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Impact of functional foods on gut health of post-weaning piglets

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CHAPTER 1

Foreword

1. Foreword

Post-weaning is the most crucial period in pig management. Associated with weaning are marked changes to the histology and biochemistry of the gut which cause decreased digestive and absorptive capacity and contribute to post-weaning diarrhoea. The piglets have poor growth rate and require more complex diets in post-weaning period, and are vulnerable in terms of health status so that they cannot efficiently cope with antigenic pressure such as enterotoxigenic *Escherichia coli* (ETEC), post-weaning-multisystemic-wasting syndrome (PMWS), circovirus, etc.
In last years, the interest in developing management and feeding strategies to stimulate gut development and health in newly-weaned pigs was increasing. In order to improve development and health of weanling piglets and to minimize incidences of diseases caused by different pathogens such *Escherichia coli* (Fairbrother et al., 2005), the combinations of functional molecules capable of modulating the development of systemic and mucosal immune system, barrier function of intestinal wall and bacterial populations in the gut need to be further examined. Among functional food and nutraceuticals, bovine milk by-products, including essential oils (EOs), diet enzymes, probiotics, prebiotics, colostrum and whey and cranberry products may be considered as good candidates.

1.1. Gut environment

Weaning is a complex step involving many stresses that interfere deeply with feed intake, GIT development and adaptation to the weaning diet (Pluske et al, 1997; Lallès et al. 2004). Gastro-intestinal tract (GIT) disorders, infections and diarrhoea increase at the time of weaning in young pigs, which causes large economic losses in the pig industry. The European ban put on in-feed antibiotic growth promoters has stimulated research on the mechanisms of GIT disorders and on nutritional approaches for preventing or reducing such disturbances.

1.1.1. Intestinal morphology

Villus:crypt ratio represents the nutrient digestion and absorption capacity of the small intestine (Pluske et al., 1996; Montagne et al., 2003). Villous atrophy after weaning is caused by either an increased rate of cell loss or a reduced rate of cell renewal (Pluske et al., 1997). The increase of cell loss rate is associated with increased crypt depth (microbial challenge, antigenic components of feedstuffs). And the decrease of cell renewal rate results in reducing cell division in the crypts. Therefore, both events are likely to operate after weaning to reduce the

villous height:crypt depth ratio.
Multiple previous literatures observed that there is a reduction in villous height (villous atrophy) and an increase in crypt depth (crypt hyperplasia) after weaning (Pluske et al., 1997). Hampson (1986) reported that the villous height was reduced to around 75% of pre-weaning values within 24 hours (940 to 694 μm) following weaning at 21 days of age. Subsequent reductions in villous height along the small intestine were smaller but continued to decline until the fifth day after weaning, at which point the villous height at most sites along the gut was approximately 50% of initial values found at weaning (Figure 1.1). Similar decreases in villous height were reported by Miller et al. (1986), while Cera et al. (1988) additionally reported a reduction in the length of micro-villi three to seven days after weaning. The villous height began to increase from five to eight days after weaning. In contrast, unweaned pigs showed only slight reductions in villous height. The longer villi present in the proximal part of the small intestine decreased in height proportionally more than the villi towards the distal part of the gut. Villous atrophy was caused by a reduction in the number of enterocytes lining the villus and was not due to villous contraction, a phenomenon suggested by Hampson (1986) representing either an increased rate of cell loss from the villous apex or a brief reduction in the rate of cell production in the crypts.

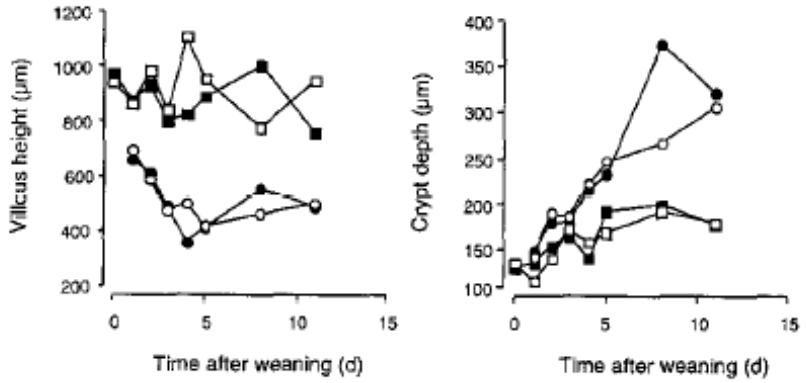

Figure 1.1. Villous height and crypt depth at a site 25% along the length of the small intestine of weaned and unweaned pigs killed between 21 and 32 days of age: ○ - ○ pigs weaned at 21 days of age and offered creep food prior to weaning; ● - ● pigs weaned at 21 days of age but not offered creep food prior to weaning; □ - □ pigs unweaned and offered creep food; ■ - ■ pigs unweaned but not offered creep food. Values are means for between two and seven pigs killed per treatment combination per day (Pluske et al., 1997).

Hall and Byrne (1989) also observed a decrease in crypt-cell production rate associated with villous atrophy and a mechanism attributed to sub-optimal intakes of energy and protein. Since crypt depth was reduced at three days after

weaning, villous stunting was due to a slowed production of new cells and not an accelerated rate of loss of mature enterocytes from the surface of the villi. Hampson (1986) reported that the number of cells in the crypts was not increased two days after weaning, but increased steadily thereafter until the eleventh day. Crypt elongation also occurred in unweaned pigs, but the increase was greater in weaned animals.
Therefore, the villous height: crypt depth ratio in weaned pigs is markedly reduced as compared to unweaned animals due to the changes in villous height and crypt depth after weaning. Hampson (1986) suggested that this represented a balance of cell production in the crypts and cell loss from the villi that began on the fifth day after weaning and persisted for at least five weeks. This is manifested in a change from the longer, finger-like villi seen in newborn and sucking pigs to wider leaf-like or tongue-like villi. These changes only occur after weaning if there is continuous absence from the sow, since pigs weaned for two days and then returned to the dam for three days showed crypt elongation only equivalent to that of pigs weaned for two days (Hampson, 1983).
Macrophages in the intestinal tract are studied mainly at the mucosal level. Their role in the gut-associated lymphatic tissue has been studied in numerous articles (Spahn et al., 2004). Macrophages are known to secrete inflammatory mediators and kill microbial pathogens on activation (Nathan, 1987; Mosser, 2003).The existence of macrophages in the intestinal smooth muscle has been mentioned first by Taxi in 1965. Their anatomical structure and presence was further studied by Mikkelsen and Rumessen (1992). In addition, macrophages act as the first line of the host defence against viral infections (Kosugi et al., 2002) and so it may be that future field studies may evidence a protective action of the studied nutraceuticals towards these pathologies (previously mentioned PRRS and PMWS).

1.1.2. Intestinal epithelium

The intestinal epithelium constitutes the major barrier that separates the external from the internal environment and represents the first line of defence against pathogens and dangerous environmental agents (Roselli et al., 2005). Tight junctions, which have barrier function and fence function, play an important role in regulating epithelial permeability and avoiding the entry of external molecules. 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). Tight junctions encircle the cells at the apical end of the lateral membrane and are composed of an array of proteins, including an integral membrane protein, the occludin, and cytosolic peripheral

membrane proteins, the *zonula occludens* proteins. Meanwhile, 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 (Sawada et al., 2003).
Gut epithelial cells play 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. Forstner and Forstner (1994) reported that the mucus layer which composed of glycoconjugates and intestinal mucins is localised on the cellular surface of the intestinal epithelium and contributes to cell defence creating a physical barrier and avoiding bacterial adhesion through glycoconjugates. Another defence mechanism against toxic substances and microorganisms is represented by proteins produced by mucosal intestinal cells, such as the "trefoil" proteins (Chen et al., 1997) and defensins (Zhang et al., 2000). Defensins are 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.

1.1.3. Gut microflora

The intestinal microflora contains numerous 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 embryo intestine is sterile, and the gastrointestinal colonisation starts after delivery. The microflora becomes similar to the adult one in one year. During life, microflora composition can transiently change in response to diet, health conditions and the environment (Akkermans et al., 2003). Hence, the intestinal microbiota takes some time before developing a stable community: in 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: the germfree gastrointestinal tract is rapidly colonized by anaerobic and facultative anaerobic bacteria after birth.

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 is obligate anaerobes, though some aerobic and facultative micro-organisms also exist (Varel and Yen, 1997). Ducluzeau (1983) reported that a large number 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 highest number of microorganisms is found in the caudal part of the intestines (Table 1.1), 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).

Table 1.1. Comparison of microflora in the stomach, small and large intestines (CFU $\log_{10}$/g chime; Vondruskova et al., 2010)

Microrganisms	Stomach	Small intestine		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

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., 2004). 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 cecum Gram-negative bacteria predominate, whilst these are outnumbered by Gram-positive bacteria in the colon (Mikkelsen et al., 2003).
The gut microflora helps the host to fight the colonisation of pathogenic bacteria and to protect against dangerous substances arriving in the colon (Roselli et al., 2005). 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 microfloras 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).

1.2. Immune system

The immune system in the young pig comprises several organs (bone marrow, thymus, spleen, and mesenteric lymphonodes) and several cell types (lymphocites-specific immune recognition of foreign antigen and phagocytes – production of innate immunity) that recognize foreign antigens. The immune defence system has two "arms" the innate or the acquired. The innate immune system is thought to have evolved before the adaptive immune system, and hence has evolved also as the first line of defence against a pathogenic/antigenic challenge.
Piglet is profoundly immunodeficient at birth 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, a major 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, 1973).
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 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).

1.2.1. Antibodies response
Lymphocytes arise continuously from progenitor cells in the bone marrow. Most functions of the immune system can be described by grouping lymphocytes into three basic types:
1. B cells
2. Cytoxic T cells (TC cells)
3. Helper T cells (TH cells)
All three types of lymphocytes carry cell surface receptors that can bind antigens. The specificity of the immune system is controlled by the fact that one cell recognises one antigen. All antigen receptors are glycoproteins and processes ensure that only one type of receptor is synthesized within any one cell.
The immune system in weaned piglet and the atopic type of immune responsiveness T helper cells have paradigmatically been labelled either T helper (Th)1 or Th2 cells depending on the cytokines they produce (Mosmann et al., 1986; Del Prete et al., 1991). Th1 cells produce predominantly interferon γ (IFN-γ) and play a central role in immune defence against intracellular pathogens. In contrast, Th2 cells produce cytokines such as interleukin (IL-4-5-13) implicated in responses to helminthic infections. Both Th1- and Th2-type responses have been recognised in the pathogenesis of human disease as well (Romagnani, 1996). It is well established that there is a counter-regulatory balance between Th1 and Th2 responses, but recently distinct mechanisms effecting both Th1 and Th2 cells have been discovered. Suppressive cytokines, such as transforming growth factor ß (TGF-ß) and IL-10, secreted by gut-derived regulatory T cells named Th3 and Tr1 cells, respectively, provide important suppressive balance and

protection from disease (Nagler-Anderson, 2000). There is an intimate interplay between different subsets of T cells and antigen-presenting cells, such as dendritic cells, in the intestine. In murine models, high basal levels of IL-4, IL-10 and TGF-ß expression have been detected in the intestinal mucosa and this cytokine milieu may be crucial for the induction of Th2 and Th3 type responsiveness (Weiner, 2001). However, as it has recently been pointed out there may be major differences between species in mucosal immune responses. In fact, there are data on record indicating a Th1-skewed cytokine profile as a constant finding in the intestine of humans (MacDonald, 2001; Nagata et al., 2000). A transient induction of IFN-γ producing Th1 cells has been detected in the early phases of oral tolerance formation (Mowat et al., 1999). Even though both Th1 and Th2 cytokines regulate the function of Th3 cells, neither are essential for the induction of peripheral tolerance in a murine model (Garside et al., 1995; Mowat et al., 1999; Shi et al., 1999). The individual role of each functional subset of T cells in the inductive phase of oral tolerance thus remains to be elucidated, but it is evident that Th3 cells provide tolerogenic suppression both in the intestine and in other target organs.

1.2.2. Inflammatory response

Inflammatory and immune processes are mediated and controlled by a diverse range of molecules follow as: proteins; the pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukins (1L)-1 and -6; derivatives from membrane phospholipids; the eicosanoids (prostaglandins (PG), leukotrienes (LT)); diacylglycerol and ceramide; miscellaneous compounds such as CAMP, inositol phosphates and reactive oxygen species (Grimble, 1998).

The pro-inflammatory cytokines are predominantly products of the immune system; however, endothelial cells and fibroblasts also have the capability for production. Biologically, TNF acts as a trigger which activates a cascade of cytolune production. The molecule is released rapidly in response to inflammatory and infective agents, and induces production of a large number of other cytokines, including IL-1 and IL-6 with which it shares a number of actions in common (Akira et al., 1990). These include generation of a fever, reactive oxygen species and acute-phase protein production, muscle proteolysis, hyperglycaemia, hyperlipidaemia, up-regulation of adhesion molecules and changes in the plasma concentrations of cations (Tracey and Cerami, 1993; Grimble, 1996). TNF, by induction of the chemokine IL-8, may also prolong the inflammatory process (Standiford et al., 1990a,b). TNF can further modulate immunological events by induction of IL-1, whch stimulates IL-2 and IL-4 production; the latter two cytokines result in increased lymphocyte proliferation and switching of immunoglobulin classes respectively (Chrétien et al., 1990). TNF, in addition to its important role as an early effector in inflammatory and

immune processes, is important in killing fungi and a number of viruses (Ito and O'Malley, 1987). However, this cytokine can enhance human immunodeficiency virus replication (Shreck et al., 1991). Excessive or biologically inappropriate TNF production is closely associated with pathological events. Such events have been closely linked with mortality from cerebral malaria, endotoxic shock, sepsis and adult respiratory distress syndrome, and with pathology in a wide range of disorders (Tracey and Cerami, 1993). These include rheumatoid arthritis, inflammatory bowel disease, psoriasis and atherosclerosis (Grimble, 1996).

1.3. *Escherichia coli* challenge in weaned piglets

Diarrhoea in neonatal and early-weaned piglets due to enterotoxigenic *Escherichia coli* (ETEC) is an important problem in the pig farming industry. However, pigs older than approximately 8 weeks appear to be resistant to infection. Strains of ETEC that cause diarrhea in pigs possess two types of virulence factors, adhesins and enterotoxins, both of which are essential for disease to occur (Francis, 2002).

Toxins produced by ETEC strains that cause diarrhea in pigs include heat labile enterotoxin (LT), heat stable enterotoxin type A (STa); heat stable enterotoxin type B (STb); Shiga toxin type 2e (Stx2e), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) (Wilson and Francis, 1986; Imberechts wt al., 1997; Choi et al., 2001). Virulence determinants are not randomly distributed among virulent strains, but typically occur in patterns associated with specific serogroups and fimbriae. Clustering of virulence determinants around serogroups and the ages of pigs commonly infected with ETEC of each virulence type are shown on Table 1.2.

Almost all strains of ETEC isolated from weaned pigs with diarrhea contain the gene for STb. Near universal presence of this gene in postweaning diarrhea isolates strongly suggests that STb plays an important role in pathogenesis that is not duplicated by other enterotoxins. However, very few ETEC strains exhibit only STb genes. This suggests that other enterotoxins are also important in postweaning diarrheal disease. In addition to STb genes, postweaning isolates also typically possess genes for STa, LT, or both.

Table 1.2. Characteristics of enterotoxigenic *Escherichia coli* (ETEC) strains associated with infections in pigs of various ages (Francis, 2002)

	ETEC characteristics			Age group affected	
Serogroup	Fimbriae	Toxins	Hemolysins	Neonatal	Weaned
O8	K99	STa	–	Yes	No
O9	K99, 987P	STa	–	Yes	No
O20	987P	STa	–	Yes	No
O101	K99	STa	–	Yes	No
O141	987P	STa	–	Yes	Yes
O8	K88	LT, STb± STa	+	Yes	Yes
O149	K88	LT, STb± STa	+	Yes	Yes
O157	K88	LT, STb± STa	+	Yes	Yes
O138	F18ab, F18ac	STa, STb ± Stx2e	+	No	Yes
O139	F18ab	STa, STb ± Stx2e	+	No	Yes
O141	F18ac	STa, STb ± Stx2e	+	No	Yes
O157	F18ac	STa, STb ± Stx2e	+	No	Yes

1.3.1. Enterotoxigenic *Escherichia coli* F4 (K88)

Enterotoxigenic *Escherichia coli* F4 (K88) is a major cause of diarrhea and death in neonatal and weaned pigs (Francis et al., 1998) and K88 is the most prevalent form of diarrhea in the pig farming industry(Fairbrother et al., 2005). Enterotoxigenic *E. coli* K88 can not only colonize in the small intestine, but also release enterotoxins to stimulate the epithelial cells to secrete fluid into the lumen of the gut to cause diarrhea (Gaastra and Graaf, 1982). Attachment of K88+ ETEC to the host epithelial cells is mediated by an interaction of K88 fimbriae with K88-specific receptors present on the brush borders of the small intestinal enterocytes, enabling colonization of the small intestine (Van den Broeck et al., 1999). Subsequently, heat-labile (LT) and heat-stable (STa/b) enterotoxins are secreted, which induce severe diarrhoea. An excessive amount of water will follow by osmosis and will accumulate in the small intestine of the pig resulting in secretory diarrhoea (Nataro and Kaper, 1998). Weaning removes young pigs from passive immune protection from the milk of the sow and increases the susceptibility of the piglets to enterotoxigenic *E. coli* infection (Yuan et al., 2006). F4 (K88) fimbriae are an important target in vaccination studies against F4+ ETEC since they are a key virulence factor involved in mediating attachment (Cox et al., 2002). Indeed, oral immunization of F4R+ piglets with purified F4 fimbriae induces an F4-specific intestinal immune response, which protects them against a subsequent ETEC challenge (Van den Broeck et al., 1999; Verdonck et al., 2004). Furthermore, the presence of the F4R is a prerequisite for the successful immunization of piglets, indicating that receptor-mediated binding is important for the induction of a protective intestinal immunity (Van den Broeck et al., 1999). The strong immunogenicity of F4 fimbriae can be explained by their

resistance to digestive enzymes, their pH stability and their polymeric nature (Snoeck et al., 2004; Verdonck et al., 2008). Indeed, oral immunization with F4 fimbriae purified from F4+ ETEC mutants, in which the polymeric stability of the fimbriae is disrupted, resulted in reduced mucosal immune responses (Joensuu et al., 2006; Verdonck et al., 2008). Intestinal epithelial cells (IEC) are pivotal for the activation of innate immunity and subsequently for the induction of adaptiveimmuneresponses (Sansonetti, 2004). IEC function as sensors detecting pathogen-associated molecular patterns (PAMPs) through pathogen-recognition receptors (PRRs), such as Toll-like receptors (TLRs). Upon recognition of these PAMPs, IEC secrete several cytokines and chemokines, thereby alerting the underlying mucosal immune cells, such as dendritic cells, to trigger innate immune defences and promote adaptive immune responses (Kagnoff and Eckmann, 1997; Neutra and Kozlowski, 2006). However, studies on the influence of F4+ ETEC on the innate immune functions of IEC are limited. This incited us to elucidate how the polymeric nature of F4 fimbriae influences bacterial adhesion to porcine IEC and subsequently, the cytokine secretion profile of IEC in an in vitro IPEC-J2 culture system. IPEC-J2 cells provide a relevant model for intestinal epithelial cells since they form apical microvilli, express tight junction proteins, produce glycocalyx bound mucins and glycoproteins for bacterial adhesins, and are known to express cytokines and chemokines after bacterial stimulation (Burkey et al., 2006; Schierack et al., 2006; Skjolaas et al., 2007). In addition, F4+ ETEC can bind to IPEC-J2 (Koh et al., 2008; Johnson et al., 2009). Moreover, the IPEC-J2 cell line was derived from the porcine jejunum and the jejunal Peyer's patches are the major inductive site for F4+ ETEC specific immune responses (Snoeck et al., 2006).

1.3.2. Lipopolysaccharides

Lipopolysaccharides (LPSs) are characteristic components of the cell wall of Gram-negative bacteria, which are usually produced by different *E. coli* strains (Raetz and Whitfield, 2002). LPS and its lipid A moiety stimulate cells of the innate immune system by the Toll-like receptor 4 (TLR4), a member of the Toll-like receptor protein family, which recognizes common pathogen-associated molecular-patterns (PAMPs). Act as an intrinsic component of the outer membrane of gram-negative bacteria, LPS has frequently been used as a model to study immune-neuroendocrine interactions in pigs (Wright et al., 2000; Kanitz et al., 2002; Tuchscherer et al., 2004). LPS provokes the synthesis and release of cytokines by macrophages and neutrophils (Feghali and Wright, 1997). LPS also activates the hypothalamus-pituitary-adrenal (HPA) axis via pro-inflammatory cytokine stimulation, resulting in increased secretions of glucocorticoids (Johnson et al., 1996; Warren et al., 1997).

In addition, LPS challenge depressed significantly feed intake, body growth rate

and efficiency of feed utilization by enhancing protein degradation rate and decreasing protein utilization for body protein retention because of the induced immune stress (Chen et al., 2008). LPS has been shown to be a potent stimulant of macrophages that produce IL-1 and TNF upon activation (Feldmann and Male, 1989). It has been reported that the administration of pro-inflammatory cytokine resulted in lower voluntary feed intake in rats (Mrosovsky et al., 1989) and in pigs (Fink et al., 1995). The reduction in voluntary feed intake is associated with an IL-1-induced release of CRH (Navarra et al., 1991) and IL-8, which serve as a potent stimulant of the lateral hypothalamus (Plata-Salaman and Borkoski, 1993). The increased protein degradation in LPS-challenged pigs is likely to meet the amino acid requirements for the synthesis of acute phase protein (Klasing, 1998). Cytokines such as IL-6, IL-1, and TNF mediate the protein synthesis and degradation by decreasing the release of anabolic hormones e.g. somatotropin (Honegger et al., 1991) and IGF-1 (Fan et al., 1994), increasing catabolic (glucocorticoid) hormone release (Navarra et al., 1991).

1.4. Functional foods

1.4.1. Essential oils

Essential oils (EOs) are volatile components of plants that have been extensively studied and used in a wide range of food systems to increase the safety and shelf life of foods (Burt, 2004). These oils are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the United States and have been used as artificial flavourings and preservatives, in the manufacture of perfume, and in over-the-counter formulations of medicines. The major components of a number of EOs with antibacterial properties are presented in Table 1.3. Recent studies on EOs as alternatives to antibiotics in feed have demonstrated that some EOs have strong antibacterial activity towards pure cultures of bacterial pathogens (including *E. coli* K88), but little inhibition to lactobacilli and bifidobacteria, even in the presence of cecal digesta (Si et al., 2006a,b). Studies are in progress to protect EOs and for effective delivery of EOs to the target region of animal guts, using encapsulation approach in microcapsules made from Ca-alginate hydrogel (Wang et al., 2009).

Impacts of EOs on growth performance of newly-weaned pigs have been inconsistent, although changes in gut microbiota composition were observed (Manzanilla et al., 2004; Gong et al., 2008). EOs are generally hydrophobic. They can be absorbed quickly after oral, pulmonary or dermal administration (Kohlert et al., 2000). In piglets, EOs were found to be absorbed nearly completely in the stomach and the proximal small intestine within 2 h after oral administration (Michiels et al., 2008). Together, these observations suggest that essential oils need protection for delivery to the target site within the pig's GIT to exert their anti-microbial activity (de Lange et al., 2010). In fact, human clinical studies have

shown that entericcoated essential oil (peppermint) capsules exhibited a better performance in treatment of irritable bowel syndrome compared to the uncoated formulation (Liu et al., 1997; Logan and Beaulne, 2002).

Table 1.3. Major components of selected[a] EOs that exhibit antibacterial properties (remade from Burt, 2004)

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]
Cilantro	*Coriandrum sativum*	Linalool	26%
	(immature leaves)	E-2-decanal	20%
Coriander	*Coriandrum sativum* (seeds)	Linalool	70%
		E-2-decanal	–
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	65%
Oregano	*Origanum vulgare*	Carvacrol	Trace-80%
		Thymol	Trace-64%
		γ-Terpinene	2 –52%
		p-Cymene	Trace-52%
Rosemary	*Rosmarinus officinalis*	a-pinene	2 –25%
		Bornyl acetate	0 –17%
		Camphor	2 –14%
		1,8-cineole	3 –89%
Sage	*Salvia officinalis*	L. Camphor	6 –15%
		α-Pinene	4 – 5%
		β-pinene	2 –10%
		1,8-cineole	6 –14%
		α-tujone	20–42%
Clove (bud)	*Syzygium aromaticum*	Eugenol	75–85%
		Eugenyl acetate	8 –15%
Thyme	*Thymus vulgaris*	Thymol	10–64%
		Carvacrol	2 – 11%
		γ-Terpinene	2 –31%
		p-Cymene	10–56%

[a] EOs which have been shown to exert antibacterial properties in vitro or in food models and for which the composition could be found in the literature.

[b] Percentages of total volatiles rounded up to the nearest whole number.

The improvements have presumably been attributed to the delayed absorption of the coated peppermint oils and the prolonged contact between peppermint oil and bacterial cells. In our recent effort, carvacrol was used as a model essential oil and successfully encapsulated in microcapsules made from Caalginate hydrogel using an emulsion-extrusion technology with high encapsulation efficiency. Encapsulated carvacrol retained its high anti-microbial activity towards E. coli K88 in a culture medium, as well as in a simulated gastrointestinal model (Wang et al., 2009). It remains to be determined if the encapsulated essential oil will reduce the burden of enteric pathogens in the gut of weaning piglets and improve pig performance.

1.4.1.1 Thymol

Thymol (2-isopropyl-5-methylphenol) is a predominant component of several essential oils derived from plant species belonging to the Lamiaceae family (figure 1.2), which is found in thyme oil, ajowan seeds (also called ajwain or carom), and horsemint. It is an isomer of carvacrol, meaning it has the same molecular formula but the atoms are arranged differently. Its antimicrobial properties urged researchers to study its ability to improve animal performance and intestinal health in pigs (Si et al., 2006b; Trevisi et al., 2007; Michiels et al., 2009, 2010). This compound has shown to possess spasmolytic and antioxidant (Youdim and Deans, 2000; Luna et al., 2010) properties and to affect the immune response by inhibition of the release of elastase (Braga et al., 2006) and cyclo-oxygenase activity (Marsik et al., 2005) which could be part of a growth-promoting claim. However, thymol, like other essential oils and their components, has a distinctive, marked flavour.

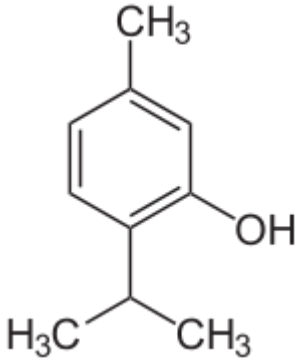

Figure 1.2. Structural formula of thymol.

