic acid at 0.5% improved growth and feed efficiency, but had no effect on reducing the number of culturable *Lactobacilli*, *Enterococci*, *Escherichia coli* and *Clostridium perfringens* from the ileum and caecum as measured with traditional culture methods (Torrallardona et al., 2007a).

1.2.2.2 Plant extracts

Plant extracts feed additive, also called phytogenic feed additives, are a diverse group of naturally growth ingredients derived from herbs, spices or other plants which could be supplemented in the diets to improve the production efficiency or maintain the health status of domestic animals. The bioactive plant extracts are essentially the secondary metabolites and they represent a wide range of mixtures of various compounds, such as terpens, phenols, glycosides, saccharide, aldehydes, esters and alcohols. Volatile or essential oils are the typical representative of plant extracts: they are distilled from non-woody parts of herbs, and contain mainly the actively aromatic and volatile ingredients of the plants (Bozin et al., 2006). The composition of the oils is highly variable within plants due to different environmental and climatic conditions (Table 1.7). Essential oils themselves make 1.5% to 4.5% of the plant (Bozin et al., 2006).

Table 1.7 - Main components of selected essential oils (%) reported in bibliography (reviewed by Lallès et al., 2009).

Essential oils	From	Carvacrol	Thymol	Eugenol	Cinnamaldehyde
Oregano oil	*Origanum vulgare*	61	14		
		76	no data		
		55	no data		
		80	2.5		
	Origanum floribundum	30	8		
		2	28		
	Origanum glandulosum	1	24		
		8	36		
	Origanum Dubium	70-71	0.1-0.3		
Thyme oil	*Thymus vulgaris*	6	48		
		no data	24		
	Thymus zygis	2	50		
	Thymus munbyanus	8	38		
	Thymus guyonii	4	11		
	Thymus pallescens	42	0.1		
	Thymus numidicus	7	15		
Clove oil	*Syzygium aromaticum*			85	
Cinnamon oil	*Cinnamon zeylanicum*			77	
	Cinnamon verum and *cassia*				85

Plant extracts used as feed additive differ from chemically synthetic ingredients or whole plants: they usually contain stable determined contents of main active ingredients and do not harm animal or people. Their use as feed additives is usually subject to restrictive regulations: in general, they are considered as products applied by the farmer to healthy animals for a nutritional purpose on a permanent basis (i.e., during the entire production period of the respective species and category), in contrast to veterinary drugs (applied under veterinarian control for a limited time period, partially associated with a waiting period). In the European Union, for example, feed additives need to demonstrate the identity and traceability of the entire commercial product, the efficacy of the claimed nutritional effects, including the absence of possible interactions with other feed additives, and the safety to the animal (e.g., tolerance), to the user (e.g., farmer, worker in feed mills), to the consumer of animal-derived products, and to the environment (Windisch et al., 2008).

Among essential oils, the most commonly used in diets fed to swine are garlic, oregano, thymol, and carvacrol. The benefits of plant extracts application in animals had been demonstrated as increasing feed intake, modulation of gastrointestinal and immune-stimulative function and enhancement of productivity.

In this section, the use of phytogenic compounds as feed additives in monogastric diets in terms of claimed antioxidative and antimicrobial actions,

beneficial effects on palatability and gut functions, and immunomodulatory or growth-promoting efficacy is discussed.

Impact on dietary palatability. Phytogenic feed additives are often claimed to improve the flavor and palatability of feed, thus enhancing production performance (Windisch et al., 2008). However, the number of studies having tested the specific effect of phytogenic products on palatability by applying a choice-feeding design is quite limited. They show dose-related depressions of palatability in pigs fed essential oils from fennel and caraway, as well as from the herbs thyme and oregano (Schöne et al., 2006). On the other hand, there are numerous reports on improved feed intake through phytogenic feed additives in swine. However, an increase in feed intake in swine is a common result of the use of growth-promoting feed additives, such as antibiotics, organic acids, and probiotics, and, in the first instance, it may be considered to reflect the higher consumption capacity of animals grown larger compared with untreated controls. Therefore, the assumption that herbs, spices, and their extracts improve the palatability of feed does not seem to be justified in general (Windisch et al., 2008).

Digestive capacity and gut modulation. A wide range of spices, herbs, and their extracts are known from medicine to exert beneficial actions within the digestive tract. Stimulation of digestive secretions (e.g., saliva), bile, and mucus, and enhanced enzyme activity are proposed to be a core mode of nutritional action (Platel and Srinivasan, 2004).

Digestion-stimulating and enzymatic activities enhancement or digestive tract development effects of plant extracts had been observed in broiler chickens: plant extract consisting of capsaicin, cinnamaldehyde and carvacrol at the rate of 100 mg/kg increased the lipase activity in pancreas and intestine wall in broiler chicks (Jamroz et al., 2003, 2005); furthermore, essential oils used as feed additives for broilers were shown to enhance the activities of trypsin and amylase (Jang et al., 2004). Phytogenic feed additives were also reported to stimulate intestinal secretion of mucus in broilers, an effect that was assumed to impair adhesion of pathogens and thus to contribute to stabilizing the microbial eubiosis in the gut of the animals (Jamroz et al., 2006). In swine, essential oil and capsaicin were reported to slow down the gastric empty rate (Manzanilla et al., 2004).

Another claim for plant extract on gut function is the modulation of gastrointestinal architecture. The intestinal morphology of animals ingested with plant extracts presented inconsistent changes. The villus height, crypt depth, ratio of villus height to crypt depth and villus surface area of different intestinal segments showed scattered responses of increased, unchanged or reduced transformation (Jamroz et al., 2006;. Manzanilla et al., 2006; Nofrarías et al.,

2006; Jin et al., 2008). The varied effect may be due to the different composition of diverse plant extracts which would exert efficacy with different mode of action on gut morphology. Thus, no definitive conclusion about the influence of plant extracts on gut function which would be plant extract specific.
Another aspect of plant extracts on gut function is to change gut microflora fermentation: modified volatile fatty acids (VFA) profile with increasing acetate, diminishing butyrate and valerate in cecum and colon were observed in piglets fed plant extract (Manzanilla et al., 2004). Changes in the ecological structure and metabolic activity of the microbial community (Castillo et al., 2006), decrease of VFA production in the cecum (Manzanilla et al., 2009) had been observed in piglets. Decreased or modified intestinal microbial metabolism was also found in chicken studies (Jamroz et al., 2003, 2005).
Finally, among plant extracts, saponins (e.g., from Yucca schidigera) are proposed to reduce intestinal ammonia formation, and thus aerial pollution of housing environment, which is considered an important health stress, especially for young animals (Francis et al., 2002).

Microbial modulation. The antimicrobial mechanisms of plant extracts are complicated and still not well definite, but it is clear that some essential oils have strong antimicrobial activity, particularly those containing phenolic structures, such as carvacrol and thymol: the capacity to modulate the microflora is attributed to their delocalized electrons and the presence of a hydroxyl group on the phenolic ring (Ultee et al., 2002). The oils initiate damage to the bacterial cell membrane, which compromises pH homeostasis and equilibrium of inorganic ions across the bacterial cell membrane (Lambert et al., 2001). This leads to the collapse of the proton motive force and depletion of the ATP pool in the microbe (Ultee et al., 2002).
Supplementation of plant extracts in diet of monogastric animals were expected to modulate the intestinal microflora to the pattern by favorably affecting the growth of beneficial bacteria and/or inhibiting the growth of pathogens. In fact, essential oils have also been shown to have a certain degree of selectivity: although essential oils extracted from *O. vulgare* and *T. vulgaris* showed activity against Gram-positive and Gram-negative bacteria (Table 1.8) and their strains multi-resistant to antibiotics (Bozin et al., 2006), Lin et al. (2000) reported more inhibition towards Gram negative *Salmonella* and *E. coli* than to Gram-positive *Listeria monocytogenes*; essential oils exhibited a high efficacy against pure cultures of *S. Typhimirium* DT104, *E. coli* O157:H7, and *E. coli* K88 with little inhibition towards *Lactobacillus* and *Bifidobacterium* in mediums that contained pig cecal digesta (Si et al., 2006a).

Table 1.8 - Bacterial species inhibited in *in vitro* studies when plant essential oils or pure oil components (from *O. vulgare* and *T. vulgaris*) were added to growth medium (reviewed by Lallès et al., 2009)

Bacterial species	**Essential oil source or pure component**
Acinetobacter baumanii	*Oregano vulgare, Syzygium aromaticum, Thymus vulgaris*
Acinetobacter calcoacetica	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Aeromonas hydrophila	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Aeromonas sobria	*O. vulgare, S. aromaticum, T. vulgaris*
Agrobacterium tumefaciens	Carvacrol
Alcaligenes faecalis	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Bacillus amyloliquefaciens	*O. vulgare, Thymbra sintensi*
Bacillus brevis	*O. vulgare, T. sintensi*
Bacillus cereus	*O. vulgare, T. sintensi,* carvacrol
Bacillus megaterium	*O. vulgare, T. sintensi*
Bacillus subtilis	*Cinnamonum zeylanicum, Eugenia caryophyllus, O. vulgare, T. sintensi, T. vulgare, S. aromaticum,* carvacrol, eugenol, thymol
Bacillus subtilis var. *niger*	*O. vulgare, T. sintensi*
Beneckea natriegens	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Bifidobacterium longum	Thymol
Bifidobacterium breve	Thymol
Brevibacterium linens	*O.vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Brocothris thermosphacta	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Citrobacter freundii	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Clostridium sporogenes	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Enterococcus faecalis	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Enterobacter aerogenes	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Erwinia carotovora	*O. vulgare, S. aromaticum, T. vulgaris*, carvacrol, eugenol
Escherichia coli (several strains)	*C. zeylanicum, E. caryophyllus, O. vulgare, S. aromaticum, Thymus mastichina, T. vulgaris, Thymus zygis,* carvacrol, cinnamon oil, clove oil, eugenol, thymol
Flavobacterium suaveolens	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Klebsiella pneumoniae	*C. zeylanicum, E. caryophyllus, O. vulgare, S. aromaticum, T. vulgaris*, carvacrol, eugenol
Lactobacillus acidophilus	Carvacrol, cinnamon oil, thymol
Lactobacillus plantarum	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol, thymol
Leuconostoc cremonis	*O. vulgare, S. aromaticum, T. vulgaris*
Listeria monocytogenes	*O. floribundum, O. glandulosum, Thymus guyonii, T. munbyanus, T. numidicus, T. pallescens*
Micrococcus flavus	*O. vulgare, T. vulgaris*
Micrococcus luteus	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Moraxella sp.	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Proteus mirabilis	Carvacrol
Proteus vulgaris	*C. zeylanicum, E. caryophyllus, O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Pseudomonas aeruginosa	*C. zeylanicum, E. caryophyllus, O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol, thymol
Pseudomonas talassi	*Carvacrol*
Rhizobium leguminosarum	*O. vulgare,* carvacrol, thymol
Salmonella choleraesuis	*O. vulgare, T. Mastichina, T. zygis*
Salmonella enteritidis	*O. vulgare, T. mastichina, T. vulgaris, T. zygis,* carvacrol
Salmonella essen	*O. vulgare, T. mastichina, T. zygis*
Salmonella pullorum	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Salmonella typhi	*O. vulgare, T. vulgaris*
Salmonella typhimurium	*O. vulgare, S. aromaticum, T. mastichina, T. vulgaris, T. zygis,* carvacrol, cinnamaldehyde, cinnamon oil, clove oil, eugenol, thymol
Sarcina lutea	*O. vulgare, T. vulgaris,* carvacrol
Serratia marcescens	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol
Shigella sonei	*O. vulgare, T. vulgaris*
Staphylococcus aureus	*C. zeylanicum, E. caryophyllus, O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol, thymol
Staphylococcus epidermidis	*O. vulgare, T. vulgaris*
Yersinia enterocolitica	*O. vulgare, S. aromaticum, T. vulgaris,* carvacrol, eugenol

Even though the anti-microbial activity of essential oils tends to be diminished when they are tested *in vivo,* many results are reported: in poultry studies, addition of 120 mg *Zingiber officinale* extract in the diet of broiler chicks increased the number of lactic acid bacteria in the jejunum (Tekeli et al., 2006); chicks fed with 100 mg/kg plant extract consisting of capsaicin, cinnamaldehyde and carvacrol had reduced counts of *E. coli*, *Clostridium perfringens* and fungi and increased counts of *Lactobacillus* spp. in small intestine (Jamroz et al., 2003; Jamroz et al., 2005). In pig research, a plant extracts mixture standardized in 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin (oregano, cinnamon and Mexican pepper) decreased ileum total microbial mass and increased the lactobacilli:enterobacteria ratio in early-weaned pig (Manzanilla et al., 2004). Protein extracted from a new potato variety "Gogu valley" had been reported to be effective in linearly reducing the populations of coliform in feces and contents of cecum, colon, and rectum (Jin et al., 2008). In cell culture model, Roselli et al. (2007) reported that yeast extract, daidzein, bromelain and allicin protected the pig intestinal IPEC-1 cells against the increased membrane permeability caused by enterotoxigenic *Escherichina coli* K88.

Antioxidant capacity. Among a variety of plants bearing antioxidative constituents, the volatile oils from the *Labiatae* family (mint plants) have been attracting the greatest interest, especially products from rosemary. Their antioxidative activity arises from phenolic terpenes, such as rosmarinic acid and rosmarol. Other *Labiatae* species with significant antioxidative properties are thyme and oregano, which contain large amounts of the monoterpenes thymol and carvacrol. Plant species from the families of *Zingiberaceae* (e.g., ginger and curcuma) and Umbelliferae (e.g., anise and coriander), as well as plants rich in flavonoids (e.g., green tea) and anthocyans (e.g., many fruits), are also described as exerting antioxidative properties. Furthermore, pepper (Piper nigrum), red pepper (Capsicum annuum L.), and chili (Capsicum frutescene) contain antioxidative components (Wimdisch et al., 2008).
The antioxidant capacity of the plant extracts was assumed to improve the antioxidant defense system of animal and the oxidative stability of animal derived products. Some studies had obtained positive and promising results. In poultry trials, dietary tea polyphenols were reported to minimize growth inhibition, hyperlipidemia and oxidative stress induced by corticosterone treatment in broiler chickens (Eid et al., 2003).
However, in many of these plants, parts of the active substances are highly odorous or may taste hot or pungent, which may restrict their use for animal feeding purposes.

Immunomodulatory function. Plant extracts had the potential to stimulate the immune function of animals: they can take effect on gut associated or general immune system. However, the investigations on immunomodulatory effects of plant extracts on monogastric animals like poultry and swine are not fully researched. In poultry studies, achyranthan, a low-molecular-weight polysaccharide extracted from *Astragalus membranaceus* had been reported to increase antibody titers against Newcastle disease virus, bursa of Fabricius index in broiler chickens in feeding trial, increased nitric oxide and interleukin-2 production of splenocytes and enhance splenocyte proliferation in a dose-dependent manner in an in vitro trial (Chen et al., 2003). In swine studies, one standardized mixture with 5% carvacrol (from *Origanum* spp.), 3% cinnamaldehyde (from *Cinnamonum* spp.), and 2% capsicum oleoresin (from *Capsicum annum*) was reported to diminish intraepithelial lymphocytes in the jejunum and increase lymphocyte presence in the colon, and not affect the productive performance in post-weanling piglets (Manzanilla et al., 2006). Sugar cane extract enhanced natural killer cytotoxicity, lymphocyte proliferation, phagocytic function of monocytes, and interferon-gamma (IFN-γ) production of CD4(+) and γδT cells in pigs challenged with pseudorabies virus (PrV) and induced in a 12% growth enhancement compared with infected controls without additive administration (Lo et al., 2006).

Production performance. Generally, massive positive productivity promotion results from the available studies suggested that well-formulated plant extracts may actually promote growth and production efficiency in poultry and swine. The mode of action may derived from one or several of the efficacies of antioxidant, microbe modulation, digestive capacity and immune function enhancement mentioned above. The core of these actions was assumed to lie in the gastrointestinal equilibrium or gut health, which would be conducive to the avoidance of pathogen incursion and immune overactivity and distribute more nutrients for growth utilization, thus leading to better growth rate under the same environment.

However, data on swine varied widely from depressions in production performance to improvements similar to those observed with common growth promoters, such as organic acids and probiotics (Manzanilla et al., 2004;2006;2009; Nofrarías et al., 2006; Jin et al., 2008). The discrepancy may arise from difference of animal growing period, physiological status, basal diet type, dosage of plant extracts, interactions among the active substances of the blends of extracts, or environmental stress factors. For poultry, the data appear to be clearer: the majority of experimental results indicate reduced feed intake at largely unchanged average daily gain or final body weight, leading to an improved Feed:Gain when feeding phytogenic compounds (Windisch et al., 2008).

1.2.2.3 Prebiotics

Prebiotics are dietary short-chain carbohydrates (oligosaccharides), which cannot be digested by monogastric animals, but are believed to enhance the beneficial activity of specific members of the microbiota, such as lactobacilli or bifidobacteria in the large intestine (Gibson and Roberfroid, 1995). They have also been referred to as the bifidus factor, because they support the growth and/ or activities of probiotic microorganisms in the gastrointestinal tract. As illustrated in Table 1.9, natural sources of Non-Digestible Oligosaccharides (NDO) exist: e.g. galactooligosaccharides in breast milk, fructans in onion (*Allium cepa*), leeks (*Allium porrum*) and garlic (*Allium sativum*), stachyose in soyabean; however, because of their nutritional interest, biotechnology (enzymic or thermal processes) has been applied to obtain new types of oligosaccharides, by either enzymic synthesis from simple sugars or enzymic hydrolysis from more complex carbohydrates.

Table 1.9 - Dietary oligosaccharides available in food products on the market and the type of source available (Delzenne et al., 2003)

Type of oligosaccharides	Natural source	Industrial production process
Fructo-oligosaccharides	Fruits and vegetables	Synthesis from saccharose; hydrolysis from chicory-root inulin
Galacto-oligosaccharides	Milk	Enzymic synthesis from lactose
Lactulose		Synthesis from lactose
Lactosucrose, glycosylsucrose		Synthesis from saccharose and/or lactose
(Iso)malto-oligosaccharides		Hydrolysis or glycosyl transfer from starch
Xylo-oligosaccharides		Hydrolysis from polyxylans
Stacchyose, raffinose	Soyabean	
Palatinose-oligosaccharides		
Gentio-oligosaccharides		
Cyclodextrin		Synthesis from starch

The following discussion focuses on the beneficial effects of prebiotics added to the diet of monogastric animals on gut microbiota and nitrogen metabolism.

Modulation of gut microbiota. As previously described, one important characteristic of NDO, once ingested, is their relative resistance to digestion by hydrolytic enzymes secreted into, or active in, the intestine (e.g. α-glucosidase, maltase and isomaltase), which is dependent on the extent of polymerization (Delzenne, 2003). Hence, escaping digestion in the upper gastrointestinal tract, they are important sources of energy for bacteria in the caeco-colon that express enzymes such as β-fructosidase, β-galactosidase, xylanase or any other hydrolases: their ingestion lead to the (re)equilibration of the colonic biotope, defined as the "prebiotic effect" (Gibson and Roberfroid, 1995).

The production of short-chain fatty acids through fermentation of oligosaccharides by colonic flora is an important outcome. The pattern of fermentation, i.e. the proportion of the different short-chain acids acetate, propionate, butyrate and lactate, produced in the caecum varies with the nature of the oligosaccharides and duration of the treatment.

Pié et al. (2007) reported that supplementation of the diet influences volatile fatty acid content (VFA), branched-chain proportion, lactic acid concentrations and ammonia concentrations in the gut in weaned piglets.

Increased concentrations of short-chain fatty acids (SCFA) have important effects in the intestinal tract, stimulating natural bacterial activity and proliferation of bifidobacteria and lactic acid bacteria: e.g., it is largely accepted that butyrate has an essential role in maintaining the metabolism, proliferation and differentiation of the different epithelial cell types (Delzenne, 2003). Even though bifidogenic effects of galactooligosaccharides, fructooligosaccharides and soybeanoligosaccharides have been repeatedly confirmed by many *in vitro* and *in vivo* experiments, where they selectively interacted with the intestinal bacterial ecosystem, however, results reported in bibliography are sometimes conflicting: table 1.10 summarises some recent studies investigating the effects of different fermentable carbohydrates on the composition of the gut microbiota.

In particular for newly-weaned piglets, the dietary supplement of fermentable carbohydrates is generally regarded as a comparatively straightforward approach to improve functionality of both the small and large intestine (Bauer et al., 2006). Some studies demonstrated that the addition of sugarbeet pulp, inulin, lactulose and wheat starch to the diet, designed to stimulate the fermentation along the entire gut, altered the composition of bacterial microbiota in the gut of newly-weaned piglets (Konstantinov et al., 2004*a*). An increase in *Bifidobacterium* and *Lactobacillus* genera numbers in the intestine, a concomitant increase in SCFA concentration and improved small intestine morphology were observed (Rayes et al., 2009). In contrast, Mikkelsen et al. (2003), while observing significantly increased numbers of *S. cerevisiae*, failed to find a stimulating effect of galactooligosacharides and fructooligosacharides on *Bifidobacterium* spp. growth in weaned piglets.

