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Stimulation of the health and productivity of nursing and weaned piglets.

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Ai mie nonni e ai miei genitori
Modello di vita, umiltà, principio

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Preface

Worldwide the swine industries are under pressure to reduce the use of antibiotics while maintaining animal health and performance. The control and prevention of infectious diseases in of basic economic importance although the occurrence of production disease has relevance not only to economic production, but also to animal welfare. The introduction of antibiotics to treat bacterial infections almost 50 years ago led to an improvement in animal production but the use of antibiotics feed additives in food production animals was linked to emergence in the food chain of multiple drug-resistance bacteria. The emergence of antibiotics resistant strains presumably demonstrated that treatment of bacterial infections can not rely on the use of antibiotics without critical considerations. In 2006 all the AGP (antibiotic growth promoters) and some feed additives with growth promoting activities (Cu, Zn) have been limited. Since the first restrictive measures were taken, and due to the beginning of the negative consequences of the ban, numerous efforts have been done to find alternatives or replacement strategies to maintain pig growth performance and controlling enteric bacterial diseases. The need of more economic and safety substances as replacers of AGP for livestock animals as probiotics, prebiotics and feed additives of natural origin, focused the attention of the research of the last years. Several studies reported the effects of probiotics, prebiotics, organic acids, bioactive milk molecules and plant extracts on pig gut and general health status. From literature they showed promising results, although still not comparable of AGP. In some cases they were supplemented as single additive, while more recently additives of different nature were pooled with the aim to evaluate possible additive or combined effects. Most of these compounds are thought to act by modulating the microbiota equilibrium, the mucosa intestinal morphometry and gut associate lymphoid tissue (GALT), while others might be active in the improvement of pig health and performance by other different mechanisms. In order to asses, the primary mode of action, metabolic pathways and optimal dosage of the additives more research will be needed. Therefore, the aims of the following trials were to study the effects of prebiotics, natural substances and bioactive milk peptides on health and performance of post-weaned piglets.

Chapter 1

General Introduction

1.1 General Introduction

1.1.1 Development of gut immune system

Weaning is a crucial phase in pig production. Piglet's immune system that is inexperienced and immature moreover it is continuously challenged by lot of pathogens. The immune system gathers experience as it is continuously faced with a myriad of antigens. Quantitatively, as well as qualitatively, the most important site of this interaction is the intestine. Encounter alone does not guarantee maturation, however, the intestinal immune system develops in intimate connection with the indigenous microbiota of the gut and has distinct features, the most prominent of which being the ability to launch suppressive, tolerogenic responses essential to maintaining a disease free state in the gut as well as systemically. Aberrant immune responsiveness with an imbalance in the composition of microbiota in the gut is characteristic to the pathogenesis of many inflammatory conditions. The development of atopic diseases in particular appears to be associated with defective tolerogenic responsiveness in the gut and alterations in the gut microbiota. The rise in incidence of atopic diseases in the industrialised pig during the last decades, attributed to changes of farm strategies (as the anticipated weaning at 21 days) in feeding and hygiene, may thus be partly explained by altered gut microecology. Piglets are nowadays weaned around three/four weeks of age because this procedure is commercially advantageous, but thus they often suffer altered gastrointestinal conditions, in part because of the sudden alteration in the intestinal microflora consequent to the rough passage from mother's milk to a solid food. This in addition may cause a strong reduction in the length of intestinal villi height and the depth of the intestinal crypts, which consequently reduces the gut digestive as well as absorptive capacities (Pluske et al., 1997; Van Beers-Schreurs et al., 1998). This in turn may cause negative effects on average daily gain (ADG), as well as on defensive responses against infectious diseases. Actually, recent scientific evidence suggests that weaning piglets at three to four weeks of age may overwhelmingly challenge a substantially immature immune system, conducting it to a possible paralysis condition and creating an ideal environment for diseases to develop (Ladekjær-Mikkelsen et al., 2002). In this fragile equilibrium the development of diseases has a great economical impact in pig production.

One critical change associated with weaning is the shift from sow's milk to a dry feed, which induces a period of fasting during the first few days following weaning. This weaning anorexia has a negative impact on growth and leads to mobilization of fat stores, as shown by the sharp increase of circulating free fatty acids levels (Barke et al., 1996). It may also be involved in the digestive problems that are frequently encountered after weaning (McCracken et al., 1999). Experimental data indicates that this period of under nutrition markedly alters the functioning of several

neuroendocrine system during the post-weaning period. For instance, the changed measured in the somatotrophic axis (increased GH and reduction IGF levels) and in the autonomic nervous system (reduced cathecolamine excretion in urine) are similar to those measured in fasting animals (Carroll et al., 1998, Hay et al., 2001). These changes may be long lasting, as in the case of weaning at 6 days of age for instance (Hay et al., 2001). Some of the metabolic response to weaning can be corrected by milk feeding, which improves the growth rate of the animals as compared to dry feed (McCracken et al.,1995, Kim et al., 2001). Indeed, piglets weaned at 2-3 days of age and subsequently fed with a milk replacer display greater growth rates by day 7-8 of lactation than piglets raised by their dam, showing that sow milk yield is a limiting factor to piglet growth (Harrel et al., 1993). The increase factor of voluntary food intake after weaning by using whole cow's milk (due to it's palatability) also improves the mucosa architecture of the small intestine (Pluske et al., 1996). However in commercial settings, weaning piglets are usually offered dry food, for economical reasons. Such a food is not accepted as well as a liquid milk replacer, and weaning alters average daily feed intake and average daily weight gain, the magnitude of which is larger with earlier weaning age (2, 3 and 4 weeks) resulted in reduced weight gain depression. All the animals nevertheless reached similar body weight at 6 weeks of age (Leibbrandt et al., 1975). There is no doubt that weaning is a period of intense stress for piglets, with profound consequences on growth, physiology and disease outbursts, which reveal severe welfare problems. Experimental data clearly show that the anorexia or, at least, the nutritional deficit due to the abrupt transition between milk and solid food, induces severe taxation of the adaptive mechanisms of piglets and may be of special relevance from a welfare point of view. Most of the problem comes from the fact that during lactation spontaneous intake of dry food remains very low up to 3 weeks of age and does not become significant until the 4^{th} week, indicating that the appetite for dry food is very low in younger animals. The large individual variation suggests that this trait may be influenced by genetic factors and could therefore respond to genetic selection. It would be valuable to gain more information on the physiological changes induced by weaning at different ages. Maintanance of gut health in the pig is probably the last emerging problem when the other pathologies are under control. Infact gut health degradation is most often observed during the post-weaning period and may in turn affect further general health through enhancement of bacterial translocation and disturbances in the maturation of the immune system including immuno-tolerance.