Thymol is able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride has been shown to have no influence on this action, suggesting a mechanism other than chelation of cations in the outer membrane (Helander et al., 1998).

Juven et al. (1994) examined the working of thymol against S. typhimurium and Staph. Aureus and hypothesised that thymol binds to membrane proteins hydrophobically and by means of hydrogen bonding, thereby changing the permeability characteristics of the membrane. Thymol was found to be more inhibitive at pH 5.5 than 6.5. At low pH the thymol molecule would be undissociated and therefore more hydrophobic, and so may bind better to the hydrophobic areas of proteins and dissolve better in the lipid phase (Juven et al., 1994).

1.4.1.2. Cinnamaldehyde

Cinnamaldehyde (3-phenyl-2-propenal), is the organic compound that gives cinnamon its flavor and odor, which has been used traditionally as condiments and flavoring agents. The natural product is trans-cinnamaldehyde and the molecule consists of a phenyl group attached to an unsaturated aldehyde (Figure 1.3). The antioxidant, antifungal and antibacterial potentials of cinnamaldehyde were investigated in the previous studies (Smith-Palmer et al., 1998; Singh et al., 2007). Researchers from the University of Illinois at Chicago (who were funded by the Wm. Wrigley Jr. Company) have found that cinnamic aldehyde, when used in Big Red, prevented oral bacterial growth by more than 50%. Although cinnamaldehyde inhibits the growth of *E. coli* O157:H7 and S. typhimurium at similar concentrations to carvacrol and thymol, it did not disintegrate the outer membrane or deplete the intracellular ATP pool (Helander et al., 1998). The carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (Wendakoon and Sakaguchi, 1995).

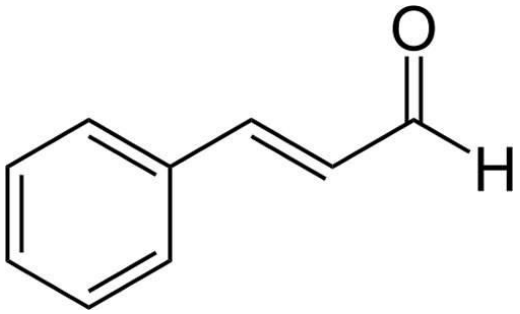

Figure 1.3. Structural formula of Cinnamaldehyde

1.4.2. Enzymes

The main goal for using exogenous feed enzymes in swine diets has been to improve the nutritive value of feedstuffs. This is achieved through several mechanisms including the breakdown of anti-nutritional factors present in feed ingredients, elimination of nutrient encapsulation effect thus increasing availability, breakdown of specific chemical bonds in raw materials that are otherwise not cleaved by endogenous enzymes, thus releasing more nutrients, and complementation of the enzymes produced by young animals (Simon, 1998; Bedford and Schulze, 1998). Majority of the vegetable feedstuffs used in swine diets contain a considerable amount of non-starch polysaccharides (NSP) whose antinutritional effects are well-established and has been a subject of intense research (de Lange et al., 2000). Thus, the use of carbohydrase enzymes in swine diets has mainly focused on eliminating the anti-nutritional activities associated with the NSP components of feed. Indeed, several studies have shown that with

appropriate enzyme preparations, these antinutritional effects can be minimized with a potential improvement in the nutritional value of feedstuffs for young pigs (Li et al., 1996; Omogbenigun et al., 2004) and that a combination of different enzyme activities is required for degradation of complex NSP to improve nutrient utilization (Meng et al., 2005).

In addition to improved nutrient utilization, enzymes may improve performance of young pigs through the production of a variety of polysaccharide hydrolysis products that have a direct effect on intestinal health by manipulating the growth of gastrointestinal microorganisms (Bedford, 2000; Williams et al., 2001; Pluske et al., 2002). Indeed, studies with nursery pigs (Kim et al., 2003) and broilers (Mathlouthi et al., 2002) suggest that the use of feed enzymes may have a positive impact on intestinal health. In a study with piglets, Inborr and Ogle (1988) reported that supplementing moistened barley with a mixture of carbohydrate degrading enzymes was effective in reducing both the incidence and severity of diarrhoea. In recent studies utilizing an in-situ model of secretory diarrhoea in piglets, Kiarie et al. (2008) reported that NSP hydrolysis products generated by incubating soybean meal and canola meal with a multicarbohydrase enzyme blend were beneficial in maintaining intestinal barrier function during enterotoxigenic *E. coli* infection. Similar observations were obtained with NSP hydrolysis products from wheat and flaxseed. These observations could be explained by various mechanisms, including the possibility that hydrolysis products interfere with the attachment of pathogens to the intestinal mucosa, which is an important first step in infection. These products may also act as prebiotics (Cummings and MacFarlane, 2002), favouring the proliferation of lactic acid-producing bacteria as has been shown by Kiarie et al. (2007) and which in turn may indirectly prohibit the growth of certain pathogenic species (Choi et al., 1994). Feed enzymes may also improve gut health by reducing the intestinal viscosity due to soluble NSP, which might reduce rate of digesta passage, diffusion of digestive enzymes, and increase endogenous gut protein secretions. This will in turn increase substrate availability in the lower gut for microbial proliferation (Omogbenigun et al., 2004), as discussed in Section 6. Among other effects, increased viscosity of intestinal digesta in weaned pigs enhances proliferation of pathogenic bacteria like enterotoxigenic *E. coli* and *Brachyspira pilosicoli* (Hopwood et al., 2002, 2004). Thus, it has been hypothesized that supplementing swine diets with enzymes to digest soluble NSP will minimize intestinal microbial load which in turn will increase nutrient availability to the host and minimize proliferation of pathogenic bacteria.

1.4.2.1 xylanase

Xylan, the major component of hemicellulose, is a heterogeneous polysaccharide with a backbone consisting of a β-D-(1 → 4) linked xylopyranoside backbone substituted with many side chains. Complete breakdown of xylans requires the synergistic action of several enzymes of which endo-β-1,4-xylanases (EC 3.2.1.8) are the crucial enzymes for depolymerisation. In recent years, xylanases have attracted considerable research interest because of their potential benefits in the animal nutrition and feed industry. The cell walls of cereals contain up to 15% NSP; exogenous enzymes can hydrolyse these carbohydrates into smaller units that can be utilised by animals (He et al., 2010).

The fungus *Trichoderma* reesei is a filamentous mesophilic fungus which has been shown to secrete large amounts of efficient xylan-degrading enzymes (Wong and Saddler, 1992). The *T. reesei* xylanase 2 (Xyn2) has been in industrial use for many years, since it represents more than 50% of the total xylanolytic activity of this fungus (Törrönen et al., 1994). However, the industrial enzymes are often used in the unpurified form. There are many side activities that can be a problem with the use of these enzymes (Wong and Saddler, 1992). For instance, the relatively crude xylanase preparation with residual cellulolytic activity requires the careful control of process parameters to avoid damage to fibres. In addition, the residual protease may degrade xylanases of interest over time (La Grange et al., 1996). The *T. reesei* Xyn2 produced in a recombinant host has an advantage of showing xylanase activity that is practically free of harmful side activities. Therefore, the recombinant production hosts are preferred (Jiang et al., 2004).

1.4.2.2 β-glucanase

β-Glucans are structural components of cereals (wheat and barley), and of fungal cell walls, which cannot be digested by mammalian enzymes, and are therefore potentially useful substrates for GI fermentation (Englyst et al. 1989; Edney et al. 1991). β-Glucan accounts for approximately 75% of the barley endosperm cell wall and may interfere with the digestion and absorption processes in the small intestine, even the production of digestive enzymes in pigs (Fan et al., 2009).

However, they may have beneficial effects on microbial composition in terms of increasing the number of *Lactobacilli*. Jonsson and Hemmingsson (1991) showed a correlation between the diet of piglets and the occurrence of cultivable faecal *Lactobacilli* with an ability to degrade β-D-glucans. Dongowski et al. (2002) investigated the effect of barley-rich diets on the GIT in young rats, and found decreased counts of coliforms and Bacteroides for rats fed the barley-based diets, with concomitant increased *Lactobacillus* counts. Specific PCR amplification of 16S rRNA gene fragments in combination with denaturing gradient gel electrophoresis was used to analyse the microbial community before and after in

vitro fermentation, using chicken caecal contents as the original inoculum. The polysaccharide extracts led to significant shifts in the bacterial community when fermented *in vitro*.
In addition, numerous researches have been carried out with growing pigs fed diets supplemented with β-glucanase. Significant improvements in performance of piglets were also reported when β-glucanase was supplemented to barley-based diets (Li et al., 1996).

1.4.3. Probiotics

Probiotics are the bacteria with beneficial effects to host health through modulating microbiota, antagonizing enteric pathogens, or enhancing intestinal barrier and immune functions (Gagnon et al., 2007; Borchers et al., 2009; Lessard et al., 2009). In addition, supplementation of probiotics may decrease the morbidity and mortality of farm animals and increase performance (Taras et al., 2005; Lodemann et al., 2006) by regulating intestinal microbial balance by increasing the activity of microbial digestive enzymes, it improves digestion, feed digestibility and nutrient utilization (Bomba et al., 2002).
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).
Probiotics may play an important role in overcoming problems related to intestinal disease and consequent diarrhea. Probable mechanisms of probiotics to improve intestinal microbiocenosis 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

The effect of probiotics depends on the combination of selected bacterial genera (Table 1.4), their doses, and on the interactions of probiotics with some pharmaceuticals, feed composition, storage conditions and feed technology.

Multiple studies have confirmed the stimulating effects of probiotics on the intestinal environment (Vondruskova et al., 2010): 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* and *Streptococcus* and their combinations. The bacteria *Enterococcus* faecium were found to be able to prevent the K88 positive ETEC strain from adhering to the intestinal mucous membrane of piglets (Scharek et al., 2005).

Table 1.4. 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	*L. lactis*
Enterococcus	*E. faecium*
Streptococcus	*S. thermophilus*
Pediococcus	*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*

Probiotics are mainly active in the caudal segments of the ileum, in the caecum and the ascending colon. Probiotics influence the digestive process in the body by increasing the activity of microbial probiotic enzymes and the digestibility of food (Roselli et al., 2005). They stimulate the immune system and the regeneration of intestinal mucosa. Probiotics can elicit an increase in

immunoglobulin a production, stimulate macrophages and NK cells (Matsuzaki and Chin, 2000), and regulate anti- and pro-inflammatory cytokine production (Roselli et al., 2005).

1.4.4. Prebiotics

Prebiotics are dietary short-chain carbohydrates (oligosaccharides), which cannot be digested by pigs, 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; Bouhnik et al., 2004). 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 1.5, natural sources of oligosaccharides exist: e.g. galactooligosaccharides in breast milk, fructans in onion (*Allium cepa*), leeks (*Allium porrum*) and garlic (*Allium sativum*), stachyose in soyabean; however, 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 because of their nutritional interest.

Table 1.5. Dietary oligosaccharides available in food products on the market and the type of source available (Delzenne et al., 2003)

Natural source	Type of oligosaccharides	Industrial production process
Fruits and vegetables	Fructo-oligosaccharides	Synthesis from saccharose
		Hydrolysis from chicory-root inulin
Human milk	Galacto-oligosaccharides	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
Soybean	Stacchyose, raffinose	
	Palatinose-oligosaccharides	
	Gentio-oligosaccharides	
	Cyclodextrin	Synthesis from starch

Prebiotics are fermented ingredients that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health. 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 is an important outcome, which is through fermentation of oligosaccharides by colonic flora. 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 1.6 summarises some studies investigating the effects of different fermentable carbohydrates on the composition of the gut microbiota (Bauer et al., 2006).

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). 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. 2004, 2006).

Table 1.6. *In vitro* and *in vivo* studies investigating effects of fermentable carbohydrates on the composition of the gastrointestinal microbiota (Bauer et al., 2006).

Fermentable carbohydrate	Origin or host species of microbial sample	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 FOS Galactosyllactose	Faecal samples of adult	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*) Herb polysaccharides (*Astragalus membranaceus*)	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 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
Galacto-oligosaccharides Galacto-oligosaccharides ± *Bifidobacterium lactis* Bb-12	Faeces, human	No effect on indigenous *Bifidobacterium* counts Transient colonisation with *B. lactis*

1.4.5. Bovine colostrum

Bovine milk has long been associated with contributing towards a balanced nutrition, and as an important foodstuff in its own right. The nutritional benefits of milk-derived proteins, vitamins and minerals have been promoted extensively by commercial dairying enterprises, and the vast range of milk-based products now available owes a lot to the continued consumer image of milk as a `healthy' product. For its physiological significance bovine milk can be considered as "functional food" for its essential components for the growth during the neonatal period and for its richness in bioactive molecules with immunological, anti-infective actions due to protect the health of the newborn (Séverin and Xia, 2005).

About 80% of bovine milk proteins consist of caseins (2.7 g/100 g milk), a

group of phosphate-containing proteins named a_s1-, a_s2-, *β*-, and *ϰ*- casein (Modler, 2000; Fox and McSweeney, 2001). Caseins are synthesized by the mammary secretory epithelium and are without any known biological activity. The bovine whey protein fraction consists of *a*-lactalbumin, *β*-lactoglobulin, bovine serum albumin, immunoglobulin, lactoferrin, transferrin, and the proteosepeptone fraction (0.08 g/100 g milk). These compounds are secreted from the blood into the milk or are synthesized in the mammary gland. A brief outline of bioactive peptides derived from bovine milk, their precursors, and possible bioactive role is shown in Table 1.7.

Table 1.7. Bioactive peptides derived from bovine milk proteins (Séverin and Xia, 2005)

Bioactive peptides	Protein precursor	Bioactivity
Casomorphins	*a*-, *β*-Casein	Opioid agonists
a-Lactorphin	*a*-, *β*-Casein	Antihypertensive
β-Lactorphin	*ϰ*-Casein	Opioid antagonists
Lactoferroxins	*ϰ*-Casein, transferrin	Antithrombotic
Casoxins	*a*-Lactalbumin	Opioid agonist
Casokinins	*β*-Lactoglobulin	Opioid agonist
Casoplatelins	Lactoferrin	Opioid antagonists
Immunopeptides	*a*-, *β*-Casein	Immunostimulants
Phosphopeptides	*a*-, *β*-Casein	Mineral carriers

Many authors also demonstrated that bovine milk by-products contain bioactive molecules with immuno-regulatory and antimicrobial properties (Cross and Gill, 2000; Schlimme et al., 2000). Both contain peptides such as lactoferrin, transforming growth factor-β, caseinoglycopeptides, epidermal growth factor (EGF) and cytokines such as interleukin (IL)-1β, IL-6, IL-10, interferon-γ (IFN-γ), osteopontin and tumor necrosis factor-α (TNF-α) (Wang and Denhardt, 2008) that can modulate bactericidal capacity of neutrophils, phagocytic function of macrophages, proliferative response of lymphocyte to mitogens and antibody responses to foreign antigens (Cross and Gill, 2000). The role of derived substances from milk or colostrum for wound repair function has been investigated (Velioglu Ogünc et al., 2008; Zava et al., 2009) and there are increased data showing that milk and colostrum components protect and promote gut development. Both colostrum and milk are also rich in oligosaccharides and there is evidence that neutral oligosaccharides present in milk are not digested or absorbed into the small intestine, but delivered into the colon instead (Boehm and Stahl, 2007). These molecules have been shown to play an important role in the establishment of different bacterial populations in the gut (Kuntz et al., 2008) and have the potential to inhibit binding of pathogenic Gram-negative bacteria such as E. coli to intestinal host cells (Newburg et al., 2005). Therefore, bovine colostrum could then be more

appropriate then plasma protein as protein source in weanling diet.

1.4.6. Cranberry

There are two kinds of cranberry (CB) fruit. The small-fruited or European CB (*Vaccinium oxycoccos*) is found in marshy land in northern North America, northern Asia, and northern and central Europe. Vinson et al. (2008) observed the order of total polyphenols on a fresh weight basis of cranberry which was shown in Table 1.8: dried CBs > frozen CBs > CB sauce > jellied sauce. CB powder usually is not considered a food, but it had the most polyphenols of the CB products and possessed the most variability (Vinson et al., 2008).

Table 1.8. Free and Total Phenols as Catechin Equivalents in cranberry (CB) Products (Fresh weight) (Vinson et al., 2008)

CB product	free phenol (mg/g)	total phenol (mg/g)
CB Sauce		
brand A whole berry	1.22	2.70
brand B whole berry	2.03	3.54
brand C whole berry	1.48	1.94
brand D whole berry organic	2.90	3.63
av	1.91 ± 0.75	2.96 ± 0.64
Jellied Sauce		
brand A	1.28	1.60
Brand B	1.45	2.64
av	1.36 ± 0.12	2.12 ± 0.73
Frozen CBs		
Massachussetts (n = 5)	5.10 ± 0.99	6.15 ± 1.45
New Jersey (n = 4)	5.25 ±1.28	6.35 ± 1.65
Wisconsin (n = 7)	5.10 ± 0.70	7.16 ± 1.19
Oregon (n = 4)	4.81 ± 0.44	5.68 ±0.64
av of all samples	5.08 ± 0.81	6.44 ± 1.31
Dried CBs		
brand A	4.26	5.77
brand B	10.2	11.9
brand C	6.00	9.89
brand D	4.96	7.25
brand E	4.32	5.28
av	6.35 ± 7.67	8.73 ± 2.78
CB Powder		
brand A	1.28	1.62
brand B	6.12	13.5
brand C	2.73	3.39
brand D	1.60	20.65
av	6.53 ± 6.61	9.77 ± 8.9

Cranberries (Vaccinium macrocarpon) contain many bioactive compounds that have antioxidant, anti-mutagenic, antihypercholesterolemic and other beneficial

health properties such as preventing urinary tract infections (Cunningham et al., 2004; Vattem et al., 2005; Neto, 2007; Vinson et al., 2008). Phenolic phytochemicals in the cranberries are now known to have potential for inhibition of development and progression of cancer and cardiovascular diseases (Reed, 2002; Vattem et al., 2005). The antibacterial activity of cranberry (V. macrocarpon) concentrate against commonly occurring foodborne pathogens in vitro was reported by Wu at 2009. Cranberry (concentrate and pulp) have been shown to have beneficial effects on health through their antioxidant property and antimicrobial activity towards food borne pathogens, such as *E. coli* and Salmonella typhimurium (Reid, 2002; Bodet. et al., 2008; Vinson et al., 2008; Wu et al., 2009).

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CHAPTER 2

Objectives

2. Objectives

The main objective of this study was to improve our knowledge on the properties of functional foods as feeding strategies, in order to increase general health in post-weaning piglets, with the aim to the alternative to in-feed antibiotic. Three trials were designed to study the strategies.

2.1. Abstract 1

The objective of the current study was to evaluate the effects of essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) either alone or in combination on growth performance, fecal score, fecal digestibility of nutrients, bacterial counts, ileal histomrphological parameters and mRNA gene expression in weaned piglets. One hundred and ninety two 24 d old weaned piglets (Stambo HBI Dalland 40) with an average initial body weight of 8.10 kg were allocated according to body weight into 4 experimental treatments (12 replicates per treatment with 4 piglets per replicate). Each group was fed the basal diet with or without supplementation with either essential oils or enzymes, or a combination of these for a 42-d period. Growth performance was measured weekly, and fecal samples for microbial population analysis were collected at 0, 14 and 42 d. The experiment with regard to digestibility was designed with two periods (d 15 to 21 and d 29 to 35) and the feces were collected at 21 and 35d. At the end of trial (42 d), six piglets from each treatment were slaughtered, the gastro-intestinal tract was removed from each animal, and the distal ileum was collected and examined for histological, histochemical, and histometrical study. Ileal gene expression was also measured. There was no effect of essential oils and/or enzymes supplementation on the growth performance of piglets. However, the combination of essential oils and enzymes decreased feed conversion ration during the last week. Meanwhile, the additives did not affect fecal score and there was no diarrhea incident. Although the fecal digestibilities of all the piglets were increased from d 21 to 35 ($P < 0.001$), no effect of essential oils or enzymes or the combination on the fecal digestibility was observed. Although dietary combination decreased population of *Lactobacilli* at d 14 ($P = 0.030$) and supplementation of EO or XB reduced *Lactobacilli* counts at d 42 ($P = 0.051$) as compared to Control, all the additives significantly decreased counts of *Coliforms* at 42 days of the trial ($P < 0.001$). Dietary enzymes improved gut health by decreasing crypt depth, increasing V: C ratio and reducing the number of macrophages ($P < 0.001$). Supplementation of essential oils and the combination with enzymes improved gut morphology by decreasing crypt depth ($P = 0.065$; $P < 0.001$), and decreasing the number of lymphatic follicles ($P = 0.002$; $P < 0.001$) and macrophages ($P < 0.001$). No effect of additives on gene

expression was observed in ileal mucosa. Results showed that diet supplementation with EO and/or XB had positive effects on intestinal bateria and gut morphology.

2.2. Abstract 2

The objective of the current study was to evaluate the possible protective effects of nutritional supplements essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) either alone or in combination on growth performance, antioxidant defense system, haematological profile, bacterial counts, ileal histomorphology and gene expression in piglets under *Escherichia coli* challenged condition. One hundred and ninety two 24 days old weaned piglets (Stambo HBI Dalland 40) with an average initial body weight of 8.64 kg were allocated according to body weight into eight experimental treatments (6 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments [a weaned piglet control diet (CTR), CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde, EO), CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase, XB), and CTR + 0.05 g/kg EO + 0.1 g/kg XB] and 2) with or without an *E. coli* challenge. At 8 d of the trial, half of the piglets in each diet group were orally inoculated with 4 ml of virulent *E. coli* 0149: F4 (K88)-positive strain (1×10^9cfu/ml). Animal weight and growth performance were measured weekly. Fecal score (FS; 1 = hard; 5 = watery diarrhea) was recorded every day. Fecal samples were collected at 9, 14 and 35 d, and blood samples were collected at 7, 9 and 35 d. At the end of trial (35 d), 6 piglets from each treatment was selected according to its body weight and slaughtered and intestinal samples were collected. Immediately after slaughtering, the gastro-intestinal tract was removed from each animal, the cecum content of each piglet was collected for the microbial assay, and the distal ileum was collected and examined for histological, histochemical, and histometrical study. Gene expression of ileal mRNA was also measured. *E. coli* challenge significantly impaired growth performance, induced severe diarrhea, increased populations of *Clostridia, E. coli* and *Coliforms*, depressed antioxidant activities, damaged gut morphology and promoted mRNA gene expression ($P < 0.05$). Supplementation of enzymes or combination with essential oils improved feed efficiency in *E. coli* challenged animals compared with control treatment during the last week ($P = 0.025$; $P = 0.020$). In the unchallenged groups, dietary XB and combination decreased *E. coli* counts in the cecum ($P = 0.007$; $P = 0.002$), and supplementation of EO reduced populations of *Clostridia* in the faeces at d 14 and decreased *Coliforms* counts in the cecum ($P = 0.024$; $P = 0.046$). The *Coliforms* populations of *E. coli* challenged piglets fed combination of essential oils and enzymes were lower than control treatment in the cecum ($P < 0.001$). The *E. coli* challenged piglets fed enzymes or

combination with essential oils had lower T-AOC activities than unsupplemented animals at d 35 ($P = 0.004$; $P = 0.003$). Supplemented essential oils and/or enzymes improved gut health by increasing villus height and V: C ratio and decreasing crypt depth ($P < 0.001$). Results showed that diet supplementation with EO and/or XB had positive effects on intestinal intestinal bateria and gut morpholgy when piglets were submitted to a bacterial challenge.

2.3. Abstract 3

In this study, the effects of dietary cocktail and body weight on production of inflammatory cytokines and time responses under Lipopolysaccharides (LPS) challenge were evaluated in early weaned piglets. Possible interaction between diets and weight were also assessed. Thirty-two Yorkshire × Landrace sows with their litters were allocated into four groups, using a randomized complete block design. After weaning at 20 ± 1 d of age, a total of 256 piglets were allocated into eight experimental treatments (8 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments: a basal weaning diet added [spray-dried plasma protein (PP) (CTR), PP + antibiotic (ATB), PP + dietary cocktail (DC), or bovine colostrum + the dietary cocktail (BC+DC)] and 2) low weight (LW) or high weight (HW). At 37 d of age, 2 piglets in each pen were injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) of LPS per animal. Piglets were bled (10 ml) before LPS injections (T0) and also bled at 4 h or 18 h post-injections. The peripheral blood mononuclear cell (PBMC) samples, which were stimulated by 0.5 or 1.0 μg/ml LPS, 1.0 μg/ml Concanavalin A (ConA), 50 ng/ml Phorbol Myristate Acetate (PMA) or none (NS), were isolated from heparinized whole blood of the piglets before inoculation of LPS at 37 days of age. Stimulations of LPS and PMA in PBMCs of piglets significantly induced TNF-α, IL-8 and IL-10 ($P < 0.05$). There was no diet effect on the concentrations of TNF-α, IL-8 and IL-10 in PBMCs of piglets ($P > 0.05$). Piglets had low weight tended to increase the concentration of IL-8 ($P = 0.106$) and IL-10 ($P = 0.098$) in the PBMCs stimulated by LPS 0.5μg/ml LPS and the concentration of IL-10 ($P = 0.097$) in PMA stimulated PMBC compared with high weight animals. Infection with LPS increased ($P < 0.001$) serum concentrations of all the cytokines four hours post inoculation, and animals recovered to basal levels at 18 h after challenge. No significant diet effect was found in the serum concentrations of cytokines ($P > 0.05$). At 4 h after challenge, low weight piglets had partially greater serum concentrations of TNF-α ($P = 0.046$), IL-6 ($P = 0.158$), IL-8 ($P = 0.179$) and IL-10 ($P = 0.185$) than high weight animals. In conclusion, dietary cocktail or combined with bovine colostrum may replace plasma protein and antibiotics and weight difference may influence the production of inflammatory cytokines after infected by LPS.