Furthermore, combining prebiotics with probiotics (symbiotics) may increase the efficacy of probiotic effects on gut health and development in newly-weaned piglets: fermentable carbohydrates enhanced colonic microbial stability and diversity, with concomitant stimulation of the growth of Lactobacillus sobrius, a novel and beneficial member of the porcine commensal microbiota (Konstantinov et al. 2004a, 2006b).

Table 1.10 – Results of some studies investigating effects of fermentable carbohydrates on the composition of the gastrointestinal microbiota (Bauer et al., 2006)

Trial	NDO	Samples	Influence on microbiota
In vitro	- Arabinoxylans - Starch	Faecal samples of children	Increase in total anaerobe counts and eubacterial rRNA concentrations; degradation of arabinoxylans associated with increased counts of *Bacteroides*
	- Inulin - Levan-type exopolysaccharides	Faecal samples of adult humans	Increase in bifidobacteria
	- FOS - Levan	Faecal samples of adult humans	No effect
	- FOS - Galactosyllactose - Inulin	Faecal samples of adult humans	Increase in bifidobacteria Concomitant reduction in *Clostridium difficile*
	- Galactosyl-melibiose mixture - FOS - Melibiose - Raffinose	Faecal samples of adult dogs	Increases in bifidobacteria and lactobacilli for all carbohydrates tested; higher increase in bifidobacteria and lactobacilli and higher decrease in clostridia for galactosyl-melibiose mixture compared with FOS, melibiose and raffinose
In vivo	Mushroom polysaccharides (*Tremella fuciformis* and *Lentinus edodes*)	Caecum, broiler chickens	Increase in bifidobacteria and lactobacilli; decrease in *Bacteroides* spp. and *Escherichia coli* highest increase in bifidobacteria and lactobacilli for *Lentinus edodes* extract
	Herb polysaccharides (*Astragalus membranaceus*)	Caecum, broiler chickens	Dose-dependent increase in *E. coli*, bifidobacteria and lactobacilli for all polysaccharides tested
	Sugarbeet pulp and FOS	Faeces, weaning piglets	Increase in *Ruminococcus*-like species; higher bacterial diversity and more rapid stabilisation of bacterial community
	Inulin, lactose, wheat starch and sugarbeet pulp	Ileum and colon, weaning piglets	Higher bacterial diversity in colon; *Lactobacillus reuteri* most prevalent in the ileum; *L. amylovorus*-like populations most prevalent in ileum and colon
	- Galactooligosaccharides - Galacto-oligosaccharides (+ *Bifidobacterium lactis* bb-12)	Faeces, human	No effect on indigenous *Bifidobacterium* population; transient colonisation with *B. lactis*

Nitrogen metabolism. the displacement of N excretion to the colon and then feces by oligosaccharide feeding is of great interest. In the absence of sufficient energy as carbohydrate, some bacteria may use protein as a source of energy, resulting in the formation of potentially toxic substances such as NH_3, amines and amides. However, if sufficient fermentable carbohydrate is available, bacteria may utilize NH_3 as an N source for their own growth. Accordingly, provision of fermentable carbohydrates can increase NH_3 uptake by gut bacteria. N would then be excreted as microbial protein via the feces instead of as urea in urine, saving energy to the host and reducing the NH_3 burden to the environment.
Canh et al. (1997), who investigated the influence of dietary non starch polysaccharides (sugarbeet pulp) on N partitioning of urine and faeces of

fattening pigs, found that the pigs fed the sugarbeet pulp-based diet excreted 22-37 % less urea in urine than the pigs fed diets with a lower content.

Among prebiotics, **mannanoligosaccharides** (MOS) have an important role: they are complex sugars (phosphorylated glucomannas) deriving from the cell wall of yeasts. The most common commercial source of MOS is *Saccharomyces cerevisiae*. The mannanoligosaccharides are not used as a substrate for microbial fermentation, but they carry out their action as growth promoters, increasing the resistance of the animal to enteric diseases: they can modify the microbial gut ecosystem by binding to the receptors present in the intestinal epithelium, thereby preventing the colonization of bacterial pathogens (Shim et al., 2005).
In fact, most enteric pathogens must attach to the intestinal wall in order to proliferate and cause disease; more specifically they attach to carbohydrates as the binding sites. Several pathogens, including some *E. coli*, attach to mannose units on the mucosal surface. It is perceived that the yeast cell wall fragment containing a mannose unit in the lumen of the intestine may bind to the pathogens, preventing the pathogens from binding to the intestinal wall. The product must survive the digestive processes and reach the lower intestine in order to function in this manner (Pettigrew, 2006).
Hence, mannanoligosaccharides isolated from the *S. cerevisiae* cell wall have a beneficial effect on the intestinal microflora (Lyons and Bourne, 1995) and animal growth (Shim et al., 2005). It was found that they suppress the growth of *E. coli*, *Salmonella typhimurium*, *Clostridium botulinum* and *C. sporogenes*, and conversely stimulate the growth of *B. longum*, *L. casei*, *L. acidophillus* and *L. delbrückei*.
MOS seem also to enhance the immune system in weaned piglets: in fact, it is well known that all animals reared under commercial conditions, are subjected to immunological stress caused by the presence of a high load of pathogens in their environment, with a consequent release of cytokines, associated with inflammatory processes. The use of MOS seems to reduce the production of pro-inflammatory cytokines allowing an increase in growth performance. Lyons and Bourne (1995) also reported that mannanoligosaccharides from the yeast cell wall stimulate the local immune system by increasing the activities of macrophages and T-lymphocytes.
However, results are sometimes in contrast: while some researches suggest that MOS supplementation can provide benefits in swine production similar to that of antimicrobial growth enhancers, others did not demonstrate their effectiveness (White et al., 2002; Le Mieux et al., 2003).
Hence, it is possible that the effect of MOS supplementation on piglet performance is influenced by different factors, such as weaning age, health status, duration of feeding and the amount of MOS addition.

1.2.2.4 Probiotics

Probiotics have been defined as "a preparation or a product containing viable, defined microorganisms in sufficient number, which alter the microflora (by implantation or colonization) in a compartment of the host, and by that exert beneficial health effects on the host" (Schrezenmeir and de Vrese, 2001). This implies that probiotics should be able to survive in the gastrointestinal tract and that an adequate dose is necessary to have beneficial effects.
They are mainly active in the caudal segments of the ileum, in the caecum and the ascending colon; their most important characteristics are the capacity to adhere to intestinal mucosa and to inhibit pathogen adhesion, transiently colonizing the intestine and preventing some intestinal diseases such as diarrhea, and the ability to modulate the immune system of the host (Teitelbaum and Walker, 2002).

Prevention of intestinal disease and inhibition of pathogen adhesion. Probiotics may represent an important mean to overcome problems related to intestinal disease and consequent diarrhea. The rationale for probiotic use is that probiotics are able to restore normal microflora. Probable mechanisms of improvement of intestinal microbiocenosis by using probiotics are based on the following (Vondruskova et al., 2010):

- ✓ competition between them and pathogenic microorganisms for binding sites in the intestinal mucosa;
- ✓ nutrient availability;
- ✓ total inhibition of pathogen growth by production of organic acids and antibiotic-like compounds.

Only a few probiotics have been tested for their capacity to prevent intestinal diseases in pigs (see table 1.11). However, multiple studies have confirmed the stimulating effects of probiotics on the intestinal environment: by lowering the pH value in the small intestine and producing organic acids and antibacterial substances probiotic supplements inhibit pathogenic microorganisms, improve the intestinal microflora and stimulate immune function (Marinho et al., 2007). Intestinal microflora can be modulated by the supplementation of feeds with probiotic bacterial species of the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Enterococcus*, *Streptococcus* and their combinations. An important role is played also by *Saccharomyces*: *S. cerevisiae* has been reported to have a varying influence on pig efficiency, such as increased feed intake and body weight gain of piglets.

Table 1.11 – Microorganisms used as probiotics (Vondruskova et al., 2010)

Genus	Bacterial species
Lactobacillus	*L. acidophilus* *L. casei* *L. rhamnosus* *L. reuteri* *L. plantarum* *L. fermentum* *L. brevis* *L. helveticus* *L. delbrückei*
Lactococcus ***Enterococcus*** ***Streptococcus*** ***Pediococcus***	*L. lactis* *E. faecium* *S. thermophilus* *P. pentosaceus*
Bacillus	*B. subtilis* *B. cereus* *B. toyoi* *B. natto* *B. mesentericus* *B. licheniformis*
Bifidobacterium	*B. bifidum* *B. pseudolongum* *B. breve* *B. thermophilum*
Saccharomyces	*S. cerevisiae*
Avirulent *Escherichia coli*	*E. coli*

Immunomodulation. Probiotics are able to prevent intestinal diseases through, both humoral and cell-mediated immune modulation (Erickson and Hubbard, 2000). Indeed, it has already been shown that probiotics may lead to an increased IgA production and stimulation of macrophage and NK activity (Matsuzaki and Chin, 2000). Moreover, several studies have reported that probiotics are able to regulate both anti- and pro-inflammatory cytokine production (Roselli et al., 2005). However the results are quite conflicting and the mechanisms of action of probiotics on cytokine expression are still not understood. All the results suggest that probiotics interfere with different steps of the inflammatory pathway, either on cytokines that can regulate the balance between lymphocyte populations, or on chemokines involved in the recruitment of inflammatory cells.

1.3 Managing gut health in the poultry

Enteric diseases are an important concern also to the poultry industry because of lost productivity, increased mortality, and the associated contamination of poultry products for human consumption (human food safety). Hence, gut health research, that has its origin in human health programs, is a major topic for research also in poultry industry. As described before, gut health is highly complex and encompasses the macro- and micro-structural integrity of the gut, the balance of the microflora and the status of the immune system. Further complexity arises from their interactions and the resulting changes in gene expression, and possibly, endocrine regulation. This, in turn, may affect the way nutrients are partitioned and used for organ development, tissue growth and immune system maturation (Kelly and Conway, 2001).
This work will discuss the link between gut health and nutrition in poultry, in particular referring to the use of probiotics as a possible alternative to antibiotic growth promoters in the feed.

1.3.1 Gut health

Not only is the gut the major organ for nutrient digestion and absorption, it also works as the first protective mechanism to exogenous pathogens which can colonize and/or enter the host cells and tissues. Thus, it is implied that a more robust gut will make a healthier animal, which, in turn, digests and uses nutrients more efficiently: e.g. Hetland et al. (2003) demonstrated that the inclusion of oat hulls in a wheat-based broiler diet increased the gizzard weight, which coincided with a significant improvement (from 97 to 99%) in the digestibility of starch (the most important energy source in broiler diets) in the ileum.
This may mistakenly lead to the notion that a heavy gut represents a healthy gut; it is not so: for instance, some works demonstrated that the size of the intestine is reduced and the mucosal layer is substantially thinned when antibiotics are added to animal diets; this suggests that gut health is related not only to the physical development as a result of stimulation by food and solid particles, but is determined by the organisms harboured in the gut (Choct, 2009).
The diversity of bacterial species in the gut is, also in the poultry, one of the most important factors for the establishment of a stable ecosystem in the intestinal tract: until the bacterial populations are fully established, young animals have fewer bacterial species in the intestinal tract than adult birds, making their gut microflora more susceptible to disturbances than that of adult animals.
Intestinal microbial populations have been in part characterized: although *Bacteroides* and *Bifidobacterium* predominate in the human intestine, *Ruminococcus*

and *Streptococcus* tend to predominate in the chicken intestinal tract (Van der Wielen et al., 2000). However, recent molecular techniques indicate that only 20 to 50% of the bacterial species present in the intestinal tract have been cultured. The naturally established protective flora is very stable, but it can be influenced by dietary, disease and environmental factors. For example, hygiene conditions (clean or dirty environment, pathogen load of the ingredients, humidity of the shed, litter type and usage, etc.), stress (change of feed, sudden disturbances, heat or water stress), and the use of feed additives can also affect gut microflora. However, diet is perhaps the most important factor influencing that. Dietary factors, such as composition, processing, digestibility and feeding method, may all disturb the balance in the gut ecosystem, especially in young animals (Choct, 2009).
Another important factor to be considered is the gut microstructure: the mucosa is a vast surface of epithelial cells of the absorptive type essential for the transport of nutrients. Minute changes may occur in the gut, which are often overlooked because the damage is subtle and usually characterized by microscopic changes in the mucosal layer, that can undermine the efficiency of nutrient assimilation. Development of the gastrointestinal tract is an important aspect of growth, especially during the early post-hatching period: close to and shortly after hatch, segments of the gut and digestive organs increase in size and weight more rapidly in relation to body weight than do other organs and tissues (Noy and Sklan, 2001).
A rapidly growing broiler devotes about 12% of newly synthesized protein to the digestive tract. An increase in cell proliferation will reduce the age and maturity of the goblet cells, which might affect the quality of mucins they produce. As a consequence, the absorption of nutrients may be reduced. In addition, a fast turnover of these cells will increase the energy requirement for maintenance of the digestive tract. Changes in intestinal morphology can lead to poor nutrient absorption, increased secretion in the gut, diarrhea, reduced disease resistance and impaired overall performance (Nabuurs et al., 1993).

1.3.2 Feed additives: probiotics

Probiotic, which means "for life" in greek, has been defined "a preparation or a product containing viable, defined microorganisms in sufficient number, which alter the microflora (by implantation or colonization) in a compartment of the host, and by that exert beneficial health effects on the host" (Schrezenmeir and de Vrese, 2001) and, as previously described, it has ideal characteristics and effects (shown in Table 1.12): numerous *in vivo* and *in vitro* studies have shown that the commensal intestinal microbiota inhibit pathogens, that disturbances of

the intestinal microbiota can increase susceptibility to infection, and that addition of probiotics can increase the resistance of the host (Patterson and Burkholder, 2003). Hence, the concept of a balanced intestinal microbiota enhancing resistance to infection (and its reduction when microbiota is disturbed) is important: sometimes, what constitutes the balanced and disturbed populations is not clear; however, it is clear that populations like *Lactobacilli* and *Bifidobacteria* are sensitive to stress and tend to decrease when a bird is under stress.

Table 1.12 – Characteristics and beneficial effects of ideal probiotics (Patterson and Burkholder, 2003)

Characteristics	**Beneficial effects**
Be of host origin	Modify intestinal microbiota
Non-pathogenic	Stimulate immune system
Withstand processing and storage	Reduce inflammatory reactions
Resist gastric acid and bile	Prevent pathogen colonization
Adhere to epithelium or mucus	Enhance animal performance
Persist in the intestinal tract	Decrease carcass contamination
Produce inhibitory compounds	Decrease ammonia and urea excretion
Modulate immune response	
Alter microbial activities	

A variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *E. coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species, and undefined mixed cultures. *Lactobacillus* and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus*, *Enterococcus*, *Saccharomyces* yeast and, more recently, *Lactobacillus* have been the most common organisms used in livestock.

In particular, lactic acid bacteria (LAB) are present in the microbiota of mammals and birds, and those originating in the intestine have undergone intensive study for their potential probiotic properties and their rapid establishment as bacterial communities for the prevention of colonization by pathogenic bacteria (e.g. Salmonella). Different studies aimed to identify the microbiota of the gastrointestinal tract of poultry pointed out the predominance of lactobacilli such as *Lactobacillus crispatus*, which was isolated from chicken crops and intestine (Beasley et al., 2004); *Lactobacillus rhamnosus* TB1, from the intestinal tract of chicken and exhibiting good adherence and *in vivo* colonization (Bouzaine et al., 2005); *Lactobacillus salivarius* with antagonism against *Escherichia coli* and *Salmonella Enteritidis* were found in gastrointestinal tracts of chicks (Garriga et al., 1998); strains of *Lactobacillus thermotolerans* G12, G22, G35T, G43, and G44 were isolated from chicken feces (Niamsup et al., 2003). The effects of the administration of such probiotics, live or inactivated, are well described: Huang et al. (2004) administered killed, cobalt-enriched *Lactobacillus casei* and *Lactobacillus acidophilus* in the feed of broiler chickens and observed increased

body weight at 6 weeks of age; Sashihara et al. (2006) applied heat-killed *Lactobacillus plantarum* and *Lactobacillus gasseri* to cultures of splenocytes and mesenteric lymph node cells, and observed an increase in production of IL-12; administration of live or dead Lactobacillus GG to cultures of Caco-2 cells resulted in a decrease of tumor necrosis factor-α induced interleukin-8 production (Zhang et al., 2005).

Particularly, *Lactobacillus* isolates from chicken origin are good sources of antimicrobial peptides, bacteriocins (Lima et al., 2007): these antimicrobial peptides are short bactericidal peptides widely present in animal intestines that have become recognized as a novel class of antibiotics to control foodborne pathogens in poultry. Bacteriocins produced by LAB belonging to the Lactobacillus genus are active against some gram-positive bacteria and occasionally gram-negative bacteria (Todorov et al., 2004). An isolate of *L. acidophilus* has been reported to produce 2 bacteriocins, which inhibited growth of nonpathogens (*Lactococcus* and *Pediococcus*), but also of several pathogenic organisms *in vitro*, from genuses including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Listeria*, *Clostridium* and *Bacillus* (Bogovič-Matijašić et al., 1998); Ocaña et al. (1999) reported isolation of a bacteriocin from a *Lactobacillus salivarius* strain that inhibited *Enterococcus* and *Staphylococcus*; Tellez et al. (2006) identified 11 lactic acid bacteria that were efficacious in the treatment of Salmonella-infected chicks.

Hence, it is clear that these products show promise as alternatives for antibiotics as pressure to eliminate growth promoters antibiotic use increases worldwide.

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CHAPTER 2

Objectives

2. Objectives

There is a wide interest in developing management and feeding strategies to stimulate gut development and health in monogastric animals. The ultimate aim of these strategies is to improve productivity, while minimizing the use of antibiotics and rather expensive feed ingredients: indeed, under practical conditions, animals don't achieve the maximum of their growth performance potential. Large amounts of research have been conducted evaluating the impact of a wide range of feed ingredients and feed additives on various aspects of gut health and development in monogastric animals.

The main objective of this thesis was to improve our knowledge on the properties of new additives as feeding strategy, in order to increase general health in piglets around weaning and poultry, with the aim to substitute antibiotics growth promoters. Three different trials were designed to study different strategies: in the first study proposed, the effects of plant extract administered through drinking water on post-weaning gut health of piglets were investigated; the aim of the second trial was to evaluate the effect of the administration of mannanooligosaccharides on growth performance, microbial population in feces and cecum and potential alteration of intestinal histomorphometric and gene expression of some intestinal inflammatory parameters of piglets fed a low digestible diet; finally, the third study was carried out to determine the effects of a probiotic mixture containing two strains of *Lactobacillus* on growth performance, carcass composition, blood lipids, digestive enzyme activity and intestinal microbiota in broiler chickens.

CHAPTER 3

Effects of plant extract administered through drinking water on post-weaning gut health of piglets.

3. Effects of plant extract administered through drinking water on post-weaning gut health of piglets.

3.1. Abstract

Phytogenic feed additives are plant-derived products used in animal feeding to improve the performance of agricultural livestock. The objective of the present work was to evaluate the effects of a novel plant extract derived from common food plants on performance and health of weaned piglets fed mixed diet. At weaning (24 d), a total of 144 piglets were allocated in two post-weaning rooms, using a 2x2 factorial arrangement; treatments were Plant Extracts, 0 (Control group) or 8 µl daily/piglet (PE group) and Feeding Regimen, *Ad Libitum* or Restricted (piglets fed from 8 AM to 8 PM). Plant Extracts were a liquid mixture administered through drinking water. Piglets were housed in pens of three; each pen represented one treatment replicate, with six pens per treatment per room. On day 9 of the trial, after an adaptation period, each piglet of room 2 was orally injected with 4 ml of a solution containing 10^9 cfu of the virulent *E. coli* 0149: F4(K88)-positive strain. Animals were weighed and growth performance were recorded weekly; fecal score was evaluated at the same time as the weighing. At 0, 14 and 35 days, fecal samples were collected for microbiological analysis, while at day 0, 6, 19 and 35, blood samples were obtained from one pig per pen. At the end of the trial (35 d), 24 animals (12 from Control groups and 12 from Plant Extract groups) among Restricted feeding piglets were selected according to their body weight and slaughtered; immediately after slaughtering, the gastro-intestinal tract was removed from each animal: the distal ileum was collected and examined to assess the ileum micro-anatomical structure, perform histometry and immunohistochemistry and determine intestinal inflammatory parameters. PE supplementation enhanced ADG during the last week of the trial (P=0.007) and reduced FCR during the second (P=0.009) and the last weeks (P=0.04), and considering the overall period (P=0.01); a lower fecal score was observed in PE piglets (P<0.01). On day 35, lower fecal *E.Coli* (P=0.02) and *Entrobacteriaceae* (P=0.009) concentrations were determined in PE animals compared to control ones. Ileum crypts from PE piglets were deeper in challenged animals in comparison with not-challenged ones (P<0.05); number of mucosal macrophages was higher in Control challenged animals (P<0.05): in particular, number of mucosal macrophages in PE challenged piglets was similar to that one identified in not challenged Controls. PE supplementation also increased

GSH-Px plasma concentration at d 6 (P=0.02) and tended to lower value of MDA at day 6 (P=0.07) and to increase value of T-AOC at the end of the trial (P=0.07). Hence, our results confirmed the possible protective functional role of the plant extracts mixture after the bacterial challenge: we can postulate that the use of plant extracts may be useful in the prevention of post-weaning diarrhea with an associated improvement in performance.