One approach to improve the gut health of the piglets is the dietary manipulation. Dietary manipulation of the gut environment by the use of feed additives, or by choice of dietary raw materials that may influence the luminal environment throughout the entire length of the intestinal tact and thereby improving "gut health"(Knud E. et al.). As discussed by Montagne et al. (2003),

the concept of gut health is complex and, at present, it is an ill-defined notion. Conway (1994) proposed that there are three mayor components of gut health, namely the diet, the mucosa and the commensal flora. The mucosa is composed of the digestive epithelium, the gut-associated lympoid tissue (GALT) and the mucus overlying the epithelium. The GALT, commensal bacteria, mucus and host epithelial cells interact with each other forming a fragile and dynamic equilibrium within the alimentary tract that ensures efficient functioning and absorption capacity of the digestive system. The feed should be selected to favour conditions in the gut that create and stabilise this balance between the host, the microflora and environment, and to prevent disturbance of the structure and function of the gut. In this respect, the relative gut health value of a dietary component or diets should rest with their capacity either to stabilise or to perturb this equilibrium.

1.1.2 Gut barrier and colonization resistance establishment

The indigenous microbiota suppresses colonization of incoming bacteria by a process named colonization resistance that is a first line of defense against invasions by exogenous bacteria, potential pathogenic organisms or indigenous opportunists (Van der Waaij et al., 1989; Hooper et al.,1998). This process involves several different complex interacting

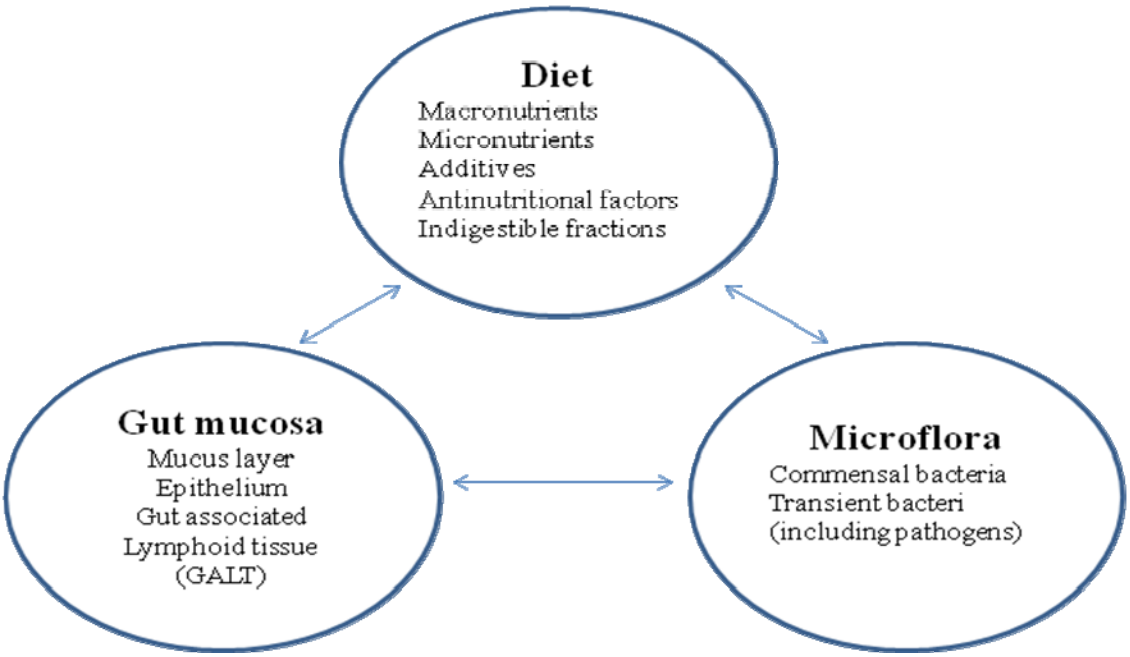

Fig. 1 **Schematic representation of the different elements in the gut ecosystem making yp the concept of gut health. Modified from Conway (1994) as discussed by Montagne et al. 2003.**

mechanisms of both the bacteria and the hosts. The host factors involved in colonization resistance are different: the peristaltic movement; the secretion of diverse digestive enzymes and electrolytes; the mucus secretion; the epithelial cell desquamation; the gut associated lymphoid tissue; and secretory IgA (Stewart et al., 1993). Besides, indigenous microbiota prevents bacterial colonization by competing for epithelium receptors (Blomberg et al., 1993; Bernet et al., 1994) and enteric nutrients (Stewart et al., 1999) producing antimicrobial compounds such as bacteriocines (Brook, 1999) and metabolizing nutrients to create a restrictive environment which is generally unfavorable for the growth of many enteric pathogens (Lievin et al., 2000). Moreover, bacterial recognition and adhesion to receptors is not only a prerequisite for colonization, which determines microbiota composition and permanent colonization, especially in the upper gastrointestinal tract (Alandet et al., 1999). It also determines antagonistic activity against enteropathogens (Coconnier et al., 1993), modulation of the immune system and also the improvement of healing in the damaged gastric mucosa (Elliot et al., 1998). Several factors are involved in the control of bacterial attachment and thus in the modulation of the indigenous microbiota profile (Freitas et al., 2002). Special interest is nowadays focused on genetic modulation of receptors by the host and the bacteria. Bacterial-mucose attachment appears consequently as a key point defining indigenous microbiota composition and different bacterial-mediated functions. Two main components are essential to the recognition between the host and the bacteria: the glucoconjugates on the gut enterocytes and bacterial adhesions metabolize cholesterol to coprostanol (Baron and Hylemon, 1997).

1.1.3 Development of immune system

Pigs have evolved many immunological characteristics in response to the environmental and infectious selection pressures they continually face. Pigs are omnivores and their enteric system must logically be adapted to respond to the range of intestinal pathogens, and therefore defence mechanisms have developed to protect the host and maintain intestinal health. Nevertheless the piglet is immunodeficient during the early phase of his life and is highly dependent upon a supply of both specific and non specific immune factors present in maternal colostrums and milk for immune protection, development and survival (Varley et al., 1987). Therefore, development of immunocompetence is an absolute requirement for optimum growth and performance (Stokes et al., 2004). Bailey et al. (2001) surmised that immunological development in the neonatal pig occurs as a balance between regulatory function and effector functions, and that GIT integrity depends on the maintenance of this balance. The immune system in the young pig comprises several organs (bone marrow, thymus, spleen, mesenteric lymphonodes) and several cell types (lymphocites-specific immune recognition of foreign antigen, 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.