CHAPTER 3

Effects of essential oils and enzymes on growth performance, nutrient digestibility, bacterial counts and gut morphology in piglets after weaning

3. Effects of essential oils and enzymes on growth performance, nutrient digestibility, bacterial counts and gut morphology in piglets after weaning

3.1. Abstract

The objective of the current study was to evaluate the effects of essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) either alone or in combination on growth performance, fecal score, fecal digestibility of nutrients, bacterial counts, ileal histomrphological parameters and gene expression of inflammatory cytokines in weaned piglets. One hundred and ninety two 24 d old weaned piglets (Stambo HBI Dalland 40) with an average initial body weight of 8.10 kg were allocated according to body weight into 4 experimental treatments (12 replicates per treatment with 4 piglets per replicate). Each group was fed the basal diet with or without supplementation with either essential oils or enzymes, or a combination of these for a 42-d period. Growth performance was measured weekly, and fecal samples for microbial population analysis were collected at 0, 14 and 42 d. The experiment with regard to digestibility was designed with two periods (d 15 to 21 and d 29 to 35) and the feces were collected at 21 and 35d. At the end of trial (42 d), six piglets from each treatment were slaughtered, the gastro-intestinal tract was removed from each animal, and the distal ileum was collected and examined for histological, histochemical, and histometrical study. Ileal gene expression was also measured. There was no effect of essential oils and/or enzymes supplementation on the growth performance of piglets. However, the combination of essential oils and enzymes decreased feed conversion ration during the last week. Meanwhile, the additives did not affect fecal score and there was no diarrhea incident. Although the fecal digestibilities of all the piglets were increased from d 21 to 35 ($P < 0.001$), no effect of essential oils or enzymes or the combination on the fecal digestibility was observed. Although dietary combination decreased population of *Lactobacilli* at d 14 ($P = 0.030$) and supplementation of EO or XB reduced *Lactobacilli* counts at d 42 ($P = 0.051$) as compared to Control, all the additives significantly decreased counts of *Coliforms* at 42 days of the trial ($P < 0.001$). Dietary enzymes improved gut health by decreasing crypt depth, increasing V: C ratio and reducing the number of macrophages ($P < 0.001$). Supplementation of essential oils and the combination with enzymes improved gut morphology by decreasing crypt depth ($P = 0.065$; $P < 0.001$), and decreasing the number of lymphatic follicles ($P = 0.002$; $P < 0.001$) and macrophages ($P < 0.001$). No

effect of additives on gene expression was observed in ileal mucosa. Results showed that diet supplementation with EO and/or XB had positive effects on intestinal bateria and gut morphology.

Key words: essential oils, enzymes, growth performance, fecal digestibility of nutrients, bacterial counts, gut morphology, weaned piglet

3.2. Introduction

Post-weaning piglets are vulnerable for gastrointestinal disorder and infection, usually leading to diarrhea and eventually losses of economic. Supplementation of antibiotics can stabilize the performance and health particular of the young animals, while the natural origin feed additives have been widely used in European Union to reduce the negative impact due to antibiotics ban since 2006. Previous studies indicated that certain essential oils and enzymes might have beneficial effects on animal performance and health status because of other properties besides their sensory characteristics. Some of these compounds have been reported to improve animal performance because of their stimulating effect on salivation and pancreatic enzyme secretions or by having a direct bactericidal effect on gut microflora (Hardy, 2002). Numerous studies have shown that thymol and carvacrol in vitro exhibit antibacterial (Sivropoulou et al., 1996; Dorman and Deans, 2000; Lambert et al., 2001; Burt, 2004), and cinamaldehyde from cinnamon has also shown antimicrobial effects (Mancini-Filho et al., 1998). Improvements in growth performance were observed when xylanase was supplemented to wheat-based diets (Dusel et al., 1997; Jeroch et al., 1999) and also supplemented β-glucanase and xylanase to barley-based diets improved performance of piglets (Fan et al. 2009; Inborr et al., 1993; Li et al., 1996). Choct and Annison (1992) reported that supplementation of xylanase and β-glucanase improved growth performance of broiler chicks by decreasing the antinutritive effects of viscous nonstarch polysaccharides. And the decrease in digesta viscosity after exogenous enzyme addition is most likely associated with an improvement in small intestine wall morphology (Yasar and Forbes, 2000). Diebold et al. (2004) observed that supplemented xylanase and phospholipase with wheat-based diet positively affected ileal nutrient and energy digestibilities while there was no effect of xylanase or the combined enzyme supplementation on the fecal nutrient digestibilities and fecal concentrations of VFA.

The aim of the present work was to assess the effects of essential oils (Thymol and Cinnamaldehyde, EO) and/or enzymes (xylanase and β-glucanase) on growth performance, fecal digestibility of nutrients, microbial counts and gut health in weaned piglets. In addition, fecal scores and gene expression were also determined.

3.3. Materials and methods

3.3.1. Animals, housing, experimental design

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 ninty-two crossbred (Stambo HBI Dalland 40) piglets (equal number of males and females; average weight, 8.10 kg) homogeneous for age and litter origin, were weighed and randomly assigned to treatments. Animals were housed in 48 pens, environmentally regulated in an isolated stable. Piglets were allocated to one of the following dietary treatment: basal weaning diet supplemented with i) No additive (Control); ii) 50 mg/kg Essential Oils (Thymol and Cinnamaldehyde, EO); iii) 100 mg/kg Enzymes (Xylanase and β-glucanase, XB) ; iv) 50 mg/kg EO + 100 mg/kg XB. 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 ºC at the start of the trial, adjusted weekly until a final temperature of 26 ºC. Piglets were hosed in pens (4 piglets/pen), located beside a 120 cm walkway with 12 pens (1.20 m × 1.00 m) each side, with a slatted floor. Each pen was equipped with two water nipples and self-feeder. Piglets were used in a 42-d experiment to assess the effect of EO and/or enzyme. Each treatment consisted of 12 replicates with 4 piglets. Each pen represented one treatment replicate.

Diet was formulated to be isonutritive, exceeding the protein requirement recommended by NRC (1998) for pigs. The approximate composition and the chemical 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. All the diets were meal and milled at 1.5 mm particle size. At 14 d of the trial, the diet was become to starter feed for the last four weeks. Chromium (III) oxide (Cr2O3, 2 g/kg) was used as an indigestible marker for the determination of apparent nutrient digestibility.

3.3.2. Performance measurements

Piglets were individually weighed at weaning (day 0) and subsequently every week until the end of trial. Feed intake was recorded daily and the residual feed was measured at the end of each period. Weight gain was measured at the beginning of each period and at the conclusion of the trial. Performance parameters (average daily gain, average daily feed intake and feed efficiency) were calculated for each pen.

Table 3.1. Ingredient and chemical composition of the diets (as fed basis)

Item	Prestarter diet[1]	Starter diet[2]
Ingredients, %		
Flacked wheat	24.52	15.00
Wheat	7.00	21.26
Barley	17.75	20.00
Flacked corn	10.00	10.40
Whey powder	9.00	4.00
Herring meal	5.50	5.00
Soycomil	9.00	4.50
Soybean meal 48 % CP	10.00	8.60
Wheat middlings	---	5.00
Dextrose	1.00	---
Soybean oil	3.30	3.30
Dicalcium phosphate	1.30	1.30
Calcium carbonate	0.50	0.70
L-Lysine	0.30	0.35
Trace elements plus vitamins [3]	0.25	0.25
L-Threonine	0.32	0.12
DL-Methionine	0.16	0.10
Tryptophan	---	0.02
Sodium chloride	0.10	0.10
Phyzyme XP 5000 TPT	0.01	0.01
Calculated energy and nutrient content		
DE, kcal/kg	3,450	3,345
Dry matter, %	90.08	88.90
CP, %	21.51	19.31
EE, %	5.45	5.75
CF, %	2.61	3.21
NDF, %	10.38	12.71
Ash, %	5.43	5.13
Lysine, %	1.47	1.37
Methionine, %	0.45	0.46
Met+Cyst, %	0.88	0.82
Threonine, %	0.96	0.89
Tryptophan, %	0.28	0.27
Ca, %	0.89	0.84
P, %	0.72	0.72

[1] Prestarter: 1st to 2nd week after weaning at 24 d of age.

[2] Starter: 3rd to 6th week after weaning at 24 d of age.

[3] Vitamin-mineral premix supplies per kg final feed: Vitamin A: 10,500 IU; Vitamin D3: 2,500 IU; Vitamin E: 15 mg; Vitamin B1: 1.5 mg; Vitamin B2: 3.8 mg; Vitamin B12: 0.025 mg; Vitamin B6: 1.6 mg; Calcium pantotenate: 12 mg; Nicotinic acid: 15 mg; Biotin: 0.15 mg; Folic acid: 0.5 mg; Vitamin K3: 3 mg; Fe: 100 mg; Cu: 6 mg; Co: 0.75 mg; Zn: 150 mg; Mn: 65 mg; I: 0.75 mg; Se: 0.4 mg; Ethoxyquin: 150 mg.

3.3.3. Fecal and tissue sampling and processing

Pen fecal score was 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. Fecal samples were

collected at day 0, 14 and 42 (end of the trial). Each sample (about 20 g) was placed in a small sterile container and immediately sent to the laboratory for the microbial assay. Microbiological counts for *Lactobacilli, Coliforms, Clostridia* and *E. coli* were calculated. 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% CO2), and Clostridia procedure had an incubation time of 48h at 37°C using TSC (Tryptose sulphite cycloserine agar).

Two experiments were performed during d 15 to 21 and d 29 to 35 for measurement of apparent fecal digestibility of nutrients, and chromium oxide (2 g/kg) was added to feed as an indicator for digestibility measurement. After two 6-d preliminary feeding periods from changing diets, feces was collected from at least 2 piglets from each pen via rectal massage at d 21 and 35, and then pooled within each pen and period. All the fecal samples (about 200 g/pen) together with the feed samples (300g/treatment) were frozen and stored at -20°C before sending to laboratory for digestibility assays.

Determination of dry matter (DM), Ash, crude protein (CP) and NDF were performed using the Association of Analytical Communities (AOAC, 2005) official method AOAC 930.15, AOAC 942.05, AOAC 2001.11 and AOAC 2002.04, respectively. The lipid content was determined according to Italian DM 21/12/1998 - GU n°31 8/2/1999 suppl n°13. The contents of calcium (Ca) and phosphorus (P) were analyzed by 05(M1) Rev.11 2009 + 05(R-P2) Rev.8 2010. The measurement of Dietary fibre (DF) and Chromium (Cr) contents in feed and feces referenced the method of 05(M1) rev11 2009 + (R-P1) rev11 2009.

Digestibility of nutrients and energy was calculated using Cr content as the indicator according to the following equation:

Digestibility = 100 − 100 × [(% Cr diet × % nutrient feces) / (% Cr feces × % nutrient diet)]

At the end of the trial, 24 animals (6 piglets per treatment), 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, the cecum content (about 10 g) of each piglet was collected and placed in a small sterile container and immediately sent to the laboratory for the microbial assay, 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-thicks) 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) (6 villi measured per section), crypt depth (C) (6 crypts measured per section), the villus height to

crypt depth ratio (V:C ratio), number of lymphatic follicles (counted in 5 fields per section at 400x and then expressed as n/mm^2 of mucosa), area of lymphatic follicles (5 follicles per section) and number of macrophages (counted in 8 fields per section at 400x and then expressed as n/mm^2 of mucosa).

3.3.4. Real-time PCR for Quantification of Cytokines, PPARγ, TLR-2 and TLR-4 mRNA

At slaughtering, ileum of each piglet was collected. Anti-inflammatory cytokine (IL-10), pro-inflammatory cytokines (TNF-Alfa, IL-6, IL-1Beta, IL-1Alfa), PPAR-Gamma, TLR-2 and TLR-4 were determined the gene expression by Quantitative analysis of PCR which was carried out in DNA Engine Opticon 2 fluorescence detection system (MJ Research) according to optimized PCR protocols and DyNAmo SYBR Green qPCR kit (Finnzymes). The PCR reaction system (10 μL) contained 5 μL DyNAmo SYBR Green mix, 2 μL primer (300 nmol/L forward and 300 nmol/L reverse), and 3 μL cDNA (< 10 μg/L). The relative standard curve methods were used for quantification of gene expression.

3.3.5. Statistical analysis

The ANOVA with repeated measurements was used to identify interactions between treatment and time in growth performance, fecal score, and microbial counts of piglets. Data were analyzed by ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Inst. Inc., Cary, NC). The Tukey's honestly significant difference test was used to measure the contrast among the four diet treatments. Mean differences between two periods for digestibility measurement were calculated using the Bonferroni t-test. An alpha level of 0.05 was used for determination of statistical significance and of 0.10 for the determination of statistical tendencies.

3.4. Results

3.4.1. Growth performance

Table 3.2 shows the results of body weight, average daily gain, average daily intake and feed conversion ratio of piglets. There was no interaction between treatment and time on growth performance was observed. Lower average daily gain of dietary combination treatment than control treatment was observed from d 14 to 21 ($P = 0.005$). Piglets fed the combination of essential oils and enzymes had less average daily feed intake than unsupplemented animals during third week, fourth week and the whole period ($P = 0.087$; $P = 0.047$; $P = 0.065$). In addition, Dietary combination decreased ADFI compared with XB during fifth week ($P = 0.041$) and EO during the last week ($P = 0.034$). Feed conversion ratio in combination treatment was partially higher than control animals from d 14 to 21 ($P = 0.065$). However, supplemented with the combination of essential oils and enzymes significantly improved growth performance by decreasing FCR compared with control, EO and XB treatments during the last week ($P < 0.001$; $P = 0.003$; $P = 0.023$).

3.4.2. Fecal scores

Effects of essential oils and/or enzymes on fecal score of piglets are showed in Table 3.3. The significant interaction between treatment and time on fecal scores was observed ($P = 0.002$) before individual analysis. At day 7, significant high fecal score in dietary EO treatment was observed compared with dietary combination treatment ($P = 0.005$). However, no diarrhea incident was observed throughout the whole period.

Table 3.2. Effects of essential oils (EO) and/or enzymes (XB) supplementation on the growth performance of piglets[1]

	Treatment[2]				Level of significance		Contrast[3]					
Item	CTR	EO	XB	EO+XB	SEM	*P*-value	1	2	3	4	5	6
d 0 to 7												
Initial weight, kg	8.10	8.11	8.10	8.10	0.48	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADG, g	125	155	138	116	19	0.515	0.699	0.966	0.985	0.924	0.483	0.847
ADFI, g	291	305	302	270	15	0.333	0.921	0.955	0.725	0.999	0.348	0.412
FCR (F:G)	3.190	2.392	2.706	4.335	0.842	0.391	0.908	0.977	0.772	0.994	0.372	0.526
d 7 to 14												
ADG, g	422	389	421	363	24	0.265	0.774	1.000	0.321	0.794	0.867	0.341
ADFI, g	594	568	602	540	27	0.357	0.907	0.996	0.488	0.810	0.873	0.363
FCR	1.425	1.476	1.473	1.493	0.048	0.768	0.872	0.893	0.740	1.000	0.994	0.990
d 14 to 21												
ADG, g	532	465	442	392	28	0.008	0.336	0.113	0.005	0.931	0.253	0.582
ADFI, g	836	766	788	701	39	0.123	0.597	0.826	0.087	0.979	0.647	0.406
FCR	1.607	1.663	1.792	1.815	0.058	0.039	0.902	0.121	0.065	0.398	0.256	0.992
d 21 to 28												
ADG, g	480	421	410	395	31	0.260	0.550	0.409	0.243	0.995	0.940	0.987
ADFI, g	871	813	823	741	34	0.073	0.632	0.754	0.047	0.997	0.446	0.334
FCR	1.841	2.046	2.124	1.894	0.100	0.177	0.477	0.204	0.982	0.946	0.709	0.377
d 28 to 35												
ADG, g	420	401	393	324	35	0.231	0.980	0.946	0.213	0.999	0.398	0.491
ADFI, g	877	871	909	744	43	0.043	1.000	0.949	0.136	0.916	0.167	0.041
FCR	2.139	2.323	2.509	2.577	0.216	0.481	0.931	0.622	0.483	0.929	0.838	0.996
d 35 to 42												
ADG, g	603	662	652	652	27	0.426	0.429	0.587	0.591	0.994	0.993	1.000
ADFI, g	1066	1103	1051	934	42	0.041	0.923	0.995	0.137	0.822	0.034	0.215
FCR	1.775	1.670	1.626	1.438	0.045	<0.001	0.351	0.098	<0.001	0.896	0.003	0.023
d 0 to 42												
ADG, g	426	409	403	366	18	0.135	0.914	0.811	0.104	0.995	0.341	0.473
ADFI, g	751	732	743	650	28	0.052	0.962	0.996	0.065	0.993	0.178	0.104
FCR	1.768	1.806	1.856	1.782	0.034	0.282	0.858	0.267	0.991	0.721	0.958	0.416

[1] n = 48 (12 pens/treatment).

[2] CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes.

[3] Contrasts by Tukey's HSD test: 1 = CTR vs. EO; 2 = CTR vs. XB; 3 = CTR vs. EO+XB; 4 = EO vs. XB; 5 = EO vs. EO+XB; 6 = XB vs. EO +XB.

Table 3.3. Effects of essential oils (EO) and/or enzymes (XB) supplementation on fecal score of piglets[1]

	Treatment[2]				Level of significance		Contrast[3]					
Item	CTR	EO	XB	EO+XB	SEM	*P*-value	1	2	3	4	5	6
Fecal score[4]												
d 0	3.02	2.94	3.15	3.15	0.11	0.463	0.949	0.850	0.850	0.539	0.539	1.000
d 7	3.29	3.77	3.50	3.06	0.14	0.007	0.087	0.719	0.655	0.524	0.005	0.135
d 14	3.06	3.02	3.10	3.15	0.05	0.412	0.948	0.948	0.702	0.702	0.376	0.948
d 21	3.06	3.13	3.04	3.10	0.04	0.457	0.696	0.983	0.885	0.472	0.983	0.696
d 28	3.06	3.15	3.17	3.04	0.07	0.498	0.824	0.705	0.996	0.996	0.705	0.572
d 35	3.08	3.15	3.08	3.08	0.07	0.880	0.908	1.000	1.000	0.908	0.908	1.000
d 42	3.04	3.13	3.02	3.00	0.05	0.288	0.615	0.990	0.928	0.429	0.271	0.990

[1] n = 48 (12 pens/treatment).

[2] CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes.

[3] Contrasts by Tukey's HSD test: 1 = CTR vs. EO; 2 = CTR vs. XB; 3 = CTR vs. EO+XB; 4 = EO vs. XB; 5 = EO vs. EO+XB; 6 = XB vs. EO +XB.

[4] Fecal score: 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).

Table 3.4. Effects of essential oils (EO) and/or enzymes (XB) on fecal nutrient digestibility (%) in weaned piglets[1]

	Treatment[2]				Level of significance		Contrast[3]					
Item	CTR	EO	XB	EO+XB	SEM	*P*-value	1	2	3	4	5	6
DM	79.34	79.79	78.71	80.08	0.48	0.215	0.911	0.795	0.693	0.398	0.973	0.195
OM	81.95	82.29	81.32	82.56	0.44	0.225	0.944	0.741	0.755	0.403	0.973	0.197
Ash	38.24	39.50	36.85	38.33	1.56	0.702	0.941	0.923	1.000	0.635	0.951	0.910
Ca	34.71	39.69	32.76	38.01	2.01	0.072	0.300	0.904	0.650	0.080	0.934	0.264
P	43.72	47.15	43.90	47.27	1.48	0.161	0.360	1.000	0.328	0.420	1.000	0.386
DF	32.24	34.74	31.31	34.60	1.44	0.247	0.611	0.968	0.652	0.344	1.000	0.380
Lipid	80.03	77.38	78.24	78.37	1.22	0.480	0.416	0.731	0.770	0.960	0.938	1.000
CP	46.07	47.56	44.44	46.87	1.56	0.537	0.869	0.653	0.985	0.978	0.976	0.848
NDF	79.27	78.18	77.60	78.77	1.01	0.678	0.906	0.882	0.983	0.498	0.989	0.692

[1] Each mean represents 24 values from twelve individually pens over two experimental periods.

[2] CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes.

[3] Contrasts by Tukey's HSD test: 1 = CTR vs. EO; 2 = CTR vs. XB; 3 = CTR vs. EO+XB; 4 = EO vs. XB; 5 = EO vs. EO+XB; 6 = XB vs. EO +XB.

3.4.3. Fecal digestibility of nutrients

There was no effect of essential oils or enzymes or the combination supplementation on the digestibilities of DM, OM, ash, Ca, P, DF, lipid, CP or NDF (Table 3.4). Enzymes tended (P = 0.080) to lower digestibility of calcium in feces than EO treatment. However, there was significant period effect on fecal nutrient digestibility (Table 3.5). Except calcium and phosphorus, the fecal digestibilities of most nutrients increased from Period I to II. The average fecal digestibilities were 2.5 to 20% units higher in Period II compared with Period I. The DF digestibilities increased from 30.05 to36.50% from Period I to Period II, and the NDF digestibilities increased from 42.12 to 50.48% in the same order as the periods.

Table 3.5. Effects of periods on fecal nutrient digestibility (%) in weaned piglets[1]

Period	I	II	SEM	*P*-value
DM	78.50	80.49	0.31	<0.001
OM	81.06	83.03	0.28	<0.001
Ash	36.73	39.80	1.08	0.047
Ca	38.27	34.35	1.43	0.056
P	44.63	46.45	1.06	0.228
DF	30.05	36.50	0.92	<0.001
Lipid	76.11	80.95	0.79	<0.001
CP	75.45	81.53	0.56	<0.001
NDF	42.12	50.48	0.92	<0.001

[1] Each mean represents values from 48 individually pens.

3.4.4. Microbial count

The effects of essential oils and/or enzymes on microbial counts are showed in table 3.6. Compared with Control, combination of essential oils and enzymes significantly decreased bacterial counts of *Lactobacilli* at 14 d of the trial (P = 0.030). At d 42, dietary XB tended to reduce population of *Lactobacilli* (P = 0.051) and *E. coli* (P = 0.104) compared with control diet, and supplemented essential oils partially decreased *Lactobacilli* counts as well (P = 0.102). Piglets fed essential oils, enzymes and combination of both additives had less *Coliforms* counts than unsupplemented animals at 42 days of the trial (P = 0.009; P < 0.001; P = 0.005). However, high population of *Coliforms* in XB treatment was observed at d 14 compared with EO treatment (P = 0.040).

3.4.5. Gut histomorphology

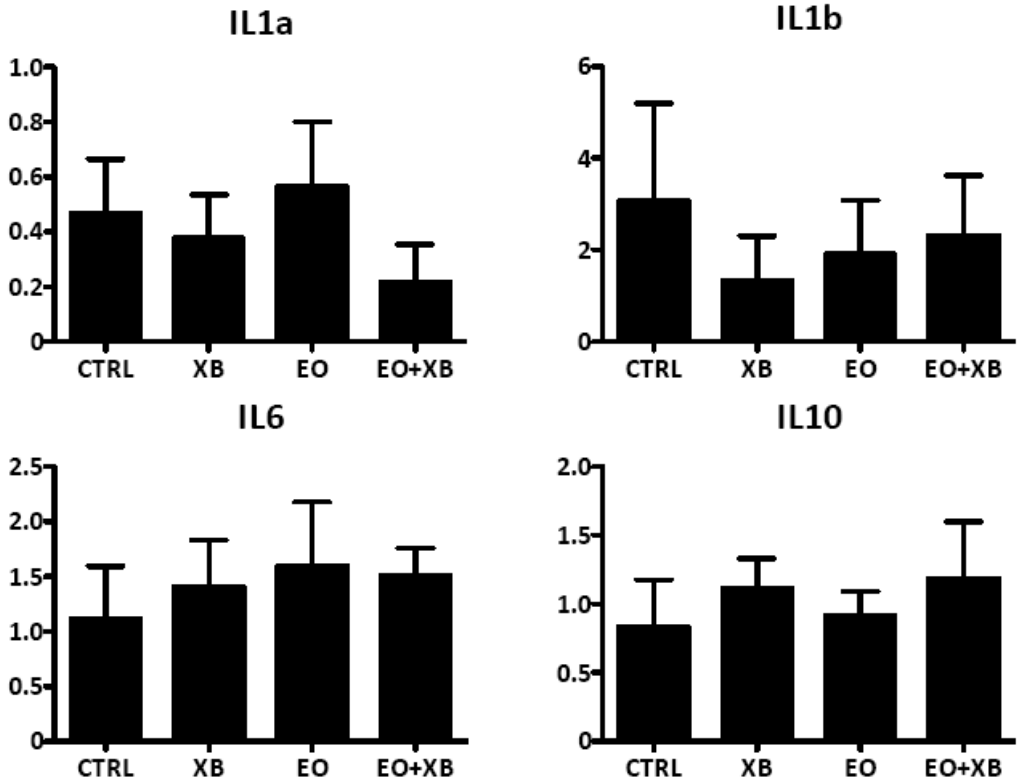
The effects of different feeding integration upon ileal histological parameters examined are showed in table 3.7. Histometrical analysis showed that villi of piglets fed enzymes were higher than dietary combination treatment (P = 0.026). Piglets fed enzymes or combination with essential oils significantly decreased crypt depth compared with unsupplemented animals (P < 0.001). Supplemented

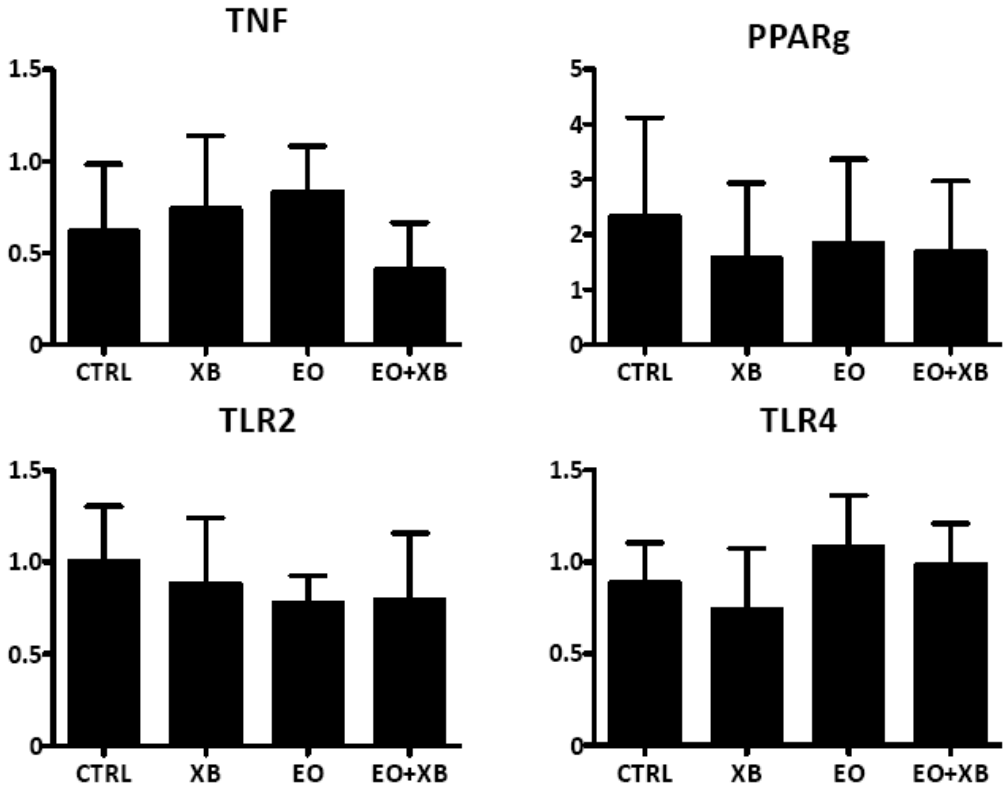
essential oils tended to reduce crypt depth as well (P = 0.065). Addition of enzymes increased V:C ratios compared with control, XB and combination treatments (P < 0.001; P = 0.015; P = 0.021). Essential oils or combination with enzymes significantly decreased the number of lymphatic follicles compared with control treatment (P = 0.002; P < 0.001). Piglets fed essential oils, enzymes and combination of both additives had less number of macrophages than unsupplemented animals (P < 0.001). In addition, dietary enzymes decreased macrophages number compared with EO treatment (P = 0.016).