3.2. Introduction

Plant extracts feed additive are a group of naturally ingredients derived from herbs, spices or other plants which could be supplemented in the diets to improve the production efficiency or maintain the health status of domestic animals. This class of feed additives has recently gained increasing interest, due to the ban on antibiotic feed additives within the European Union, because of risk for generating antibiotic resistance in pathogenic microbiota. Phytobiotics (term used to describe plant-derived natural bioactive compounds) are essentially secondary metabolites and represent a wide range of mixtures of various compounds, such as terpens, phenols, glycosides, saccharides, aldehydes, esters and alcohols. Nowadays, they are discussed possibly to add to the set of nonantibiotic growth promoters, such as organic acids and probiotics, which are already well established in animal nutrition . However, they are a relatively new class of feed additives and our knowledge is still rather limited regarding their modes of action and aspects of their application. Further complications arise because phytogenic feed additives may vary widely with respect to botanical origin, processing, and composition.
The objective of the present study was to determine the effects of dietary supplementation with Plant Extracts on growth performance and health of weaned piglets fed mixed diet, challenged with *E. coli* (EC).

3.3. Materials and methods

The animal protocol of this research was approved by the Animal Care and Use Committee of University of Milan, Italy.

3.3.1. Animals, housing, experimental design and E.coli challenge

The trial was carried out at the facility of the "Centro Zootecnico Didattico Sperimentale", Azienda Polo di Lodi, University of Milan, Italy. At weaning (24 days), a total of one hundred forty-four crossbred piglets (average weight, 6.5 kg) homogeneous for age and litter origin, were weighed and randomly assigned to

treatments. Animals were housed in 2 rooms, equipped with 24 pens each, environmentally regulated in an isolated stable. A combination of daylight (through skylights) and artificial light (non-programmable) was used. Ventilation was achieved by single, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28 °C at the start of the trial, adjusted weekly until a final temperature of 26 °C. Pens, located beside a 120 cm walkway with 12 pens (1.20 m x 1.00 m) each side, had a slatted floor. Each pen was equipped with two water nipples and self-feeder. The pigs were housed in groups of three. Piglets were used in a 35 day experiment to assess the effect of Plant Extract (PE) supplemented in combination with two feeding regimen, *ad libitum* or restricted, and in presence of a challenge with *E. coli* (EC). A 2x2x2 factorial arrangement of treatments was employed. Each treatment consisted of 6 replicates/room with 3 piglets. Each pen represented one treatment replicate. Plant Extract test material (LiveLeaf Bioscience) was stored at room temperature and away from direct sunlight. Plant Extracts were a blend of pomegranate peel and green tea powder and was administered from 8 PM to 8 AM through drinking water. A graduated tank with treated water was linked to the water system in each pen; the amount of the dilute product was based on animal average weight and was estimated at 0.008 ml/kg/d. Following the *E.coli* challenge (day 9 of the trial), PE supplementation was increased: on day 8, 9 and 12 of trial, challenged piglets of the Treated groups received 200µl/kg of the product, while on day 10 and 11 they received 400 µl/kg. The tested product was diluted daily and manually supplemented in the water tank to adjust perfectly the dosage with the weight of the piglets. Diet was formulated to be isonutritive, exceeding the protein requirement recommended by NRC (1998) for pigs. The approximate composition and the chimical analysis of the diet are presented in Table 3.1. Diet was formulated and manufactured before the trial start, without the inclusion of any antibiotic growth promoters or antibiotic growth promoter alternatives. Starter diet was a meal, milled at 1.5 mm particle size. Feed was stored in a cool dry place until required. In *Ad libitum* groups, animals were allowed *ad libitum* access to feed, while in Restricted ones, piglets were allowed a restrictive access to feed (from 8 AM to 8 PM). Feeding troughs were removed at 8 PM, weighed and contents stored. The morning after, feeding troughs were filled, weighed and placed in pens by 8 AM.

On day 9 of the trial, after an adaptation period, piglets housed in one room were submitted to an *E. coli* challenge. Piglets were orally injected 4 ml of a solution containing 10^9 cfu of the virulent *E. coli* 0149: F4(K88)-positive strain. Precautions were taken to minimize the confounding room effect on intestinal microbiota and development of the immune system of piglets.

Table 3.1 - Percentage composition and chimical analysis (as-fed) of the diet

Composition of basal diet (%)		Chemical analyses (as fed)	
Wheat	59.34	Dry matter, %	89.63
Whey powder	9.5	ME, Mcal/kg	3,530
Soycomil	7.0	Crude protein, %	19.85
Herring meal	5.5	Ether extract, %	5.07
Wheat bran	5.0	Crude fibre, %	2.38
Soybean meal	4.78	NDF, %	9.89
Soybean oil	2.5	Ash, %	5.87
Dextrose monohydrate	2.2	Total lysine, %	1.53
Dicalcium phosphate	1.26	Total SAA, %	0.94
Pig lard	0.8	Threonine, mg	1.14
Lysine HCl 78	0.5	Tryptophan, mg	0.33
Calcium carbonate	0.47	Calcium, %	0.88
L-Threonine	0.4	Phosphorus, %	0.75
DL-Methionine	0.25	Sodium, %	0.22
Vitamins premix	0.25		
Salt	0.15		
L-Tryptophan	0.1		

3.3.2. Sampling and observations

Piglets were individually weighed at weaning (day 0) and subsequently every week until the end of trial. Feed disappearance was measured on a daily basis for Restricted groups, on d 0, 7, 14, 21, and 28 for *Ad libitum* ones. The ADFI and FCR were calculated for each pen.

3.3.3. Fecal, blood and tissue sampling and processing

Pen fecal scores were recorded using a subjective scale, as follows: 1= hard, dry pellet; 2 = firm, formed stool; 3= soft, moist stool that retains shape; 4= soft, unformed stool; 5= watery liquid that can be poured. Feces samples were collected at day 0, 11 (two days after challenge) and 35 (end of the trial). Each sample (about 20 g) was placed in a small sterile container and immediately sent to the laboratory for microbial assay. Microbiological counts for *Lactobacilli*, *Enterobacteriaceae*, *Clostridia* and *E.coli* were calculated: plates for enumeration of *Enterobacteriaceae* (Violet-Red Bile Dextroseagar) were incubated aerobically at 37°C for 24h; *E. coli* was grown in Tryptic Soy agar at 37°C; *Lactobacilli* faecal content was determined by MRSA (*Lactobacillus* Agar) with an incubation time of 72h at 37°C (10% CO_2) and *Clostridia* procedure had an incubation time of 48h at 37°C using TSC (Tryptose sulphite cycloserine agar).

On d 0, 6, 19 and 35 of the experiment, blood samples were obtained from one pig per pen (a total of 48 samples). Blood was collected via jugular puncture into 10 ml vacutainers containing no anticoagulant. Blood was allowed to clot at room temperature for 45 min and stored overnight at 4°C before serum was

harvested at room temperature by centrifugation for 10 min at 1,800 x g. The collected serum was aliquoted and frozen at -20°C (80°C), and later analyzed for antioxidant indicators. Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxygenic capability (T-AOC) in serum were determined by using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China). All measurements were done in duplicate.
At the end of the trial, 24 animals from Restricted groups (3 piglets per treatment per room), selected as being most representative of pen performance in terms of weight gain and health, were carried to a local slaughter house. Immediately after slaughtering, the gastro-intestinal tract was removed from each animal, and the distal ileum (2 cm prior to its opening into the cecum) was collected and promptly fixed in neutral buffered formalin for 24 h at 4°C. The specimens were then dehydrated in graded ethanol series, cleared with xylene and embedded in paraffin. After dewaxing and re-hydration, microtome sections (4 μm-thick) were stained with hematoxylin and eosin (HE) and examined to either assess the ileum micro-anatomical structure or perform histometry. For histometry, the following parameters were evaluated per section: villus height (V) (5 villi measured per section), crypt depth (C) (5 crypts measured per section), the villus height to crypt depth ratio (V:C ratio), number of lymphatic follicles (counted in 3 fields per section at 400x and then expressed as n/mm2 of mucosa), area of lymphatic follicles and their compartments (cortex, medulla, corona; 5 follicles per section). Other ileum sections were processed by immunohistochemistry to identify mucosal macrophages using a macrophage monoclonal antibody (1:400, ab22506, abCAM), after antigen retrieval with K-protease (20μg/ml of buffer solution). For each section, the number of immuno-positive mucosal cells was counted in 8 fields at 400x and subsequently expressed as n/mm2 of mucosa.
After slaughtering, ileal mucosa was obtained also for myeloperoxidase (MPO), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) content determinations by commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).Three 5-cm segment from the ileum were collected, opened longitudinally and cleaned with PBS. Intestinal mucosa was collected by scratching with a glass slide for the next step. Nine milliliters of 4°C PBS was added to 1 g of intestinal mucosa, followed by homogenization. The homogenates were centrifuged (4,000 × g for 5 min at 4°C), and supernatant fluid was used following the manufacturer instructions.

3.3.4. Statistics

The data were analysed as a completely randomised block design with a 2x2x2 factorial treatment arrangement by ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Institute Inc., Cary, NC). The model included effect of

treatment (PE), feeding regime, *E. coli* challenge, treatment x feeding regime, treatment x challenge, feeding regime x challenge and treatment x feeding regime x challenge interaction. Three subject died at the beginnig of the trial, thus they were not considered for performance analyses.
Data relative histometry were analyzed by the ANOVA mixed model that included treatment as fixed effect and piglet as random effect. Differences between least squared means were considered significant at $P < 0.05$.

3.4. Results and Discussion

3.4.1. Growth performance

Phytogenic feed additives have attracted increasing interest as an alternative feeding strategy to replace antibiotic growth promoters; however, literature on their biological efficacy presents a scattered picture. These feed additives are often claimed to improve the flavor and palatability of feed, thus enhancing production performance.
The effects of PE administration on growth performance are shown in table 3.2. No differences among treatments were found for piglet body weight and ADG and ADFI calculated on the overall experimental period. However, piglet average daily gain (ADG) was different across treatments and feeding regimens for the last week of the trial. Piglets receiving the supplementation with PE and piglets fed *ad libitum* had greater ADG than their controparts (P=0.007; P=0.006). ADFI was significantly higher in pens of piglets fed *ad libitum* than restricted during the second and third weeks (P=0.02 and P=0.03, respectively). PE significantly improved piglet FCR during the second (P=0.009) and last weeks of the trial (P=0.04), and considering the overall period (P=0.01). *E coli* challenge significantly affected piglet growth performance. ADG was significantly lower in challenged animals than non challenged during the third week of the trial (P=0.01) and considering the overall period (P=0.03). ADFI was significantly lower in challenged animals than non challenged during the third (P=0.05), last weeks of trial (P=0.03) and considering the overall period (P=0.04).
Our results indicate that PE administration affect piglet growth performance The number of studies having tested the specific effect of phytogenic products on palatability by applying a choice-feeding design is quite limited and, as previously discussed, data on growth performance in the swine are not consistent and similar to those observed with common growth promoters, such as organic acids and probiotics. Ilsley et al. (2005) did not observe any effect on piglet growth performance after quillaja saponin and curcumin supplementation.Similarly, Manzanilla et al. (2004) did not show differences for

ADFI, ADG and feed:gain ratio after a dietary supplementation with a plant extract mixture containing carvacrol, cinnamaldehyde and capsicum oleoresin. The same substances, in a standardized mixture with 5% carvacrol (from *Origanum* spp.), 3% cinnamaldehyde (from *Cinnamonum* spp.), and 2% capsicum oleoresin (from *Capsicum annum*), had no significant effect on the growth performance of piglets (Manzanilla et al., 2009; Manzanilla et al., 2006), or were correlated to a 82% greater ADG and a 39% higher ADFI during the first week after weaning, although the difference were not significant (Nofrarias et al., 2006). On the contrary, pigs fed with chinese traditional herbal medicine Bazhen displayed a reduced feed intake, but, according to our results, their average daily gain was greater than that of the control group, and consequently, their feed to gain ratio was improved (Lien et al., 2007). TheAuthors conclude that it could be associated to the fact that Bazhen extract can strengthen the immune system. Hong et al. (2004) demonstrated that supplementation with 0.2% of a citrus fruit and chestnut tree extract mixture determined, from day 10 to 20 after weaning, a better ADG (365 vs. 323 g/d), but no differences were found among the treatments for ADFI and gain:feed. Mavromatis and Kyriakis (1998), in a study to determine the effects of Origanum essential oil as a growth promoter in weaned and growing-finishing pigs, found that feed intake and gain:feed of PE groups were significantly improved, in comparison to the control. Also, Kyriakis et al. (1998) suggested that ADG was faster for the PE groups than for the control. In our study, although possible beneficial effects of herb or botanicals in farm animals may arise from activation of feed intake and secretion of digestive enzymes and immune stimulation properties (Wenk, 2003), we didn't find a difference in ADFI (that could be related to the way of administration of the PE we used), but we evidenced an improvement of ADG and FCR, in particular at the end of the trial. Our positive results confirmed by numerous examples of experimental results in literature, indicate that phytogenic feed additives, in general, may actually exert growth-promoting activity. The stabilizing effects on the ecosystem of gastrointestinal microbiota and morphological changes in gastrointestinal tissues caused by phytogenic feed additives may provide benefits to the digestive tract and, consequently, have growth-promoting efficacy (Windisch et al., 2008).

For other species, such as poultry, the data appear to be clearer. The majority of experimental results indicate reduced feed intake at largely unchanged body weight gain or final body weight, leading to an improved feed:gain when feeding phytogenic compounds. Of course, the wide variation in biological effects induced by phytogenics reflects the experimental approaches used to test the suitability of these substances as growth-promoting feed additives and also includes failures in selecting the proper plants, active components, and efficacious dietary doses (Windisch et al., 2008).

Table 3.2 - Effects of PE supplementation on growth performance of challenged (+) and non-challenged (-) piglets fed *ad libitum* or restricted diet

Treatment:	**Control**				**Plant Extracts**				**SEM**	**P value**						
Regimen:	***Ad libitum***		**Restricted**		***Ad libitum***		**Restricted**			**Treatment**	**Regimen**	**Challenge**	**Treatment × Regimen**	**Treatment × Challenge**	**Regimen × Challenge**	**Treatment × Regimen × Challenge**
Challenge:	-	+	-	+	-	+	-	+								
Weight, kg																
Day 0	6.41	6.63	6.44	6.58	6.37	6.53	6.39	6.59	0.28	0.83	0.94	0.36	0.89	1.00	0.96	0.87
Day 7	6.85	7.15	7.02	7.05	6.87	7.00	6.68	6.92	0.29	0.48	0.81	0.41	0.68	0.95	0.85	0.65
Day 14	8.09	8.31	8.28	8.17	8.28	8.29	7.98	7.97	0.36	0.76	0.58	0.92	0.52	0.92	0.74	0.77
Day 21	10.66	10.28	10.62	9.96	10.70	10.44	10.06	9.96	0.50	0.81	0.30	0.33	0.60	0.63	0.93	0.76
Day 28	13.41	12.80	13.40	12.29	13.12	12.91	12.99	12.75	0.62	0.95	0.65	0.23	0.90	0.48	0.77	0.80
Day 35	16.67	15.68	16.17	15.12	17.19	16.41	16.17	15.70	0.79	0.42	0.22	0.15	0.77	0.73	0.91	0.87
ADG, g/d																
Day 0-7	63.49	74.03	83.03	67.14	71.68	67.70	41.03	46.98	11.95	0.08	0.26	0.92	0.06	0.83	0.63	0.29
Day 7-14	177.5	165.6	179.7	160.0	201.5	184.1	186.3	149.5	18.49	0.47	0.32	0.11	0.38	0.67	0.61	0.83
Day 14-21	366.4	281.3	334.3	255.9	345.0	307.4	296.8	284.6	29.29	0.96	0.13	0.01	0.87	0.18	0.70	0.82
Day 21-28	392.9	360.3	397.0	333.3	346.2	353.1	418.3	398.3	31.78	0.72	0.30	0.23	0.13	0.36	0.53	0.96
Day 28-35	465.5	411.4	395.9	404.5	581.4	499.2	455.0	421.0	35.41	0.007	0.006	0.11	0.21	0.49	0.28	0.89
Day 0-35	293.1	258.6	278.0	244.2	309.2	282.3	279.5	260.1	18.46	0.28	0.13	0.03	0.67	0.68	0.88	0.90
ADFI, g/d/pen																
Day 0-7	438.6	491.0	525.1	403.6	502.3	406.0	436.6	365.4	38.96	0.19	0.34	0.04	0.34	0.38	0.18	0.08
Day 7-14	945.2	1005	909.5	847.6	987.6	931.3	868.4	770.3	68.40	0.44	0.02	0.42	0.66	0.44	0.40	0.68
Day 14-21	1535	1427	1433	1216	1611	1416	1376	1243	115.7	0.91	0.03	0.05	0.77	0.99	0.88	0.60
Day 21-28	2065	1790	1858	1632	1948	1840	1839	1696	138.2	0.96	0.12	0.06	0.78	0.53	0.97	0.83
Day 28-35	2506	2062	2238	2100	2533	2330	2405	2151	165.2	0.28	0.26	0.03	0.87	0.79	0.59	0.45
Day 0-35	1498	1355	1393	1240	1516	1385	1385	1245	95.72	0.87	0.08	0.04	0.85	0.93	0.95	0.99
FCR																
Day 0-7	3.25	2.70	2.22	2.10	2.51	2.13	4.35	2.82	0.58	0.36	0.58	0.13	0.02	0.46	0.67	0.34
Day 7-14	1.80	2.26	1.75	1.80	1.60	1.75	1.57	1.72	0.13	0.009	0.12	0.03	0.23	0.55	0.27	0.26
Day 14-21	1.42	2.41	1.44	1.60	1.55	1.59	1.62	1.46	0.31	0.46	0.35	0.25	0.41	0.16	0.25	0.47
Day 21-28	1.78	1.73	1.55	1.67	1.91	1.76	1.46	1.45	0.09	0.56	0.0003	0.73	0.08	0.37	0.27	0.87
Day 28-35	1.98	1.69	1.91	1.77	1.46	1.59	1.77	1.73	0.13	0.04	0.21	0.37	0.24	0.16	0.94	0.38
Day 0-35	1.73	1.78	1.68	1.70	1.62	1.65	1.65	1.60	0.05	0.01	0.28	0.72	0.41	0.50	0.44	0.69

Table 3.3 – Effects of administration of plant extracts on fecal score of challenged (+) and non-challenged (-) piglets fed *ad libitum* or restricted diet

Treatment:	**Control**				**Plant Extracts**				**SEM**	**P value**						
Regimen:	***Ad libitum***		**Restricted**		***Ad libitum***		**Restricted**			**Treatment**	**Regimen**	**Challenge**	**Treatment × Regimen**	**Treatment × Challenge**	**Regimen × Challenge**	**Treatment × Regimen × Challenge**
Challenge:	-	+	-	+	-	+	-	+								
Day 0	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Day 7	3.00	3.17	3.00	3.33	2.83	3.00	3.00	3.00	0.11	0.04	0.30	0.04	1.00	0.30	1.00	0.30
Day 14	4.00	4.17	3.17	4.17	2.67	3.67	2.83	3.67	0.37	0.02	0.53	0.01	0.35	0.53	0.53	0.35
Day 21	3.33	3.50	3.17	3.50	2.33	3.17	2.50	3.17	0.24	0.001	1.00	0.01	0.62	0.15	1.00	0.62
Day 28	3.00	3.33	3.00	3.00	2.17	3.00	2.33	3.00	0.12	<0.0001	0.63	<0.0001	0.15	0.002	0.15	0.63
Day 35	2.83	3.00	2.83	3.00	2.17	3.00	2.17	3.00	0.12	0.0003	1.00	<0.0001	1.00	0.0003	1.00	1.00

A second objective of our study, was to evaluate the potential protective effect of PE in *E. coli* challenged piglets. Our results confirmed that *E. coli* challenge was successful in creating a chronic infection that negatively influenced growth performance in piglets. Comparative results have been reported with different infective challenge. *Salmonella typhimurium* challenge resulted in reduced piglet ADG, ADFI and gain:feed during week 3 after weaning (Turner et al., 2002). Our results did not show any PE x challenge interaction.

3.4.2. Fecal score and microbiological populations

Three piglets died at the beginning of the trial; all the others were clinically normal during the first period of the experiment (days 0 to 9) and at the time of the bacterial challenge. The *E. coli* challenge was successful in creating a clinical response: infected pigs showed diarrhea, while the non challenged piglets remained healthy during the course of the experiment.

The effects of PE administration on the fecal score of the piglets are shown in table 3.3. PE group showed a better fecal score from day 7 to day 35 ($P<0.05$) *E. coli* administration determined an higher fecal score from the day of the challenge to the end of the trial ($P<0.05$).