Development of the immune system in early 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 and Anderson, 2000). Notwithstanding the ample evidence indicating the involvement of Th2-skewed immune responsiveness in fully established atopic disease, the events leading to the establishment of such pathological skewness early in life remain less well understood. It is of note that due to the immunological balance prevailing in utero the immune responses early in life are physiologically predominantly of the Th2-type (Piccinni et al., 1998). Recently, it has been observed that neonatal CD8 T cells produce large amounts of IL-13 which may account for the Th2 bias in the newborn (Ribeiro-do-Couto et al., 2001). Interestingly, however, a previous study indicated that infants who later develop atopic disease exhibit impaired IL-13 production at birth (Williams et al., 2000). The same phenomenon has been demonstrated with regard to IL-4 as well, as neonatal IL-4 responses were lower in infants who developed atopic disease as compared to those who remained healthy (Prescott et al., 1999). Consequently, a significant overlap in the concentrations of Th2 cytokines, such as IL-4, and IgE antibodies prevails between atopics and non-atopics at an early age (Kulig et al., 1999; Prescott et al., 1999). Thus the concentration of IgE antibodies in cord blood, thought to reflect sensitisation in utero, predicts poorly the development of atopic disease (Bergmann et al., 1997). An age-dependent decline in Th2 responses during the early postnatal period in non-atopic children and a converse pattern in atopic children has been observed, suggesting that infants who develop atopic disease may exhibit defective suppression of Th2-type responsiveness (Prescott et al., 1999). Recent observations have lead to the conclusion that such suppressive mechanisms at least in part originate in the gut as a result of stimulation by dietary and microbial antigens. Whilst there is a strong hereditary component in the development of atopic

disease, the rapid increase in morbidity can only be explained by environmental factors (Bergmann et al., 1997). The hygiene hypothesis of allergy, first introduced by Strachan, suggests a causal relationship between reduced exposure to microbes at an early age and the increase in the prevalence of atopic disease (Strachan et al., 1989). There is epidemiological evidence indicating that infectious diseases early in life may protect the individual from atopy. However, as it has recently been pointed out, the most important source of tolerogenic stimuli may be the indigenous microbiota of the intestine (Monteleone et al., 2001). The immunomodulatory events produced by the interaction between the intestinal immune system and microbiota as well as the induction and maintenance of oral tolerance is complex and surpass the Th1/Th2 paradigm. The intestinal immune system must be able to discriminate between potentially pathogenic microbial antigens and the non-pathogenic dietary and indigenous microbial antigens in order to avoid both invasive infections and chronic inflammatory conditions. The unresponsiveness to dietary proteins and indigenous microbiota, known as oral tolerance, is the result of several distinct processes. Furthermore, there is growing evidence suggesting that resident microbiota provide the intestinal immune system with endogenous stimuli which are essential for its normal maturation and function (Toms et al., 2001). There are somewhat contradictory data on the type of immune responsiveness preferentially elicited by the intestinal immune system although it is apparent that T lymphocytes are the main effector cells in responses to luminal antigens. Results obtained from animal models suggest that mucosal T cell responses are favourably of the Th2 type (Xu-Amano et al., 1992). This Th2-bias is thought to originate in utero and as a result, transient sensitisation to dietary antigens is common at an early age. On the other hand, however, Th2-type responsiveness, such as IgE antibody production seems to be most readily susceptible to suppression (Nagler-Anderson, 2000). Furthermore, dietary antigens appear to induce Th1-type responsiveness in the human intestine, which may down-regulate Th2-type responsiveness (MacDonald et al., 2001). Oral tolerance has traditionally been defined as the induction of peripheral unresponsiveness as the result of oral administration of soluble protein antigens (Nagler- Anderson, 2000). It is well documented that there are distinct mechanisms, including clonal deletion, clonal anergy and active suppression, by which tolerance, defined recently not only as unresponsiveness but more broadly as any mechanism by which a potentially injurious immune response is prevented, suppressed, or shifted to a noninjurious class of immune response, is induced and maintained in the intestinal mucosa (Weiner, 2001). The relative contributions of these mechanisms appears to be dependent on the dose of the antigen: High doses of oral antigen result in clonal anergy/deletion, a phenomenon often explained by small amounts of antigen bypassing the intestinal immune system, gaining access to circulation and thus inducing anergy due to the lack of sufficient costimulatory signalling (Weiner, 2001). Oral administration of

antigen leads to the generation of a unique subset of T cells named Th3 cells in both animal models and humans (Chen et al., 1996; Fukaura et al., 1996). Th3 cells produce predominantly TGF-ß and are thought to be pivotal in the active suppression leading to oral tolerance after low doses of oral antigen. After the priming of Th3 cells has taken place in the Peyer's patches, the gut-originating cells migrate to the periphery and are capable of suppressing inflammatory responses upon reactivation thus mediating tolerance in sites other than the gut as well (Weiner, 2001). In addition to down-regulating both Th1- and Th2 type responses, TGF-ß elicits immunomodulatory effects on antigen-presenting cells (Takeuchi et al., 1998). Furthermore, TGF-ß may favour the development of IL-10-secreting regulatory Tr1 cells (Toms et al., 2001), another subset of suppressive T cells characteristic of the intestinal immune system. IL-10 has a central role in maintaining intestinal homeostasis, especially by suppressing inflammatory responses towards intestinal microbial agents. 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.

1.1.4 Modulation of intestinal equilibrium through the feed

Weaning is characterized by significant nutritional, social and environmental changes that can impose a significant penalty on subsequent growth and production. A marked feature of weaning is the transient decrease in macronutrient and micronutrient intake that occurs as piglets are switched abruptly from a mostly all milk diet to a solid diet. Despite to the studies conducted in the past years concerning nutrition and weaning under commercial farm conditions, there appears to have been very little research directed specifically towards immune system (Pluske J.R., 2007). There are surprisingly few studies examining the roles specifically of feed additives/feed ingredients on the immune system GIT in young pigs, although an increasing number of studies deriving

predominantly from Europe have occurred more recently in view of the ban on antibiotic feed additives in diets. The use of nutraceuticals or functional foods plays an interesting role in the intestinal equilibrium of the early weaned piglet. Functional foods have been defined as food with ingredients (either naturally occurring or added) that provide a health benefit beyond the traditional nutrient value of the food. The following sections discuss several feed additives and functional foods with an important role on the GIT immune function.