3.4.6. Ileal gene expression

Figure 3.1 shows the effects of EO and/or XB on ileal gene expression of the piglets. Piglets fed the combination of essential oils and enzymes seemly decreased gene expression of IL-1α and TNF, and IL-1β in dietary enzymes treatment tended to be lower. However, those tendencies were not significant (P > 0.05).

Figure 3.1. Effect of Essential oils (EO) and/or Enzymes (XB) supplementation on gene expression of piglets1





[1] n = 24 (6 pens/treatment).

Table 3.6. Effects of essential oils (EO) and/or enzymes (XB) supplementation on bacterial counts (Log10 cfu/g) in the feces of piglets[1]

	Treatment[2]				Level of significance		Contrast[3]					
Item	CTR	EO	XB	EO+XB	SEM	*P*-value	1	2	3	4	5	6
d 0												
Clostridia	6.02	5.57	5.53	5.72	0.32	0.689	0.747	0.695	0.906	1.000	0.987	0.975
Lactobacilli	8.55	8.26	8.54	8.45	0.21	0.721	0.741	1.000	0.984	0.757	0.912	0.988
E.Coli	9.02	8.50	8.64	8.97	0.24	0.346	0.413	0.680	0.999	0.972	0.504	0.770
Coliforms	10.00	9.39	10.26	9.91	0.30	0.238	0.491	0.927	0.997	0.189	0.621	0.842
d 14												
Clostridia	5.31	5.46	6.23	5.89	0.31	0.163	0.986	0.176	0.554	0.318	0.761	0.874
Lactobacilli	8.94	8.69	8.43	8.21	0.18	0.035	0.761	0.200	0.030	0.734	0.243	0.819
E.Coli	6.94	7.18	7.49	7.44	0.23	0.307	0.873	0.333	0.419	0.779	0.858	0.999
Coliforms	8.38	7.80	8.64	8.22	0.21	0.059	0.240	0.828	0.954	0.040	0.513	0.522
d 42												
Clostridia	6.38	6.88	6.55	6.58	0.28	0.655	0.595	0.972	0.955	0.845	0.880	1.000
Lactobacilli	8.86	8.32	8.24	8.44	0.16	0.049	0.102	0.051	0.282	0.989	0.949	0.829
E.Coli	6.71	6.44	6.00	6.72	0.21	0.073	0.815	0.104	1.000	0.468	0.797	0.096
Coliforms	8.20	7.29	6.91	7.23	0.19	<0.001	0.009	<0.001	0.005	0.513	0.998	0.631

[1] n = 48 (12 pens/treatment).

[2] CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes.

[3] Contrasts by Tukey's HSD test: 1 = CTR vs. EO; 2 = CTR vs. XB; 3 = CTR vs. EO+XB; 4 = EO vs. XB; 5 = EO vs. EO+XB; 6 = XB vs. EO +XB.

Table 3.7. Effects of essential oils (EO) and/or enzymes (XB) supplementation on histomorphometry parameters of piglets[1]

	Treatment[2]				Level of significance		Contrast[3]					
Item	CTR	EO	XB	EO+XB	SEM	*P*-value	1	2	3	4	5	6
Villus height, μm	397	391	410	372	9	0.043	0.977	0.749	0.258	0.496	0.480	0.026
Crypt depth, μm	337	313	296	297	7	<0.001	0.065	<0.001	<0.001	0.249	0.292	1.000
V:C	1.20	1.26	1.40	1.26	0.03	<0.001	0.548	<0.001	0.481	0.015	1.000	0.021
Lymphatic Follicles Number[4]	1.23	0.99	1.10	0.93	0.05	<0.001	0.002	0.181	<0.001	0.382	0.790	0.061
Total area of follicles (mm^2)	0.50	0.50	0.43	0.51	0.03	0.162	1.000	0.314	0.992	0.268	0.998	0.192
Macrophages Number[4]	283	170	87	110	20	<0.001	<0.001	<0.001	<0.001	0.016	0.141	0.834

[1] n=144 for villus height, crypt depth and V:C; n=120 for Lymphatic Follicles Number; n=120 for Lymphatic Follicles Total area, n=192 for Macrophages

[2] CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Contrasts by Tukey's HSD test: 1 = CTR vs. EO; 2 = CTR vs. XB; 3 = CTR vs. EO+XB; 4 = EO vs. XB; 5 = EO vs. EO+XB; 6 = XB vs. EO +XB.

[4] The number in every mm^2 mucosa

3.5. Discussion and conclusion

The goal of this experiment was to determine whether adding essential oils and/or enzyme supplement to the solid diet of weaned piglets would produce some positive effects when the piglets consumed a diet with no supplemental antibiotics. Previous literatures observed that essential oils and enzyme improved growth performance mainly related to feed efficiency (Fan et al., 2009; Maenner et al., 2011). In the current study, diet supplementation did not affect ADG and ADFI, but the results indicated that improvement of growth performance was mainly embodied in decrease of feed convention ratio by supplementation of the combination of essential oils and enzymes during the last week. However, piglets fed combination of essential oils and enzyme had high FCR during third week, so the pattern of response in this study suggested that dietary combination reduced growth rate early and increased it later.

No effects of essential oils and/or enzymes were observed on fecal score, there was also no diarrhea incident in the current study. Weary et al. (1999) concluded that separation distress and frustration of suckling motivation were significant problems for young weaned piglets. The higher fecal score at d 7 might be explained by the adaptation period of animals to adapt to new environment and the solid diets. Therefore, we assumed that essential oils or enzymes or the combination of them did not affect fecal score as the piglets were healthy.

Plenty of literatures reported the effects of essential oils or enzymes on the apparent ileal digestibility of nutrients of weaned piglets (Yin et al., 2000; Wenk, 2003; He et al., 2010; Owusu-Asiedu et al., 2010; Maenner et al., 2011). There was no effect of essential oils or enzymes or the combined additive supplementation on fecal digestibility values in current study. Similarly, Diebold et al. (2004) observed that supplemented xylanase and phospholipase with wheat-based diet positively affected ileal nutrient and energy digestibilities while there was no effect of xylanase or the combined enzyme supplementation on the fecal nutrient digestibilities. Dierick and Decuypere (1996) stated that measurements in ileal digesta were more sensitive than those in feces for detecting the possible effects of enzyme supplementation. Our study also observed that the fecal digestibility values of most nutrients (except for calcium) increased from Period I to II. The differences between Period I and II might be explained, in part, by the growing microbial population in the large intestine. According to Graham et al. (1986), older pigs have a more mature gastrointestinal system and are able to better digest cereal components of the diet through the effects of both enzyme secretion and bacterial fermentation. Consequently, as the pig ages, the potential for responses to essential oils and enzymes supplementation decreases.

In vitro studies, the antibacterial activity of essential oils was demonstrated (Sivropoulou et al., 1996; Mancini-Filho et al., 1998; Dorman and Deans, 2000; Lambert et al., 2001; Burt, 2004). Essential oils comprise a large number of components and it is likely that their mode of action involves several targets in the bacterial cell. The hydrophobicity of EO enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents. As the important exogenous enzymes, Xylanase and β-glucanase might exert their beneficial action by influencing the intestinal microflora (Bedford, 2000). Our study observed that essential oils and/or enzymes induced significant decreases of Coliforms counts in the faeces, indicating the antibacterial activities. Although dietary essential oils and/or enzymes tended to reduce population of Lactobacilli, the increases of Lactobacilli to Coliforms ratio were observed in those groups. The results might be reinforced the observations in vitro and indicated the potential for the positive effects of essential oils and enzymes on gut health.

Villus:crypt ratio represents the nutrient digestion and absorption capacity of the small intestine (Pluske et al., 1996; Montagne et al., 2003). In our study, dietary enzymes increased V/C ratio in the ileum of piglets by increasing villus height and decreasing crypt depth, which might due to the nutrient digestion activity of enzymes. 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. Mathlouthi et al. (2002) found that addition of xylanase and β-glucanase increased villus size and the villus height-to-crypt depth ratio of broilers. Supplementation of essential oils may improve gut health by reducing the burden of enteric pathogens in the gut of weaning piglets (De Lunge et al., 2010). Macrophages are known to secrete inflammatory mediators and kill microbial pathogens on activation (Nathan, 1987; Mosser, 2003). Wehner et al. (2005) observed that depleted and inactivated muscularis macrophage markedly decreased expression of inflammatory responses and prevented postoperative ileus. So the significant decrease of the number of lymphatic follicles and macrophages in the piglets fed essential oils and/or enzymes might be the possibility that those animals did not activate the lymphatic follicles and macrophages as many as unsupplemented piglets because the guts were healthier.

Cytokines play an important role in immunoregulation. Tumour necrosis factor (TNF), interleukins (1L)-1 and IL-6 derivative from membrane phospholipids, and TNF acts as a trigger which activates a cascade of cytolune production (Grimble, 1998). Toll-like receptor (TLR) 4 is the main signal transducer of classical LPS (i.e. Escherichia coli LPS), while TLR2 is used by certain non-classical LPSs. Lai et al. (2005) observed that Lipopolysaccharide from E. coli induced symptoms of acute inflammation responses and increased gene

expressions of IL-1β, IL-6, TNF-α, IL-10 and PPARγ in spleen and thymus of weaned piglets. Our study observed that no additive affected the expression of the genes, which might be indicated that those ileal mRNA were not sensitive in healthy animals for promoting the gene expression in the end the trial.
In conclusion, the current observation demonstrated that essential oils and/or enzymes improved gut morphology in the piglets especially in XB and combination treatments. In addition, all the supplemented treatments decreased the populations of *Coliforms* in the feces of animals. Combination of essential oils and enzymes increased FCR early but improved the feed efficiency by the end of the trial. Meanwhile, no effects of diets on fecal score, fecal digestibility of nutrients and gene expression were observed in the study.

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CHAPTER 4

Supplementation of essentials oils and enzymes in *Escherichia coli* challenged piglets: effects on gut health

4. Supplementation of essentials oils and enzymes in *Escherichia coli* challenged piglets: effects on gut health

4.1. Abstract

The objective of the current study was to evaluate the possible protective effects of nutritional supplements essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) either alone or in combination on growth performance, antioxidant defense system, haematological profile, bacterial counts, ileal histomorphology and gene expression of inflammatory cytokines in piglets under *Escherichia coli* challenged condition. One hundred and ninety two 24 days old weaned piglets (Stambo HBI Dalland 40) with an average initial body weight of 8.64 kg were allocated according to body weight into eight experimental treatments (6 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments [a weaned piglet control diet (CTR), CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde, EO), CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase, XB), and CTR + 0.05 g/kg EO + 0.1 g/kg XB] and 2) with or without an *E. coli* challenge. At 8 d of the trial, half of the piglets in each diet group were orally inoculated with 4 ml of virulent *E. coli* 0149: F4 (K88)-positive strain (1×10^9cfu/ml). Animal weight and growth performance were measured weekly. Fecal score (FS; 1 = hard; 5 = watery diarrhea) was recorded every day. Fecal samples were collected at 9, 14 and 35 d, and blood samples were collected at 7, 9 and 35 d. At the end of trial (35 d), 6 piglets from each treatment was selected according to its body weight and slaughtered and intestinal samples were collected. Immediately after slaughtering, the gastro-intestinal tract was removed from each animal, the cecum content of each piglet was collected for the microbial assay, and the distal ileum was collected and examined for histological, histochemical, and histometrical study. Gene expression of ileal mRNA was also measured. *E. coli* challenge significantly impaired growth performance, induced severe diarrhea, increased populations of *Clostridia, E. coli* and *Coliforms*, depressed antioxidant activities, damaged gut morphology and promoted mRNA gene expression ($P < 0.05$). Supplementation of enzymes or combination with essential oils improved feed efficiency in *E. coli* challenged animals compared with control treatment during the last week ($P = 0.025$; $P = 0.020$). In the unchallenged groups, dietary XB and combination decreased *E. coli* counts in the cecum ($P = 0.007$; $P = 0.002$), and supplementation of EO reduced populations

of *Clostridia* in the faeces at d 14 and decreased *Coliforms* counts in the cecum (P = 0.024; P = 0.046). The *Coliforms* populations of *E. coli* challenged piglets fed combination of essential oils and enzymes were lower than control treatment in the cecum (P < 0.001). The *E. coli* challenged piglets fed enzymes or combination with essential oils had lower T-AOC activities than unsupplemented animals at d 35 (P = 0.004; P = 0.003). Supplemented essential oils and/or enzymes improved gut health by increasing villus height and V: C ratio and decreasing crypt depth (P < 0.001). Results showed that diet supplementation with EO and/or XB had positive effects on intestinal intestinal bateria and gut morpholgy when piglets were submitted to a bacterial challenge.
Key words: essential oils, enzymes, *Escherichia coli* challenge, bacterial counts, gut morpholgy, performance, weaned piglet

4.2. Introduction

Post-weaning piglets have poorer growth rate and require more complex diets, and are vulnerable in terms of health status so that they cannot efficiently cope with antigenic pressure such as enterotoxigenic Escherichia coli (ETEC), post-weaning-multisystemic-wasting syndrome (PMWS), circovirus.

Previous studies indicated that certain essential oils and enzymes might have beneficial effects on animal performance and health status because of other properties besides their sensory characteristics. Some of these compounds have been reported to improve animal performance because of their stimulating effect on salivation and pancreatic enzyme secretions or by having a direct bactericidal effect on gut microflora (Hardy, 2002). In vitro, numerous studies have shown that thymol and carvacrol exhibit antibacterial (Sivropoulou et al., 1996; Dorman and Deans, 2000; Lambert et al., 2001; Burt, 2004), antioxidative (Lagouri et al., 1993) and antifungal properties (Adam et al., 1998; Manohar et al., 2001) and cinamaldehyde from cinnamon has also shown antioxidant and antimicrobial effects (Mancini-Filho et al., 1998). Improvements in growth performance were observed when xylanase was supplemented to wheat-based diets (Dusel et al., 1997; Jeroch et al., 1999) and also supplemented β-glucanase and xylanase to barley-based diets improved performance of piglets (Fan et al. 2009; Inborr et al., 1993; Li et al., 1996).

The aim of the present work was to assess the effects of essential oils (Thymol and Cinnamaldehyde, EO) and/or enzymes (xylanase and β-glucanase) on growth performance, microbial counts and gut health in weaned piglets under *Escherichia coli* challenged condition. In addition, antioxidant defences, haematological profile and gene expression were also determined.

4.3. Materials and methods

4.3.1. Experimental Animals and Housing

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 and ninety-two crossbreed (Stambo HBI Dalland 40, 8.64kg) piglets (equal number of males and females) homogeneous for age and litter origin, were weighed and randomly assigned to treatments. Animals were housed in 48 pens, environmentally regulated in an isolated stable. Piglets were allocated to one of the following dietary treatment: basal weaning diet supplemented with i) No additive (Control); ii) 50 mg/kg Essential Oils (Thymol and Cinnamaldehyde, EO); iii) 100 mg/kg Enzymes (Xylanase and β-glucanase, XB) ; iv) 50 mg/kg EO + 100 mg/kg XB. 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 ºC at the start of the trial, adjusted weekly until a final temperature of 26 ºC. Piglets were hosed in pens (4 piglets/pen), located beside a 120 cm walkway with 24 pens (1.20 m × 1.00 m) each side, with a slatted floor. Each pen was equipped with two water nipples and self-feeder. Piglets were used in a 35-d experiment to assess the effect of essential oils (Thymol and Cinnamaldehyde, EO) and/or enzymes (Xylanase and β-glucanase, XB) supplemented in presence of a challenge with *E. coli* (EC). A 4 x 2 factorial arrangement of treatments was employed (4 different dietary treatments with or without *E. coli* challenge treatment). Each treatment consisted of 6 replicates with 4 piglets. Each pen represented one treatment replicate.

Diet was formulated to be isonutritive, exceeding the protein requirement recommended by NRC (1998) for pigs. The approximate composition and the chemical analysis of the diet are presented in Table 4.1. Diet was formulated and manufactured before the trial start, without the inclusion of any antibiotic growth promoters or antibiotic growth promoter alternatives. Both prestarter and starter diets were meal and milled at 1.5 mm particle size, which were stored in a cool dry place. At 14 d of the trial, the diet was become to starter feed for the last three weeks.

At d 8 of the trial, after an adaptation period, half of the piglets in each diet group 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 4.1. Ingredient and chemical composition of the diets (as fed basis)

Item	Prestarter diet[1]	Starter diet[2]
Ingredients, %		
Barley	35.23	38.21
Wheat	20.50	26.50
Whey powder	8.00	6.00
Soycomil	7.00	5.00
Herring meal	5.00	4.00
Wheat bran	5.00	5.00
Soybean meal	4.78	5.50
Soybean oil	4.00	4.00
Fatfilled Whey powder	4.00	--
Dextrose monohydrate	2.20	2.00
Dicalcium phosphate	1.26	0.90
Pig lard	0.80	0.80
Lysine HCl 78	0.55	0.45
Calcium carbonate	0.47	0.60
L-Threonine	0.40	0.35
DL-Methionine	0.30	0.20
Vitamins premix[3]	0.25	0.25
Salt	0.15	0.15
L-Tryptophan	0.10	0.08
Phyzyme XP 5000 TPT	0.01	0.01
Feed enzyme Premix[4]	--	--
Calculated energy and nutrient content		
Dry matter, %	90.10	89.30
DE, kcal/kg	3290	3380
CP, %	19.10	18.10
EE, %	6.60	6.70
CF, %	3.00	3.30
NDF, %	12.10	13.20
Ash, %	5.50	5.10
Total lysine, %	1.52	1.31
Total Threonine, %	1.09	0.99
Total Tryptophan, %	0.31	0.28
Ca, %	0.80	0.72
P, %	0.69	0.61

[1] Prestarter: 1st to 2nd week after weaning at 24 d of age.

[2] Starter: 3rd to 5th week after weaning at 24 d of age.

[3] Vitamin-mineral premix supplies per kg final feed: Vitamin A: 10,500 IU; Vitamin D3: 2,500 IU; Vitamin E: 15 mg; Vitamin B1: 1.5 mg; Vitamin B2: 3.8 mg; Vitamin B12: 0.025 mg; Vitamin B6: 1.6 mg; Calcium pantotenate: 12 mg; Nicotinic acid: 15 mg; Biotin: 0.15 mg; Folic acid: 0.5 mg; Vitamin K3: 3 mg; Fe: 100 mg; Cu: 6 mg; Co: 0.75 mg; Zn: 150 mg; Mn: 65 mg; I: 0.75 mg; Se: 0.4 mg; Ethoxyquin: 150 mg.

[4] Feed enzyme premix: EO group: premix 250g/ton (50 g EO/ton); XB group: premix 250 g/ton (100g XB/ton); EO+XB group: premix 250 g/ton (50g EO/ton + 100g XB/ton).

4.3.2. Performance Measurements

Piglets were individually weighed at weaning (day 0) and subsequently every week until the end of trial. Feed intake was recorded each day and the residual feed was measured at the same day as weighing piglets. Performance parameters

(average daily gain, average daily feed intake and feed efficiency) were calculated for each pen.

4.3.3. Fecal, Blood and Tissue Sampling and Processing

Pen fecal score was 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. Fecal samples were collected at day 9 (one day after the challenge), 14 (six days after the 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 the microbial assay. Microbiological counts for *Lactobacilli*, *Coliforms*, *Clostridia* and *E. coli* were calculated. *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% CO2), and Clostridia procedure had an incubation time of 48h at 37°C using TSC (Tryptose sulphite cycloserine agar).

At d 7 (one day before the challenge), 9 (one day after the challenge) and 35 (end of the trial), 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 and 10 ml vacutainers containing anticoagulant. Blood in no anticoagulant vacutainers 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 -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. Blood in anticoagulant vacutainers was to evaluate counts of white blood cell (WBC), red blood cell RBC and platelet (PLT) and percentages of Neutrophils, Lymphocytes, Monocytes, Eosinophils and Basophils.

At the end of the trial, 48 animals (6 piglets per treatment), 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, the cecum content (about 10 g) of each piglet was collected and placed in a small sterile container and immediately sent to the laboratory for the microbial assay, 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-thicks) 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) (6 villi measured per section), crypt depth (C) (6 crypts measured per section), the villus height to crypt depth ratio (V:C ratio), number of lymphatic follicles (counted in 5 fields per section at 400x and then expressed as n/mm^2 of mucosa), area of lymphatic follicles (5 follicles per section) and number of macrophages (counted in 8 fields per section at 400x and then expressed as n/mm^2 of mucosa).

4.3.4. Real-time PCR for Quantification of Cytokines, PPARγ, TLR-2 and TLR-4 mRNA

At slaughtering, ileum of each piglet was collected. Anti-inflammatory cytokine (IL-10), pro-inflammatory cytokines (TNF-Alfa, IL-6, IL-1Beta, IL-1Alfa), PPAR-Gamma, TLR-2 and TLR-4 were determined the gene expression by Quantitative analysis of PCR which was carried out in DNA Engine Opticon 2 fluorescence detection system (MJ Research) according to optimized PCR protocols and DyNAmo SYBR Green qPCR kit (Finnzymes). The PCR reaction system (10 μL) contained 5 μL DyNAmo SYBR Green mix, 2 μL primer (300 nmol/L forward and 300 nmol/L reverse), and 3 μL cDNA (< 10 μg/L). The relative standard curve methods were used for quantification of gene expression.

4.3.5. Statistical Analysis

The data were analysed as a completely randomised design with a 4 x 2 factorial treatment arrangement by ANOVA using the MIXED procedure of SAS v. 9.2 (SAS Inst. Inc., Cary, NC), the model included effects of diet (CTR, EO, XB or EO+XB), challenge (sham or *E. coli*) and their interaction. The Dunnett's test was used to measure the contrast between control group and the additives treatments collectively within each challenge treatment. An alpha level of 0.05 was used for determination of statistical significance and of 0.10 for the determination of statistical tendencies.

4.4. Results

4.4.1. Growth Performance

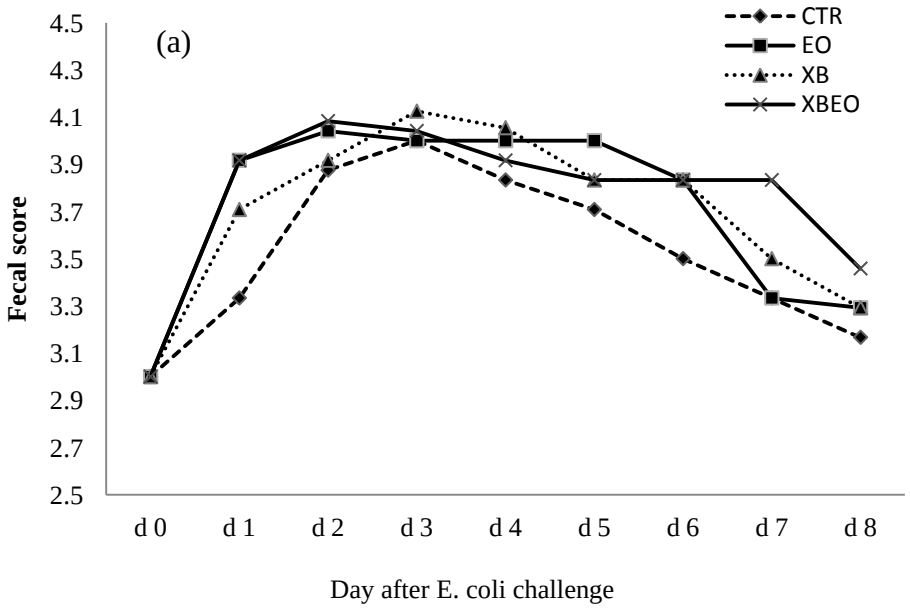
Table 4.2 shows the results of average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) of piglets. During the last week of trial, supplementation enzymes or combination with essential oils tended to improve feed efficiency in challenged animals but not in controls (interaction, P = 0.056), because *E. coli* challenged piglets fed XB and combination had lower FCR than unsupplmented animals (P = 0.025; P = 0.020). *E. coli* challenge significantly decreased ADG in challenge piglets compared with unchallenged animals during second week, fourth week, last week and whole period (P < 0.001;

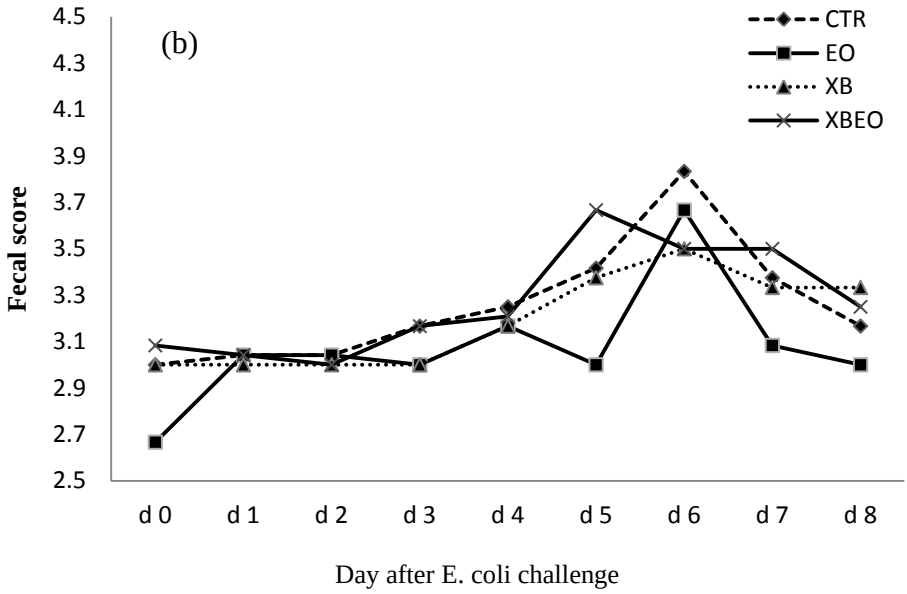
$P < 0.001$; $P < 0.001$; $P = 0.002$). *E. coli* challenge also resulted in lower ADFI than unchallenged treatments during d 7 to14 ($P < 0.001$), d 21 to 28 ($P = 0.003$), d 28 to 35 ($P < 0.001$) and d 0 to 35 ($P = 0.010$). Significant increase of FCR values were observed in challenged piglets during second week, fourth week and over the entire experimental period ($P = 0.022$; $P = 0.006$; $P = 0.052$; $P = 0.011$).