A treatment x challenge interaction was also shown from day 28: fecal score of challenged piglets supplemented with PE was higher than that of non-challenged PE animals, but still comparable to (or, during the last week, better than) that of non-challenged control piglets ($P<0.01$). The positive effects we found after plant extracts administration on fecal score of weaned piglets are in accordance with other results reported in literature. The amount of pigs with diarrhea and the diarrhea scores were decreased after dietary supplementation with blended essential oil (abstracts of *Cinnamomum verum*, *Origanum vulgare* spp., *Syzygium aromaticum*, *Thymus vulgaris* and *Rosmarinus*) when compared to the control group (Huang et al., 2010). Kyriakis et al. (1998) suggested that Origanum essential was effective at controlling post weaning diarrhea syndrome.

The effects of the administration of plant extracts on the microbiological populations in the feces are shown in table 3.4. PE group had lower *Enterobacteriaceae* ($P=0.009$) and *E.coli* ($P=0.02$) counts at the end of the trial (day 35); in addition, number of *Clostridia* in the feces was lower in the PE group at the end of the trial compared to control animals, although the difference was not significant ($P=0.17$). On the other hand, no effects of the administration of plant extracts on *Lactobacilli* count were recorded. Furthermore, piglets fed *ad libitum* had lower *Lactobacilli*, *Enterobacteriaceae* and *E.coli* counts at the end of the trial. *E coli* challenged piglets recorded higher *E.coli* count few days after the challenge (day 14) ($P=0.02$) and *Enterobacteriaceae* count at the end of the trial ($P=0.0005$). Finally, an interaction treatment x feeding regimen x challenge was evidenced at day 14 on the number of *Lactobacilli* ($P=0.03$), that was lower in control

challenged piglets fed restricted, and of *E.coli* (P=0.05), that was higher in control challenged animals fed *ad libitum*, and at the end of the trial on *Enterobacteriaceae* count (P=0.02), lower in PE non-challenged animals fed *ad libitum*.

Our results confirm that PE exert a modulation of the microbial gut population. Herbs and spices are well known to exert antimicrobial actions *in vitro* against important pathogens (Burt, 2004; Si et al., 2006; Özer et al., 2007). Among the active substances, phenolic compounds are the principal active components (Burt, 2004). The plant family of *Labiatae* has received the greatest interest, with thyme, oregano, and sage as the most popular representatives (Burt, 2004). The antimicrobial mode of action is considered to arise mainly from the potential of the hydrophobic essential oils to intrude into the bacterial cell membrane, disintegrate membrane structures, and cause ion leakage. High antibacterial activities are also reported from a variety of nonphenolic substances, for example, limonene and compounds from *Sanguinaria Canadensis* (Newton et al., 2002; Burt, 2004). Less results on the effects of green tea or pomegranate peel extracts are available. Tea polyphenols, which are constituents of tea extracts, have been shown to have antibacterial activities against human and animal disease-related bacteria, phytopathogenic bacteria and food-borne bacteria (Sakanaka et al. 2000). Their main constituent responsible for antibacterial action is EGCG, epigallocatechin gallate. Yoda et al. (2004) affirmed that, compared to the minimum inhibitory concentrations of EGCG against *S. aureus*, *S. epidermidis*, *S. hominis* and *S. haemolyticus* (50-100μg/ml), higher values (≥800μg/ml) were observed against Gram negative rods, including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia marcescens*, and difference was also observed between the binding abilities of EGCG with viable *S. aureus* (binding rate of 38.2%) and with *E. coli* (binding rate of 18.8%), due to the different component of the bacterial cells. Furthermore, Su et al. (2008) reported that green tea extract exerted an inhibitory effect on the growth of two major human pathogens *Staphilococcus aureus* and *Streptococcus pyogenes*, while, in contrast, *E. coli* and probiotic strains *L. acidophilus* L10, *B. animalis* B94 and *L. casei* L26 were unaffected by the presence of the green tea extract. Differently, Archana and Abraham (2011), in a comparative analysis of antimicrobial activity of green tea *Camellia sinensis* fresh leaves, commercial green tea leaves and dust tea against enteropathogens, found that the more sensitive organisms to fresh green tea extracts were *E.coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa*.

Taguri et al. (2006) have reported that the antimicrobial potency of polyphenols was dependent upon the bacterial species and was not correlated with the Gram stain. Moreover, Goto et al. (1999) who evaluated the effects of tea catechins on faecal contents and metabolites of elderly residents in a long-term care facility,

Table 3.4 - Effects of PE on fecal microbiological counts (log_{10} cfu/g) of challenged (+) and non-challenged (-) piglets fed *ad libitum* or restricted diet

Treatment:	Control				Plant extracts				SEM	P value						
Regimen:	*Ad libitum*		Restricted		*Ad libitum*		Restricted			Treatment	Regimen	Challenge	Treatment × Regimen	Treatment × Challenge	Regimen × Challenge	Treatment × Regimen × Challenge
Challenge:	-	+	-	+	-	+	-	+								
Lactobacilli																
Day 0	7.89	8.23	8.52	7.30	7.81	7.67	7.63	7.56	0.38	0.25	0.59	0.32	1.00	0.54	0.18	0.14
Day 11	10.95	11.55	10.92	9.42	11.34	11.05	10.64	11.34	0.49	0.27	0.07	0.73	0.21	0.34	0.43	0.03
Day 35	8.46	8.57	9.02	8.90	8.75	8.67	8.97	8.96	0.21	0.51	0.02	0.86	0.51	0.89	0.79	0.62
Clostridia																
Day 0	6.08	6.64	6.29	6.43	6.05	7.07	5.69	5.41	0.50	0.40	0.16	0.32	0.17	0.97	0.23	0.54
Day 11	2.47	2.52	2.37	2.20	2.23	2.21	1.96	2.11	0.40	0.37	0.49	0.99	0.96	0.83	0.96	0.73
Day 35	2.30	3.28	2.20	2.42	2.40	2.19	1.96	2.10	0.40	0.17	0.19	0.32	0.71	0.27	0.72	0.33
Enterobacteriaceae																
Day 0	6.60	7.57	7.48	6.63	7.40	7.17	5.99	6.88	0.54	0.58	0.25	0.61	0.29	0.73	0.65	0.06
Day 11	7.88	8.90	7.81	7.92	8.12	8.01	7.55	8.27	0.54	0.72	0.38	0.26	0.62	0.73	0.96	0.26
Day 35	4.92	6.21	6.38	6.36	4.63	5.28	4.97	6.52	0.32	0.009	0.001	0.0005	0.98	0.31	0.65	0.02
E.Coli																
Day 0	6.35	6.13	6.53	6.81	6.03	6.26	5.91	6.03	0.45	0.21	0.69	0.74	0.34	0.82	0.76	0.62
Day 11	4.86	6.94	5.36	6.17	6.42	5.85	4.52	6.29	0.62	0.89	0.33	0.02	0.50	0.34	0.55	0.05
Day 35	4.17	4.32	5.27	5.19	2.64	3.69	4.36	4.53	0.53	0.02	0.004	0.39	0.70	0.45	0.46	0.67

Table 3.5 - Effects of plant extracts supplementation on ileum of challenged (+) and non-challenged (-) piglets fed restricted diet

Treatment :	Control		Plant extracts		P value
Challenge:	-	+	-	+	
Villus height (V; µm)	361.34 ± 11.09	355.12 ± 11.09	350.82 ± 11.09	374.53 ± 11.09	0.47
Crypt depth (C; µm)	295.92 ± 7.16	285.52 ± 7.16	277.14± 7.16	305.96 ± 7.16	0.05
V:C	1.23 ± 0.04	1.26 ± 0.04	1.27 ± 0.04	1.24 ± 0.04	0.92
Total area (μm^2)	457192 ± 40864	441239 ± 40864	357818 ± 40864	403213 ± 40864	0.34
Cortex area (μm^2)	169184 ± 7293	169363 ± 7262	153395 ± 7360	173025 ± 7241	0.27
Medulla area (μm^2)	143927 ± 7967	41634 ± 7939	153759 ± 8020	140353 ± 7923	0.64
Corona area (μm^2)	102896 ± 6872	105092 ± 6847	109381± 6920	102799 ± 6833	0.90
Lymphatic Follicles Number/mm^2 of mucosa	1.44 ± 0.10	1.52 ± 0.10	1.44 ± 0.10	1.45 ± 0.10	0.94
Macrophages number/mm^2 of mucosa	105.19 ± 14.80	174.61± 14.80	126.66± 14.80	128.56± 14.80	0.02

Values are means ± SEM (n=120 for villus height, crypt depth and V:C; n=72 for Lymphatic Follicles Number; n=120 for Lymphatic Follicles Total area, Cortex area, Medulla area, Corona area; n=192 for Macrophages).

demonstrated that consumption of green tea selectively promoted the growth of *Bifidobacterium* and *Lactobacillus* in the gut wall.
Concerning the effects in swine, however, the few studies available have sometimes failed to demonstrate the efficacy of phytogenic compounds on shedding of specific pathogens. Hagmüller et al. (2006), giving Thymi Herba (*Thymus vulgaris*, rubbed) as feed additive to weanling piglets, didn't find significant differences in the shedding of haemolysing *E. coli*; Jugl-Chizzola et al. (2005) have found no differences concerning isolation of haemolytic *E. coli* serotypes in an *in vivo* trial. However, the Authors investigated in addition the antibacterial activity of the essential oil of Thymi herba against 39 haemolytic *E. coli* isolated from the same weaners *in vitro*, and they showed antibacterial activity against all haemolytic *E. coli* investigated, suggesting possible different mechanisms or interactions that could happen *in vivo*. In broilers, instead, some studies demonstrated *in vivo* antimicrobial efficacy of essential oils against *Escherichia coli* and *Clostridium perfringens* (Mitsch et al., 2004; Jamroz et al., 2006). Overall, our results and the available literature suggest a possible antimicrobial potential of phytogenic compounds in vivo that needs more investigations.

3.4.3. Ileum histology evaluation and histometry

The effect of PE on ileum histological parameters was examined considering only the restricted feeding group, challenged and non-challenged.
All the slaughtered piglets showed a moderate to severe degree of catarrhal enteritis, chronic in type, except animals of PE challenged group, which showed a slight to moderate degree of catarrhal enteritis, chronic in type (Fig. 2-5).
The histometry results are summarized in Table 3.5. The ileum crypts were deeper in PE challenged piglets in comparison with PE non-challenged animals (P=0.05), thus suggesting a possible reparative action of the plant extract on the small intestinal mucosa following the *E. coli* challenge. The number of mucosal macrophages was higher in Control challenged animals than in all the others three groups (P=0.02). In particular, the number of mucosal macrophages in PE challenged piglets was comparable to that one identified in Control non-challenged animals. On the contrary, the other studied parameters (villus height, V:C ratio, number of lymphatic follicles, area of lymphatic follicles and theirs compartments: cortex, medulla, corona) were not influenced by PE administration. Hence, despite the presence of a moderate to severe chronic inflammatory status in the ileum of all the studied animals. Overall, our results could confirm the possible protective functional role of the plant extract after a bacterial challenge.
A wide range of spices, herbs, and their extracts are known from medicine to exert beneficial actions within the digestive tract and morphological changes in gastrointestinal tissues caused by phytogenic feed additives may provide further

information on possible benefits. Associated with weaning, thereare marked changes to the histology and biochemistry of the small intestine, such as villous atrophy and crypt hyperplasia, which cause decreased digestive and absorptive capacity and contribute to post-weaning diarrhea (Pluske et al., 1997); hence, in this context, a better villus structure may enhance nutrient uptake.

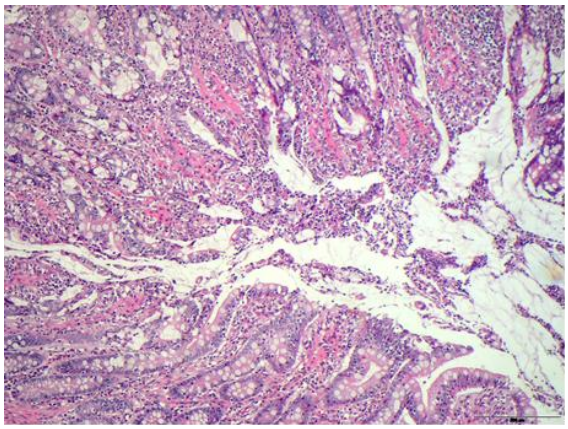

Fig. 2 - Ileum of a control non-challenged animal (HE, 100x).

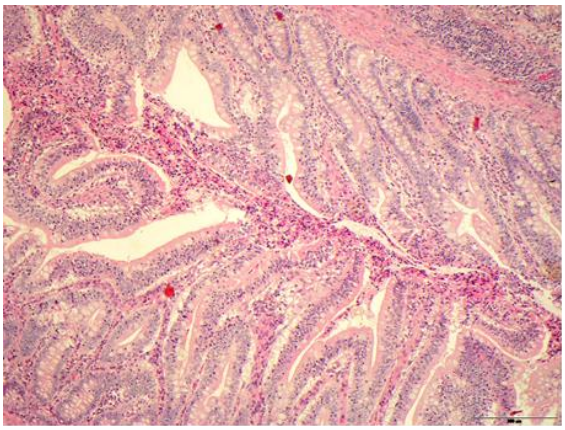

Fig. 3 - Ileum of a control challenged animal (HE, 100x).

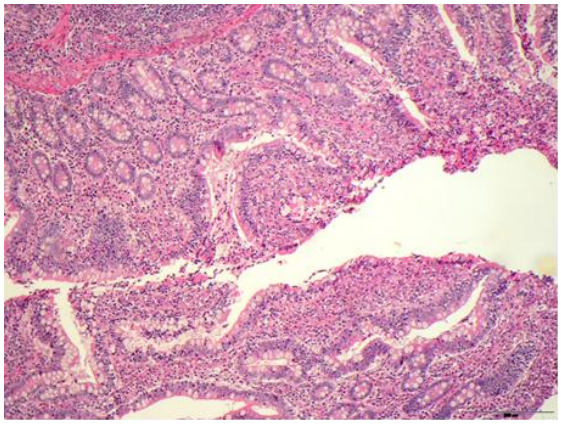

Fig. 4 - Ileum of a PE non-challenged animal (HE, 100x).

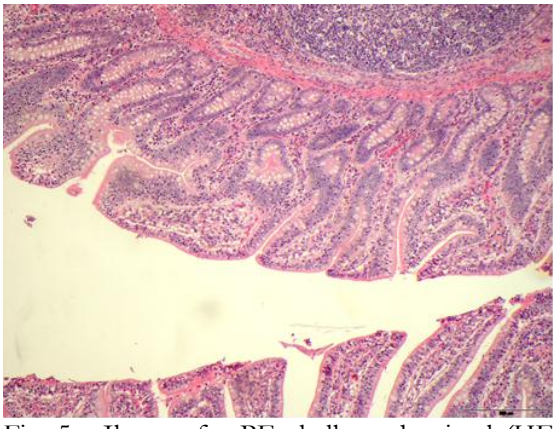

Fig. 5 - Ileum of a PE challenged animal (HE, 100x).

However, the available literature does not provide a consistent picture on the possible benefits of this substances on gut morphology and development and available reports have shown some inconsistent results. Namkung et al. (2004) found no treatment effects on intestinal morphology after the inclusion in the diet of 0.75% herbal extracts (containing cinnamon, thyme and oregano extract), although there was numerically higher proliferation of goblet cells in crypts and lower crypt depths. Nofrarias et al. (2006), and similarly Manzanilla et al. (2006), didn't evidence any differences in villus height and crypt depth in the proximal jejunum, ileum, and colon, but a reduction of the counts of intraepithelial lymphocyte (which are mainly T cells) in the proximal jejunum of animals fed

with 5% carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin, suggesting a possible inhibition of activation or proliferation of T cells and a modulation of their differentiation. Differently, Fang et al. (2009) evidenced an increase of the villus height in the duodenum, jejunum and ileum and a decrease of the crypt depth in response to dietary supplementation of *Acanthopanax senticosus* extract. Sehm et al. (2007) found no effect on the gut morphology in the ileum after administration of apple pomace or red-wine pomace, but a significant influence on the GALT (gut-associated lymphoid tissue): the ileum incorporating Peyer's patches were 21% enlarged in the control group, and this size increase indicates an activation of the Peyer's patches and the GALT activation in the PE lacking feeding regimen.
In our study, we didn't evidence a difference in the lymphatic follicles number, but an higher number of mucosal macrophages in control challenged animals (P=0.02). These results suggest that dietary plant extracts may increase the animal's ability to cope with stress, reducing the effects of *E.coli* challenge. And in fact, our results also showed a lower degree of inflammation in the ileum of PE challenged group, suggesting that dietary supplementation with plant extracts may modulate immune function (Kong et al., 2007b), thereby reducing the inflammation in the small-intestinal mucosa that often occurs in weanling piglets. These results do not allow for conclusions to be drawn on the relevance of changes in intestinal morphology in view of a growth promoting potential of phytogenic feed additives, however our observations could give further support to the hypothesis that phytogenic feed additives may stabilize gut structure and functions.

3.4.4. Plasma antioxidant parameters

The effect of PE administration on plasma antioxidant parameters are shown in table 3.6. Piglets receiving the administration of plant extract had higher value of GSH-Px at day 6 compared to control animals (P=0.02), tended to have lower value of malondialdehyde (MDA) at day 6 (P=0.07) and higher value of T-AOC at the end of the trial (P=0.07). No effect after PE administration was evidenced for the other tested parameters. Piglets fed restricted diet had a higher value of SOD at the end of the trial (P=0.0002). *E.coli* challenge strongly increased GSH-Px value at day 19 (P=0.0001). and tended to increase it at the end of the trial (P=0.08). Moreover, in challenged animals higher value for MDA at the end of the trial was evidenced (P=0.04). Finally, our results also showed a treatment x challenge interaction for T-AOC value at day 19, with lower value in control challenged animals (P=0.02).
The antioxidative status of an animal depends on many different factors. The animal itself represents a homeostatic system with the available enzymes. With the feed it ingests nutrients with a variable potential for oxidation, the

polyunsaturated fatty acids (PUFA) representing the highest risk. With the feed it also ingests substances like iron, copper or phytase that can catalyze the nutrient oxidation. It has been generally recognized that SOD and GSHPx are main antioxidant enzymes in scavenging the oxygen free radical; consequently, total antioxidant capacity (TAOC), superoxide dismutase (SOD) activity and glutathione peroxidase (GSH-Px) activity are the main parameters to assess oxidative status (Wang et al., 2008). Malondialdehyde is usually formed as an end product of lipid peroxidation and therefore the extent of lipid peroxidation by reactive oxygen species can be monitored by MDA levels. Our results indicate that PE administration could improve the oxidative status of piglets and allow piglets to better cope with an oxidative stress. This confirms the antioxidative properties of herbs and spices well described in literature(e.g., Craig, 1999; Wei and Shibamoto, 2007). Among a variety of plants bearing antioxidative constituents, the volatile oils from the *Labiatae* family (mint plants) have been attracting the greatest interest (especially products from rosemary), whose antioxidative activity arises from phenolic terpenes, but also plant species from the families of *Zingiberaceae* (e.g., ginger and curcuma) and *Umbelliferae* (e.g., anise and coriander), as well as plants rich in flavonoids (e.g., green tea) and anthocyans (e.g., many fruits) are described as exerting antioxidative properties (Windisch et al., 2008).

In particular, concerning pomegranate, its peel contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid. It has been used in the preparation of tinctures, cosmetics, therapeutic formulae and food recipes. The presence of antioxidants has been reported in pomegranate juice (Gil et al., 2000); Negi et al. (2003) showed that the pomegranate peel extracts have both antioxidant and antimutagenic properties and may be exploited as biopreservatives in food applications and neutraceuticals.