1.1.5 Functional properties of milk and its Physiological Characteristics

The discovered of functional molecules in food with extra-nutritional activities opened the way to a nutrition not just only with the aim of to meet the nutritional needs, but also to improve the health of humans and animals. Bioactive substances in functional foods able to improve the health of the animals are defined as nutraceutical. For its physiological significance *in primis* milk can be considered "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. Among the native components of milk, of particular importance and interest are attributable to some lipid and protein fraction. The importance of these compounds is due not only for their content but also for their bioavailability and bioaccessibility often positively affected by their inclusion in a milk matrix (Baldi and Pinotti, 2008). In the evaluation of the functional activity of milk it is important remember the protective activity towards the oligosaccharide component against intestinal infectious diseases. The protective role of the bioactive components of milk has an action on intestinal ecosystem, developing newborn immune system, reducing the risk of developing of health problems improving performance. These components may have a potential use as nutraceuticals to improve health, both in relation to their presence in food or as food additives. In this field, knowledge is more developed with regard to human or bovine milk, while less known or are missing data on the sow's milk and other species. The importance of milk in the nutrition of all mammals has been recognized from time immemorial. The biosynthetic capacity of the mammary gland, both in terms of the number of substances synthesized and the magnitude of the total synthetic ability is remarkable. Mammary gland synthesizes a number of proteins that have unique aminoacids composition and highly desirable physicochemical properties. Caseins and whey proteins are the two main protein groups in milk. Caseins designate the predominant group of proteins and they are characterized by ester-bound phosphate, high proline content, few or no cysteine residues and low solubility at pH 4.0–5.0. Cow’s milk contains 4 individual caseins: as1-casein, comprising of 199 amino acids (aa), b-casein (209 aa), k-casein (169 aa) and as2-casein (207 aa). The major whey proteins in milk include b-

lactoglobulin, a-lactalbumin, immunoglobulins and bovine serum albumin (Ng Kwai Hang, 2003). In addition to the major milk proteins, milk contains a wide group of biologically active proteins, involved in development and healthfulness of the neonate. Among these bioactive components the 80 KDa iron-binding glycoprotein lactoferrin is recognized as one of the most versatile. Its role was originally considered to be essentially antimicrobial, but several findings have demonstrated that the protein has nutritional and physiological significance (Lo¨nnerdal & Iyer, 1995). Milk proteins can be subjected to inadvertent proteolysis during storage before or after milking. Proteolysis in the mammary gland can be attributed to native proteases, such as the plasmin/plasminogen system (Baldi et al. 1996; Fantuz et al. 2001; Politis, 2007). Proteins can be subjected to deliberate proteolysis such as the proteolysis occurring during manufacture of fermented dairy products (yoghurt) and cheese manufacture and/or ripening. Lastly, proteins are subjected to hydrolytic breakdown during gastric processing and later upon exposure of the proteins with indigenous or intestinal bacteria-derived enzymes in the gut. Proteolytic breakdown of milk proteins generates a number of peptides endowed with various biological properties (antimicrobial, antihypertensive, antithrombotic) including those that have the ability to modulate immune function (Hill et al. 2000). Immunomodulatory peptides are inactive within the sequence of the parent protein and they can be released by enzymatic proteolysis. One good example is the proteolysis occurring by the action of proteinases and peptidases from lactic acid bacteria (LAB). LAB utilize milk proteins, mainly caseins, as their prime source of essential aminoacids. The proteolytic machinery of LAB includes a cell wall bound proteinase and several intracellular peptidases. The transport system of LAB enables them to internalize oligopeptides up to 18 aa in length. Longer oligopeptides can become a major source of bioactive peptides in fermented milk when further degraded by gastrointestinal enzymes or by intracellular peptidases of lysed LAB after consumption of these products (Law & Haandrikman,1997).

1.1.5.1 Immunomodulatory Milk peptides

Suppression or Induction Milk proteins represent the exclusive protein supply for the newborn; all human and animals neonates depend upon milk proteins until they enter the fifth month of their life (Table 1). Evidence will be presented regarding the ability of milk peptides to regulate gastrointestinal immune function leading us to hypothesize that these peptides may guide the local immune system until it develops its full functionality. The first few weeks is a very critical period in life because the immune system should develop oral tolerance to nutrient molecules and avoid tolerance to pathogen-derived antigens. The term immunomodulation was purposely adopted to indicate that suppression of the immune system may be required in certain instances (oral tolerance)

or induction towards pathogen-derived antigens. The latter is very important in order to avoid the well recognized susceptibility of newborns to infection (Hill, 1987).

Table 1 Biological function of the principle bioactive milk component (adapted from Séverin e Wenshui, 2005).

Protein	*Function*
Caseine (α-, β-, κ-caseine)	Ionic Carrier (Ca, PO_4, Fe, Zn, Cu), precursors of bioactive peptides
Serum proteins	
β-Lattoglobuline	Carrier del retinolo, legante acidi grassi, possibile antiossidante
α-Lattalbumine	Lactose synthesis in mamary gland, Ca carrier, immunomodulator, anticarcinogenic
Immunoglobuline (A, M & G)	Immunoprotection
Lactoferrine	Antimicrobial, antioxidant, immunomodulator, iron absorption, anticarcinogenic
Lactoperossidase	Antimicrobial
Lysozyme	Antimicrobial, synergic action with immunogglobuline and lactoferrine
Glicomacropeptide	Antivirus, biphydogenic

1.1.5.2 Milk peptides – Suppression of immune response

The mucosal immune system of infants is stimulated daily by the continuous passage of milk proteins. Breakdown of proteins in the gut and activation of suppressor T-cells lead to a systemic hypo-responsiveness to ingested protein antigens, a phenomenon known as 'oral tolerance'. Pecquet et al. (2000) reported that peptides obtained from tryptic hydrolysis of bovine b-lg down regulate various immune functions and induce specific oral tolerance in mice. Mice fed with either b-lg hydrolyzates or fractions of the hydrolyzate were tolerized against b-lg. Specific serum and intestinal IgE levels were reduced. Delayedtype hypersensitivity and proliferative responses were inhibited. In a more recent study, Prioult et al. (2004) examined the possibility that the microorganism Lactobacillus paracasei induces oral tolerance by generating a number of immunomodulatory peptides from the hydrolysis of b-lg. They showed that Lb. paracasei peptidases were capable of further hydrolyzing, tryptic-chymotryptic peptides of b-lg. Furthermore, they reported that these peptides repressed lymphocyte proliferation and up regulated interleukin-10 (IL-10) production. Based on these results, they concluded that Lb. paracasei induces oral tolerance to b-lg in vivo by degrading its acidic peptides and releasing immunomodulatory peptides capable of stimulating regulatory T-cells, which function as major immunosuppresants by secreting IL-10.