4.4.2. Fecal Scores

There were no diet effect and interaction of diet and challenge on fecal score of piglets after *E. coli* challenge (table 4.3). Although fecal score in EO treatment was lower than others treatment at d 7 ($P = 0.047$), there was no frequency of diarrhea. Significant increase ($P < 0.001$) of fecal score in challenge animals compared to unchallenged piglets had been observed since one day after injecting *E. coli*, and the challenge resulted in increase of severe diarrhea and the fecal score peaked at 3 days after challenge (11 d) (Figure 4.1). However, diarrhea animals started to recover at 6 days after challenge (14 d), and the differences between challenged and unchallenged animals were not significant ($P > 0.05$) except 28 d ($P = 0.041$).

Figure 4.1. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on fecal score of piglets challenged with (a) or without (b) *E. coli*





4.4.3. Microbial Counts

The effects of EO and/or XB administration on microbial counts of the piglets are shown in Table 4.4. One day after *E. coli* injection, microbial counts of *Clostridia, E. coli* and *Coliforms* in the feces were significantly increased ($P = 0.001$; $P < 0.001$; $P < 0.001$). Although the populations of *E. coli* ($P < 0.001$) and *Coliforms* ($P = 0.010$) in the feces were still higher than unchallenged groups at d 14, the *E. coli* challenged animals were recovering gradually. Among the unchallenged piglets, dietary enzymes or combination with essential oils decreased *E. coli* counts in the cecum ($P = 0.007$; $P = 0.002$), and supplementation of EO reduced populations of *Clostridia* in the feces at d 14 and decreased *Coliforms* counts in the cecum ($P = 0.024$; $P = 0.046$). In addition, the unchallenged piglets fed enzymes seemly had lower *E. coli* counts in the faeces at d 14 ($P = 0.066$) and *Coliforms* counts in the feces and cecum at d 35 ($P = 0.058$; $P = 0.083$) than control treatments. The *Coliforms* populations of *E. coli* challenged piglets fed combination of essential oils and enzymes were higher than unsupplemented piglets in the feces at 6 d postchallenge ($P = 0.025$) while the counts were lower than control treatment in the cecum ($P < 0.001$). Dietary XB tended to decrease *E. coli* counts in the feces of challenged piglets at 35 d ($P = 0.090$). Significant interaction between treatment and challenge on *Coliforms* in the feces at 14 d and in the cecum at slaughtering were observed ($P = 0.016$; $P < 0.001$).

Table 4.2. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on performance of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		CTR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
d 0 to 7																		
Ini. weight, kg	8.63	8.63	8.64	8.63	8.59	8.68	8.66	8.63	0.66	1.000	0.993	1.000	1.000	0.999	1.000	1.000	1.000	1.000
ADG, g	267	296	250	265	256	269	311	255	23	0.649	0.853	0.222	0.521	0.973	0.823	0.352	1.000	1.000
ADFI, g	394	392	344	367	357	377	410	378	17	0.912	0.613	0.026	0.999	0.824	0.041	0.176	0.391	0.807
FCR (F:G)	1.517	1.333	1.400	1.398	1.433	1.480	1.320	1.587	0.109	0.596	0.581	0.455	0.309	0.987	0.641	0.870	0.633	0.736
d 7 to 14																		
ADG, g	397	436	399	410	322	342	273	243	42	0.468	<0.001	0.715	0.873	0.969	1.000	0.718	0.993	0.379
ADFI, g	640	652	627	652	540	586	531	530	31	0.605	<0.001	0.836	0.982	0.646	0.971	0.996	0.983	0.994
FCR	1.860	1.553	1.642	1.614	1.697	1.749	2.168	2.427	0.204	0.312	0.022	0.111	0.573	0.996	0.783	0.282	0.719	0.056
d 14 to 21																		
ADG, g	336	353	390	415	380	439	369	407	39	0.558	0.362	0.498	0.987	0.353	0.746	0.988	0.501	0.834
ADFI, g	719	737	736	782	729	791	762	762	40	0.657	0.530	0.826	0.986	0.457	0.987	0.840	0.648	0.844
FCR	2.416	2.258	1.979	1.913	1.928	1.807	2.137	1.898	0.176	0.517	0.118	0.191	0.932	0.674	0.421	0.273	0.313	0.991
d 21 to 28																		
ADG, g	624	623	618	687	541	568	529	554	30	0.427	<0.001	0.631	1.000	0.850	0.998	0.983	0.356	0.975
ADFI, g	1061	1091	1081	1144	987	997	1028	995	42	0.742	0.003	0.697	0.940	0.995	0.982	0.791	0.446	0.997
FCR	1.703	1.763	1.756	1.665	1.857	1.765	1.944	1.809	0.059	0.284	0.006	0.427	0.669	0.694	0.736	0.720	0.884	0.936
d 28 to 35																		
ADG, g	678	679	678	668	536	597	614	624	30	0.504	<0.001	0.397	1.000	0.406	1.000	0.226	0.990	0.155
ADFI, g	1216	1241	1206	1241	1084	1148	1083	1091	45	0.679	<0.001	0.934	0.954	0.649	0.997	1.000	0.953	0.999
FCR	1.808	1.832	1.780	1.870	2.036	1.940	1.768	1.759	0.063	0.095	0.239	0.056	0.985	0.620	0.976	0.025	0.802	0.020
d 0 to 35																		
ADG, g	438	455	443	465	388	422	398	397	21	0.621	0.002	0.869	0.897	0.487	0.996	0.968	0.715	0.975
ADFI, g	774	788	763	800	709	748	735	722	28	0.766	0.010	0.792	0.965	0.617	0.986	0.837	0.843	0.975
FCR	1.791	1.736	1.723	1.721	1.840	1.776	1.849	1.828	0.043	0.562	0.011	0.682	0.680	0.594	0.531	0.998	0.519	0.995

[1] n = 48 (6 pens/treatment).

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

Table 4.3. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on fecal score of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		TR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
Fecal score[5]																		
d 0	3.00	3.17	3.17	3.00	3.00	3.00	3.17	3.04	0.10	0.379	0.671	0.753	0.633	1.000	0.633	0.398	1.000	0.973
d 7	3.00	2.67	3.00	3.08	3.00	3.00	3.00	3.00	0.08	0.047	0.257	0.047	0.104	-	1.000	-	0.906	-
d 9	3.04	3.04	3.00	3.04	3.33	3.92	3.71	3.92	0.14	0.129	<0.001	0.132	1.000	0.109	0.754	0.394	1.000	0.109
d 10	3.04	3.04	3.00	3.00	3.88	4.04	3.92	4.08	0.09	0.606	<0.001	0.515	1.000	0.637	0.633	0.989	0.633	0.475
d 11	3.17	3.00	3.00	3.17	4.00	4.00	4.13	4.04	0.09	0.660	<0.001	0.341	0.633	1.000	0.633	0.051	1.000	0.736
d 12	3.25	3.17	3.17	3.21	3.83	4.00	4.06	3.92	0.15	0.970	<0.001	0.736	0.969	0.692	0.969	0.494	0.996	0.941
d 13	3.42	3.00	3.38	3.67	3.71	4.00	3.83	3.83	0.16	0.474	<0.001	0.070	0.244	0.399	0.997	0.886	0.622	0.886
d 14	3.83	3.67	3.50	3.50	3.50	3.83	3.83	3.83	0.20	0.965	0.371	0.287	0.895	0.445	0.542	0.445	0.542	0.445
d 21	3.21	3.00	3.04	3.00	3.04	3.00	3.04	3.17	0.09	0.515	1.000	0.308	0.223	0.975	0.384	1.000	0.223	0.633
d 28	3.00	3.00	3.00	3.00	3.04	3.00	3.08	3.04	0.03	0.534	0.041	0.534	-	0.799	-	0.799	-	1.000
d 35	3.00	3.00	3.00	3.00	3.00	3.00	3.04	3.00	0.01	0.403	0.323	0.403	-	1.000	-	0.375	-	1.000

[1] n = 48 (6 pens/treatment).

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

[5] Fecal score: 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)

Table 4.4. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on bacterial counts (Log_{10} cfu/g) of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		CTR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
d 9 in the feces																		
Clostridia	4.33	4.17	4.33	5.00	5.01	4.92	5.23	5.24	0.26	0.166	0.001	0.629	0.907	0.995	1.000	0.919	0.096	0.902
Lactobacilli	9.19	8.90	8.89	8.69	9.33	8.92	8.80	8.66	0.23	0.088	0.938	0.961	0.691	0.456	0.679	0.268	0.298	0.126
E.Coli	7.16	6.90	6.69	6.37	8.44	8.79	8.25	8.18	0.22	0.039	<0.001	0.531	0.829	0.384	0.460	0.757	0.110	0.561
Coliforms	9.49	9.04	8.62	8.77	9.96	9.76	9.59	10.43	0.35	0.323	<0.001	0.373	0.708	0.952	0.242	0.779	0.381	0.641
d 14 in the feces																		
Clostridia	7.70	5.54	6.90	6.43	7.19	6.73	5.76	5.92	0.49	0.033	0.496	0.117	0.024	0.816	0.585	0.091	0.238	0.146
Lactobacilli	9.49	8.99	8.84	8.84	9.18	9.00	9.08	8.94	0.22	0.207	0.943	0.655	0.326	0.876	0.147	0.971	0.150	0.767
E.Coli	7.32	7.27	6.64	6.93	8.40	8.19	8.16	8.30	0.23	0.263	<0.001	0.578	0.996	0.901	0.066	0.856	0.388	0.988
Coliforms	9.48	8.35	8.62	8.37	8.85	9.14	9.40	10.09	0.35	0.520	0.010	0.016	0.125	0.839	0.298	0.454	0.135	0.025
d 35 in the feces																		
Clostridia	5.88	7.31	6.40	6.08	7.07	6.48	7.27	6.77	0.51	0.725	0.192	0.209	0.187	0.690	0.845	0.982	0.989	0.945
Lactobacilli	9.44	9.27	8.91	9.14	9.66	8.87	9.19	9.42	0.29	0.272	0.652	0.575	0.906	0.261	0.246	0.644	0.664	0.934
E.Coli	6.90	6.75	6.38	6.35	6.89	6.98	5.85	6.75	0.28	0.023	0.922	0.374	0.917	0.994	0.219	0.090	0.189	0.982
Coliforms	8.80	8.20	7.94	8.04	8.36	8.76	7.61	7.37	0.39	0.056	0.433	0.431	0.232	0.899	0.058	0.581	0.100	0.410
d 35 in the digesta[5]																		
Clostridia	7.67	6.96	6.85	7.23	7.31	7.34	7.25	7.22	0.38	0.678	0.709	0.712	0.557	1.000	0.446	0.997	0.833	0.993
Lactobacilli	9.25	9.18	9.32	9.01	9.49	8.82	9.17	9.11	0.30	0.598	0.845	0.756	0.996	0.380	0.995	0.839	0.847	0.789
E.Coli	7.50	7.10	6.33	6.11	6.77	7.08	6.79	6.70	0.27	0.019	0.704	0.076	0.512	0.796	0.007	1.000	0.002	0.997
Coliforms	9.09	8.00	8.13	8.63	9.22	8.81	8.80	6.96	0.29	<0.001	0.940	<0.001	0.046	0.626	0.083	0.610	0.568	<0.001

[1] n = 48 (6 pens/treatment).

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

[5] Digesta collected from cecum at slaughtering

4.4.4. Serum antioxidant Property

There were significant interactions between diet and *E. coli* challenge on T-AOC at 9 d ($P = 0.002$) and 35 d ($P = 0.022$) of the trial (Table 4.5). *E. coli* challenge significantly increased MDA content at 1 d postchallenge ($P < 0.001$), and decreased T-AOC activity at 9 and 35 d of the trial ($P = 0.004$; $P < 0.001$). Among the unchallenged piglets, EO treatment declined T-AOC activity at d 7 ($P = 0.011$) and dietary EO+XB reduced SOD and GSH-Px activities at the end of the trial ($P = 0.007$; $P = 0.006$). The *E. coli* challenged piglets fed enzymes or combination with essential oils had lower T-AOC activities than unsupplemented animals at d 35 ($P = 0.004$; $P = 0.003$).

4.4.5. Haematological Profile

No significant effects of diet and interaction between diet and challenge on haematological profile were observed after challenge (table 4.6). At 35 d of the trial, *E. coli* challenge significantly decreased the number of total WBC, PLT and percentage of neutrophils ($P = 0.023$; $P = 0.043$; $P = 0.004$), meanwhile, percentages of lymphocytes, monocytes, eosinophils and basophils in *E. coli* challenged treatments were higher than unchallenged animals ($P = 0.007$; $P = 0.055$; $P = 0.036$; $P = 0.077$).

4.4.6. Intestinal Morphology

Effects of EO and/or XB administration on histomorphometry parameters of the piglets are shown in Table 4.7. *E. coli* challenge significantly increased crypt depth ($P = 0.021$) and decreased total area of lymphatic follicles ($P = 0.002$). Among unchallenged piglets, dietary EO increased villus height and V:C ratio ($P < 0.001$; $P = 0.001$), supplementation of enzymes increased villus height ($P = 0.05$) and combination of essential oils and enzymes can improve gut health by increasing villus height and V:C ratio and reducing crypt depth ($P < 0.001$). The *E. coli* challenged piglets fed essential oils had higher villus height and V:C ratio ($P < 0.001$) than unsupplemented piglets, and high villus height and V:C ratio were also observed in the *E. coli* challenged piglets fed enzymes compared with control treatment ($P = 0.087$; $P = 0.015$). In addition, supplemented the combination of essential oils and enzymes with diet of the *E. coli* challenged piglets resulted in significant increase of villus height and V:C ratio and decrease of crypt depth ($P = 0.024$; $P < 0.001$; $P < 0.001$), and tended to decrease lymphatic follicles number and area ($P = 0.058$; $P = 0.073$).

Table 4.5. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on antioxidant property of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		CTR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
d 7																		
MDA, nmol/ml	2.49	2.04	2.04	1.91	2.01	2.60	2.31	2.10	0.21	0.503	0.367	0.121	0.417	0.109	0.429	0.563	0.243	0.973
SOD,U/ml	95.32	106.6	93.08	91.19	93.46	80.21	79.98	79.32	4.06	0.072	<0.001	0.046	0.173	0.093	0.961	0.087	0.817	0.071
GSH-Px, U	567.7	536.9	546.3	550.1	475.6	508.9	492.1	459.5	27.0	0.901	0.002	0.585	0.428	0.835	0.684	0.973	0.789	0.975
T-AOC, U/ml	2.29	2.13	2.30	3.07	3.39	2.92	2.31	2.40	0.24	0.137	0.080	0.005	0.922	0.428	1.000	0.025	0.075	0.040
d 9																		
MDA, nmol/ml	1.73	1.35	1.63	1.64	2.25	2.10	1.97	2.03	0.17	0.486	<0.001	0.658	0.339	0.853	0.958	0.538	0.972	0.687
SOD,U/ml	107.7	112.4	111.1	104.8	110.3	101.7	104.5	101.1	3.4	0.333	0.066	0.277	0.448	0.346	0.672	0.629	0.777	0.295
GSH-Px, U	545.5	471.3	499.7	501.1	506.8	567.7	534.7	542.0	33.2	0.994	0.168	0.268	0.133	0.585	0.447	0.925	0.470	0.867
T-AOC, U/ml	3.36	2.61	3.83	2.89	3.19	2.61	2.41	2.84	0.18	0.007	0.004	0.002	0.011	0.180	0.108	0.056	0.110	0.518
d 35																		
MDA, nmol/ml	1.77	1.97	1.73	1.65	1.75	1.82	1.48	2.35	0.22	0.351	0.677	0.172	0.623	0.996	0.995	0.849	0.889	0.353
SOD,U/ml	124.2	114.9	112.5	98.56	119.6	113.8	117.7	110.5	3.9	0.002	0.320	0.191	0.412	0.381	0.248	0.938	0.007	0.105
GSH-Px, U	636.3	584.3	568.3	508.5	655.4	631.0	584.9	555.7	24.8	0.001	0.077	0.874	0.303	0.851	0.141	0.193	0.006	0.051
T-AOC, U/ml	4.10	4.30	3.98	4.47	3.34	2.87	2.23	2.18	0.23	0.059	<0.001	0.022	0.907	0.250	0.975	0.004	0.645	0.003

[1] n = 48 (6 piglets/treatment).

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

Table 4.6. Effects of Essential oils (EO) and/or Enzymes (XB) on haematological profile of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		CTR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
d 7																		
WBC, × 10^3/μL	14.62	17.65	16.29	16.81	16.73	14.34	13.50	12.72	1.63	0.849	0.088	0.237	0.521	0.502	0.856	0.275	0.736	0.141
RBC, × 10^6/μL	6.29	6.07	6.94	6.19	5.81	6.29	5.66	5.94	0.52	0.957	0.227	0.533	0.993	0.398	0.843	0.953	0.999	0.966
PLT, × 10^3/μL	390.8	400.0	365.2	387.8	307.8	284.3	275.5	315.3	72.8	0.972	0.088	0.992	1.000	0.983	0.993	0.958	1.000	0.999
Neut, %	79.38	81.52	82.02	81.88	78.92	75.50	72.82	80.17	2.85	0.641	0.037	0.406	0.813	0.824	0.706	0.468	0.736	0.988
Lymp, %	14.00	13.55	12.23	12.70	15.50	16.85	20.45	13.87	1.76	0.388	0.007	0.181	0.991	0.939	0.696	0.246	0.844	0.900
Mono, %	2.18	1.97	2.32	2.10	2.92	2.28	2.70	2.17	0.47	0.688	0.264	0.911	0.958	0.764	0.989	0.984	0.997	0.640
Eos, %	3.70	2.38	2.77	2.62	1.80	2.53	2.93	2.73	0.79	0.966	0.514	0.478	0.585	0.798	0.790	0.530	0.712	0.666
Basos, %	0.73	0.58	0.67	0.70	0.87	1.05	1.10	1.07	0.14	0.906	0.001	0.656	0.448	0.829	0.893	0.710	0.984	0.791
d 9																		
WBC, × 10^3/μL	14.32	14.82	17.75	14.23	13.79	13.50	13.94	10.82	1.67	0.281	0.062	0.715	0.994	0.999	0.365	1.000	1.000	0.454
RBC, × 10^6/μL	6.17	5.77	5.75	6.03	5.74	6.49	6.14	6.04	0.29	0.913	0.394	0.241	0.378	0.339	0.344	0.767	0.927	0.882
PLT, × 10^3/μL	247.8	241.2	280.3	355.5	369.5	329.3	273.3	244.2	74.7	0.974	0.667	0.410	1.000	0.977	0.960	0.782	0.438	0.628
Neut, %	80.08	79.95	80.17	78.73	78.77	79.10	79.40	79.45	2.25	0.992	0.729	0.972	1.000	0.999	1.000	0.993	0.961	0.991
Lymp, %	14.50	14.62	14.02	14.58	16.08	15.60	16.10	15.08	1.84	0.996	0.327	0.975	1.000	0.995	0.995	1.000	1.000	0.962
Mono, %	2.27	2.02	2.25	2.25	1.92	1.98	1.53	1.82	0.36	0.955	0.141	0.823	0.947	0.998	1.000	0.722	1.000	0.992
Eos, %	2.12	2.40	2.40	3.52	2.18	2.40	2.25	2.57	0.57	0.427	0.522	0.793	0.979	0.979	0.979	0.999	0.302	0.900
Basos, %	1.03	1.02	1.17	0.92	1.05	0.92	0.72	1.08	0.21	0.969	0.546	0.522	1.000	0.957	0.927	0.623	0.949	0.999
d 35																		
WBC, × 10^3/μL	14.74	13.83	17.66	15.78	15.29	12.55	14.22	11.84	1.21	0.121	0.023	0.239	0.927	0.214	0.298	0.828	0.900	0.093
RBC, × 10^6/μL	6.84	6.99	6.76	6.92	6.88	7.31	6.78	6.63	0.26	0.429	0.887	0.708	0.908	0.644	0.986	0.992	0.982	0.894
PLT, × 10^3/μL	443.3	399.8	408.5	476.5	244.7	332.0	351.7	398.3	67.9	0.563	0.043	0.702	0.941	0.695	0.968	0.559	0.972	0.285
Neut, %	84.05	85.40	84.52	85.02	80.73	80.32	78.37	81.88	2.07	0.800	0.004	0.864	0.894	0.999	0.995	0.827	0.956	0.974
Lymp, %	11.92	10.80	11.85	11.40	13.72	14.93	15.73	13.47	1.48	0.822	0.007	0.801	0.887	0.908	1.000	0.704	0.986	0.999
Mono, %	1.28	0.90	0.93	1.27	1.33	1.33	1.73	1.77	0.32	0.665	0.055	0.705	0.478	1.000	0.546	0.814	1.000	0.778
Eos, %	1.87	1.65	1.88	1.33	2.93	2.35	2.93	1.75	0.53	0.316	0.036	0.913	0.966	0.845	1.000	1.000	0.689	0.412
Basos, %	0.88	1.25	0.82	0.98	1.28	1.07	1.23	1.13	0.15	0.842	0.077	0.183	0.328	0.511	0.986	0.986	0.955	0.752

[1] n = 48 (6 piglets/treatment).

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

Table 4.7. Effects of Essential oils (EO) and/or Enzymes (XB) supplementation on ileal histomorphometry of weaned piglets challenged with *Escherichia coli*[1]

	Treatment[2]									*P*-value								
	Sham				*E. coli*					Main effect[3]			CTR vs. EO[4]		CTR vs. XB[4]		CTR vs. EX[4]	
Item	CTR	EO	XB	EO+XB	CTR	EO	XB	EO+XB	SEM	Diet	*E. coli*	D × E	Sham	*E. coli*	Sham	*E. coli*	Sham	*E. coli*
Villus height, μm	381.9	421.1	403.5	417.4	388.0	440.5	411.6	417.4	7.13	<0.001	0.098	0.584	<0.001	<0.001	0.050	0.087	<0.001	0.024
Crypt depth, μm	275.8	267.9	271.0	242.6	282.1	276.2	275.7	250.1	4.08	<0.001	0.021	0.973	0.364	0.625	0.729	0.562	<0.001	<0.001
V:C	1.40	1.58	1.50	1.73	1.38	1.60	1.50	1.68	0.03	<0.001	0.508	0.720	0.001	<0.001	0.130	0.015	<0.001	<0.001
Folli. Number[5]	0.96	1.04	0.89	1.02	1.08	1.00	0.94	0.93	0.05	0.111	0.788	0.135	0.550	0.505	0.665	0.097	0.720	0.058
Folli. (mm^2) area	0.50	0.57	0.52	0.54	0.49	0.49	0.47	0.42	0.03	0.359	0.002	0.211	0.285	0.999	0.933	0.819	0.672	0.073
Macro. Number[5]	254.1	205.6	199.4	199.4	191.8	183.2	200.2	215.8	16.31	0.324	0.145	0.088	0.153	0.949	0.091	0.953	0.091	0.484

[1] n=288 for villus height, crypt depth and V:C; n=240 for Lymphatic Follicles Number and Total area, n=384 for Macrophages

[2] Sham = unchallenged; *E. coli* = *E. coli* challenged; CTR = basal diet without additive; EO = CTR + 0.05 g/kg essential oils (Thymol and Cinnamaldehyde); XB = CTR + 0.1 g/kg enzymes (Xylanase and β-glucanase); EO+XB = CTR + 0.05 g/kg essential oils + 0.1 g/kg enzymes

[3] Diet = diet effect; *E. coli* = *E. coli* challenge effect; D × E = interaction between diet and *E. coli* effects.

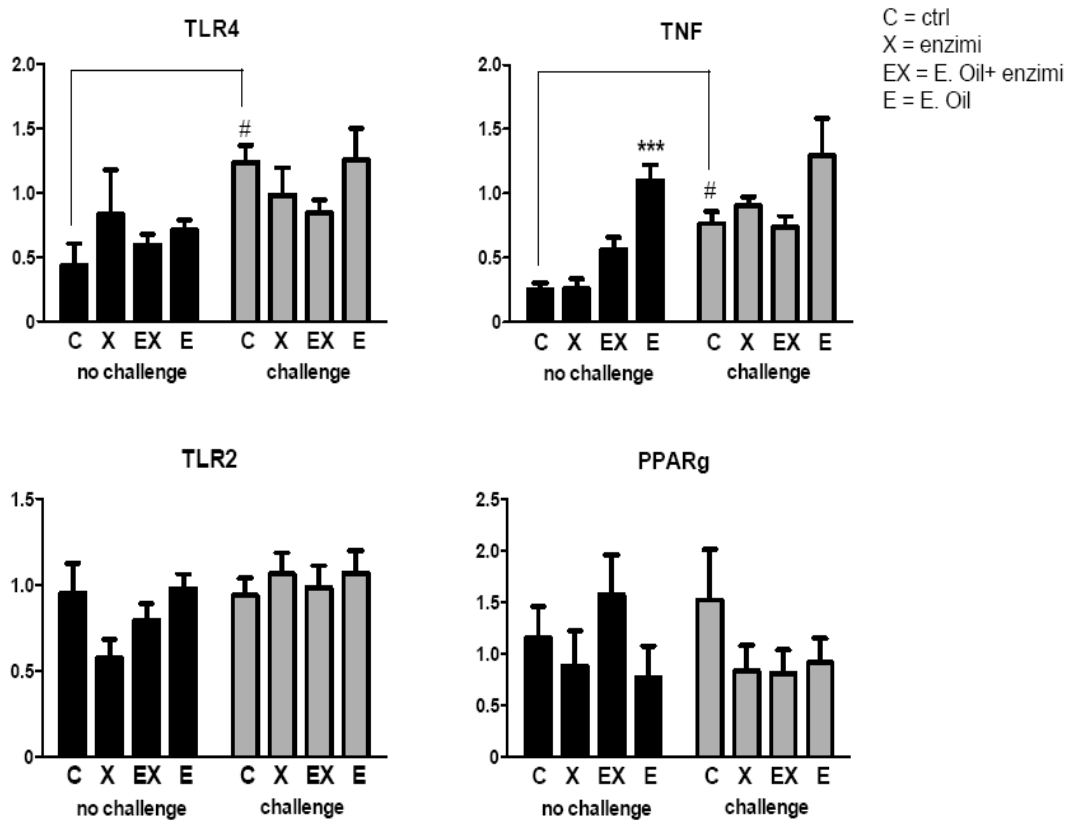
[4] Contrast between control diet and EO and/or XB treatments within challenge treatments.