Green tea (*Camellia sinensis*) and its extracts, rich sources of catechins such as Epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate and epigallocatechin (EGC), are known to possess potent antioxidant activity (Leung et al., 2001) and may thus directly quench free radicals or regenerate endogenous antioxidants like vitamin E: e.g., i*n vitro* these catechins spared α-tocopherol by inhibiting its free radical-mediated oxidation and by regenerating it from its α-tocopheroxyl radical (Frank et al., 2006). Tea catechins have been found to be efficient scavengers of free radicals in a number of *in vitro* systems (Higdon and Frei, 2003). The ability of a compound to act as a free radical scavenger is related to: 1) its one-electron reduction potential, a measure of reactivity of antioxidants as hydrogen or electron donors: a lower reduction potential indicates that lower energy is required for hydrogen or electron donation and is one factor in determining antioxidant activity; EGCG and EGC have lower reduction potentials than

Table 3.6 - Effects of PE administration on antioxidant parameters of challenged (+) and non-challenged (-) piglets fed *ad libitum* or restrided diet

Treatment:	**Control**				**Plant extracts**				**SEM**	**P value**						
Regimen:	***Ad libitum***		**Restricted**		***Ad libitum***		**Restricted**			**Treatment**	**Regimen**	**Challenge**	**Treatment × Regimen**	**Treatment × Challenge**	**Regimen × Challenge**	**Treatment × Regimen × Challenge**
Challenge:	-	+	-	+	-	+	-	+								
GSH-Px,U																
Day 0	510.2	522.1	510.0	506.4	557.2	553.2	487.5	568.4	31.17	0.19	0.43	0.34	0.66	0.44	0.44	0.26
Day 6	660.5	515.7	677.0	558.2	730.3	629.3	684.6	603.0	33.77	0.02	0.89	<0.0001	0.18	0.40	0.64	0.95
Day 19	563.7	664.3	498.9	648.5	529.6	621.3	518.1	613.5	36.36	0.37	0.34	0.0001	0.55	0.54	0.61	0.66
Day 35	575.1	623.0	523.7	585.5	549.8	610.9	590.0	575.0	31.11	0.83	0.34	0.08	0.30	0.47	0.48	0.31
SOD, U/ml																
Day 0	124.7	134.0	130.5	128.5	129.4	136.6	123.6	133.5	3.76	0.62	0.42	0.03	0.39	0.36	0.42	0.19
Day 6	113.6	122.3	116.0	123.4	101.2	128.4	117.7	126.0	5.31	0.89	0.25	0.001	0.49	0.20	0.19	0.25
Day 19	108.0	108.9	108.0	112.0	110.9	111.9	119.2	110.9	3.34	0.10	0.28	0.79	0.66	0.20	0.51	0.19
Day 35	103.9	109.6	117.3	114.3	104.9	112.1	117.1	116.8	2.96	0.49	0.0002	0.26	0.89	0.62	0.06	0.89
MDA, nmol/ml																
Day 0	4.60	3.82	3.75	4.81	4.48	4.04	3.56	3.87	0.47	0.45	0.49	0.91	0.36	0.76	0.06	0.42
Day 6	3.09	3.84	2.67	3.92	3.10	2.76	3.43	2.04	0.42	0.07	0.53	0.82	0.97	0.003	0.65	0.20
Day 19	2.12	2.24	2.31	2.39	2.07	2.42	1.95	2.22	0.25	0.58	0.99	0.24	0.34	0.54	0.87	0.96
Day 35	0.71	1.46	1.11	1.61	0.67	1.31	1.30	1.09	0.28	0.52	0.24	0.04	0.85	0.32	0.17	0.46
T-AOC, U/ml																
Day 0	8.72	6.51	4.75	5.28	7.04	3.52	5.57	5.42	1.43	0.37	0.25	0.20	0.18	0.63	0.14	0.88
Day 6	7.60	7.50	5.94	6.74	5.29	7.60	6.28	7.18	1.35	0.71	0.63	0.31	0.44	0.51	0.89	0.55
Day 19	3.42	2.38	4.49	2.30	3.14	4.01	1.70	3.36	0.84	0.87	0.64	0.77	0.21	0.02	0.88	0.42
Day 35	4.82	4.67	3.21	3.81	5.74	6.14	7.80	3.40	1.25	0.07	0.38	0.32	0.62	0.22	0.26	0.13

Table 3.7 - Effects of PE administration on intestinal inflammatory parameters of challenged (+) and non-challenged (-) piglets fed restricted diet

Treatment:	**Control**		**Plant extracts**		**SEM**	**P value**		
Challenge:	-	+	-	+		**Treatment**	**Challenge**	**Treatment × Challenge**
MPO, U/g	1.11	2.31	1.66	2.26	0.24	0.31	0.001	0.22
NO content, µmol/gprot	10.85	8.62	11.14	9.62	1.47	0.67	0.22	0.81
iNOS content, U/mgprot	0.62	0.65	0.73	0.71	0.06	0.12	0.94	0.63

vitamin E, suggesting they are superior electron donors to vitamin E; 2) the rate of its reaction with free radicals in a given system (scavenging rate constant) and the stability of the resulting antioxidant radical: tea catechins were found to be efficient scavengers of singlet oxygen ($^1O_2$), $O_2^{\cdot -}$, ·OH and peroxyl radicals (·OOH).

Numerous human studies have reported health benefits associated with tea consumption (Higdon and Frei, 2003). Less data on the effects of tea extracts and, in general, herbs or spices, on farm animals are present in literature (in particular, the effect of green tea catechins on biomarkers of oxidative stress and antioxidant status has yet not been systematically studied in pigs). In our study, we evidenced a significant increase of GSH-Px at day 6, a lower value of MDA at day 6 and a higher value of T-AOC at the end of the trial in PE supplemented animals. The increased activity of GSH-Px would subsequently enhance the capacity of piglets to clear out the oxygen free radicals. Consistent with that, T-AOC tended to be enhanced and MDA concentration in the serum tended to be reduced by inclusion of PE in the diet; hence, the reduced serum MDA level in PE-supplemented as compared with control animals indicated that lipid peroxidation was reduced by PE via enhancing antioxidative action.

Similarly to our results, supplementation of ginger increased activities of total superoxide dismutase and glutathione peroxidase and reduced concentrations of malondialdehyde in broilers (Zhang et al., 2009), while administration of *Forsythia suspensa* determined greater total antioxidant capacity and superoxide dismutase activity and lower malondialdehyde activity in the serum of broilers (Wang et al., 2008). Moreover, sweet chestnut wood extract (whose active components are hydrolysable tannins, such as ellagitannins) significantly decreased the 24 h urinary excretion of MDA, whereas was not able to lower the concentration of MDA in plasma of growing pigs (Frankič and Salobir, 2011). Plant extracts may also have the potential to improve the oxidative stability of animal-derived products. Mason et al. (2005) found that lipid oxidation was highest in meat from pigs fed diets with great energy restriction and lowest in meat from pigs fed diets supplemented with green tea catechins.

In the current study, challenge led to a significant higher production of MDA generation in plasma and higher value of GSH-Px. It has ben reported that bacterial lipopolisaccharides are able to cause production of free radicals, and consequently increase the oxidant status. Türközkan et al. (2005) found a significant increase in MDA levels after *E. coli* administration. It is believed that NO itself or highly reactive and cytotoxic reactive nitrogen species formed from NO and superoxide anion account for cellular injury and cellular dysfunction. The higher value of GSH-Px after the challenge coul be related to the activation of the antioxidant system of the host to compensate this imbalance.

Dietary restriction may also have an impact. For example, decline in cytosolic glutathione, GSH-reductase, GSH-transferase, GSH- peroxidation and catalase are each modulated differentially by dietary restriction.Moreover, a dietary restriction attenuates declines in mitochondrial oxidation of malondialdehyde, suggesting a more efficient removal of oxidative byproducts in restricted rats (Yu, 1996).
All together, these results demonstrated that supplementation of PE was able to improve the antioxidant status of weaned piglets.

3.4.5. Intestinal inflammatory parameters

The effect of PE supplementation on intestinal inflammatory parameters are shown in table 3.7. No effect on myeloperoxidase (MPO), nitric oxide (NO) or inducible nitric oxide synthase (iNOS) contents were evidenced after PE administration, while the challenge with *E. coli* determined a higher value of MPO (P=0.01).
The ileum is frequently involved in diseases related to bacterial endotoxins: during the early phase of infection, endotoxin and other bacterial by-products activated the primed neutrophiles, monocyte and macrophages, initiating a signal transduction cascade leading to the release of a number of pro-inflammatory cytokines and inducible enzymes (such as iNOS, that consequently produces NO), which would lead to a release of massive amounts of oxidants and cause further tissue damage. The iNOS is calcium independent and inducible by bacterial endotoxins and cytokines in various cell types including macrophages, hepatocytes and endothelial cells. Nitric oxide (NO) is cytotoxic at high concentrations, and thus the level of NO generated by macrophages after iNOS induction contributes to their ability to kill intracellular and extracellular microorganisms. But the iNOS-dependent synthesis of large amounts of NO may be dangerous for the host (Tsukahara et al., 2001). Consequently, the reduction of NO production is important in some aspects (Napolitano et al., 2005): massive amounts of NO generated by activated macrophages may be responsible not only for immunological defense against tumor cells and pathogens, but also may cause injury in cells, damaging several tissues. Furthermore, it is known that the simultaneous production of superoxide and NO results in the rapid formation of peroxynitrite by macrophages, responsible of diverse chemical reactions in biological systems, including lipid peroxidation, and promotes oxidative damage to macromolecules and tissues; finally, free radical and NO or their derivatives are the key denominators in carcinogenesis.
In response to microbial invasion, stimulated phagocytes secrete also MPO at inflammatory sites, where it generates a powerful reactive oxygen species, hypochlorous acid, at physiological chloride concentrations, that can in turn serve as a metal-independent oxidizing agent in vivo (Sugiyama et al. 2001). As

an important component in degranulation material of leukocytes, MPO exerts a critical activity in animal innate host defenses and is used as a marker of inflammation.
Diverse plant extracts have been reported to have anti-inflammatory activity (Kim et al., 2004): they possess antioxidative and radical scavenging activities. They could regulate cellular activities of the inflammation-related cells, mast cells, macrophages, lymphocytes, and neutrophils; they could inhibit histamine release from mast cells and others inhibit T-cell proliferation; they can modulate the enzyme activities of arachidonic acid metabolizing enzymes such as phospholipase A2 (PLA2), cyclooxygenase (COX), and lipoxygenase (LOX) and the nitric oxide (NO) producing enzyme, nitric oxide synthase (NOS). Napolitano et al. (2005) evidenced a strong inhibition of NO production in murine macrophages treated with brazilian plant extracts. Flavonoids, including catechins, contained in *Artocarpus gomezianus* have been found to efficiently scavenge NO *in vitro* (Sritularak et al., 2010). Oral administration of the antiinflammatory spice principles-capsaicin (from red pepper) and curcumin (from turmeric) lowered the paw inflammation in arthritic rats (Joe et al., 1997a). However, data on farm animals are inconsistent and the inhibitory activity found is not always exhibited *in vivo*. In our study, no effects of the administration of plant extracts were evidenced on the tested parameters but challenge with *E.coli* induced increase of MPO, indicating the involvement of inflammatory response.
Similarly, Steadman et al. (1988) have shown that human polymorphonuclear leukocytes challenged with defined strains of *E. coli* could release significant amounts of MPO.

3.5. Conclusions

In our study, PE supplementation improved growth performance in the last phase of the trial. These results were associated with fecal *Enterobacteriaceae* and *E.coli* reductions in PE group at the end of the trial. PE administration also resulted in a lower crypt depth in non-challenged piglets compared to challenged ones, suggesting a possible reparative action of the studied product on the small intestinal mucosa following the challenge. In addition, the number of mucosal macrophages in challenged piglets from PE group was similar to that one identified in control piglets, thus confirming the possible protective functional role of the plant extracts mixture after the bacterial challenge. Finally, dietary PE favourably affected the systemic antioxidant capacity by enhancing the GSH-Px activity and T-AOC and reducing MDA.
Hence, we can postulate that PE interact in the intestine with feed components, microbiota, and the mucosa in a very complex and dynamic way. The effect

could be greatest under an infectious pressure, such as occurs at certain ages, in certain husbandry conditions, and in certain regions. Therefore, the use of plant extracts may be useful in the prevention of postweaning diarrhea with an associated improvement in performance, but further studies are warranted to clarify the mechanisms underlying the role of dietary PE.

3.6. References

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CHAPTER 4

Dietary supplementation of mannanoligosaccharides in nutritionally stressed piglets: effects on gut health.

4. Dietary supplementation of mannanoligosaccharides in nutritionally stressed piglets: effects on gut health.

4.1 Abstract

The aim of the trial was to evaluate the effect of the administration of mannanoligosaccharides (MOS) on growth performance, microbial population in feces and cecum and potential alteration of intestinal histomorphometric and gene expression of some intestinal inflammatory parameters of piglets fed a low digestible diet. Forty-eight weaned piglets (6.72 ± 0.32 kg of BW, 24 d of age) were used in a 35-d experiment and randomly allotted to 2 dietary treatments: basal diet (Control) and basal diet + 0.2 % MOS. Growth performance were recorded weekly, fecal samples were collected at 0, 14 and 35 d. At the end of trial, 10 piglets from each group were slaughtered and intestinal samples were collected. Data were analysed by a General Linear Model (GLM) procedure of SAS. BW, ADG, ADFI were not influenced by MOS supplementation; FCR was lower in treated animals in the last 2 weeks ($P<0.05$). Mean fecal score was improved in MOS piglets ($P<0.01$). At the end of trial treated piglets had higher *Lactobacilli* fecal count ($P<0.05$). No difference was detected among groups for Coliforms, while lower *Clostridia* occurred on day 14 in MOS piglets ($P<0.05$). Intestinal villi height in the duodenum was higher in MOS than Control ($P<0.05$). MOS supplementation also led to significant increase of NO production in ileal mucosa ($P<0.05$); finally, MOS suppressed mRNA relative expression of pro-inflammatory genes for IL-1α, IL-1β, IL-6 and TLR2 ($P<0.05$), for TLR4 ($P<0.01$) and for TNF ($P<0.001$), while there was no effect on IL-10 and PPARγ expression. Results indicate that MOS supplementation improved feed efficiency and intestinal morphometry of piglets fed low digestible diet.

4.2 Introduction

Early weanling is the common practice in commercial swine farms nowadays; it is usually cause of gastrointestinal disturbances and an increased susceptibility to infection in piglets. To prevent gastrointestinal disorders and reduce mortality ratio feed-grade antibiotics have been used regularly in the past decades. Since the ban of antibiotics as feed additive in 2006, nutritionist and animal producers

in Europe are seeking for alternatives. Mannanoligosaccharides (MOS) are ones of the most widely studied oligosaccharides as alternatives to antimicrobials in swine diets; they derived from the outer cell wall of yeast and consist of a mannan and a glucan component. The mannans act as high-affinity ligands for the mannose- specific type-1 fimbriae of pathogenic bacteria such as *Escherichia coli* and salmonellae that may bind to the mannan component of MOS rather than adhere on the mucosal surface of the intestine being flushed from the intestinal tracts, enhancing in this way the health and growth performance of the piglets: MOS have been reported to result in better body weight gain and feed efficiency in piglets (Castillo et al., 2008; LeMieux et al., 2005) and to mediate immunomodulatory effects in gut-associated lymphoid tissue (Janardhana et al. 2009; Castillo et al., 2008). Sometimes, modest increases in growth rates of weanling pigs have been supported when MOS is added to the diet (Miguel et al., 2004). If the perceived action of MOS is as just described, the benefit of the product may be greater in the presence of greater disease challenges. In fact, there is support for a greater response to MOS in situations in which growth rate is slower (Miguel et al., 2004). Similarly, antimicrobials enhance growth and decrease mortality in young pigs, with even greater response under high-disease, stressful conditions (Cromwell, 2001).
The aim of the trial was to evaluate the effect of the administration of MOS on growth performance, villi height and crypts depth of gastrointestinal tracts, intestinal inflammatory responsive parameters and microbial population in faeces and cecum in piglets fed a low digestible diet in order to determine a nutritional stress in the intestine.

4.3 Material and methods

4.3.1 Animals and housing

The trial was performed at the Teaching and Research Zootechnical Center of the Faculty of Veterinary Medicine of the Università degli Studi di Milano, Lodi, Italy. Forty eight male piglets (Stambo HBI Dalland 40), 24 days of age (initial body weight 6.73kg±0.32), coming from the same herd and vaccinated at two days of age for *Mycoplasma hyopneumoniae* were used for the trial. All piglets were allocated in the same post-weaning room on slatted floor and had free access to water. Room temperature and ventilation were electronically controlled over a 24h period. Starting temperature was 28°C with a ventilation equal to 10m3/h/head. The temperature program decreased values of 1°C/week until 25°C reached at 28 days from the arrival of the piglets.
All animals in the experiment were randomly allotted to one of two dietary treatments at the arrival to the experimental facilities: Control (C), piglets fed a

basal diet, and Treated (MOS, Lallemand SAS, France), piglets fed the basal diet supplemented with Mannanoligosaccharides at a concentration of 0.2g/100g feed. The basal diet was a dry feed fed *ad libitum* and was formulated in order to provide a low digestibility and high protein content (Table 4.1). Each experimental group was composed by 12 pens (replicates), 2 piglets per pen. Treatments lasted 35 days, starting on day 24 of age until day 59 of age. Control animals were always fed first followed by animals of the treated group. Risk was reduced by carefully cleaning boots and other relevant equipment that might be contaminated.

Table 4.1 - Diet composition and chemical analyses.

Composition of basal diet (kg/100kg as fed)		**Chemical analyses (%DM)**	**Group**	
			Control	**MOS**
Wheat	33.80	Dry matter	89.08	89.65
Mais meal	23.15	Crude protein	22.33	22.11
Soybean meal	9.90	Ether Extract	4.82	4.90
Soy protein concentrate*	3.30	Crude fiber	3.05	2.97
Soybean oil	3.00	Ash	6.90	7.01
Calcium carbonate	1.50	ME (kcal/kg)	3,825	3,780
Dicalcium phosphate	1.10	DE(kcal/kg)	3,858	3,875
Animal fat	1.00			
HCl-Lysine	0.35			
Sodium chloride	0.30			
Vitamins+Minerals	0.30			
L-Threonine	0.15			
Sweetening (strawberry flavour)	0.10			
DL-Metionine	0.10			
Tryptophane	0.10			
Flavour	0.05			

*chemical composition: CP 65%, Lys 6.5%, Ash 7%, EE 1%, CF 4%.

4.3.2 Measurement of growth performance and sample collection

Individual piglets live weight was recorded on 0, 7, 14, 21, 28, 35 days on trial by an electronic scale (Ohaus ES100L). Feed intake was recorded weekly per pen.
The presence and severity of diarrhea was monitored using a 1-3 scoring system, as follows: 3 (normal feces), 2 (soft, moist feces), 1 (diarrhea).
Fecal samples from 12 piglets per group (1 piglet per pen) were collected at the beginning and subsequently on days 14 and 35. Samples were refrigerated and immediately analysed for Anaerobic, Aerobic, Coliforms, *Lactobacilli*, and *Clostridia* bacteria populations. Aerobic and anaerobic faecal populations were performing using PCA (plate count agar) with an incubation time of 48h at

37°C; Coliforms were determined by VRBA (Violet Red Bile Agar) with an incubation time of 24h at 37°C. *Lactobacilli* faecal content was determined by MRSA (*Lactobacillus* Agar) with an incubation time of 72h at 37°C (10% CO_2) and Clostridia procedure had an incubation time of 48h at 37°C using TSC (Tryptose sulphite cycloserine agar).

At the end of trial, 10 animals per group were sacrified. At slaughtering, carcass weight and dressing percentage were individually determined. Individual cecum content was collected and analyzed for microbial populations as for previously described fecal analyses. Individual samples of duodenum, jejunum, ileum, and proximal colon, were collected, stored and subsequently analyzed for villi height, villi width, crypts depth, crypts width, villi:crypts ratio, tunica propria thickness, and goblet cells for each gut district: the segments were immediately fixed in 4% para-formaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Serial microtome sections (4 μm-thick) were obtained from each sample, were processed in low-melt paraffin and stained with hematoxylin and eosin. The 10 straightest villi and their associated crypts from each segment were measured. The villus height was measured from the tip to the base, and then the crypt depth was measured from the base of the villus to the base of the crypt. The villus height-to-crypt depth ratio (VCR) was calculated. The number of mucosal macrophages in the diffuse lymphatic system and the number and size of lymphatic follicles of Peyer's patches were also calculated. Moreover ileum mucosa was obtained by scarification for total protein, myeloperoxidase (MPO), nitric oxide (NO), total nitric oxide (NOS) content, and inducible nitric oxide synthase activity (iNOS) determination by commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China): three 5-cm segment from the ileum were collected, opened longitudinally and cleaned with PBS. Intestinal mucosa was collected by scratching with a glass slide for the next step. Nine milliliters of 4°C PBS was added to 1 g of intestinal mucosa, followed by homogenization. The homogenates were centrifuged (4,000 × g for 5 min at 4°C), and supernatant fluid was used following the manufacturer instructions.

Intestinal samples from proximal part of ileum were also obtained for the determination of gene expression profile; the samples obtained, approximately 10 mg, were stored frozen in RNAlater (Invitrogen) and subsequently analyzed for TNF, IL6, IL1α, IL1β, TLR2, TLR4, PPARγ, IL10 and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA was double extracted with TRIzol Reagent® (Invitrogen), purified with a commercial kit (Macherey-Nagel), and quantified using a Nanodrop (Thermo Scientific). Specific mRNAs were amplified and quantified using the iScriptTM One Step RT-PCR for Probes reagent (Bio-Rad), according to the manufacturer's instructions. RT Q-PCR analysis was performed with CFX384

Real-Time System (BIO-RAD, CA, USA). The thermal protocol was: 50°C for 10 minutes for reverse transcription and then 95°C for 10 seconds/60°C 30 seconds for 40 cycles. Proper amplification was checked by performing a melting curve of each PCR product at the end of the last cycle of amplification. Primers and probes for real-time qPCR were purchased from Applied Biosystems. Oligonucleotides used for real-time qPCR. The relative expression levels were determined by normalizing the Ct of the indicated mRNA with the Ct of S. scrofa beta-actin, here used as the housekeeping gene for normalization, and their primers and probe were designed with IDT software available online, optimized to work in a one-step protocol and were synthesized by Eurofin MWG Operon. Ct values of beta-actin were comparable in all samples from different animal groups treated with different diets.