Hydrolysis of casein with pepsin and trypsin or additionally with enzymes derived from Lb. casei strain GG generates molecules capable of inhibiting lymphocyte proliferation (Sutas et al. 1996b). Thus, indigenous enzymes in the gut together with enzymes of bacteria origin have been proven beneficial in the down regulation of hypersensitivity reactions to ingested proteins in human neonates. Pessi et al. (2001) investigated the effect of digests of bovine casein by enzymes derived from Lb. rhamnosus GG on T-cell activation. They found that the digests reduced expression of IL-2 and inhibited protein kinase C translocation. Both effects indicate suppression of T-cell activation by casein digests. Peptides obtained from the hydrolysis of casein by trypsin induce oral tolerance in mice (Hachimura et al. 1993). A synthetic peptide corresponding to residues 142–149 of as1-casein was proven to be an effective inducer of CD8(+) T-cells which recognize the parent peptide and secrete interferon-gamma (IFNc) , a potent inhibitor of Th-2 dependent events, including IgE production (Totsuka et al. 1998). Otani & Hata (1995) reported that intact bovine k-casein inhibited proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells to various mitogens. Pancreatin and trypsin digests of k-casein, as1-casein and b-casein inhibited cell proliferation in response to mitogens. Based on these results, they suggested that peptides obtained from various caseins by the action of trypsin may suppress immune response of neonates. In further support of this notion, Kayser & Meisel (1996) reported that pepsin and trypsin digests of bovine as1-casein and b-casein inhibited mitogen-induced proliferation of peripheral blood mononuclear cells. The k-caseinoglycopeptide corresponding to residues 106–169 of bovine k-casein is a potent inhibitor of phytohaemagglutinin-induced and lipopolysaccharide-induced proliferation of murine splenic lymphocytes and rabbit Peyer's patch cells and well as antibody production in various spleen cell cultures (Otani et al. 1995). They suggested that the k-caseinoglycopeptide down regulates immune response by acting on both T- and B-lymphocytes. Furthermore, they showed that the inhibitory effect was abolished after neuraminidase treatment which indicates that both the peptide and the carbohydrate parts of the molecule are necessary for the inhibitory effect. Elitsur & Luk (1991) investigated the effect of b-casomorphins (fragments of b-casein, residues 60–70) on immune function. They reported that casomorphins inhibited proliferation of human lamina propria-derived lymphocytes in vitro. However, these results should be interpreted with caution because casomorphins appear to stimulate resistance of mice to infection (Klebsiella pneumoniae), thus, having the opposite effect in vivo. Despite the fact that casomorphins are rapidly degraded once they enter the blood stream, casomorphins were found in the plasma of new born calves after their first milk intake (Umbach et al. 1985). Thus, the neonatal intestine appears to be permeable to pre-casomorphins (Clare & Swaisgood, 2000). Recent data obtained at the Agricultural University of Athens indicate that the effect of casein digests on immune parameters may depend upon the

maturity of the immune system. We have examined the effect of two synthetic peptides on membrane-bound urokinase plasminogen activator (uPA) and expression of major histocompatability complex (MHC) class II antigens by porcine blood neutrophils. The two peptides utilized were the tripeptide LLY which corresponds to residues 191–193 of bovine b-casein and the hexapeptide PGPIPN which corresponds to residues 63–68 of bovine b-casein. The uPA is a critical enzyme for neutrophil diapedesis and the overall ability of neutrophils to resist against various pathogens (Politis et al.2003). In this model system, neutrophils are obtained at weaning which is considered a period of immaturity of the immune system and 4 weeks later when the immune system is thought to have gained its full functionality (Fragou et al. 2004). Results indicated that both peptides reduced the amount of u-PA present on the cell membrane as well as the expression of MHC class II antigens only at the time of weaning and not 4 weeks later. Thus, b-casein synthetic peptides appear to be effective only at a time period which coincides with immaturity of the immune system and they are not effective when the immune system has presumably gained its full functionality.

1.1.5.3 Milk peptides – Induction of immune response

The main mechanism of protection against pathogen derived antigens provided by mucosal immunity is mediated through IgA-producing cells and secretory IgA which neutralize and, thus, prevent the entry of potentially harmful antigens in the host. Thus, stimulation of local immune response can be effective in preventing certain diseases caused by microorganisms entering the host through the oral route. Feeding mice with milk fermented with Lb. helveticus increased the number of IgA(+) B cells in the small intestine and bronchial tissues. A protease deficient derivative of Lb. helveticus was ineffective (Matar et al. 2001). These results taken together indicate that peptides generated by the action of proteases are responsible for this effect.

Furthermore, it appears that the peptides can stimulate local immune response in the gut and activate the IgA cycle as shown by the increase of the IgA(+) B cells in the bronchial tissues. In another study, Perdigon et al. (1999) demonstrated that feeding mice with milk fermented by LAB resulted in increases in IgA(+) cells in the gut, macrophage activity and specific antibody responses during infection. However, it was not certain whether the effect was mediated by milk peptides and/or the presence of the LAB. To further elucidate the mechanism, Leblanc et al.(2002) investigated the effect of specific peptides generated following fermentation of milk with Lb. helveticus on humoral immunity. Three specific peptide fractions were isolated from fermented milk by chromatography and then were fed to mice. All fractions increased the number of the IgA(+) B cells in the gut which indicates that bioactive peptides are generated by the action of