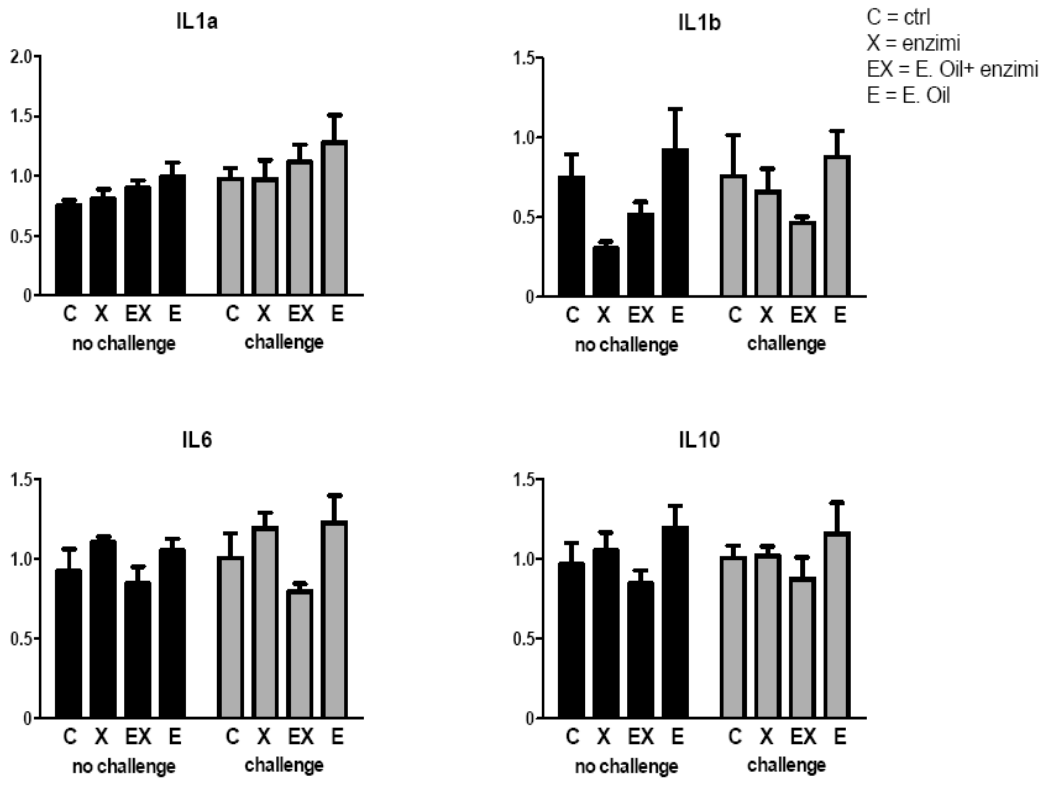
[5] The number in every mm^2 mucosa

4.4.7. Gene Expression

Figure 4.2 shows the effects of EO and/or XB on ileal gene expression of the piglets. Only TLR4 and TNF mRNA levels were significantly altered in piglets after injection of *E. coli* ($P < 0.01$). However, none of the additives alone or in combination decreased the expression of these two genes. Notably, dietary EO increased the TNF mRNA levels in animals not challenged with *E. coli* ($P < 0.01$) and the expression of TNF in piglets after injection of *E. coli* and then treated with the diet supplemented with EO was even higher than that of challenged animals.

Figure 4.2. Effects of Essential oil and/or Enzymes supplementation on ileal gene expression of *E. coli* challenged and non-challenged piglets[1]





[1] n = 48 (6 piglets/treatment).

4.5. Discussion and conclusion

The goal of this experiment was to determine whether adding essential oil and/or enzyme supplement to the solid diet of weaned piglets would protect against *E. coli* infection when the piglets consumed a diet with no supplemental antibiotics. In the current study, *E. coli* challenge successfully created a chronic infection that negatively influenced growth performance in piglets, which was in agreement with previous observations showing that the performance of weaned pigs was impaired by enterotoxigenic *E. coli* challenge (Bosi et al., 2004; Ding et al., 2006; Jensen et al., 2006). No differences in ADG and ADFI after diet supplementation were found in our study, but the results indicated that improvement of growth performance was mainly embodied in decrease of feed convention ratio by supplementation of enzymes or combination with essential oils in presence of *E. coli* challenge during the last week. This finding is in agreement with previous observations that essential oil and enzyme improved growth performance mainly related to feed efficiency (Fan et al., 2009; Maenner et al., 2011). However, *E. coli* challenged piglets fed XB and EO+XB had high FCR during second and third weeks, so the pattern of response in this study suggested that XB and EO+XB reduced growth rate early after inoculation and increased it later.

The increase of diarrhea scores and *Clostridia, E. coli* and *Coliforms* populations also indicated the success of *E. coli* infection model in this study, which was in accordance with the observations reported by Song et al. (2012). Diarrhea caused by infectious disease is a serious problem in weaned piglets, which usually leads to an increased mortality rate (Glass et al., 1991; Osek, 1999). Several studies observed that *E. coli* K88 challenge increased diarrhea incident of weaned piglets but fecal scores were waned to normal level at 5 or 6 days after challenge (Yokoyama et al., 1997; Liu et al., 2010). In the present experiment, *E. coli* infection achieved a major diarrhea episode within 6 days postchallenge and then animals recovered gradually.

In vitro studies, the antibacterial activity of essential oils was demonstrated (Sivropoulou et al., 1996; Mancini-Filho et al., 1998; Dorman and Deans, 2000; Lambert et al., 2001; Burt, 2004). Essential oils comprise a large number of components and it is likely that their mode of action involves several targets in the bacterial cell. The hydrophobicity of EO enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents. As the important exogenous enzymes, Xylanase and β-glucanase might exert their beneficial action by influencing the intestinal microflora (Bedford, 2000). Supplementation with essential oils and/or enzymes in present study did not change the situation of severe diarrhea post *E. coli* infection which may be related to the degree of pathogenicity of stain used. However, *E. coli* challenged piglets fed enzymes or combination with essential oils reduced populations of *E. coli* or *Coliforms* at 35 d, which reinforces the observation of growth performance. In addition, essential oils and/or enzymes induced decreases of *E. coli* or *Coliforms* counts in both faeces and cecum, indicating the antibacterial activities.

Reactive oxygen species (ROS) and free radicals are generated and scavenged continuously within normal animal body and concentrations of ROS and free radicals maintain homeostatic. If ROS or free radicals are not removed in a timely manner by the antioxidant system, an imbalance between free radical generation and removal would lead to oxidative stress. Mammalian cells may encounter oxidative stress that causes destruction of macromolecules and abnormal function (Evans et al., 1997). The animals may show alteration of physiology and behavior and poor growth performance, or even suffer from various kinds of diseases under the condition of excessive ROS presence. Antioxidant enzymes such as glutathione superoxide dismuthase (SOD) and peroxidase (GSH-Px) play most important role in the antioxidant protective system. In this study, oral inoculation of *E.coli* induced significant increase of malondialdehyde (MDA) content and decrease of total antioxygenic capability (T-AOC) activity at 2 d postchallenge, which indicated that the excessive release of ROS or free radical were beyond the scavenging capacity of antioxidant

defense system. Several literatures reported that essential oils had antioxidant activity (Lagouri et al., 1993; Mancini-Filho et al., 1998). In contrary, our results observed that dietary XB and EO+XB weakened serum total antioxidant capacity, which might be the possibility that enzymes fail to improve serum antioxidant defense system in particular with *E. coli* challenge.

Haematological analysis is one important adjuvant tool in the diagnosis of infection and its evolution. Oral inoculation of *E. coli* induced a significant increase of lymphocytes, monocytes, eosinophils and basophils percentages, indicating the duration of transitional effects of infection, but there was no difference with all the diet treatments.

Villus: crypt ratio represents the nutrient digestion and absorption capacity of the small intestine (Pluske et al., 1996; Montagne et al., 2003). *E. coli* challenge in the current study tightly damaged ileal morphology only by increasing crypt depth and decreasing follicles area but no influence in V/C ratio. The reason may be attributed to the period from injection to slaughtering when was enough for animals to get rid of the negative effect of *E. coli* challenge. Mathlouthi et al. (2002) found that addition of xylanase and β-glucanase increased villus size and the villus height-to-crypt depth ratio of broilers. Supplementation of essential oils may improve gut health by reducing the burden of enteric pathogens in the gut of weaning piglets (De Lunge et al., 2010). Obviously in our work, the V/C ratio was higher in essential oils and/or enzymes fed both *E. coli* challenged or unchallenged animals, especially in EO and EO+XB treatments. The change in V/C ratio was accompanied with enlarged villi or reduced crypts, villus height in all the treatments were higher than control group while only dietary combination of EO and XB decreased crypt depth. The results of gut morphology in the ileum also evidence that supplemented the combination of essential oils and enzymes with the diet of *E. coli* challenged piglets had the tendency to decrease mucosal lymphatic follicles number and area, which suggest that dietary combination may increase the animals' ability to cope with stress, reducing the negative effects of *E.coli* challenge.

Cytokines play an important role in immunoregulation. Tumour necrosis factor (TNF), interleukins (1L)-1 and IL-6 derivative from membrane phospholipids, and TNF acts as a trigger which activates a cascade of cytolune production (Grimble, 1998). Toll-like receptor (TLR) 4 is the main signal transducer of classical LPS (i.e. Escherichia coli LPS), while TLR2 is used by certain non-classical LPSs. Lai et al. (2005) observed that *Lipopolysaccharide* from *E. coli* induced symptoms of acute inflammation responses and increased gene expressions of IL-1β, IL-6, TNF-α, IL-10 and PPARγ in spleen and thymus of weaned piglets. Our study observed that *E. coli* challenge increased TLR4 and TNF mRNA level, which reinforces the typical acts of them. No additive decreased the expression of TLR4 and TNF. Dramatically, dietary EO increased

the gene expression of TNF and the unchallenged piglets fed EO were even higher than challenged animals fed without EO.
In conclusion, the current observation demonstrated that essential oils (Thymol and Cinnamaldehyde) and/or enzymes (Xylanase and β-glucanase) improved gut morphology in the piglets with/without *E. coli* K88 challenge, especially in EO and combination treatments. In addition, all the supplemented treatments decreased the populations of *Clostridia, E. coli* and *Coliforms* in unchallenged animals. Enzymes or combination with essential oils increased FCR and *E. coli* counts of faeces early after challenge but improved by the end of the trial. Meanwhile, no positive effects of diets on antioxidant defense system, haematological profile and gene expression were observed in the study.

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CHAPTER 5

Effects of dietary cocktail of fuctional foods and body weight on production of inflammatory cytokines and time responses in early weaned piglets challenged with lipopolysaccharides

5. Effects of dietary cocktail of fuctional foods and body weight on production of inflammatory cytokines and time responses in early weaned piglets challenged with lipopolysaccharides

5.1. Abstract

In this study, the effects of dietary cocktail and body weight on production of inflammatory cytokines and time responses under Lipopolysaccharides (LPS) challenge were evaluated in early weaned piglets. Possible interaction between diets and weight were also assessed. Thirty-two sows with their litters were allocated into four groups, using a randomized complete block design. After weaning at 20 ± 1 d of age, a total of 256 piglets were allocated into eight experimental treatments (8 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments: a basal weaning diet added [spray-dried plasma protein (PP) (CTR), PP + antibiotic (ATB), PP + dietary cocktail (DC), or bovine colostrum + the dietary cocktail (BC+DC)] and 2) low weight (LW) or high weight (HW). At 37 d of age, 2 piglets in each pen were injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) of LPS per animal. Piglets were bled (10 ml) before LPS injections (T0) and also bled at 4 h or 18 h post-injections. The peripheral blood mononuclear cell (PBMC) samples, which were stimulated by 0.5 or 1.0 μg/ml LPS, 1.0 μg/ml Concanavalin A (ConA), 50 ng/ml Phorbol Myristate Acetate (PMA) or none (NS), were isolated from heparinized whole blood of the piglets before inoculation of LPS at 37 days of age. Stimulations of LPS and PMA in PBMCs of piglets significantly induced TNF-α, IL-8 and IL-10 ($P < 0.05$). There was no diet effect on the concentrations of TNF-α, IL-8 and IL-10 in PBMCs of piglets ($P > 0.05$). Piglets had low weight tended to increase the concentration of IL-8 ($P = 0.106$) and IL-10 ($P = 0.098$) in the PBMCs stimulated by LPS 0.5μg/ml LPS and the concentration of IL-10 ($P = 0.097$) in PMA stimulated PMBC compared with high weight animals. Infection with LPS increased ($P < 0.001$) serum concentrations of all the cytokines four hours post inoculation, and animals recovered to basal levels at 18 h after challenge. No significant diet effect was found in the serum concentrations of cytokines ($P > 0.05$). At 4 h after challenge, low weight piglets had partially greater serum concentrations of TNF-α ($P = 0.046$), IL-6 ($P = 0.158$), IL-8 ($P = 0.179$) and IL-10 ($P = 0.185$)

than high weight animals. In conclusion, dietary cocktail or combined with bovine colostrum may replace plasma protein and antibiotics and weight difference may influence the production of inflammatory cytokines after infected by LPS.
Key Words: body weight, dietary cocktail, inflammatory cytokines, lipopolysaccharides, piglets, time response

5.2. Introduction

Early weaned piglets have poor growth rate (Pluske et al., 1997) and are vulnerable in terms of health status so that they cannot efficiently cope with antigenic pressure such as enterotoxigenic Escherichia coli (ETEC), post-weaning-multisystemic-wasting syndrome (PMWS), circovirus, etc. (Meehan et al., 1998; Ramamoorthy and Meng, 2009; Schroyen et al., 2012). Supplementation of antibiotics can relief lower infection rates, but widespread application is restricted due to possible antibiotic residues and the development of bacterial resistance (Frydendahl et al. 2002; Roselli et al., 2005). Therefore, functional food and nutraceuticals may be considered as good alternatives to in-feed antibiotic.

Bovine milk by-products contained bioactive molecules with immuno-regulatory and antimicrobial properties (Cross and Gill, 2000; Schlimme et al., 2000). Cranberry (concentrate and pulp) have been shown to have beneficial effects on health through their antioxidant property and antimicrobial activity towards food borne pathogens, such as *E. coli* (Reid et al., 2002; Bodet et al., 2008; Vinson et al., 2008; Wu et al., 2009). Essential oils are used in a wide range of food systems to increase the safety and shelf life of foods (Burt, 2004). Probiotics are the bacteria with beneficial effects to host health through modulating microbiota, antagonizing enteric pathogens, or enhancing intestinal barrier and immune functions (Gagnon et al., 2007; Borchers et al., 2009; Lessard et al., 2009).

Therefore, to investigate the inflammatory roles, the influence of body weight and action of diet in early weaned piglets, we created a model of acute inflammation using lipopolysaccharides (LPS) challenge in pigs and determined the effects of dietary cocktail (DC) containing cranberry extract, essential oil, yeast-derived products, probiotics, premix supplemented with vitamins and organic Se with/without bovine colostrum on the production of inflammatory cytokines profiles and the comparison between low and high body weight piglets.

5.3. Materials and methods

5.3.1. Experimental Design, Housing and Dietary Treatments

Thirty-two Yorkshire × Landrace sows housed at the Dairy and Swine Research and Development Centre, Agriculture and Agri-Food Canada (Sherbrooke, Quebec, Canada) were used. Two weeks before parturition, sows were housed by blocks (groups of 4 litters) in two maternity rooms. Experiment was performed by lots of two blocks (eight sows by lot). Oestrus was synchronized before to inseminate the sows. Litter size was adjusted to 12 piglets within the first two days after birth and piglets were weighed. Overall, a total of 32 sows and their litters were divided in four groups of 8 litters, using a randomized complete block design. The experiment was a 4 × 2 factorial arrangement. At weaning (day 20 ± 1d), 8 piglets per litter were kept and were divided in two groups based on their weight gain at 14 days of age: 4 low weight gain (LW) and 4 high weight gain (HW) piglets within each litter were respectively housed in two different pens. Litters were allocated to one of the following dietary treatment: basal weaning diet supplemented with i) 35 g/kg spray-dried plasma protein (PP) (CTR); ii) 35 g/kg PP and antibiotics (ATB); iii) 35 g/kg PP and a dietary cocktail composed of: cranberry extract, encapsulated essential oil, yeast-derived products (mannans, glycans), the probiotic *Pediococcus acidilactici* MA18/5M, vitamins A, D, E and B complex, seleno-methionine (DC); iv) 50 g/kg bovine colostrum and the dietary cocktail (BC+DC).

Diet was formulated to be isonutritive, exceeding the protein requirement recommended by NRC (1998) for pigs. The approximate composition and the chemical analysis of the diet are presented in Table 5.1. Diet was formulated and manufactured before the trial start, all the diets were meal and milled at 1.5 mm particle size.

5.3.2. LPS Challenge

At 37 days of age, 2 piglets (one piglet per batch) of each pen were used to evaluate their potential to cope with inflammatory challenge under stress condition by giving them intra-peritoneal injection a single dose of 200 μg (in 5 mL PBS) of LPS per piglet. Twenty-four hours before LPS injections, room temperature was reduced to 20° C to induce a stress. Piglets were bled (10 ml) before LPS injections (T0). The 1st batch or 2nd batch of piglets was also bled before being euthanatized at 4 h or 18 h post-injections.

Table 5.1. Ingredient and chemical composition of the diets (as fed basis).

Item	Basal diet	ATB	DC	BC + DC
Ingredient, %				
Wheat	20	20	20	20
Corn	30	30	30	30
Soybean meal, 48 % CP	20	20	20	20
Whey	15	15	15	15
Soybean oil	5.00	5.00	5.00	5.00
Plasma protein	3.50	3.50	3.50	---
Bovine colostrum	---	---	---	5.00
Cranberry extract	---	---	1.00	1.00
Essential oils (carvacrol + cinnamon)	---	---	0.10	0.10
yeast-derived products (mannans + glycans)	---	---	0.50	0.50
Pediococcus acidilactici MA18/5M (CFU/kg)	---	---	2×10^9	2×10^9
Antibiotics	---	1.00	---	---
Micro-premix	0.30	0.30	---	---
Micro-premix + vitamins A, D, E, B, Se-Met	---	---	0.30	0.30
Calcium carbonate	1.40	1.40	1.40	1.40
Dicalcium phosphate 21%	1.83	1.83	1.83	1.83
Salt	0.48	0.48	0.48	0.48
Methionine	0.17	0.17	0.17	0.17
Lysine HCl 98%	0.32	0.32	0.32	0.32
L-Threonine	0.13	0.13	0.13	0.13
L-Tryptophan	0.03	0.03	0.03	0.03
Calculated chemical composition				
DE, kcal/kg	3,600	3,600	3,600	3,600
Protein, %	21.50	21.50	21.50	21.50
Fat, %	7.50	7.50	7.50	7.50
Fiber, %	2.50	2.50	2.50	2.50
Calcium,%	1.00	1.00	1.00	1.00
Phosphorus, %	0.76	0.76	0.76	0.76
Sodium, %	0.20	0.20	0.20	0.20
Copper, mg/kg	30	30	30	30
Zinc, mg/kg	80	80	80	80
Vitamin A, IU/kg	10,000	10,000	30,000	30,000
Vitamin D, IU/kg	1,100	1,100	5,000	5,000
Vitamin E, IU/kg	55	55	250	250
Vitamin B6 (mg/kg)	2.50	2.50	4.00	4.00
Vitamin B9 – folic acid (mg/kg)	3.00	3.00	10.00	10.00
Vitamin B12 (µg/kg)	25	25	100	100
Niacin (mg/kg)	30	30	60	60
Choline (mg/kg)	300	300	1,000	1,000
Menadione (mg/kg)	2.50	2.50	4.00	4.00
Thiamine (mg/kg)	2.70	2.70	7.00	7.00
Riboflavin (mg/kg)	5.00	5.00	15.00	15.00
Seleno-Methionine (mg/kg)	---	---	0.30	0.30
Selenite (mg/kg)	0.30	0.30	---	---

5.3.3. Isolation and Stimulation of Peripheral Blood Mononuclear Cells (PBMCs)

The PBMCs were isolated from heparinized whole blood of the 1st batch of piglets which were bled before inoculation of LPS at 37 days of age by Ficoll-Paque density gradient centrifugation. Briefly, blood samples mixed with Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+} (HBSS) were centrifuged (750 × g, 40min), and the mononuclear cell layer was recovered and washed twice with HBSS . Subsequently, the cells were resuspended in complete RPMI-1640 medium supplemented with 7.5% (v:v) heat-inactivated fetal bovine serum (FBS) and 1X Penicillin Streptomycin (Pen-Strep) (Sigma). The cells were counted using the Countess® automated cell counter (LIFE technologies). Then 2.5 x 10^6 cells were stimulatedwith one of the following: 0.5μg/ml LPS, 1.0 μg/ml LPS, 1.0 μg/ml Concanavalin A (ConA), 50 pg/ml Phorbol Myristate Acetate (PMA) or non-stimulated (NS).

5.3.4. Determination of Cytokines

PBMCs and serum samples were assayed in duplicates with commercial porcine ELISA kits according to the protocols provided by the manufacturers. Standards of known recombinant porcine cytokine were used to make standard curves. The ELISA kits used for quantification of TNF-α, IL-6, IL-8 and IL-10 were bought from R & D Systems (Minneapolis, USA).

The PBMCs samples were analyzed for TNF-α, IL-8 and IL-10. The intra-assay coefficients of variation for TNF-α, IL-8 and IL-10 were 3.8, 4.6 and 4.9 %, respectively. The inter-assay coefficients of variation (average of category) for TNF-α, IL-8 and IL-10 were 3.8, 4.6 and 5.0%, respectively. The results were expressed in pictograms per milliliter based on a standard curve.

The cytokines of serum samples were analyzed at 1:2 dilutions. The intra-assay coefficients of variation for TNF-α, IL-6, IL-8 and IL-10 were 5.1, 2.9, 5.2 and 7.2%, respectively. The inter-assay coefficients of variation (average of category) for TNF-α, IL-6, IL-8 and IL-10 were 5.0, 2.9, 5.2, 7.2%, respectively. The results were expressed in pictograms per milliliter based on a standard curve.

5.3.5. Statistical Analysis

Data were analysed as a 4 × 2 factorial arrangement of treatments by ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC). The model included the effects of diet and body weight as well as the diet × body weight interaction. Fixed effects were diet and body weight, and random effect was block. A Log2 transformation was performed for the cytokine concentration in PBMCs. Tukey's Honestly Significant Difference test was used to measure the contrast between diets treatments. A *P*-value of less than 0.05 was considered significant.

5.4. Results

5.4.1. Concentration of Inflammatory Cytokines in the Stimulated PBMCs

TNF-α. Stimulation with 0.5 μg/ml LPS, 1.0 μg/ml LPS and 50 pg/ml PMA resulted in significant unregulation ($P < 0.05$) of TNF-α, there was no effect of 1.0 μg/ml ConA stimulation (Figure 5.1). Dietary cocktail combined with colostrum increased ($P = 0.044$) the concentrations of TNF-α in non-stimulated PBMC as compared to dietary antibiotic (Table 5.2). No significant difference among all the dietary treatments in stimulated PBMCs was observed. Although the TNF-α concentration of low weight piglets seems higher than high weight piglets in the stimulated PBMCs samples, the differences were not significant. There was no interaction between diet and weight was found.

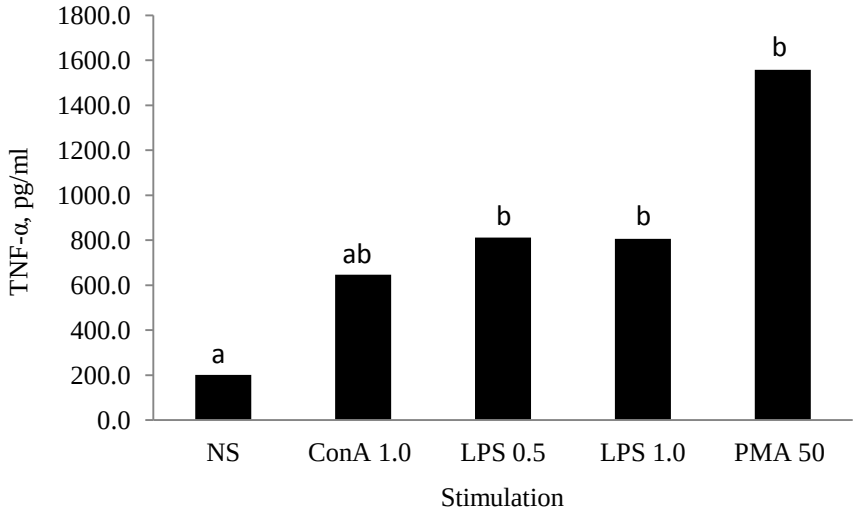

Figure 5.1. TNF-α production of PBMCs (n = 256) stimulated by 1.0 μg/ml ConA, 0.5 or 1.0 μg/ml LPS, 50 ng/ml PMA or none (NS). [a,b] Bars with different letters differ at $P < 0.05$.

IL-8. IL-8 was significantly induced in PBMC of piglets after stimulation with 0.5 μg/ml LPS, 1.0 μg/ml LPS and 50 pg/ml PMA ($P < 0.05$), but stimulated with 1.0 μg/ml ConA did not change the IL-8 level (Figure 5.2). There was no dietary effect on IL-8 concentration of non-stimulated and stimulated PBMC (Table 5.3). However, the IL-8 concentrations of PMBC stimulated by 0.5μg/ml LPS in low weight piglets partial higher than high weight animals ($P = 0.106$). No interaction between diet and weight was observed.

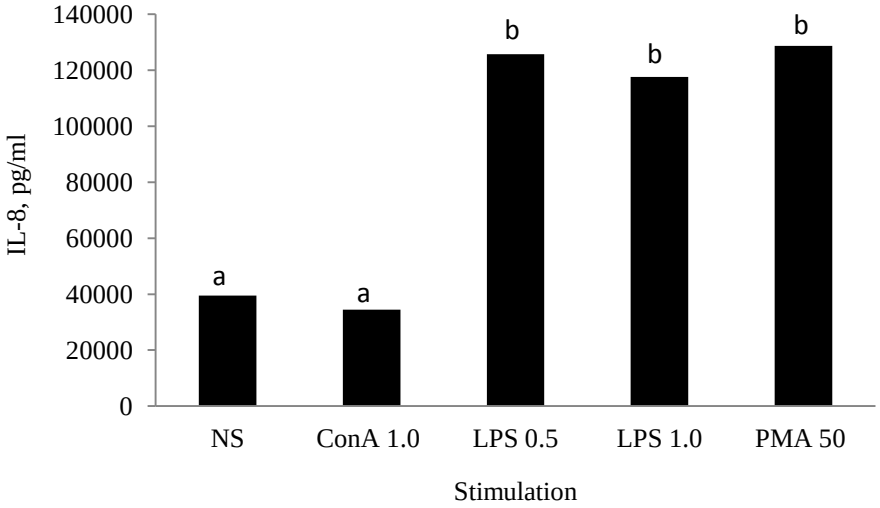

Figure 5.2. IL-8 production of PBMCs (n = 256) stimulated by 1.0 μg/ml ConA, 0.5 or 1.0 μg/ml LPS, 50 ng/ml PMA or none (NS). [a,b] Bars with different letters differ at $P < 0.05$.