4.3.3 Statistics

Data relative to live body weight (BW), average daily gain (ADG), feed conversion ratio (FCR) and feed intake were analyzed by a General Linear Model (GLM) procedure of SAS (2006) including the effect of treatment. The pen was considered as the experimental unit. Slaughtering performance were analyzed by a General Linear Model (GLM) procedure od S.A.S. (2006) including the effect of treatment and considering the piglet as the experimental unit. One subject from MOS group died during the trial, thus it was not considered for performance analyses, moreover the corresponding pen was removed from the statistical evaluation on feed intake and feed conversion rate. Data relative to slaughtering day were analyzed by a General Linear Model (GLM) procedure of S.A.S. (2006).

Histometric data about villi height, crypt depth, V:C ratio, villi width, crypts width and tunica propria thickness were analyzed by GLM of SPSS package. The model included treatment as fixed effect and piglets as random effect. The data were presented as least squared means ± SEM.

4.4 Results and discussion

4.4.1 Growth performance and health status

Results of growth performance are presented in table 4.2. Feed intake, BW, average daily gain, carcass weight and dressing percentage were not influenced by MOS supplementation. FCR was significantly improved in treated animals in the last two weeks of trial ($P<0.05$).

Table 4.2 - Effects of MOS administration on growth and slaughtering performance

Item	Days	Group		SEM
		Control	MOS	
Body weight (kg)	0	6.63	6.82	0.32
	7	7.35	7.43	0.36
	14	8.23	8.51	0.43
	21	10.95	10.97	0.56
	28	13.51	13.82	0.58
	35	16.84	17.32	0.71
ADG (kg/d)	0-7	0.10	0.09	0.01
	7-14	0.13	0.15	0.02
	14-21	0.39	0.35	0.03
	21-28	0.37	0.41	0.02
	28-35	0.47	0.50	0.03
Feed intake (kg/d/pen)	0-7	2.80	2.96	0.20
	7-14	4.58	5.24	0.29
	14-21	7.62	7.32	0.26
	21-28	9.32	8.28	0.64
	28-35	13.10	12.27	0.50
FCR	0-7	2.63	2.56	0.38
	7-14	2.81	3.24	0.53
	14-21	1.46	1.51	0.07
	21-28	1.85[a]	1.48[b]	0.13
	28-35	2.12[a]	1.81[b]	0.10
Carcass weight (kg)		13.61	13.47	0.74
Dressing percentage (%)		76.93	75.90	0.97

[a,b] Different superscript letters within rows indicate differences at $P < 0.05$.

The total number of treated piglets with antibiotic was equal in both experimental groups, while one death occurred in MOS group.
Mean days on diarrhea were similar in both groups (5.25 *vs* 4.81 for MOS), while treated animals showed a better fecal score compared to Control.
Analysis of the literature shows that the effect of MOS on piglet growing performance are somewhat contrasting: Lazarevic et al. (2010) found, in addition to increased piglet blood plasma IgG concentrations by an average of 23% (that, according to the authors, could be used as a gauge of increased health status, which translated into improved growth performance), also an improvement in body weight by 9% at 30 days of age (8.18 kg for MOS group vs. 7.5 kg for Control group); similarly, Davis et al. (2004) reported that pigs fed diets supplemented with mannans had greater ($P<0.05$) ADG and Gain:Feed than pigs fed the basal diet from d 0 to 14 post-weaning and, although growth performance were unaltered as a result of dietary treatment from d 14 to 21

post-weaning, the improvement in ADG and Gain:Feed was maintained in the overall experiment ($P<0.05$) and was reflected by the greater ($P<0.05$) body weight of pigs fed mannans on d 14 and 21 of the experiment.

Table 4.3 - Effects of MOS administration on health status during the trial

Item	Group		SEM
	Control	MOS	
Piglets treated (n°)	13	13	-
Piglets died (n°)	-	1	-
Days of treatment*	4.4	4.6	-
Days of diarrhea**	5.25	4.81	-
Fecal score***	1.99^{A}	2.09^{B}	0.04

* mean value related to 13 animals in both group
** mean value related only to treated animals
*** Fecal Score: 3 (normal feces), 2 (soft, moist feces), 1 (diarrhea)
^{A,B} Different superscript letters within rows indicate differences at $P< 0.01$.

In present study no significant effect of MOS administration was observed in feed intake, BW, average daily gain and slaughtering performance, in accordance with Sauerwein et al. (2007). The Authors reported that the effects of cell wall extracts from *Saccharomyces cerevisiae* dietary administration on growth performance were mostly negligible and, if anything, numerically lower gains were observed; similarly, Nochta et al. (2009), did not observed any significant difference among treatments in ADG, feed intake or FCR, stating that the older weaning age (28 days) and the extremely good conditions could be the reason for the lack of statistically proved growth promoting effects. Indeed, it is reported that MOS and most beneficial additives (Hooge, 2004) are most effective under disease and stress conditions, such as extremes of ambient temperature, crowding, and poor management, which are invariably present in commercial production.
In the present study, animals were fed a low digestible diet in order to exacerbate the weaning nutritional stress. Nevertheless, growth performance did not differ smong the groups, except feed conversion rate, that was found to be improved in MOS treated piglets compared to controls during the last two weeks on trial, suggesting a better capacity of treated animals to manage the nutritional stress; that is in accordance with Castillo et al. (2008), which found that average daily gains and ADFI were not affected by treatment, whereas the Gain:Feed was improved by the addition of MOS compared to the pigs fed the control diet during the starter period; to the Authors, the effects appeared to be due to decreased jejunal numbers of *Eterobacteria,* associated with an increase in

villus:crypt ratio. In addition, the Authors found also a better fecal score for the entire period after administration of MOS.

4.4.2 Microbiological populations

The effect of the administration of MOS on microbial populations of feces and cecum are shown in table 4.4.

Table 4.4 - microbiological content of feces and cecum

Item (log_{10})	Group		SEM
	Control	MOS	
Anaerobic bacteria			
Day 0	9.42	9.01	0.16
Day 14	9.04	9.50	0.20
Day 35	8.77^{b}	9.29^{a}	0.14
Slaughtering (cecum)	8.96	8.88	0.70
Aerobic bacteria			
Day 0	9.06	8.80	0.53
Day 14	9.45^{b}	9.97^{a}	0.29
Day 35	8.71^{B}	9.50^{A}	0.36
Slaughtering (cecum)	8.82	8.93	0.07
Coliforms			
Day 0	8.20	8.26	1.41
Day 14	6.70	7.53	1.56
Day 35	6.43	6.70	0.81
Slaughtering (cecum)	7.12	7.16	0.46
Lactobacilli			
Day 0	9.41	9.44	0.46
Day 14	9.51^{b}	10.02^{a}	0.30
Day 35	8.86^{b}	9.39^{a}	0.27
Slaughtering (cecum)	8.87	8.87	0.05
Clostridia			
Day 0	5.57	5.49	1.23
Day 14	3.83^{a}	3.00^{b}	0.68
Day 35	3.28	3.00	0.27
Slaughtering (cecum)	3.23	3.11	0.34

^{a,b} Different superscript letters within rows indicate differences at $P< 0.05$.
^{A,B} Different superscript letters within rows indicate differences at $P< 0.01$.

MOS piglets had higher anaerobic bacteria fecal count at the end of trial ($P<0.05$), while aerobic content was found to be higher on day 14 ($P<0.05$) and 35 ($P<0.01$). No differences were detected among the experimental groups for

Coliforms. Higher *Lactobacilli* content was determined in MOS animals during the trial ($P<0.05$), while *Clostridia* fecal count was decreased in MOS group on day 14 respect to Control ($P<0.05$). No differences were found in microbial cecal count at slaughtering among the two groups.
Mannanoligosaccharides have been proposed to promote growth by modifying the gastrointestinal ecosystem and reducing intestinal pathogen colonization: in swine and poultry, it appears that MOS attach to mucosa-binding proteins on the cell surface of some bacteria, preventing colonization of intestinal epithelium. However, results are somehow variable, and factors contributing to this variability in the effects on population of beneficial bacteria in the gut may include differences in experimental conditions, diet formulation, seasonal effects and health status.
In the present study, we found a lower *Clostridia* fecal content in MOS group; in agreement with Kim et al. (2011) a significantly lower population of *C. perfringens* in chickens fed 0.05% MOS was also found. Castillo et al. (2008) showed a lower concentration of Coliforms in the feces of pigs fed diets with MOS, suggesting that the decline in their numbers is important because of their relationship to outbreaks of post-weaning diarrhea; however, *Lactobacilli* counts in jejunum digesta did not differ among diets. In the present study, no effect on the Coliforms concentration was evidenced, whereas we found a higher concentration of *Lactobacilli* in feces of MOS piglets at the end of the trial (day 35).
The lactobacilli:enterobacteria ratio has routinely been used as an indicator of the gut health, with an increase in the ratio considered to be beneficial. An inhibition of enterobacteria and/or an improvement of *Lactobacilli* could prevent or decrease the severity of diarrhea that appears after weaning (Melin et al., 2004).
Similarly to our work, Khalaji et al. (2011), did not find any significant effect of MOS on *E.coli* ceca population of broiler chicks; also Poeikhampha and Bunchasak (2011), did not observe any difference in *E. coli* counts of caecum or rectum digesta in nursery pigs fed MOS.

4.4.3. Gut histometry and immunohistochemistry

The effects of MOS administration on intestinal histometry and immunohistochemistry are shown in table 4.5.
The experimental treatment affected the intestinal villi height in the duodenum, which was higher in MOS treated piglets ($P<0.05$); villi width, crypths depth and width, villi:crypts ratio and number of goblet cells in lamina propria didn't show any significant difference among the treatments. No differences were detected for the tested parameters in jejunum, ileum or colon.

Table 4.5 - Effects of MOS treatment on gut histometry and immunohistochemistry

Item	Control	MOS
DUODENUM		
Villi height (μm)	296.60 ± 7.82[b]	328.78 ± 7.82[a]
Villi width (μm)	125.19 ± 3.92	133.30± 3.72
Crypts depth (μm)	469.30 ± 7.37	468.59 ± 11.26
Crypts width (μm)	46.24 ± 1.01	48.06 ± 0.97
V:C ratio	0.64 ± 0.02	0.70 ± 0.02
Tunica propria (μm)	511.90 ± 8.64	519.15 ± 12.80
Goblet cell (nr/villi height)	11.68 ± 1.63	11.68 ± 0.74
Goblet cell (nr/crypts depth)	30.21 ± 0.96	30.21 ± 1.27
JEJUNUM		
Villi height (μm)	287.61 ± 7.48	293.34 ± 6.88
Villi width (μm)	115.53 ± 3.46	115.58 ± 3.51
Crypts depth (μm)	380.71 ± 6.13	396.06 ± 5.67
Crypts width (μm)	44.69 ± 0.89	44.62 ± 0.84
V:C ratio	0.76 ± 0.02	0.75 ± 0.02
Tunica propria (μm)	417.47 ± 7.28	430.65 ± 6.63
Goblet cell (nr/villi height)	6.88 ± 0.97	6.48 ± 0.91
Goblet cell (nr/crypts depth)	17.94 ± 2.53	18.67 ± 2.66
ILEUM		
Villi height (μm)	290.67 ± 6.55	289.75 ± 5.24
Villi width (μm)	122.65 ± 2.93	119.45 ± 3.03
Crypts depth (μm)	340.83 ± 5.92	334.95 ± 5.71
Crypts width (μm)	46.85 ± 0.98	45.25 ± 0.81
V:C ratio	0.83 ± 0.02	0.89 ± 0.02
Tunica propria (μm)	383.87 ± 6.73	371.08 ± 5.62
Goblet cell (nr/villi height)	13.06 ± 1.84	10.74 ± 1.51
Goblet cell (nr/crypts depth)	25.45 ± 3.71	23.60 ± 3.40
COLON		
Crypts depth	437.27 ± 13.56	419.16 ± 8.93
Crypts width	60.21 ± 1.35	61.81 ± 1.20
Tunica propria	453.68 ± 13.35	440.01 ± 9.27
Goblet cell (nr/crypts depth)	20.69 ± 3.19	22.00 ± 3.27

[a,b] Different superscript letters within rows indicate differences at $P < 0.05$

Piglet weaning stress events change small intestine morphology, resulting in intake and digestive disorders; consequently, animal performance is prejudiced. There are some evidences that prebiotics may provoke beneficial changes in digestive anatomical traits. Budiño et al. (2005) observed higher villous density in duodenum of piglets fed MOS, while no significant difference were found in jejunum. Rekiel et al. (2007) reported a significant lower depth of crypts in duodenum and a higher depth in the jejunum after administration of MOS. Iji et al. (2001) also observed an increase of villi height:crypt depth ratio in poultry due

to a significant increase in villi height rather than an effect on crypt depth. The Authors stated that the beneficial effect of MOS on intestinal morphology may be due to a reduction in the enterobacteria population. On the other hand, Van der Peet-Schewering et al. (2003) found that villi and crypts were unaffected by the administration of MOS. The present study shows that the administration of MOS to post-weaning piglets was able to affect intestinal villi in the duodenum, in agreement with Budiño et al. (2005), while no differences were found for the other tested parameters and the other intestinal tracts.

4.4.4. Total protein and intestinal inflammatory responsive parameters

The effects of MOS administration on total protein and intestinal inflammatory responsive parameters are showed in table 4.6.

The administration of dietary MOS led to a significant increase of NO production (P=0.01), while no significant difference were detected for the other tested parameters.

Table 4.6 - Effect of MOS administration on total protein, MPO (myeloperoxidase), NO (nitric oxide), tNOS (total nitric oxide synthase activity), iNOS (inducible nitric oxide synthase activity)

Item		Group		SEM
		Control	MOS	
Protein	(mg/mL)	8.13	8.40	0.28
MPO	(U/g)	1.98	2.06	0.37
NO	(μmol/g protein)	0.88[b]	1.41[a]	0.17
tNOS	(U/mg protein)	0.53	0.47	0.03
iNOS	(U/mg protein)	0.19	0.16	0.02

[a,b] Different superscript letters within rows indicate differences at P< 0.05

Myeloperoxidase (MPO) is present in the primary granules of neutrophils and monocytes, and plays an important role in animal innate host defense responsible for microbiocidal activity against a wide range of organisms (Williams, 2001). In addition, MPO is used as a marker of inflammation caused by disease such as asthma, systemic vasculitis or environment irritants (Sugiyama et al. 2001). During the primary stage of infection, endotoxin and other bacterial by-products can activate neutrophils, monocyte and macrophages, initiating a signal transduction cascade and leading to the release of a number of pro-inflammatory cytokines and inducible enzymes such as inducible nitric oxide synthase, iNOS (Lazarov et al. 2000).

Some earlier studies demonstrated that excessive or prolonged induction of iNOS can lead to gut inflammation and systemic inflammatory response syndrome (Miller and Clark, 1994; Ungureanu-Longrois et al., 1995). In the

present study, no significant difference of MPO activity among MOS and Control groups indicated that MOS did not influence host defense responsible for microbicidal activity against pathogenic microorganism. In the same way, the lack of response on iNOS indicates that MOS were not able to exert an effect on that.
Nitric oxide (NO) is a central effector molecule in the innate immune system, which is mainly derived from iNOS produced by activated neutrophils and macrophages: it is a short-lived, labile gas produced in most biological systems through the conversion of L-arginine(L-arg) to L-citrulline (L-cit) and represent a key factor in gastrointestinal physiology and gut responses to injury; however, excessive amounts of NO is involved in cellular damage, tissue and organ dysfunction (Liang et al. 1999).
Anyway the primary function of NO in host defense is damaging and destroying pathogens (Nathan et al. 2000). Thus, this molecule is produced by the body to fight infections, whereas the excess production of NO by activated neutrophils and macrophages plays a key role in infection pathogenesis (Tsukahara et al. 2001). In the present study, dietary MOS induced increased values of NO production, but no significant variations were found for iNOS activity. NO levels are in the suggested ranges (Devaux et al, 2001), thus the significant differences in NO production among experimental groups could be due to the induction of other innate immune system factors by MOS.

4.4.5. Gene expression profile

The effects of MOS administration on mRNA levels for TNF, IL6, IL1α, IL1β, TLR2, TLR4, PPARγ, IL10 and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the ileal mucosa are shown in Figure 1. MOS supplementation significantly reduced mRNA levels of TNF, IL6, IL1α, IL1β, TLR2 and TLR4 in intestinal mucosa, while no difference where detected for PPARγ and IL10 among the animals. A reduction in the mRNA level for TNF, IL6, IL1α, IL1β, TLR2 and TLR4 is expected to reduce the translation of the gene and, therefore, the production of the corresponding proteins.
Apart from their effect on the intestinal microbiota, little is known concerning the possible implications of prebiotics on the host response, especially in pig. It has been demonstrated that weaning is associated with increased proinflammatory cytokine mRNA content in the intestine of piglets (Pié et al., 2004). Cytokines are known to mediate the inflammatory response. They act through complex mechanisms: by promoting the proliferation and differentiation of thymocytes and mature T-cells; by inducing T-cells to generate IL-2 and activating Th cells to release factors required for the function of B-cells; by enhancing B-cell differentiation and, therefore, promoting the production of antibodies, and by inhibiting the growth of tumor cells and killing

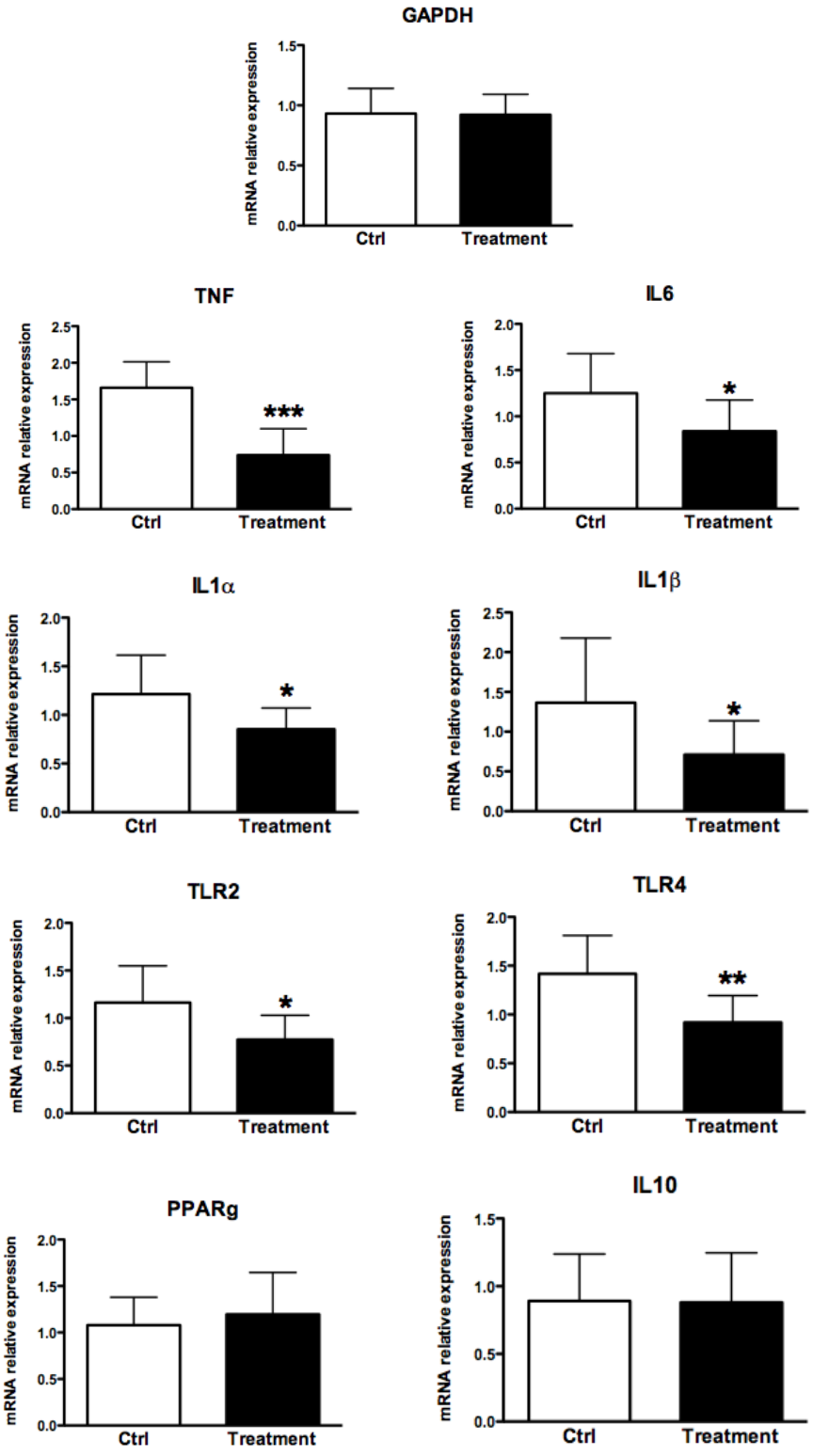

Figure 1 - Ileal mRNA relative expression of TNF, IL6, IL1α, IL1β, TLR2, TLR4, PPARγ, IL10 and GAPDH genes in Control (Ctrl) and MOS (Treatment) groups.
* Means differ significantly (P<0.05).
** Means differ significantly (P<0.01).
*** Means differ significantly (P<0.001).

them (Blok et al., 2002). Many studies have shown that diet can influence the immune response: Pié et al., 2007 reported that diet enriched with fermentable carbohydrates induced an up-regulation of IL-6 mRNA level in the colon of piglets at 4 d post-weaning; according to the Authors, these results indicate that the regulation of IL-6 mRNA in colon depend on dietary factors, or at least on microbiological factors that have been influenced by diet. They also demonstrated that lactic acid, in ileum, was positively correlated with IL-6 mRNA, and this may indicate that the IL-6 mRNA content is linked in some way to bacterial fermentation. Yin et al. (2008) found that dietary supplementation with oligosaccharides had no effect on IL-1β mRNA in peripheral blood mononuclear cells (PBM), while supplementation with either galacto-mannan-oligosaccharide (GMOS) and chitosan oligosaccharide (COS) increased IL-1β mRNA levels in PBM or IL-1β mRNA levels in jejunal mucosa and mesenteric lymph nodes. According to the Authors, it also should be noted that if inflammatory cytokines increased with the indigestible oligosaccharides, it is possible that the effect might result from the activation of some intestinal bacteria which then stimulated an immune defense response.