LAB. There was no increase in the number of the IgA(+) B cells in the bronchial tissues which indicates that the effect of the peptides was restricted in the gut and there was no activation of the IgA cycle. A great number of studies demonstrating the ability of milk peptides to enhance the function of immune system were performed in the 80s and early to middle 90s and some excellent reviews of these studies are available (Clare & Swaisgood, 2000; Hill et al. 2000). Therefore, emphasis in the present review will be given towards recent studies or those not adequately covered in the previous reviews. A recent study investigated the effect of bovine glycomacropeptide on proliferation and phagocytic activity of the U937 human macrophages like cell line (Li & Mine, 2004). They showed that the glycomacropeptide to be a potent enhancer of cell proliferation and phagocytic ability. Furthermore, they showed that both the peptide and the carbohydrate portions were essential for the stimulatory effect. Politis (1995-2007) investigated the effect of a synthetic hexapeptide corresponding to residues 63–68 of bovine b-casein on several immune parameters of bovine macrophages and neutrophils. Results showed that the hexapeptide up regulated interleukin-1 production and expression of MHC class II molecules by macrophages and superoxide production by neutrophils. In contrast, the hexapeptide had no effect on chemotactic responsiveness of bovine neutrophils. It is clear that the effectiveness of milk peptides is cell-specific. It is difficult to speculate as to why the hexapeptide differentially affects two functions of bovine neutrophils (superoxide production, chemotactic responsiveness). A study performed in the 90s demonstrates the dichotomy between suppression and/or induction of immune response by milk peptides. Sutas et al. (1996a) examined the effect of milk peptides generated by an intestinal Lactobacillus strain on various immune functions. They showed that purified casein peptides up regulated IL-4 and IFN-gamma production by blood peripheral mononuclear cells. In contrast, Lactobacillus-degraded caseins up regulated IL-4 production but they had no effect on IFNgamma. It is clear that the overall effect of milk peptides on the immune system might be peptide-specific. Non-nutrient milk components modulating gastrointestinal development: Colostrum and milk contain a high number of biologically active compounds that modulate gastrointestinal activity and function and promote the development of the newborn (Blum & Baumrucker, 2002). A significative number of studies demonstrated the efficacy of these peptides to modulate the intestinal immune response of the newborn (Baldi et al., 2005). Chronopoulou et al. (2006) demonstrated an increase of superoxide anion production from macrophages and neutrophils isolated from weaning piglets treated with casein peptides. This research group tested hanno testato *in vivo* the effect of casein peptides on phagocytic cells of 27 piglets in the first weaning phase in three experimental groups: control (basal diet), low integration (300 mg/die), high integration (600 mg/die) (Politis e Chronopoulou 2008).

The production of sueroxide anions from serum monocytes, macrophages and neutrophils at days 7, 14, 21 increased compared to the level of the integration (**Figure 2**).

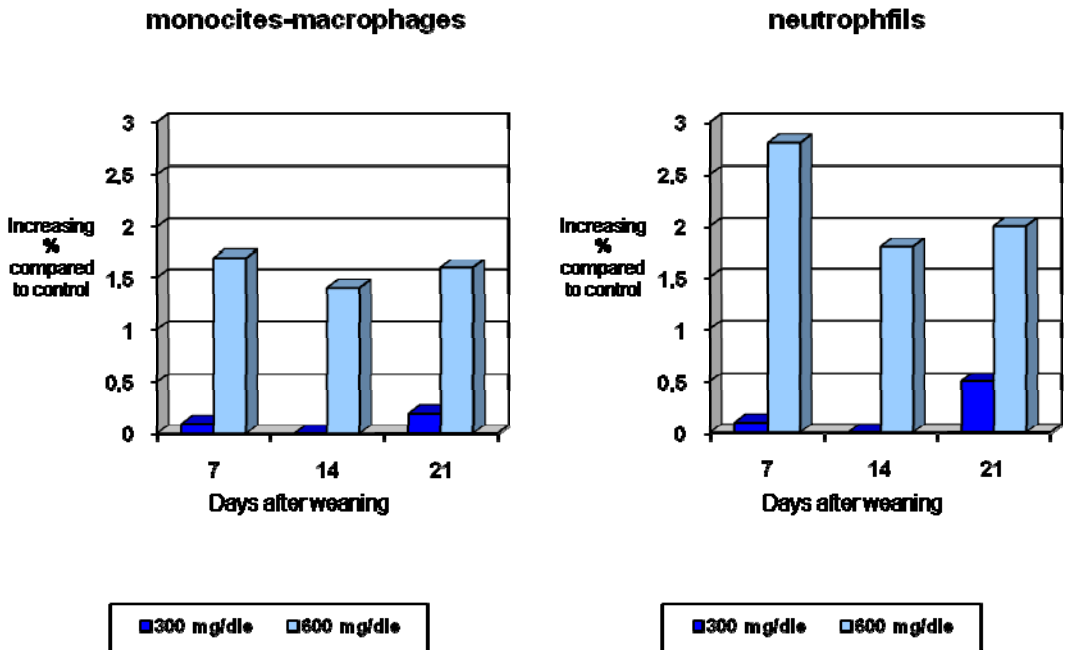

Figure 2 **Milk peptides and immune response in the neonate adapted from (Politis I., Chronopoulou R. 2008).**

Among these compounds there are: **lactoferrin,** a multifunctional protein involved in non-specific immune defence against bacteria and viruses, immunoregulation, anti-inflammation and iron metabolism (Brock, 2002). From a nutritional point of view lactoferrin may be of interest not so much as a dietary protein source of amino acids but for iron bioavailability (Lo¨nnerdal & Iyer, 1995). Although amino acid composition of lactoferrin suggests a high biological value of the protein, some evidence indicated that the major function is not as a nutritional support of amino acids for the newborn. In fact, lactoferrin is extremely resistant to proteolytic enzymes and the ironsaturated form is more resistant to enzymatic digestion than the apoprotein (Brines & Brock, 1983; Kuwata et al. 2001). As a consequence, ingested lactoferrin is not highly degraded in the gastrointestinal tract and nearly intact protein is delivered in the gut (Hutchens et al. 1991). This argument is supported by the fact that intact lactoferrin can be found in the faeces of breastfed neonate; thus, the protein undergoes incomplete proteolysis and it does not represent a source of utilizable amino acids (Davidson & Lo¨nnerdal, 1987). These data suggested that intact lactoferrin should have a physiological significance for the gut of the neonate. Following oral ingestion, survival of lactoferrin and other milk-borne bioactive compounds in the gastrointestinal tract is a prerequisite for direct action in the gut or for activity on peripheral targets after absorption into the