IL-10. After stimulation with 0.5 μg/ml LPS, 1.0 μg/ml LPS, 1.0 μg/ml ConA and 50 pg/ml PMA in PBMC of pathogen free piglets, significant increases ($P < 0.001$) of IL-10 were observed (Figure 3). There was no main effect of either dietary treatment on IL-10 concentration (Table 4). Dietary cocktail and colostrums or low weight might increase the concentrations of IL-10 as compared to dietary antibiotic or high weight, while there was no significant difference. In addition, no interaction between diet and weight was observed.

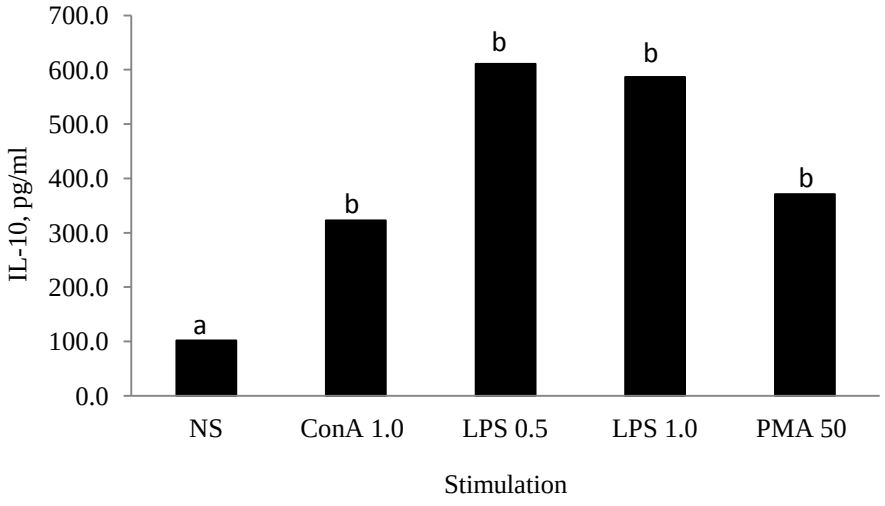

Figure 5.3. IL-10 production of PBMCs (n = 256) stimulated by 1.0 μg/ml ConA, 0.5 or 1.0 μg/ml LPS, 50 ng/ml PMA or none (NS). [a,b] Bars with different letters differ at $P < 0.05$.

Table 5.2. Effects of cocktail diet or combination with bovine colostrum on concentration of tumor necrosis factor-alpha (TNF-α) in stimulated PBMCs of high weight or low weight piglets[1]

Diet[2]	CTR		ATB		DC		BC+DC		*P*-value[4]		
Weight[3]	HW	LW	HW	LW	HW	LW	HW	LW	Diet	Weight	Diet × Weight
ConA 1.0 μg/ml	402.9 [223.8, 725.5]	631.3 [350.6, 1136.7]	502.4 [279.1, 904.6]	367.1 [196.4, 685.9]	333.1 [191.7, 578.9]	462.3 [258.3, 827.6]	476.0 [273.9, 827.1]	625.0 [359.7, 1085.9]	0.657	0.233	0.340
LPS 0.5μg/ml	357.3 [185.0, 689.9]	482.9 [250.1, 932.5]	439.8 [227.7, 849.2]	361.6 [178.9, 730.7]	428.4 [230.9, 794.7]	622.4 [323.5, 1197.5]	530.4 [285.9, 983.9]	909.5 [490.3, 1687.1]	0.306	0.176	0.554
LPS 1.0μg/ml	335.6 [173.9, 647.9]	450.3 [233.2, 869.2]	451.3 [233.8, 871.3]	373.9 [184.4, 758.0]	411.4 [222.4, 761.3]	556.5 [289.2, 1071.2]	513.4 [277.5, 950.0]	912.6 [493.2, 1688.6]	0.378	0.210	0.578
PMA 50 pg/ml	847.4 [436.2, 1646.4]	1205.2 [620.4, 2341.3]	1118.1 [575.5, 2172.2]	820.2 [404.8, 1662.0]	967.9 [516.8, 1812.9]	1550.1 [800.3, 3002.2]	1013.8 [541.3, 1898.8]	1302.2 [695.3, 2438.9]	0.834	0.298	0.475
NS	81.34 [34.5, 191.8]	117.4 [49.8, 276.7]	72.55 [30.8, 171.0]	51.37 [20.5, 128.6]	75.97 [33.9, 170.4]	118.2 [50.2, 278.1]	194.9 [86.9, 437.1]	190.5 [84.9, 427.1]	0.044	0.671	0.694

[1] Least-square means values (n = 6 to 8) were presented together with confidence intervals [Lower, Upper].

[2] CTR: basal weaning diet supplemented with 35 g/kg spray-dried plasma protein (PP); ATB: CTR + antibiotics; CCT: CTR + a dietary cocktail; CCT+CLS: 50 g/kg bovine colostrum + the dietary cocktail.

[3] High weight and low weight groups were divided based on weight gain of piglets at 14 days of age.

[4] *P*-value was obtained on the transformed variable using Log2 transformation.

Table 5.3. Effects of cocktail diet or combination with bovine colostrum on concentration of interleukin-8 (IL-8) in stimulated PBMCs of high weight or low weight piglets[1]

Diet[2]	CTR		ATB		DC		BC+DC		*P*-value[4]		
Weight[3]	HW	LW	HW	LW	HW	LW	HW	LW	Diet	Weight	Diet × Weight
ConA 1.0 μg/ml	23704 [11641, 48270]	26005 [12770, 52954]	19271 [9464, 39243]	17010 [7963, 36338]	21305 [10905, 41621]	29258 [14413, 59394]	25758 [13185, 50322]	31891 [16324, 62304]	0.573	0.536	0.883
LPS 0.5μg/ml	91505 [66212, 126460]	110074 [79649, 152122]	117767 [85215, 162753]	112778 [79551, 159882]	102336 [75613, 138503]	132911 [96207, 183618]	115616 [85425, 156476]	153652 [113529, 207956]	0.401	0.106	0.681
LPS 1.0μg/ml	82218 [58344, 115860]	91615 [65013, 129103]	105234 [74677, 148294]	105065 [72550, 152152]	99697 [72333, 137414]	125878 [89341, 177357]	109997 [79805, 151610]	146320 [106159, 201675]	0.188	0.172	0.803
PMA 50 pg/ml	84416 [49124, 145061]	113173 [65859, 194478]	76215 [44352, 130970]	105793 [59789, 187195]	126120 [75208, 211494]	139164 [81092, 238825]	107829 [64301, 180822]	106256 [63363, 178184]	0.295	0.225	0.800
NS	11801 [3729, 37345]	19305 [6100, 61088]	12699 [4013, 40184]	11113 [3264, 37832]	17404 [5845, 51826]	21626 [6852, 68256]	26920 [9040, 80161]	25355 [8515, 75500]	0.401	0.698	0.906

[1] Least-square means values (n = 6 to 8) were presented together with confidence intervals [Lower, Upper].

[2] CTR: basal weaning diet supplemented with 35 g/kg spray-dried plasma protein (PP); ATB: CTR + antibiotics; CCT: CTR + a dietary cocktail; CCT+CLS: 50 g/kg bovine colostrum + the dietary cocktail.

[3] High weight and low weight groups were divided based on weight gain of piglets at 14 days of age.

[4] *P*-value was obtained on the transformed variable using Log2 transformation.

Table 5.4. Effects of dietary cocktail or combination with bovine colostrum on concentration of interleukin-10 (IL-10) in stimulated PBMCs of high weight or low weight piglets[1]

Diet[2]	CTR		ATB		DC		BC+DC		*P*-value[4]		
Weight[3]	HW	LW	HW	LW	HW	LW	HW	LW	Diet	Weight	Diet × Weight
ConA 1.0 μg/ml	310.9 [188.7, 433.1]	319.3 [197.0, 441.5]	271.4 [149.2, 393.6]	317.0 [186.8, 447.3]	329.3 [213.8, 444.8]	325.4 [203.4, 447.4]	312.1 [196.6, 427.6]	342.3 [226.8, 457.8]	0.911	0.579	0.963
LPS 0.5μg/ml	543.3 [278.7, 807.9]	581.2 [316.6, 845.8]	556.0 [291.4, 820.6]	520.0 [240.2, 799.9]	511.3 [259.5, 763.2]	691.7 [427.8, 955.7]	549.7 [297.9, 801.6]	859.9 [608.1, 1111.8]	0.385	0.098	0.343
LPS 1.0μg/ml	508.5 [230.0, 787.0]	530.8 [252.3, 809.3]	543.2 [264.7, 821.7]	489.8 [195.5, 784.0]	509.1 [243.7, 774.4]	659.0 [381.2, 936.9]	523.5 [258.1, 788.9]	849.6 [584.2, 1114.9]	0.352	0.148	0.307
PMA 50 pg/ml	273.8 [138.6, 408.9]	405.4 [270.3, 540.6]	272.6 [137.4, 407.7]	418.6 [272.9, 564.2]	425.7 [299.1, 552.4]	376.6 [241.6, 511.7]	348.6 [221.9, 475.2]	421.6 [295.0, 548.3]	0.712	0.097	0.397
NS	16.7 [3.9, 71.4]	36.1 [8.5, 154.4]	15.6 [3.6, 66.6]	15.0 [3.2, 70.0]	31.9 [8.0, 126.9]	27.5 [6.5, 117.3]	66.1 [16.6, 262.9]	46.6 [11.7, 185.4]	0.192	0.884	0.768

[1] Least-square means values (n = 6 to 8) were presented together with confidence intervals [Lower, Upper].

[2] CTR: basal weaning diet supplemented with 35 g/kg spray-dried plasma protein (PP); ATB: CTR + antibiotics; CCT: CTR + a dietary cocktail; CCT+CLS: 50 g/kg bovine colostrum + the dietary cocktail.

[3] High weight and low weight groups were divided based on weight gain of piglets at 14 days of age.

[4] *P*-value was obtained on the transformed variable using Log2 transformation.

5.4.2. Concentration and Time Response of Inflammatory Cytokines in Serum

TNF-α. Four hours after inoculation, LPS challenge resulted in significant increase ($P < 0.001$) of serum TNF-α concentrations (Figure 5.4a, b). Although supplementation of cocktail or combination with bovine colostrum seemly decreased serum TNF-α concentrations as compared to control and antibiotic treatments, the differences were not significant (Figure 5.4a). There was an effect of weight ($P = 0.050$), time ($P < 0.001$) and weight by time interaction ($P = 0.058$) on serum concentrations of TNF-α in the 1st batch of piglets, low weight piglets had higher ($P = 0.046$) serum concentrations of TNF-α than high weight piglets at 4 h post challenge (Figure 5.4b). In addition, no effect of time or diet or weight was found in serum TNF-α concentration of the 2nd batch of piglets. LPS challenged piglets returned to basal levels at 18 hours post injection.

IL-6. There was clearly an effect of the LPS infection ($P < 0.001$) on serum IL-6 concentrations in the 1st batch of piglets. Other than that, only the values at four hours after inoculation can be analyzed due to too many zero values of IL-6 concentrations in both batches of piglets at 0 h or 18 h after challenge (Table 5.5, 5.6). No dietary effect on the concentrations of serum IL-6 was observed at 4 h post challenge (Figure 5.5a). However, LPS infected piglets with high weight tended to decrease ($P = 0.158$) serum IL-6 concentrations compared with low weight animals (Figure 5.5b).

IL-8. Infection with LPS increased ($P < 0.001$) serum concentrations of IL-8 four hours post inoculation, and animal recovered to basal levels by 18 h (Figure 5.6a, b). Piglets fed control seemly had higher IL-8 concentrations than others dietary treatments at 4 h after inoculation, while the differences were not significant (Figure 5.6a). Treatment tended to have an effect of weight ($P = 0.195$) and time by weight interaction ($P = 0.155$) on serum IL-8 concentration, and an effect of time ($P < 0.001$) was also found (Figure 5.6b). High weight piglets tended to have lower IL-8 concentration than low weight piglets at 4 h after injection of LPS ($P = 0.169$).

IL-10. LPS challenge resulted in significant increase ($P < 0.001$) of serum TNF-α concentrations four hours after inoculation (Figure 5.7a, b). Administration of LPS increased serum concentrations of IL-10 from 0 h to 4 h after injection ($P < 0.001$), and then IL-10 concentration were reduced to basal levels by 18 h. Although there was no significant differences between all the dietary treatments, diet supplemented any additive might decrease IL-10 concentrations of serum as compared to control at 0 and 4 h post challenge (Figure 5.7a). There was an effect of time ($P < 0.001$), and treatment tended to have an effect of time by weight interaction ($P = 0.129$) on serum IL-10 concentration (Figure 5.7b). Increase of body weight potentially decreased the concentration of IL-10 at 4 h after injection of LPS ($P = 0.185$).

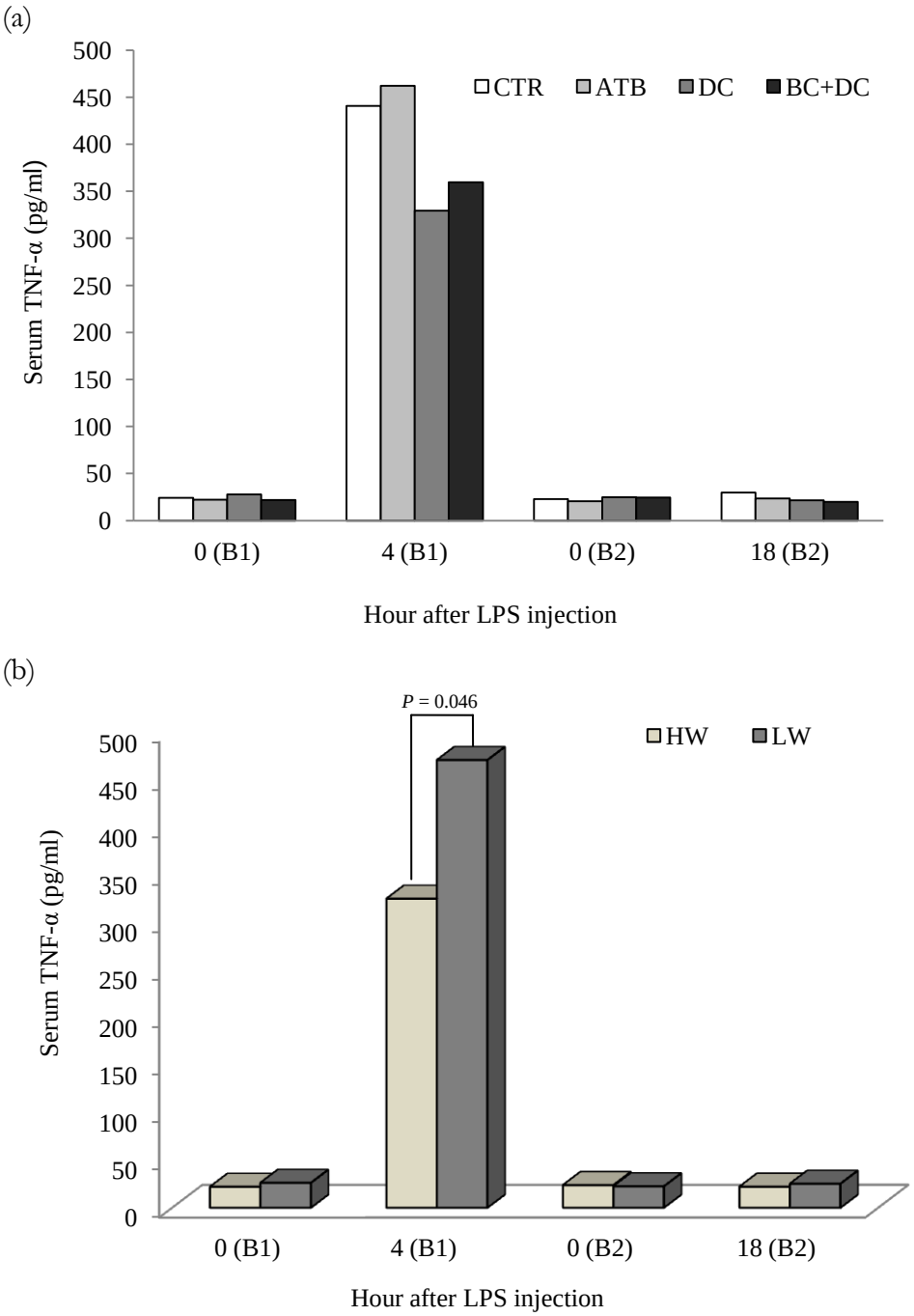

Figure 5.4. Serum concentration of tumor necrosis factor-alpha (TNF-α) in piglets following lipopolysaccharide (LPS) challenge, where two batches of piglets (B1 and B2) were injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) at 37 days of age. (a) Effect of diet and time response on serum TNF-α levels (n = 14 to 16). (b) Effect of body weight and time response on serum TNF-α levels (n = 29 to 32).

Table 5.5. Frequency of zero values of serum concentration of interleukin-6 (IL-6) in 1st batch of piglets at 37 days of age [1]

	Frequency (0 h)[2]		Frequency (4 h)[2]		
Item	0	1	0	1	Total
Numbers					
CTR×HW	5	3	0	8	8
CTR×LW	4	4	0	8	8
ATB×HW	7	1	0	8	8
ATB×LW	7	0	0	7	7
DC×HW	7	1	1	7	8
DC×LW	6	1	0	7	7
(BC+DC)×HW	7	1	1	7	8
(BC+DC)×LW	7	1	0	8	8
Total	50	12	2	60	62
Percentage (%)					
CTR×HW	62.50	37.50	0	100	100
CTR×LW	50.00	50.00	0	100	100
ATB×HW	87.50	12.50	0	100	100
ATB×LW	100	0	0	100	100
DC×HW	87.50	12.50	12.50	87.50	100
DC×LW	85.71	14.29	0	100	100
(BC+DC)×HW	87.50	12.50	12.50	87.50	100
(BC+DC)×LW	87.50	12.50	0	100	100
Total	80.65	19.35	3.23	96.77	100

[1] Serum samples were collected at 0 and 4 hours after LPS injection.

[2] Binary data (absence = 0; presence = 1) were used to calculate the percentage of zero data.

Table 5.6. Frequency of zero values of serum concentration of interleukin-6 (IL-6) in 2nd batch of piglets at 37 days of age [1]

	Frequency (0 h)[2]		Frequency (18 h)[2]		
Item	0	1	0	1	Total
Numbers					
CTR×HW	5	3	3	5	8
CTR×LW	5	3	5	3	8
ATB×HW	7	1	7	1	8
ATB×LW	6	1	6	1	7
DC×HW	5	1	5	1	6
DC×LW	6	2	6	2	8
(BC+DC)×HW	6	1	6	1	7
(BC+DC)×LW	8	0	7	1	8
Total	48	12	45	15	60
Percentage (%)					
CTR×HW	62.50	37.50	37.50	62.50	100
CTR×LW	62.50	37.50	62.50	37.50	100
ATB×HW	87.50	12.50	87.50	12.50	100
ATB×LW	85.71	14.29	85.71	14.29	100
DC×HW	83.33	16.67	83.33	16.67	100
DC×LW	75.00	25.00	75.00	25.00	100
(BC+DC)×HW	85.71	14.29	85.71	14.29	100
(BC+DC)×LW	100	0	87.50	12.50	100
Total	80.00	20.00	75.00	25.00	100

[1] Serum samples were collected at 0 and 18 hours after LPS injection.

[2] Binary data (absence = 0; presence = 1) were used to calculate the percentage of zero data.

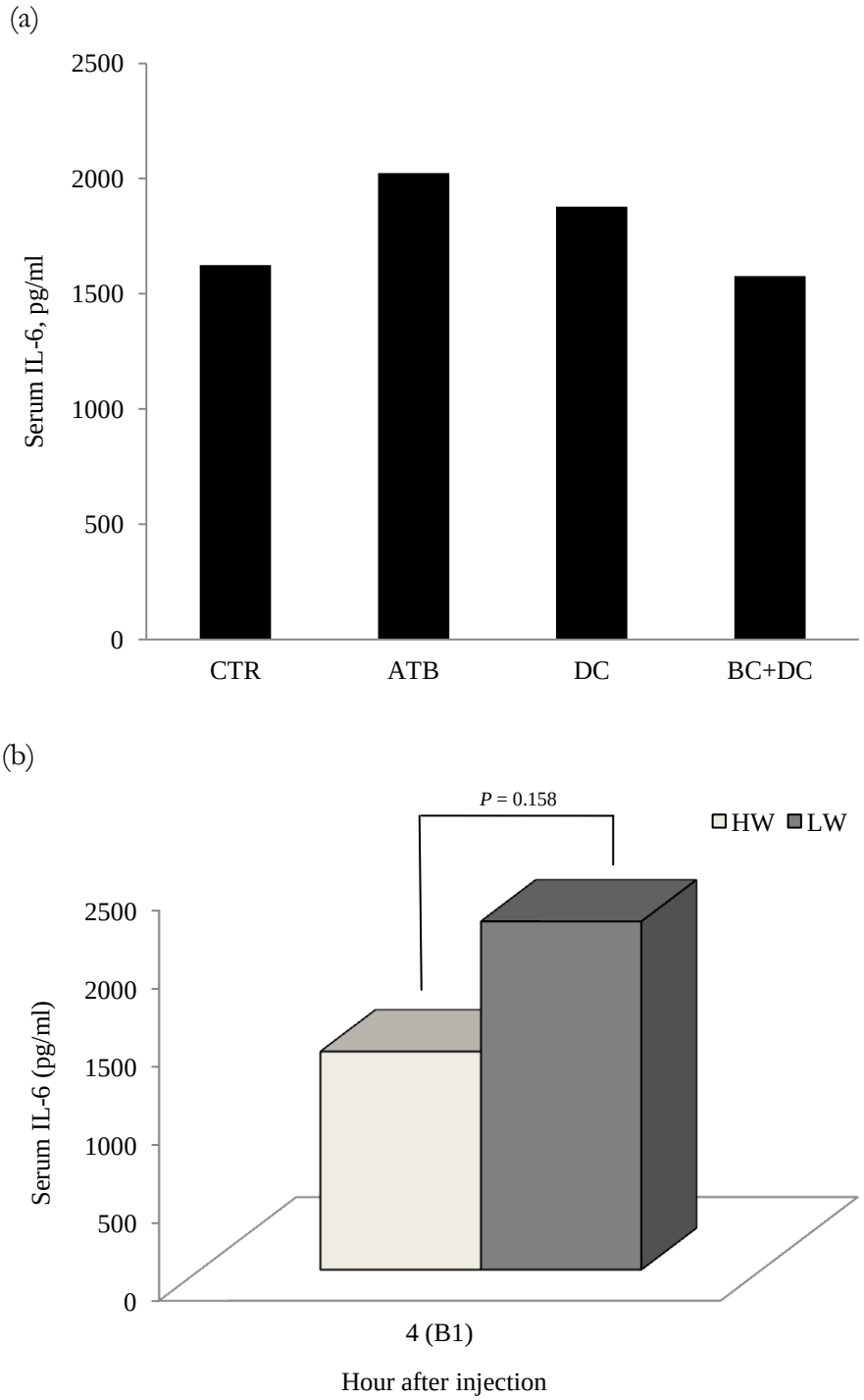

Figure 5.5. Serum concentration of interleukin-6 (IL-6) in piglets at 4 hours after lipopolysaccharide (LPS) challenge, where the 1st batch of piglets was injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) at 37 days of age. (a) Effect of diet on serum TNF-α levels (n = 14 to 16). (b) Effect of body weight on serum TNF-α levels (n = 29 to 32).

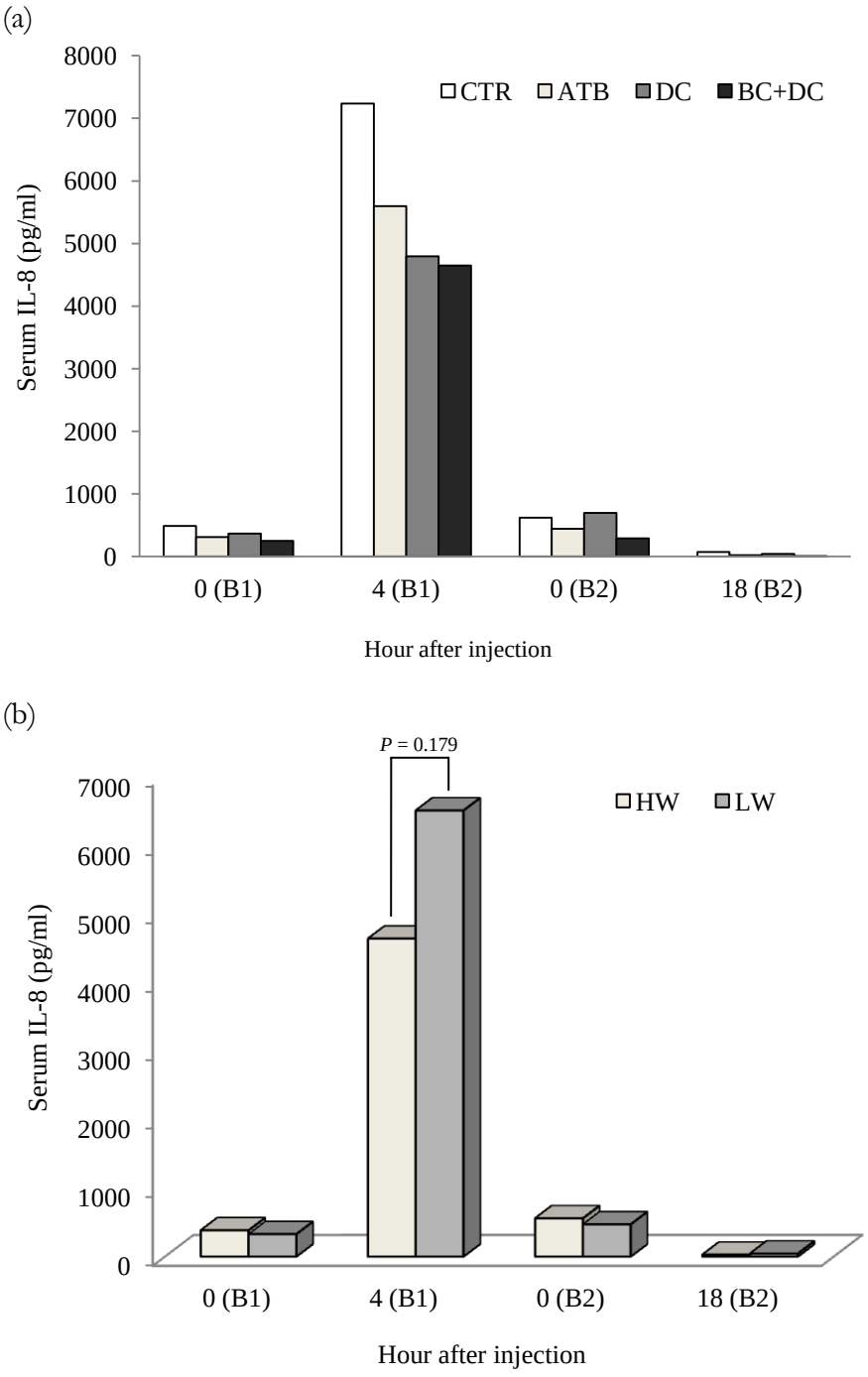

Figure 5.6. Serum concentration of interleukin-8 (IL-8) in piglets following lipopolysaccharide (LPS) challenge, where two batches of piglets (B1 and B2) were injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) at 37 days of age. (a) Effect of diet and time response on serum IL-8 levels (n = 14 to 16). (b) Effect of body weight and time response on serum IL-8 levels (n = 29 to 32).