Differently, in our study we evidenced a suppression of mRNA relative expression of proinflammatory genes (TNF, IL6, IL1α, IL1β, TLR2 and TLR4), accordingly to several reports indicating that prebiotics tend to suppress inflammatory responses (Kanauchi et al., 1998; Videla et al., 2001; Cherbut et al., 2003). We found a reduction in IL-1β mRNA in piglets fed MOS compared to the control animals. An increase of this cytokine is a common gut phenomenon at the time of weaning, and may be considered to be a general and early immunological change associated with weaning (or other stress) in piglets (Pié et al., 2004); consequently, the reduction of this cytokine evidenced in our study could suggest a possible antinflammatory effect of the tested prebiotics. That is particularly noteworthy if we consider that the animals were fed a low digestible diet, in order to exacerbate the nutritional stress. According to our results, Guigoz et al. (2002) described some changes in non-specific immunity after administration of fructooligosaccharides (FOS) to humans, with a decreased phagocytic activity of granulocytes and monocytes, as well as a decreased expression of interleukin-6 mRNA in peripheral blood monocytes (suggesting a possible decrease in inflammatory process). Daddaoua et al. (2006) evidenced that administration of some oligosacchatides isolated from goat milk to colitis-induced rats was associated with a significantly lower expression of IL-1β, one of the predominant cytokines in rat colitis. Hosono et al. (2003) reported that IFN-γ production from spleen $CD4^{+}$T cells was increased in mice supplemented with FOS, while IL-5 and IL-6 production were decreased.

Our results demonstrate that a diet enriched in fermentable carbohydrates can modulate the cytokines mRNA content during the weaning period in piglets fed

a low digestible diet; cytokines play a major role in the maintenance of gastrointestinal homeostasis, and their regulation by nutritional factors may be an important consideration; the different effects of prebiotic supplementation reported in literature upon cell-mediated immunity in the GALT may be dependent upon the site of origin of the cells and the animal model used.

4.5 Conclusions

In the present study, the administration of MOS in post-weaning piglets fed low digestible diet from 24 to 59 days of age significantly improved feed conversion during week 4th and 5th of the trial. These results could be related to gut changes for duodenal villi height that increased absorption surface for nutrients, fecal *Lactobacilli* count that was found to be higher than Control in the same weeks and mRNA level for TNF, IL6, IL1α, IL1β, TLR2 and TLR4 in intestinal mucosa, that was significantly reduced in piglets fed MOS.

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CHAPTER 5

Third trial: Dietary inclusion of *Lactobacillus rhamnosus* and *Lactobacillus farciminis* improved growth performance and intestinal microbial population in broilers

5 Dietary inclusion of *Lactobacillus rhamnosus* and *Lactobacillus farciminis* improved growth performance and intestinal microbial population in broilers

5.1 Abstract

This study was carried out to determine the effects of a probiotic mixture containing two strains of *Lactobacillus* on growth performance, carcass composition, blood lipids, digestive enzyme activity and intestinal microbiota in broiler chickens. Two dietary treatments, consisting of basal diet (control) and basal diet supplemented with combination of *L. farciminis* and *L. rhamnosus* were fed to 392 one day-old Ross 708 broiler chicks for 7 weeks. Each treatment had 28 replicates of 7 broilers.

The results showed that body weight gain was improved in broilers fed probiotics diet compared to controls during 0-49 d ($P<0.001$) but not 43-49 d of age. Probiotic fed chicks had transitorily higher serum total cholesterol ($P=0.02$) and high-density lipoprotein cholesterol ($P<0.008$) at 28 d of age. Serum total protein was higher at 28 d of age ($P=0.02$) and lower at 49 d of age ($P=0.001$) in probiotics fed chicks compared to controls. Probiotics tended to increase abdominal fat percentage at 49 d of age ($P<0.10$). No difference in enzyme activity of small intestine digesta was observed. Dietary probiotics markedly increased *Lactobacilli* ($P=0.01$) and total Anaerobes ($P\leq0.01$) counts and decreased Coliform ($P=0.01$) and total Aerobe counts ($P\leq0.01$) in small intestine and caecum. The overall results demonstrated that dietary inclusion of a mixture of *L. farciminis* and *L. rhamnosus* could promote the growth and positively modulate intestinal microbiota in broiler chickens.

5.1 Introduction

The use of sub-therapeutic antibiotics has been a cornerstone to prevent the microorganism infection and promote growth in poultry industry for many years. As new antibiotic-resistant strains of pathogens emerge and since the ban of non-therapeutic uses of antibiotics in feed in Europe in 2006, poultry rearing is searching for new strategies for preventing and treating bacterial infection common to poultry.

Probiotics present a promising alternative and are increasingly being used in poultry feed. A probiotic is a culture of live microorganisms supplement that benefits the host animal by improving its intestinal microbial balance, improving the health of man or animals (including growth promotion in animals) and can have effect on all host mucosal surfaces including the mouth and gastrointestinal tract. (Fuller et al., 1992). The application of probiotic in poultry production has grown continuously during the past years (Jin et al., 1997; Patterson and Burkholder, 2003; Flint and Garner, 2009; Kabir, 2009). Products of probiotic culture had been reported to improve the production efficiency (Jin et al., 1996a, 2000; Panda et al, 2006; Mountzouris et al. 2007; Awad et al. 2009), modulate gut microflora (Watkins et al., 1982; Mountzouris et al., 2007) and promote health status in broilers.

Most microbial species being used or tested today for probiotic efficacy belong to lactic acid bacteria (LAB). LAB has been proposed as probiotics for the prevention of various enteric diseases and the improvement of overall health for many years (Tellez et al, 2006). Many attempts have been made to use *Lactobacillus* as commercial poultry probiotics. *Lactobacillus farciminis*, a newly approved bacterial for use as a feed additive in poultry by European Food Safety Authority recently, has been demonstrated to protect against experimentally induced colitis (Lamine et al., 2004) and have the potential to modulate visceral stress status (Ait-Belgnaoui et al., 2006). *Lactobacillus rhamnosus* has been reported to increase enterocyte production (Banasaz et al., 2002), protect against pathogen infection (Gill et al. 2001; Hirano et al., 2003) in rodents. Multistrain probiotics had the advantage over monostrian probiotic with regard to growth and particularly mortality in broilers (Timmerman et al., 2004). It is worthwhile to examine the efficacy of the combination of the two *lactobacilli* in broiler chickens.

The objective of this work was to evaluate the efficacy of a probiotic mixture of *Lactobacillus farciminis* and *Lactobacillus rhamnosus* in broiler production. Growth performance, carcass composition, blood lipids, digestive enzymes and intestinal microbial population were investigated.

5.2 Material and Methods

5.3.1 Animals and diets

Three hundred and ninety two day-old Ross 708 male broiler chicks obtained from a local hatchery were randomly divided into two groups (196 birds/group) with 28 pens (replicates) of 7 chicks each. Chicks were assigned to cages of same size (1.2 × 2.45 m) in a deep litter system with a wood shaving floor. Cages contained self-feeder and waterer inside to provide free access to feed and water. The two groups were separated by a 5 m walkway, and separate boots and

isolation clothes for each group were used by the person that entered the pens so as to avoid the cross contamination. The environment conditions and lighting program were automatically operated in computer system. The room temperature was 35°C during the first 3 days and decreased by 2°C during the succedent weeks and maintained 25°C until the end of the experiment. During the first week, 22 h of light was provided with a reduction to 20 h afterwards. All animal management and sampling procedures were in accordance with the guidelines of the Consortium Guide.Chicks were fed either a basal diet (control) or basal diet supplemented with 2.0 kg probiotic/t of feed to provide 6.07×10^6 VFU (viable fluorescent unit)/g feed. The probiotic product consisted of a mixture of *L. rhamnsous* and *L. farciminis*. The diet was supplied according to a 4-phase feeding program, starter (1-14 d), grower (15-28 d), grower second phase (29-42 d) and finisher (43-49 d). The ingredient composition of and nutrient levels of basal diet are listed in Table 5.1.

Table 5.1- Ingredient Composition and Nutrient levels of the diets

Ingredients, %	Starter	Grower	Grower II phase	Finisher
Wheat	28	27.71	28.1	28
Sorghum	10	11	10.5	10.4
Maize	25.52	24.52	25	24.52
Extruded soybean	12.53	12.8	13.9	15
Soybean meal, 48 % CP	20	20	18.7	18.6
Lysine HCl 78	0.3	0.3	0.27	0.16
DL-Methionine	0.22	0.22	0.28	0.26
Calcium carbonate	0.85	0.85	0.85	0.66
Dicalcium phosphate	1.72	1.72	1.54	1.54
Sodium bicarbonate	0.2	0.2	0.2	0.2
Salt	0.16	0.16	0.16	0.16
Mineral & Vitamin Premix[1]	0.5	0.5	0.5	0.5
L. rhamnsous - L. farciminis	+/-	+/-	+/-	+/-
Nutrient levels:				
ME, kcal/kg	3090	3150	3200	3350
Crude protein, %	23	22.5	21.4	19
Crude fibre, %	2.9	2.9	2.87	2.85
Ether extract, %	6.7	6.7	9.4	8.9
Linoleic acid, %	1.25	1.20	1.00	1.00
Total lysine, %	1.44	1.20	1.00	0.95
Total methionine, %	0.51	0.44	0.37	0.36
Total threonine, %	0.93	0.79	0.68	0.64
Total tryptophan, %	0.25	0.21	0.18	0.16
Total valine, %	1.09	0.92	0.67	0.74
Calcium, %	1.00	0.90	0.90	0.85
Available phosphorus, %	0.50	0.45	0.45	0.42
Sodium, %	0.16	0.16	0.16	0.16
Chloride, %	0.16	0.16	0.16	0.16

[1]Mineral and vitamin premix provided per kilogam of diet: Mn, 80 mg; Zn, 90 mg; Fe, 60 mg; Cu, 12 mg; Se, 0.147 mg; sodium chloride, 2.247 g; retinyl acetate, 8,065 IU; cholecalciferol, 1,580 IU; 25-hydroxycholecalciterol, 31.5 µg, dl-α-tocopheryl acetate, 15 IU; vitamin B12, 16 µg; menadcre, 4 mg; riboflavin, 7.8 mg; pantothenic acid, 12.8 mg; niacin, 75 mg; Choline chloride, 509 mg; folic acid, 1.62 mg; biotin, 0.27 mg.

During the experiment, the basal diet was prepared every week and stored in bags in a cool place. The probiotic product was added and mixed in the basal diet on a weekly basis. The treatment feed was produced after the control feed to prevent cross contamination.

5.3.2 Measurement of growth performance

All birds were weighed after arrival to experimental farm (initial body weight) and at every week of the experiment. Average daily gain for each period was calculated. Feed consumption was recorded every day for each treatment, and the feed conversion ratios were calculated correspondingly. Mortality was recorded as it occurred and mortality percentage was determined at the end of the experiment.

5.3.3 Sample collection and preparation

At 28 and 49 d of age, 15 chicks with the average body weight near that of each group were selected to collect blood by wing vein puncture. Sera were prepared by centrifugation at 1500×g at 4°C for 15 minutes and then stored at -20°C pending analysis for serum lipids, total protein and lysozyme content.

At 49 d of age, after blood collection, the chicks were sacrificed by cervical dislocation. Carcasses were then plucked and eviscerated to determine carcass weight, as a percentage of total weight, and abdominal fat (the fat extending within the ischium, surrounding the cloaca, and adjacent to the abdominal muscle) and breast (pectoralis major and pectoralis minor) and boneless leg muscle weight as a percentage of carcass weight.

A homogenous intestinal content was collected by massaging the tract from the distal end of duodenum to the middle of jejunum. The digesta samples were immediately stored at – 70°C until used.

Small intestinal content from middle jejunum to middle of ileum and caecum contents were collected by bondage of the both ends and placed in ice and immediately sent to the preparation lab for microbe culture.

5.3.4 Assay of the parameters

Serum lipids, total protein and lysozyme content

Serum lipids including total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and total triglyceride were determined using commercial kits (purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by colorimetric methods conforming to the manual instructions. Serum total protein was measured by method of Lowry et al. (1951). Serum lysozyme activity was measured according the method of Kreukniet et al. (1994) using *Micrococcus lysodeikticus* cells as a substrate.

Digestive enzymes activity

Activity of α-amylase was determined by using gelatinized maize starch as the substrate. The amounts of glucose released after incubation with portions of digesta were presumed to be directly proportional to the activity of α-amylase.

Lipase activity was determined on the theory that the amount of standard sodium hydroxide required to neutralize fatty acids released from olive oil triacylglycerols is directly proportional to lipase activity. Digesta supernatant fractions (1 ml) were incubated with 3 ml olive oil triacylglycerols for 6 h at 37°C. The reaction was stopped by the addition of alcohol (950 ml/l) and titration performed with 0.05 M-sodium hydroxide solution.

The pepsin content of the small intestine digesta was assayed by the method of Anson (1938). One unit of pepsin activity was equivalent to an increase in extinction at 280 nm of 0.001/min at 37°C. Trypsin activity was estimated in digesta of small intestine as described by Hummel (1959).

Microbial population

The following population densities were determined in small intestinal and caecal content. Coliform bacteria counts were assessed on CC Coliform agar and incubated aerobically at 37°C for 24 h.

Lactobacilli counts were assessed by plating on the Man, Rogosa, and Sharp agar and incubated anaerobically at 37°C for 48 h.

Total aerobe counts were determined using Brain Heart Infusion agar and incubated for 24 h. Total anaerobe counts were assayed using FM98-5 medium in an agar roll tube and incubated for 6 d. The microbial counts of intestinal content were expressed as log10 colony-forming units per gram.

5.3.5 Statistics

Data were reported as means and pooled SEM and were analyzed with independent samples t-test of SPSS 15.0 to determine if variables differed between the 2 groups. The Kolmogorov-Smirnov test was used to test the normal distribution of the data before statistical analysis was performed. Probability values of less than 0.05 ($P < 0.05$) were considered significant.

5.4 Results and discussions

5.4.1 Growth performance

Data of body weight (BW) and average daily gain of the chicks are presented in table 5.2. BW was higher in control birds compared with birds fed with probiotic at d 14 ($P<0.05$); subsequently, birds fed probiotic had greater BW compared with controls at d 28, 42 and 49 ($P<0.05$).

Table 5.2 - Body weight (BW) and average daily gain (ADG) of chicks in experiment

	Control	Probiotic	SEM	P value
BW (g):				
d 0	49.23	49.21	2.55	N.S.
d 14	439.14	420.71	3.01	0.017
d 28	1334.14	1382.71	7.89	0.029
d 42	2498.86	2762.71	22.31	0.001
d 49	3123.43	3434.86	26.48	0.001
ADG (g/d):				
d 0-14	27.85	26.54	0.21	0.002
d 15-28	63.93	68.71	0.66	0.000
d 29-42	83.19	98.57	1.43	0.000
d 43-49	89.22	96.02	2.70	N.S.
d 0-49	62.74	69.09	0.54	0.000

N.S: not significant

Average daily gain (ADG) was higher in control birds compared with birds fed with probiotic during the first 2 weeks ($P<0.05$). Birds fed probiotic diet had greater ADG compared with control birds from the 15 to 42 d of age, and during the whole period of 7 weeks ($P<0.05$). It was noteworthy that probiotic fed chicks had 15 gram greater growth rate of ADG compared with the controls during d 29 to 42 ($P<0.05$). There were no growth rate differences during the 7th week ($P>0.05$).

Table 5.3 - Feed conversion rate (FCR) of chicks of the experiment

FCR:	Control	Probiotic	P value
d 0-14	1.115	1.316	N.S.
d 15-28	1.549	1.551	N.S.
d 29-42	1.848	1.680	N.S.
d 43-49	1.905	2.147	N.S.
d 0-49	1.417	1.485	N.S.

N.S: not significant

FCR was higher in probiotic group compared to control group during periods of 0-14 d and 43-49 d, while chicks fed probiotic diet had decreased FCR during periods of 29-42 d, 15-42 d and 0-42 d; however, differences among the groups were not significant (Table 5.3).

The mortality rate was lower in probiotic group (2.95%) than control group (3.47%) during the 49 d of experiment.

5.4.2 Performance at slaughtering

The effect of the administration of *Lactobacilli* on performance at slaughtering are shown in table 5.4. In the current study, no significant change on carcass composition was observed. Probiotic did not cause lower absorption and deposition of abdominal fat. This result disagrees with those from Santoso et al. (2001) and Kalavathy et al. (2003), where broilers fed *Bacillus subtilis* or *Lactobacilli*

showed decreased abdominal fat content. In our trial, the comparable increase of abdominal fat percentage in probiotics fed chicks maybe due to the greater body weight: the probiotics fed chicks had 300 grams more BW than the controls at 49 d of age. It is well known that animal begins to deposit more fat after the development of bone and muscle during the growing phase.

Table 5.4 - Performance at slaughtering of the chick of the experiment

(%)	Control	Probiotic	SEM	P value
Dressing percentage	76.09	76.33	0.519	N.S.
Breast	20.47	20.61	0.362	N.S.
Boneless leg muscle	15.21	14.90	0.392	N.S.
Abdominal Fat	1.43	1.76	0.125	N.S.

N.S: not significant

5.4.3 Lipids, total protein and lysozyme

The effect of the administration of *Lactobacilli* on serum lipids are shown in table 5.5. This study observed a transitory increase of total cholesterol and HDL-C in probiotic fed chicks at 28 d of age. HDL-C delivers cholesterol to the liver for excretion, conveys a direct beneficial effect on the arterial wall and protection against atherosclerosis (Sacks, 2003). The elevation of total cholesterol was due to the increase of HDL-C, which would not harm the metabolism of lipids and cholesterol of the broilers.

Table 5.5 - Serum lipid profile (mmol/L)

	Control	Probiotic	SEM	P value
Total cholesterol:				
d 28	2.69	3.47	0.17	0.023
d 49	2.98	3.14	0.11	NS
HDL-cholesterol:				
d 28	1.50	2.05	0.07	0.008
d 49	1.63	1.82	0.10	NS
LDL-cholesterol:				
d 28	1.13	1.36	0.09	NS
d 49	1.29	1.38	0.08	NS
Total triglyceride:				
d 28	0..97	1.14	0.06	NS
d 49	0.99	1.08	0.06	NS

NS : not significant

This increase of cholesterol was not in accordance to what found from other studies (Mohan et al., 1996; Jin et al., 1998) which recorded a significant decrease of cholesterol in probiotics fed broilers. The decrease of cholesterol was probably ascribed to cholesterol assimilation by the *Lactobacilli* cells, or to the co-precipitation of cholesterol with deconjugated bile salts (Buck and Gilliand,

1994; Klaver and Van der Meer, 1993; Jin et al., 1998). Still, no variations of blood cholesterol in chicks fed mixture of different strains of probiotics (*Lactobacillus* spp, *Bacillus* spp, *Enterococcus faecium*, *Streptococcus thermophilus*) were observed in studies of Kanashiro et al (2001) and Djouvinov et al. (2005). Effect of the specific strains of *L. rhamnosus* and *L. farciminis* on cholesterol metabolism is pending for further research.

It was interesting that probiotics fed chicks had more serum protein on 28 d of age, while had lower serum protein upon marketing age compared to controls (table 5.6). A possible reason may be related to the different growing speed of the two groups. Probiotics fed chicks grew faster and had higher serum protein at early age, while accumulated more fat during the finishing period due to higher body weight, which would possibly contribute to the relatively lower blood protein content.

In the current study, dietary *lactobacilli* did not affect the serum lysozyme content in broilers (table 5.6), which was similar to the results of Pirarat et al. (2008) who found no change of serum lysozyme in *L. rhamnosus* fed tilapia. While Panigrahi et al. (2004) demonstrated that the *L. rhamnosus* JCM 1136 could modulate the lysozyme activity in fish. More parameters should be investigated to elucidate the precise influence on innate immune responses of *L. rhamnosus* and *L. farciminis* in chicks.