blood circulation (Xu et al. 2000). Lactoferrin is involved in iron uptake by the newborn; this potential role for the bioavailability of this micronutrient in milk arises from the observation that its bioavailability in breast milk is high, although its content is low (Lo¨nnerdal & Iyer, 1995). Studies by Kawakami & Lo¨nnerdal (1991) and Suzuki et al. (2001) showed that lactoferrin facilitates iron uptake by human intestinal cell cultures and this biological effect is mediated by the interaction with a receptor localized on enterocytes. Lactoferrin receptors were identified in the small intestine brush-border membrane of several species, such as mice (Hu et al. 1990), piglets (Gislason et al. 1993) and cows (Talukder et al. 2003). The binding of lactoferrin to the receptor is highly species-specific. Studies by Gislason et al. (1993) showed that human lactoferrin, bovine lactoferrin and pig transferrin do not interact with the receptor for lactoferrin localized on the small intestine of piglets. Another interesting aspect of the interaction of lactoferrin with its specific receptor is that the protein can be internalized after binding. Recent findings have showed that lactoferrin has an import signal for translocation into the nucleus (Penco et al. 2001) and surface nucleolin is involved in the binding and endocytosis of lactoferrin in target cells (Legrand et al. 2004). Ashida et al. (2004) studied the cellular internalization of lactoferrin in the intestinal epithelial cell line Caco-2; they showed that lactoferrin is localized to the nuclei after uptake. Baumrucker & Erondu (2000) demonstrated that bovine lactoferrin is involved in both the interaction and entry of IGFBP-3 in the nucleus of mammary cells. The nuclear localization of lactoferrin suggested its involvement as a transcription factor after binding to specific D In cow's milk, more than 100 nutrients are included. Preisely, milk is categorized as perfect food containing protein, lactose, calcium, phosphorus, magnesium and trace elements, and varius vitamins. And it is also mentioned as a natural food with biological function and significant meaning to the body. The history of milk as a food started from B.C 6000. According to the many studies since then, we can say that milk is a great food involving various physiological functions, such as bone-growth and immune control.

Immunoglobulin (Ig). The structure of the Ig present in milk is exactly same as that in the serum or other excreta. But the content of each Ig is totally different in between serum and milk. IgG is the most common Ig in serum of human, bovine and porcine (80-90 % of total Ig). In colostrum the most common Ig is Ig A in human, and for bovine and pig's colostrum the most represente is the IgG. Lactoferrin: Is a glycoprotein in milk containing 2 molecules of iron. It presents relatively higher in human breast milk, and especially 10-20 times higher than cow's milk (6-8 mg/l in colostrum and 2-4 mg/l in normal milk). This glycoprotein plays an important role in resistance toward E.coli infection and its function in colon is known to be strong. In addition, the content of lactoferrin is generally high in mammal colostrums, which is helpful in restricting the growth of pathogenic bacteria and E.coli in order to make Bifidobacteria settle down in large intestine in the

young. Due to its strong adherence to metal ions, this glycoprotein may provide the function of suppressing the pathogenic bacteria growth by transforming the active minerals into inactive ones which is essential for bacteria growth, and the function of preventing anemia with controlling iron absorption. In addition, its known functions are immune restoration, anti-inflammation, cell proliferation and anti-oxidation.

Lysozyme: has anti-bacterial anti-viral and immune enhancing effects, and involves in blood clotting and stryptic process, anti-inflammation and milk digestion. The content of lysozyme is around 170 μg % in milk. When infants were fed with formula containing lysozyme shown an increasy of the growth of Bifidobacteria and a decrease of E.coli in the feces.

The changes in bacterial composition can be explained by N-acetylglucosamin, produced when lysozyme degrades the mucus polysaccharides on the cell walls of bacteria, that increases the growth of Bifidobacteria.

Mucin: is chemical compound of olighosaccarides and glycoprotein and has lubricative and protectie functions, existing in pituauitary gland, gastric mucosa, cartilage, saliva, blood, nasal mucosa in the body. Especially, it is high in breast milk and cow's mil and can protect stomach from gastric juice such as pepsin and and hydrochloride.

1.1.6 Vegetable milks as functional foods

Compared to cow's milk, vegetable milks contains more carbohydrates, but does not contain significant amounts of calcium or protein, and no cholesterol or lactose. Commercial brands of vegetable milks such as rice milk and soy milk, however, are often fortified with vitamins and minerals, including calcium, vitamin B12, vitamin B3, and iron. Soy milk for example has about the same amount of protein as cow's milk. Natural soy milk contains also little digestible calcium as it is bound to the bean's pulp. To counter this, many manufacturers enrich their products with calcium carbonate available to human digestion. Unlike cow's milk, these kind of vegetable milks have little saturated fat and no cholesterol. Soy products contain sucrose as the basic disaccharide, which breaks down into glucose and fructose. Since soy doesn't contain galactose, a product of lactose breakdown, soy-based infant formulas can safely replace breast milk in children with galactosemia (Franck M et al., 2006). Soy milk is promoted as an healthy alternative to cow's milk for reasons including: source of lecithin and vitamin E, lacks casein, safe for people with lactose intolerance or milk allergy, contains far less saturated fat than cow's milk, contains isoflavones, organic chemicals that may possibly be beneficial to health. Rice milk is a kind of grain milk processed from rice. It is mostly made from brown rice and commonly unsweetened. The sweetness in most rice milk varieties is generated by a natural enzymatic process, cleaving the carbohydrates into sugars,

especially glucose, similar to the Japanese amazake. Some rice milk kinds may nevertheless be sweetened with sugarcane syrup or other sugars.

1.1.6.1 Some components of vegetable milks

1.1.6.2 Inositol

Inositol is not-in the group of vitamin B, but is closely associated with choline and biotin. Animal studies have shown that vitamin B6, folic acid, pantothenic acid and PABA also have very close activity association with inositol. The myoinositol is the only active constituent from the nutritional point of view of phosphatidylinositol. Inositol is active in cell membranes and in sending messages to the nervous system to control the cellular functions. Like choline, inositol is also present in lecithin. The animal tissues and some plants contain inositol. In animal tissues is presented as a component of phospholipids, substances containing phosphorus, fatty acids and nitrogen bases. In plant cells is in the form of phytic acid, an organic acid that binds calcium and iron in an insoluble complex and interfere with their assimilation. Is effective to stimulate the production of lecithin in the body. Fats are mobilized from the liver cells with the help of lecithin and inositol, therefore, contributes to the metabolism of fats and helps reduce cholesterol in the blood. Combined with choline, prevents hardening of the fat in the arteries and protects the liver, kidneys and heart. It was also reported its usefulness in nutrition of brain cells. Large amounts of inositol are found in the nerves of the spinal cord, brain and cerebro-spinal fluid. And 'necessary for the growth and survival of bone marrow cells, for the eye membranes and intestines. Is important for hair growth and may prevent their weakening and baldness. Of all the isomers present in nature only mio-inositol is the active form of Inositol.

Inositol and a number of its mono and polyphosphates function as the basis for a number of signaling and secondary messenger molecules. They are involved in a number of biological processes, including: insulin signal transduction, cytoskeleton assembly, nerve guidance (Epsin), intracellular calcium (Ca^{2+}) concentration control, cell membrane potential maintenance, serotonin activity modulation, breakdown of fats and reducing blood cholesterol, gene expression.