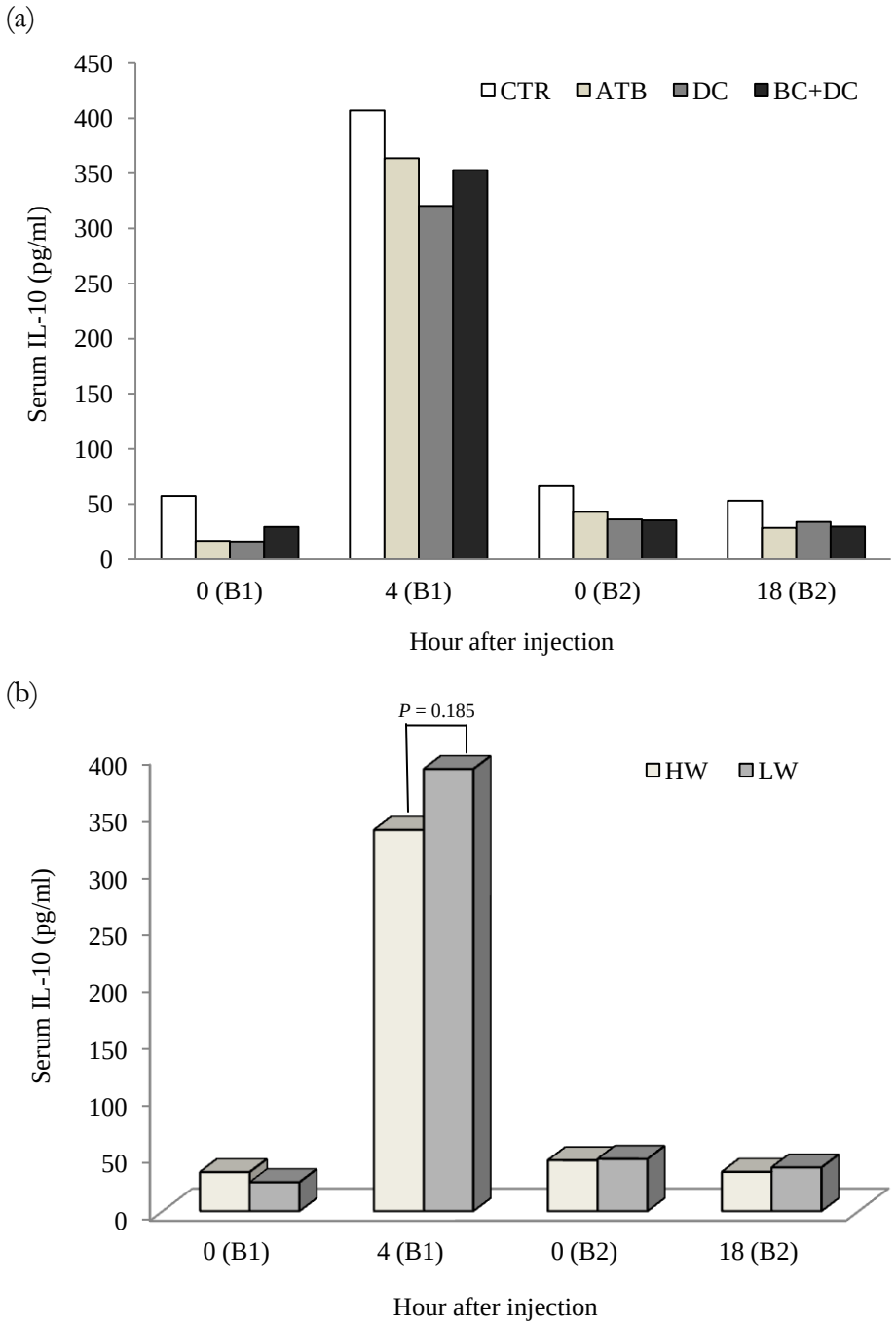

Figure 5.7. Serum concentration of interleukin-10 (IL-10) in piglets following lipopolysaccharide (LPS) challenge, where two batches of piglets (B1 and B2) were injected intraperitoneally with a single dose of 200 μg (in 5 mL PBS) at 37 days of age. (a) Effect of diet and time response on serum IL-10 levels (n = 14 to 16). (b) Effect of body weight and time response on serum IL-10 levels (n = 29 to 32).

5.5. Discussion and conclusion

In present study, several inflammatory cytokines including TNF-α, IL-8 and IL-10 were detected in the supernatant of PBMC obtained from pathogen free piglets after stimulation with LPS and PMA, which is in agreement with previous findings in pigs or human (Janský et al., 2003; Gao et al., 2010). No difference between ConA stimulated and non-stimulated PBMCs in TNF-α and IL-8 were observed in this study, which is similar with the finding of Friebe et al. (2004). The PBMC stimulated by ConA had higher IL-10 production than PBMC without stimulation. As one of most important anti-inflammatory cytokines, IL-10 is produced by Th2 (Friebe et al., 2004; Lai et al., 2005). The upregulation of IL-10 may be part of a homeostatic mechanism to balance the action of Th1 (Hontecillas et al., 2002), and the ConA-stimulation in porcine PBMC exhibited a Th1 bias in cytokine expression profiles (Wilkinson et al., 2012).

Our results indicated that LPS challenge induced symptom of acute inflammation response by increasing production of proinflammatory cytokines (IL-6 and TNF-α) and chemokine (IL-8) at 4 h after inoculation, which agrees with previously reported results showing that a low dose of LPS increased plasma concentrations of the pro-inflammatory cytokines during 2 and 4 h after injection (Weble et al., 1997; Llamas Moya et al., 2006). They also found that plasma IL-6 and TNF-α level of LPS treated piglets returned to normal by 12 h after injection, which coincided with our observation that there was no difference of serum TNF-α, IL-6 and IL-8 concentrations between 18 h after LPS challenge and before challenge. Lai et al. (2005) observed that injection of LPS increased the concentrations of plasma pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokine (IL-10). Similarly, the increase of serum IL-10 concentration in the current study was simultaneous with the acute inflammation response.

Antibiotics have been used over decades to inhibit pathogen infection, and many findings have established that antibiotics may generate anti-inflammatory effects (Chin et al., 1998; Diarra et al., 1999; Buret, 2010). But the possible antibiotic residues and the development of bacterial resistance (Frydendahl et al. 2002; Roselli et al., 2005) are potentially harmful for human health. Therefore, various functional food and nutraceuticals such as bovine milk by-products, cranberry products, essential oil (EO), probiotics, prebiotics, essential fatty acids and vitamins were evaluated currently to alternatives to in-feed antibiotics. Some studies demonstrated that bovine milk by-products contained bioactive molecules with immuno-regulatory and antimicrobial properties (Cross and Gill, 2000; Schlimme et al., 2000). Bodet et al. (2006) reported that the cranberry NDM fraction inhibited the production of pro-inflammatory cytokines (IL-1β,

IL-6, and TNF-*a*) and chemokines (IL-8 and RANTES) by macrophages stimulated with lipopolysaccharides (LPS) from E. coli and major periodontopathogens, including *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *T. denticola*, and *T. forsythia*. Probiotics interfere with different steps of the inflammatory pathway, either on cytokines that can regulate the balance between Th1 and Th2 lymphocyte populations, or on chemokines involved in the recruitment of inflammatory cells (Roselli et al., 2005). Lessard et al. (2009) reported that ingestion of *Pediococcus acidilactici* might modulate establishment of lymphocyte populations and IgA secretion in the gut. Other studies have also reported that essential oils, yeast-derived products and vitamins supplementations had benefits to modulate immune function, or enhancing intestinal barrier (Lessard et al., 1991; Burt, 2004; Bontempo et al., 2006; De Lange et al., 2010). Although supplementation of dietary cocktail or combination with bovine colostrum in this study did not inhibit the release of inflammatory cytokines, there were no differences among those two diets and dietary antibiotics. It might reinforce the possibility that dietary cocktail and bovine colostrum in replacement of plasma protein and antibiotics.

Some factors have been also reported that female piglets had lower plasma concentrations of TNF-*a* and IL-1β than males due to the inhibitory actions of glucocorticoids after challenged with a low dose of LPS (Llamas Moya et al., 2006) and cold environmental temperature induced the increases of serum TNF-*a*, IL-6 and IL-1β concentrations in the neonatal piglets after LPS challenge (Frank et al., 2003). However, weight differences of LPS infected piglets in productions of inflammatory cytokines were seldom reported in the previous studies. The potential increases in serum levels of TNF-*a*, IL-6, IL-8 and IL-10 following weight decrease of the LPS infected piglets were found in the current study. Also stimulation with LPS and PMA in PBMCs revealed a potential relationship between high body weight and low levels of TNF-*a*, IL-8 and IL-10. To our knowledge, this might be the first report evaluating the effect of body weight under LPS challenge on cytokines TNF-*a*, IL-6, IL-8 and IL-10 in the early weaned piglets. High weight and low weight groups in our study were separated based on weight gain of piglets at 14 days of age when they were still fed by mother's milk. There may be two reasons for the difference between high and low weight piglets of every litter: 1) the birth weight of piglets were different and 2) received different quantity milk. The piglet profoundly deficient in immune at birth and is highly dependent upon maternal colostrums and milk for immune protection, development and survival (Stokes et al., 2004). In addition, birth weight has a substantially great impact on pig growth performance after weaning (Wolter et al., 2002). Therefore, the high weight piglets might be healthier and have stronger immunity than low weight animals, which resulted in the less release of inflammatory cytokines.

In conclusion, results from this study indicate that the stimulations of LPS and PMA can induce the release of TNF-α, IL-8 and IL-10 in the PBMCs from pathogen free piglets. The current study also shows that LPS administration modulated the inflammation response of piglets. Although dietary cocktail or combined with bovine colostrum as well as antibiotic had no efficacy to inhibit the concentration of inflammatory cytokines in the presence of LPS challenge, it may support including dietary cocktail and bovine colostrum in piglet weaning diet in replacement of plasma protein and antibiotics. Finally, weight differences exist in the inflammatory cytokines response, probably due to high weight animals have stronger immunity under LPS administration in the present work, which may have reference value for commercial farms that smaller size piglets need more care than heavier animals because of the susceptibility to inflammation.

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CHAPTER 6

General Discussion

6. General discussion

Weaning period is usually characterized by a marked reduction of volume of feed intake, poor growth and development, diarrhoea and increased risk of disease, particularly from enterotoxigenic *E.coli* (ETEC). The risk of diarrhoea in post-weaning period is the cause of serious economic losses in pig herds. Antibiotics have been widely used in animal production for decades worldwide for the considerable commercial purposes. While since January 1st 2006, 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 marketing and use of antibiotics as growth promoters in animal feed have been banned by the European Commission. Therefore, the measure led to the investigation of alternative suitable feed supplements that would be reasonably efficient in protecting and sustaining animal health, increasing nutrient availability and guaranteeing performance of the young pigs to the use of antibiotic for prevention of diarrhoeal diseases in weaned piglets.

In the first trial of this work, we intended to determine whether adding essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) supplement to the solid diet of weaned piglets would produce some positive effects when the piglets consumed a diet with no supplemental antibiotics. Although diet supplementation of essential oil and/or enzyme did not affect ADG and ADFI, the results indicated that improvement of growth performance was mainly embodied in decrease of feed convention ratio by supplementation of the combination of essential oils and enzymes during the last week. Similar results were reported by Fan et al. (2009) and Maenner et al. (2011) that essential oil and enzyme improved growth performance mainly related to feed efficiency. In additon, piglets fed the combination of essential oil and enzyme had high FCR during third week, so the pattern of response suggested that the combination of essential oils and enzymes may reduce growth rate early and increased it later. There was no effect of essential oils or enzymes or the combined additive supplementation on fecal digestibility values. Similarly, Diebold et al. (2004) observed that supplemented xylanase and phospholipase with wheat-based diet positively affected ileal nutrient and energy digestibilities while there was no effect of xylanase or the combined enzyme supplementation on the fecal nutrient digestibilities. Dierick and Decuypere (1996) stated that measurements in ileal digesta were more sensitive than those in feces for detecting the possible effects of enzyme supplementation. Our results also observed that the fecal digestibility values of most nutrients (except for calcium) increased from Period I to II. The differences between Period I and II might be

explained, in part, by the growing microbial population in the large intestine. According to Graham et al. (1986), older pigs have a more mature gastrointestinal system and are able to better digest cereal components of the diet through the effects of both enzyme secretion and bacterial fermentation. Consequently, as the pig ages, the potential for responses to essential oils and enzymes supplementation decreases. The trial also observed that essential oils and/or enzymes induced significant decreases of *Coliforms* counts in the faeces, indicating the antibacterial activities. Although dietary essential oils and/or enzymes tended to reduce population of *Lactobacilli*, the increases of *Lactobacilli* to *Coliforms* ratio were observed in those groups. The results might be reinforced the observations in vitro and indicated the potential for the positive effects of essential oils and enzymes on gut environment. Villus:crypt ratio represents the nutrient digestion and absorption capacity of the small intestine (Pluske et al., 1996; Montagne et al., 2003). In our study, dietary enzymes increased V/C ratio in the ileum of piglets by increasing villus height and decreasing crypt depth. In addition, the significant decrease of the number of lymphatic follicles and macrophages in the piglets fed essential oils and/or enzymes might be the possibility that those animals did not activate the lymphatic follicles and macrophages as many as unsupplemented piglets because the guts were healthier. No additive affected the expression of the genes, which might be indicated that those ileal mRNA were not sensitive in healthy animals for promoting the gene expression in the end the trial. The overall results demonstrated that diet supplementation with essential oil and/or enzyme had positive effects on intestinal bacterial counts and gut enviorment, although there was no significant diet effect on grow performance or digestibility.

The aim of our second trial was to evaluate the possible protective effects of nutritional supplements essential oils (Thymol and Cinnamaldehyde, EO) and enzymes (Xylanase and β-glucanase, XB) either alone or in combination on growth performance and health of piglets under *Escherichia coli* challenged condition. *E. coli* challenge successfully created a chronic infection that negatively influenced growth performance in piglets, which was in agreement with literatures that the performance of weaned pigs was impaired by enterotoxigenic *E. coli* challenge (Bosi et al., 2004; Ding et al., 2006; Jensen et al., 2006). The results indicated that improvement of growth performance was mainly embodied in decrease of feed convention ratio by supplementation of enzymes or combination with essential oils in presence of *E. coli* challenge during the last week. This finding is in agreement with previous observations that essential oil and enzyme improved growth performance mainly related to feed efficiency (Fan et al., 2009; Maenner et al., 2011). However, *E. coli* challenged piglets fed XB and the combination had high FCR during second and third weeks, so the pattern of response in this study suggested that XB and the

combination reduced growth rate early after inoculation and increased it later. Diarrhea caused by infectious disease is a serious problem in weaned piglets, which usually leads to an increased mortality rate (Glass et al., 1991; Osek, 1999). We found the increases of diarrhea scores and bacteria counts of *Clostridia*, *E. coli* and *Coliforms*, which indicated the success of *E. coli* infection model in this study. However, *E. coli* infection achieved a major diarrhea episode within 6 days postchallenge and then animals recovered gradually. In our study, supplemented with essential oils and/or enzymes failed to change the situation of severe diarrhea post *E. coli* infection which may be related to the degree of pathogenicity of stain. However, *E. coli* challenged piglets fed enzymes tended to reduce counts of *E. coli* in feces and the combination decreased populations of *Coliforms* in digesta at 35 d. And for all the animals, essential oils and/or enzymes induced decreases of *E. coli* or *Coliforms* counts in both feces and digesta of cecum, indicating the antibacterial activities and improvement of gut environment. *E. coli* challenge also had negative effects on haematology and gut morphology. Obviously, the V/C ratio was higher in essential oils and/or enzymes fed both *E. coli* challenged or unchallenged animals, especially in EO and the combination treatments. The results of gut morphology in the ileum also evidenced that supplemented the combination of essential oils and enzymes with the diet of *E. coli* challenged piglets had the tendency to decrease mucosal lymphatic follicles number and area, which suggest that dietary combination may increase the protective ability to cope with stress, reducing the negative effects of *E.coli* challenge. Our study also observed the reduction of mRNA levels of proinflammatory genes such as TLR4 and TNF in ileal mucosa post *E. coli* challenge, which reinforces the typical acts of them, while no additive decreased the expressions of TLR4 and TNF. All the results showed that supplementation of essential oils and/or enzymes might improve the protective capacity against pathogenic bacteria when piglets were submitted to a bacterial challenge through improve the modulation of gut bacteria and morphology.

The last trial was desined to determine the effects of dietary cocktail which contains bovine colostrums, cranberry extract, encapsulated essential oil, yeast-derived products, the probiotic *Pediococcus acidilactici* MA18/5M, vitamins A, D, E and B complex, seleno-methionine and body weight on inflammatory cytokines and time responses under *Lipopolysaccharides* challenge in early weaned piglets. Stimulation with LPS and PMA in the supernatant of PBMC obtained from pathogen free piglets induced several inflammatory cytokines such as TNF-α, IL-8 and IL-10 were detected, which is in agreement with previous findings in pigs or human (Janský et al., 2003; Gao et al., 2010). Similarly with the observation of many literatures (Weble et al., 1997; Llamas Moya et al., 2006), our results also indicated that LPS challenge induced symptom of acute inflammation response by increasing production of proinflammatory cytokines (IL-6 and TNF-α),

chemokine (IL-8) and anti-inflammatory cytokine (IL-10) at 4 h after inoculation, while there was no difference of inflammatory cytokines concentrations between 18 h after LPS challenge and before challenge. Although multiple authors evidenced that functional foods such bovine milk by-product, cranberry, essential oils, probiotics, prebiotics, yeast-derived products and vitamins supplementations have benefits to modulate immune function, or enhancing intestinal barrier (Cross and Gill, 2000; Schlimme et al., 2000; Roselli et al., 2005; Bodet et al., 2006; Bontempo et al., 2006; Lessard et al., 2009; De Lange et al., 2010), supplementation of dietary cocktail or combination with bovine colostrum in this study did not inhibit the release of inflammatory cytokines. However, there were also no differences among those two diets and dietary antibiotics, which might reinforce the possibility that dietary cocktail and bovine colostrum in replacement of plasma protein and antibiotics. In our study, we observed the potential increases in serum levels of TNF-*a*, IL-6, IL-8 and IL-10 following weight decrease of the LPS infected piglets. In addition, stimulation with LPS and PMA in PBMCs revealed a potential relationship between high body weight and low levels of TNF-*a*, IL-8 and IL-10. Therefore, the high weight piglets might be healthier and have stronger immunity than low weight animals, which resulted in the less release of inflammatory cytokines. In this trial, dietary cocktail or combined with bovine colostrum may replace plasma protein and antibiotics and weight difference may influence the production of inflammatory cytokines after infected by LPS.

In conclusion, piglets are susceptibility to enterotoxigenic *Escherichia coli* (ETEC) in post-weaning period which is very complex. We observed some improvement of gut bacteria and morphology through the supplementation of fuctional foods as essential oils and/or enzymes, and also the possibility of fuctional foods as the alternative to in-feed antibiotic. However, our knowledge is insufficient in understanding the complexity at the interaction between natrition and gut health, we still need future works to identify other modes to improve gut health of piglets in post-weaning period.

CHAPTER 7

Summary

7. Summary

Post-weaning is the most crucial period in pig management. Associated with weaning are marked changes to the histology and biochemistry of the gut which cause decreased digestive and absorptive capacity and contribute to post-weaning diarrhoea. In last years, the interest in developing management and feeding strategies to stimulate gut development and health in newly-weaned pigs was increasing.

In order to increase general health in post-weaning piglets and be alternative to in-feeding antibiotic, three trials were created in this thesis to determine the utilizations of some functional foods which have been widely used to improve growth performance while minimizing the use of antibiotics and rather expensive feed ingredients in weaned piglets.

We utilized essential oils (Thymol and Cinnamaldehyde, EO) and/or enzymes (Xylanase and β-glucanase, XB) in the first 2 trials. The first trial mainly focused on the effects of those additives on general parameters such as performance and digestibility of weaned piglets. To investigate the possible protective effects of EO and/or XB on health status in weaned piglets, we created a model of challenge using *Escherichia coli* in the second trial. After first two investigations, we intended to enlarge the categories of functional foods and determine the effects on regulation of systemic inflammatory reaction and, in addition, we supposed that initial body weight might also influence the regulation. Therefore, the third trial was desined to determine the effects of dietary cocktail (bovine colostrums, cranberry extract, encapsulated essential oil, yeast-derived products, the probiotic *Pediococcus acidilactici* MA18/5M, vitamins A, D, E and B complex, seleno-methionine) and body weight on inflammatory cytokines and time responses under *Lipopolysaccharides* challenge in early weaned piglets.

In the first trial, a total of 192 weaned piglets (Stambo HBI Dalland 40, 24 d) with an average initial body weight of 8.10 kg were allocated according to body weight into 4 experimental treatments (12 replicates per treatment with 4 piglets per replicate). Each group was fed the basal diet alone or supplemented with either essential oils or enzymes, or their combination. There was no effect of essential oils and/or enzymes supplementation on the growth performance of piglets. However, the combination of essential oils and enzymes decreased feed conversion ratio during the last week. Although the fecal digestibilities of all the piglets were increased from d 21 to 35 ($P < 0.001$), no effect of essential oils or enzymes or the combination on the fecal digestibility was observed. All the additives significantly decreased counts of *Coliforms* at 42 days of the trial ($P < 0.001$). Dietary enzymes improved gut morphology by decreasing crypt depth, increasing villus:crypt ratio and reducing the number of macrophages ($P <$

0.001). Supplementation of essential oils and the combination with enzymes also improved gut morphology by decreasing crypt depth ($P = 0.065$; $P < 0.001$), and decreasing the number of lymphatic follicles ($P = 0.002$; $P < 0.001$) and macrophages ($P < 0.001$). No effect of additives on mRNA expression of inflammatory cytokines was observed in ileal mucosa. Results showed that diet supplementation with EO and/or XB had positive effects on intestinal bacterial counts and gut morphology, although there was no significant diet effect on grow performance or digestibility.

In the second trial, 192 weaned piglets (Stambo HBI Dalland 40, 8.64 kg) were allocated according to body weight into eight experimental treatments (6 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments [a weaned piglet control diet (CTR), CTR + 0.05 g/kg essential oils (EO), CTR + 0.1 g/kg enzymes (XB), and CTR + 0.05 g/kg EO + 0.1 g/kg XB] and 2) with or without an *E. coli* challenge. On d 8, half of piglets in each dietary group were challenged with *E. coli*. *E. coli* challenge significantly impaired growth performance, induced severe diarrhea, increased populations of *E. coli, Clostridia* and *Coliforms*, depressed antioxidant activities, damaged gut morphology and promoted TLR-4 and TNF-α mRNA expression in ileal mucosa ($P < 0.05$). In the *E. coli* challenge group, dietary enzymes or combinated with essential oils improved feed efficiency compared with control treatment during the last week ($P = 0.025$; $P = 0.020$). The *Coliforms* populations in the cecum of challenged piglets fed combination of essential oils and enzymes were lower than control treatment ($P < 0.001$). In the *E. coli* challenge group, supplemented essential oils and/or enzymes improved gut morphology by increasing villus height and villus:crypt ratio and decreasing crypt depth ($P < 0.001$). The positive effects on intestinal bacterial counts and gut morphology suggests that supplementation of EO and/or XB might improve the protective capacity against pathogenic bacteria when piglets were submitted to a bacterial challenge.

In the third study, a total of 256 Yorkshire × Landrace weaned piglets (20 ± 1 d) were allocated into eight experimental treatments (8 replicates per treatment with 4 piglets per replicate). The treatments were in a factorial arrangement: 1) dietary treatments: a basal weaning diet added [spray-dried plasma protein (PP) (CTR), PP + antibiotic (ATB), PP + dietary cocktail (DC), or bovine colostrum + the dietary cocktail (BC+DC)] and 2) low weight (LW) or high weight (HW). At 37 d of age, 2 piglets in each pen were injected with LPS. Stimulations of LPS and PMA in PBMCs of piglets significantly induced TNF-α, IL-8 and IL-10 ($P < 0.05$). There was no diet effect on the concentrations of TNF-α, IL-8 and IL-10 in PBMCs of piglets ($P > 0.05$). Piglets had low weight tended to increase the concentration of IL-8 ($P = 0.106$) and IL-10 ($P = 0.098$) in the PBMCs stimulated by LPS 0.5μg/ml LPS and the concentration of IL-10 ($P = 0.097$) in

PMA stimulated PMBC compared with high weight animals. Infection with LPS increased ($P < 0.001$) serum concentrations of all the cytokines four hours post inoculation, and animals recovered to basal levels at 18 h after challenge. No significant diet effect was found in the serum concentrations of cytokines ($P > 0.05$). At 4 h after challenge, low weight piglets had partially greater serum concentrations of TNF-α ($P = 0.046$), IL-6 ($P = 0.158$), IL-8 ($P = 0.179$) and IL-10 ($P = 0.185$) than high weight animals. Dietary cocktail or combined with bovine colostrum may replace plasma protein and antibiotics and weight difference may influence the production of inflammatory cytokines after infected by LPS.

In conclusion, we observed that supplementation of functional foods as essential oils and enzymes might strengthen protective capacity of weaned piglets against pathogenic bacteria by decreasing negative intestinal bacterial counts and improving gut morphology. Dietary cocktail or combined with bovine colostrums, at the amount used in this work, may replace plasma protein and antibiotics. Besides, weight difference may influence the production of inflammatory cytokines after infected by LPS.

CHAPTER 8

Acknowledgements

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