Table 5.6 - Serum total protein and lysozyme content

	Control	Probiotic	SEM	P value
Total protein (mg/mL):				
d 28	25.92	37.19	2.536	0.022
d 49	50.10	35.59	2.347	0.001
Lysozyme (µg/mL):				
d 28	3.57	3.79	0.089	NS
d 49	3.72	3.78	0.076	NS

NS : not significant

5.4.4 Digestive enzymes

The effects of administration of *Lactobacilli* on the activity of digestive enzymes in small intestine are shown in table 5.7. In the current study, no change in the activity of digestive enzymes was observed. Results reported in literature are sometimes contrastant: similarly to our results, the intestinal lipolytic and proteolytic activities of broilers did not change after feeding diets supplemented with *Lactobacillus* (Jin et al., 2000); the Authors reported that this result is not surprising as extracellular protease and lipase of the *Lactobacillus* spp. were not detected by a previous *in vitro* test; other Authors have reported some changes in amylase activity: some *Lactobacillus* strains had been shown to produce digestive enzymes, especially amylase *in vitro* (Szylt et al. 1980; Jin et al. 1996b); Jin et al.

(2000) reported that *Lactobacillus* culture increased amylase activity in small intestine in broilers.
The different strains of the probiotics and the sample time of study may contribute the discrepancy.

Table 5.7 - Activities of enzymes in small intestine digesta on d 49 (U/mg prot)

	Control	Probiotic	SEM	P value
Lipase	33.93	41.79	2.95	N.S.
Trypsin	195.74	217.6	16.70	N.S.
Amylase	132.63	97.27	23.65	N.S.
Pepsin	1.60	1.94	0.29	N.S.

N.S: not significant

5.4.5 Microbial population in the gut

Results about the microbial populations in the gut are presented in table 5.8. The consistently higher *lactobacillus* counts in small intestinal and caecal content indicated that the *lactobacilli* mixture used in the present study had a strong capability to colonize in the gut in chickens. The decreased coliform counts and aerobes in gut were supportive of the concept of competitive exclusion, which implied that the *lactobacilli* mixture was probably able to antagonize and be competitive against some pathogenic bacteria. The competitive exclusion of *Lactobacillus* product had been reported in lot of previous reports (Francis et al, 1978; Watkins et al. 1982; Watkins and Kratzer, 1983; Kabir et al., 2005; Higgins et al., 2007; Mountzouris et al., 2007). Jin et al. (1996c) reported that different strains of *lactobacilli* isolated from chicken intestine were able to decrease significantly *E.coli* population in vitro. Kabir et al. (2005) evidenced that probiotic organisms inhibited some non-beneficial pathogens by occupying intestinal wall space.
The observed decrease in Coliforms in the caecum could be due to the higher level of volatile fatty acids (VFA) that are the major end products of microbial fermentation. Jin et al. (1998a) reported that *lactobcillus* cultures resulted in lower pH in the caecum and increased concentration of total VFA in ileal and caecal contents. This study also showed the fact that the microbial population is larger in caecum than that in small intestine and the total number of microbes decreased slightly with the age increasing in chicks (Jin et al., 1997, 1998b).
The two strains of *Lactobacilli* used in this study had been reported to possess the capacity to exclude the pathogens or exert gut immumodulatory effects. Milk fermented with *L. rhamnosus* was found to exert protective effect in mice challenged with *Salmonella typhimurium*, as evidenced by a significant reduction in *Salmonella* counts in liver and spleen and an almost twofold higher serum antibody titre (Perdigon et al. 1990). *L. rhamnosus* fed mice had significantly lower

pathogen burdens in visceral organs (spleen, liver) compared to controls after *Salmonella typhimurium* infection (Gill et al., 2001). The *in vitro* internalization of enterohemorrhagic *Escherichia coli* into human intestinal cells was markedly suppressed by *L. rhamnosus* (Hirano et al., 2003). It was reported that *L. farciminis* inhibited the tissue injury in experimental model of rat colitis (Lamine et al., 2004), prevented stress induced visceral hypersensitivity in rats (Ait-Belgnaoui et al., 2006). The improved growth performance and the positive shift in gut microbial composition indicated that the dosage of *lactobacilli* mixture was sufficient to present viable numbers to elicit beneficial effects on broilers.

Table 5.8 - Microbial populations in small intestinal and caecal content (Log cfu/g)

	Coliforms	*Lactobacilli*	Total aerobes	Total anaerobes
Small intestine				
Control	6.33	7.48	7.18	6.82
Probiotic	5.88	8.02	6.98	6.93
SEM	0.043	0.052	0.023	0.024
P value	0.01	0.01	0.01	0.01
Cecum				
Control	7.13	7.96	7.84	9.74
Probiotic	6.80	8.33	7.64	9.74
SEM	0.043	0.036	0.043	0.017
P value	0.01	0.01	0.01	N.S.

N.S: not significant

5.5 Conclusions

The growth promotion and competitive exclusion efficacy of a probiotic product containing *Lactobacillus farciminis* and *Lactobacillus rhamnosus* were observed in male broiler chickens. The *lactobacilli* successfully colonized the intestine of broilers and beneficially modulate the microflora which was characterized by decreased coliform and aerobes and increased anaerobes population. The positive effect of dietary probiotics on growth was distinct from 28 to 42 d of age. Broilers fed *lactobacilli* had faster growth rate and would reach the market size around 3 days earlier than the controls.

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CHAPTER 6

General Discussion

6. General discussion

In modern society the way of human food production is intensively discussed and questioned. We expect food from plants, farm animals and microorganisms to be of good quality, healthy and inexpensive. In addition, we are increasingly concerned about environmental matters and look for low energy and nutrient input production systems. Arguments for food that is produced as naturally as possible come primarily from consumers that, on the other hand, do not always feel the impact of the steady growth of world population.

In this context, antimicrobial feed additives have been used worldwide in animal production for many decades because of their favourable economic effects in livestock production: added in low doses to the feed of farm animals, they improved growth and performance and hence were known as antimicrobial growth promoters. Due to the emergence of microbes resistant to antibiotics ('antimicrobial resistance') that are used to treat human and animal infections and, in addition, to the this greater interest of the consumers for a healthier and more natural food, the European Commission decided to phase out, and subsequently ban since 1 January 2006, the marketing and use of antibiotics as growth promoters in animal feed. Since then, the use of antibiotics is only allowed on veterinary prescription. What are the consequences? Available data suggest that the growth-promoter ban has driven an increase in infections and therefore a substantial increase in the use of therapeutic antibiotics for food animals in Europe, but the ban also has reduced overall antibiotic use in animals (Casewell et al., 2003).

Consequently, there is the need of exploring new ways to improve and protect the health status, to guarantee animal performance and to increase nutrient availability, particularly for young animals. This goal can be attained by good production practices, but also by formulating diet for its effects on gut health. This is because gut health is essential for the welfare and productivity of animals. Today, gut health is a major topic for research, but it is generally conceded that its maintenance or enhancement is really complex (Choct, 2009): this is not surprising considering that the gut harbours hundreds of different species of bacteria, contains over 20 different hormones, digests and absorbs the vast majority of nutrients, and accounts for 20% of body energy expenditure. It is also the largest immune organ in the body (Kraehenbuhl and Neutra, 1992). Thus, anything that affects the health of the gut will undoubtedly influence the animal as a whole and consequently alter its nutrient uptake and requirements. That it is true in particular for young animals, more susceptible to infections: in poultry, close to and shortly after hatch, animals are selected for an intensive growth rate, and they can increase their body weight by 25% overnight; as the

growth period is progressively shortened and feed efficiency continuously improved, the health care and nutrition of the bird are becoming more demanding. This makes it more important to pay attention to the minute changes that occur in the gut. Similarly in the swine around weaning, which has always been a source of losses for the farmer: piglets can undergo gastrointestinal underdevelopment, reduced nutrient utilization, reduced disease resistance due to an immature immune system, reduced intestinal barrier function, undesirable morphological changes in the small intestine and increased susceptibility to environmental stress.

Young farm animals, hence, need time to develop a complex gut community as well as their immune system and, until such developments have taken place, are vulnerable to the presence of potential pathogens in their gastrointestinal tract. These considerations have led to intensive research over Europe to explore the possibilities offered by various natural materials to be added to the diets as efficient alternatives to antibiotic growth promoters, for their ability to optimize the digestive process and to improve the health of the animal. Among them, our interest was focused in particular on probiotics, prebiotics and plant extracts and on their potential for protecting the gut, albeit through very different ways, including anti-bacterial properties, mucosal growth, intestinal barrier function strengthening, anti-oxidant and anti-inflammatory capacities.

Hence, in our first trail we intended to evaluate the effects of a novel plant extract derived from common food plants on performance and health of weaned piglets, in association with a *E. coli* challenge; plant extracts, also called phytobiotics, are plant-derived natural bioactive compounds, which are known to affect animal growth and health (Kim et al., 2008): piglets receiving diet supplemented with plant extracts showed better performance (better average daily gain and feed conversion rate), in particular in the last phase of the trial; it is reported that beneficial effects of herbs or botanicals in farm animals may arise from activation of feed intake, although literature on the biological efficacy of phytogenic feed additives presents a scattered picture and data on the effects on growth performance in swine varied widely (Windisch et al., 2008). However, in our study we administered the tested product through drinking water and we didn't evidence an improvement in the average daily feed intake; it is also known that herbs and spices can affect digestion processes, in different ways: most of them stimulate the secretion of saliva, others enhance the synthesis of bile acids in the liver and their excretion in bile (what beneficially effects the digestion and absorption of lipids), stimulate the function of pancreatic enzymes (lipases, amylases an proteases), or increase the activity of digestive enzymes of gastric mucosa; besides the effect on bile synthesis and enzyme activity, plant extracts can accelerate the digestion and shorten the time of feed passage through the digestive tract (Frankič et al., 2009). We can suppose that those beneficial effects

had a role in the improvement of the performance evidenced in our trial, and that the supplementation with plant extracts growth promoting feed additives relieved the host animals from immune defense stress during the critical situation of the weaning associated with a challenge and increase the intestinal availability of essential nutrients for absorption, thereby helping animals to grow better within the framework of their genetic potential (Windisch et al., 2008). What is sure is that improved growth performance were associated to other actions, such as modulation of gut environment and antioxidant activity: a lower concentration of *Enterobacteriaceae* and *E. coli*, associated with a better fecal score, a possible reparative action and protective role of the plant extract on the small intestinal mucosa following the *E. coli* challenge were indeed evidenced. It is known, in fact, that plant extracts beneficially influence the gastrointestinal ecosystem mostly through growth inhibition of pathogenic microorganism's growth: due to improved health status of digestive system, animals are less exposed to the toxins of microbiological origin, and consequently herbs and spices help to increase the resistance of the animals exposed to different stress situations and increase the absorption of essential nutrients, determining also morphological changes in gastrointestinal tissues (Windisch et al., 2008). Finally, our data also showed an improvent in the antioxidant activity, in particular through an improvement of GSH-Px and T-AOC and a reduction of MDA: many active components of herbs and spices can in fact prevent lipid peroxidation through quenching free radicals or through activation of antioxidant enzymes, as reported in literature (Frankič et al., 2009).

Although plants and, where they have been indentified, their bioactive components are very different and their potential to enhance pig health and immunity has only been scarcely evaluated *in vivo*, the overall results demonstrated that dietary plant extracts had the potential to improve growth performance through a modulation of the gut environment and an enhancement of the systemic antioxidant capacity.

In the second trial, we had the aim to evaluate the effect of the administration of mannan-oligosaccharides in piglets fed a low digestible diet in order to determine a nutritional stress in the intestine. MOS are believed to improve pig health and performance by binding to specific lectin ligands on the surface of epithelial cells, thus preventing pathogenic bacteria from binding to these ligands, resulting in a "flushing" effect on pathogenic bacteria (LeMieux et al., 2003; Rozeboom et al., 2005): in fact, we found a higher *Lactobacilli* content in MOS animals during the trial, while *Clostridia* fecal count was decreased. In our study, we also evidenced gut changes for duodenal villi height, that increased absorption surface for nutrients; in literature, it is sometimes reported that prebiotics may provoke health changes in digestive anatomical traits; e.g., similarly to our study, Budiño et al. (2005) observed higher villous density in duodenum of piglets fed

MOS. Furthermore, although supplementation with MOS determined an increase of nitric oxide, whose first function in host defense is damaging and destroying pathogens (but no significant variations were found for iNOS activity, and NO levels were in the suggested ranges), we also evidenced a reduction of mRNA levels of proinflammatory genes such as TNF, IL6, IL1α, IL1β, TLR2 and TLR4 in intestinal mucosa, accordingly to several reports indicating that prebiotics tend to suppress inflammatory responses (Cherbut et al., 2003); little is known concerning the possible implications of prebiotics on the host response, especially in the pig, but our results showed that MOS were able to exert an effect on the intestinal barrier and gut immune system, that is sometimes reported to be mediated by the SCFAs produced by microbiota (Gourbeyre et al., 2011). All these results had a ripercussions on the growth of the animals: feed conversion rate was significantly improved in treated piglets in the last two weeks of the trial, in accordance with results from several experiments, in which mannanoligosaccharides showed an increase in pig performance (LeMieux et al., 2003; Rozeboom et al., 2005).

The last trial was carried out to determine the effects of the administration of two strains of *Lactobacillus* (*Lactobacillus farciminis* and *Lactobacillus rhamnosus*) in broiler chickens. As reported by Roselli et al. (2005), the most known characteristics of probiotics are the capacity to adhere to intestinal mucosa and to inhibit pathogen adhesion, ability to transiently colonise and proliferate in the intestine, prevention of some intestinal diseases such as diarrhea, modulation of the immune system of the host. In fact, we found a decrease of the Coliforms and Aerobes counts and an increase the Anaerobes population in small intestinal and caecal content; these results suggest that *Lactobacilli* successfully colonized the intestine of broilers, beneficially modulating the microflora, similarly to other results reported in literature: probiotic organisms can in fact inhibit some non-beneficial pathogens by occupying intestinal wall space (Kabir et al., 2005). Consequently, there was a positive repercussion on growth: broilers fed *Lactobacilli* had faster growth rate and reached the market size around 3 days earlier than the controls. That is in accordance with other results reported in literature: it is clearly evident from the result of Kabir et al. (2004) that the live weight gains were significantly higher in experimental birds as compared to control ones, and other studies demonstrated increased live weight gain in probiotic fed birds (Kabir, 2009).

In conclusion, we can affirm that health and nutrition are interdependent and the interaction between the two occurs largely in the gut. However important progress has been made in understanding this complexity, our knowledge is still not complete and future developments likely will focus on identifying other means to improve gut health in farm animals.

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CHAPTER 7

Summary

7. Summary

There is a wide interest in developing management and feeding strategies to stimulate gut development and health in monogastric animals. The ultimate aim of these strategies is to improve productivity, while minimizing the use of antibiotics and rather expensive feed ingredients: indeed, under practical conditions, animals don't achieve the maximum of their growth performance potential. Large amounts of research have been conducted evaluating the impact of a wide range of feed ingredients and feed additives on various aspects of gut health and development in monogastric animals.

The main objective of this thesis was to improve our knowledge on the properties of new additives as feeding strategy, in order to increase general health in piglets around weaning and poultry, with the aim to substitute antibiotics growth promoters. Three different trials were designed to study different strategies.

In the first study proposed, the effects of plant extract administered through drinking water on post-weaning gut health of piglets were investigated. Phytogenic feed additives are plant-derived products used in animal feeding to improve the performance of agricultural livestock. The objective of the present work was to evaluate the effects of a novel plant extract derived from common food plants on performance and health of weaned piglets fed mixed diet. At weaning (24 d), a total of 144 piglets were allocated in two post-weaning rooms, using a 2x2 factorial arrangement; treatments were Plant Extracts, 0 (Control group) or 8 µl daily/piglet (PE group) and Feeding Regimen, *Ad Libitum* or Restricted (piglets fed from 8 AM to 8 PM). Plant Extracts were a liquid mixture administered through drinking water. Piglets were housed in pens of three; each pen represented one treatment replicate, with six pens per treatment per room. On day 9 of the trial, after an adaptation period, each piglet of room 2 was orally injected with 4 ml of a solution containing 10^9 cfu of the virulent *E. coli* 0149: F4(K88)-positive strain. Animals were weighed and growth performance were recorded weekly; fecal score was evaluated at the same time as the weighing. At 0, 14 and 35 days, fecal samples were collected for microbiological analysis, while at day 0, 6, 19 and 35, blood samples were obtained from one pig per pen. At the end of the trial (35 d), 24 animals (12 from Control groups and 12 from Plant Extract groups) among Restricted feeding piglets were selected according to their body weight and slaughtered; immediately after slaughtering, the gastro-intestinal tract was removed from each animal: the distal ileum was collected and examined to assess the ileum micro-anatomical structure, perform histometry and immunohistochemistry and determine intestinal inflammatory parameters. PE supplementation enhanced ADG during the last week of the trial (P=0.007) and reduced FCR during the second (P=0.009) and the last weeks (P=0.04), and

considering the overall period (P=0.01); a lower fecal score was observed in PE piglets (P<0.01). On day 35, lower fecal *E.Coli* (P=0.02) and *Entrobacteriaceae* (P=0.009) concentrations were determined in PE animals compared to control ones. Ileum crypts from PE piglets were deeper in challenged animals in comparison with not-challenged ones (P<0.05); number of mucosal macrophages was higher in Control challenged animals (P<0.05): in particular, number of mucosal macrophages in PE challenged piglets was similar to that one identified in not challenged Controls. PE supplementation also increased GSH-Px plasma concentration at d 6 (P=0.02) and tended to lower value of MDA at day 6 (P=0.07) and to increase value of T-AOC at the end of the trial (P=0.07). Hence, our results confirmed the possible protective functional role of the plant extracts mixture after the bacterial challenge: we can postulate that the use of plant extracts may be useful in the prevention of post-weaning diarrhea with an associated improvement in performance.

The aim of the second trial was to evaluate the effect of the administration of mannanooligosaccharides (MOS) on growth performance, microbial population in feces and cecum and potential alteration of intestinal histomorphometric and gene expression of some intestinal inflammatory parameters of piglets fed a low digestible diet. Forty-eight weaned piglets (6.72 ± 0.32 kg of BW, 24 d of age) were used in a 35-d experiment and randomly allotted to 2 dietary treatments: basal diet (Control) and basal diet + 0.2 % MOS. Growth performance were recorded weekly, fecal samples were collected at 0, 14 and 35 d. At the end of trial, 10 piglets from each group were slaughtered and intestinal samples were collected. Data were analysed by a General Linear Model (GLM) procedure of SAS. BW, ADG, ADFI were not influenced by MOS supplementation; FCR was lower in treated animals in the last 2 weeks (P<0.05). Mean fecal score was improved in MOS piglets (P<0.01). At the end of trial treated piglets had higher *Lactobacilli* fecal count (P<0.05). No difference was detected among groups for Coliforms, while lower *Clostridia* occurred on day 14 in MOS piglets (P<0.05). Intestinal villi height in the duodenum was higher in MOS than Control (P<0.05). MOS supplementation also led to significant increase of NO production in ileal mucosa (P<0.05); finally, MOS suppressed mRNA relative expression of pro-inflammatory genes for IL-1α, IL-1β, IL-6 and TLR2 (P<0.05), for TLR4 (P<0.01) and for TNF (P<0.001), while there was no effect on IL-10 and PPARγ expression. Results indicate that MOS supplementation improved feed efficiency and intestinal morphometry of piglets fed low digestible diet.

The third study was carried out to determine the effects of a probiotic mixture containing two strains of *Lactobacillus* on growth performance, carcass composition, blood lipids, digestive enzyme activity and intestinal microbiota in broiler chickens. Two dietary treatments, consisting of basal diet (control) and

basal diet supplemented with combination of *L. farciminis* and *L. rhamnosus* were fed to 392 one day-old Ross 708 broiler chicks for 7 weeks. Each treatment had 28 replicates of 7 broilers.

The results showed that body weight gain was improved in broilers fed probiotics diet compared to controls during 0-42 d ($P<0.001$) but not 43-49 d of age. Probiotic fed chicks had transitorily higher serum total cholesterol ($P=0.02$) and high-density lipoprotein cholesterol ($P<0.008$) at 28 d of age. Serum total protein was higher at 28 d of age ($P=0.02$) and lower at 49 d of age ($P=0.001$) in probiotics fed chicks compared to controls. Probiotics tended to increase abdominal fat percentage at 49 d of age ($P<0.10$). No difference in enzyme activity of small intestine digesta was observed. Dietary probiotics markedly increased *Lactobacilli* ($P=0.01$) and total Anaerobes ($P\leq0.01$) counts and decreased Coliform ($P=0.01$) and total Aerobe counts ($P\leq0.01$) in small intestine and caecum. The overall results demonstrated that dietary inclusion of a mixture of *L. farciminis* and *L. rhamnosus* could promote the growth and positively modulate intestinal microbiota in broiler chickens.