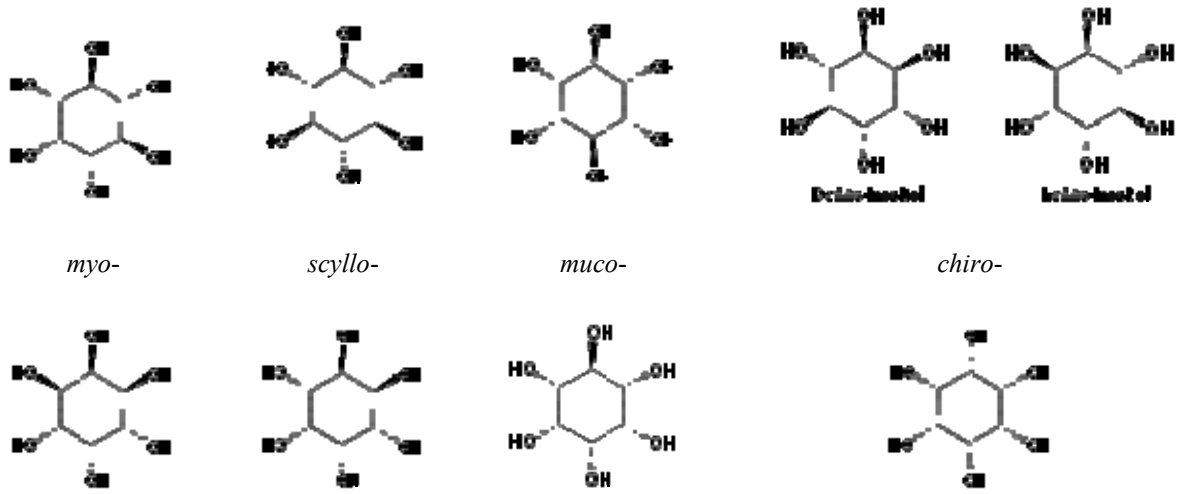

Fig.3 **Isomers of Inositol**

1.1.6.3 γ-Oryzanol

Gamma-oryzanol, a mixture of ferulic acid esters of sterol and triterpene alcohols, it occurs in rice bran oil at a level of 1 to 2%, where it serves as natural antioxidant. Recent research has shown that gamma-Oryzanol can lower the cholesterol levels in the blood, lowering the risk of coronary heart disease, besides that, also has been used in Japan like natural antioxidant in foods, beverages and cosmetics. Gamma-Oryzanol plays an important role for its physiochemical properties, its presence in the rice bran oil, its antioxidant and hypocholesterolemic activity, as well as, identification, quantitation and extraction methods. (Scavariello EM et al. 1998). Gamma-oryzanol may be a more important antioxidant of rice bran in the reduction of cholesterol oxidation than vitamin E, which has been considered to be the major antioxidant in rice bran. The antioxidant function of these components against cholesterol oxidation may contribute to the potential hypocholesterolemic property of rice bran (Xu Z et al. 2001).

1.1.7 Prebiotics

In 1995 Gibson and Robertfroid defined a prebiotic as a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon. Prebiotics are non-digestible dietary components that have a positive effect on intestinal well-being, selectively stimulating the growth and activity of a limited

number of useful bacterial species already present in the intestine. These kinds of products are not only presents in the plant derived products but also in the maternal milk **(Table 2).** Among the food additives the activity of these compounds is very interesting for two classes of oligosaccharides: fruit-oligosaccharides (FOS) and mannan-oligosaccharides (MOS). Both of the two classes of oligosaccharides can be a good alternative to the use of AGP, in fact they facilitate and support the symbiotic relationship between the host and the intestinal microflora.

Table 2. Human milk: glicoconiugates and oligosaccharides pathogens inhibitorsi (adapted from Newburg et al., 2005; Séverin e Wenshui, 2005).

Glicoconiugates	Pathogens	Action	Concentration(*)
GM1	*Vibrio cholera* *Campylobacter jejuni*	Legame con tossina	180 µg/L
GM3	*E. coli* enteropathogenic	Bind the toxin	13 mg/L
Gb3	Shiga toxin	Bind the toxin	100-150 µg/L
Condroitin sulphate	HIV	Bind the virus	6 mg/L
Lactaderine	Rotavirus	Bind the virus	100 µg/L
Mucine	*E. coli*	Bind the pathogen	1 g/L
Mannosilate Glycopeptides	*E. coli* enteroemorragic	Bind the toxin	60 mg/L
Oligosaccharides	*Streptococcus pneumoniae* *E. coli* enteropathogenic *Listeria monocytogenes* *Clostrium spp.* *Campylobacter jejuni* Rotavirus	Bind the pathogen	0.2-10 g/L 3 g/L 3 g/L
Fucosilate Oligosaccharides	*Campylobacter jejuni* *Vibrio cholera* *E. coli* (enterotoxin)	Bind the pathogen Bind the toxin	1-25 mg/L 1-25 mg/L 40 µg/L
Glicoproteines associated to macromolecules	Norovirus *Pseudomonas aeruginosa*		370 mg/L
Sialillactose	Cholera toxin *E. coli* *P. aeruginosa* Influenza virus Poliomavirus *Helicobacter pylori*		200 mg/L

(*) Concentration of the active fraction/component in the milk

Inulin and Mannano oligo saccharides are the most studied and well-established prebiotics in pig diets. They escape digestion in the upper gastrointestinal tract and reach the large intestine virtually intact where they are fermented. Indeed, in many studies it was shown the selective stimulation of growth of the beneficial flora, namely bifidobacteria, and to a lesser extent, lactobacilli and possibly other species like *Clostridium coccoides*, *Eubacterium rectale*, cluster known to be a butyrate producer. (Klesser et al., 2001). The mechanism of the bifidogenic effect of inulin and MOS is thought to be their selective fermentation by the bifidobacteria that produce an intracellular inulinase necessary to hydrolyze the β-(2 $\rightarrow$ 1) fructosyl-fructose osidic linkages. But these data

also call attention to the facts that not all inulin derivates have, qualitatively, the same effects on intestinal microflora and that for each inulin-type fructan, the different segments of the large bowel (including the feces), might be differently influenced (Apajalahti et al., 2002). In this regard, two different questions have attracted attention concerning the qualitative aspects of the prebiotic effect. These questions can be formulated as follows: are the different inulin-type fructans and MOS equally effective? Can a dose-effect relationship be established? At the population level it is the fecal flora composition characteristic to each individual that determines the efficacy of a prebiotics but not necessarily the dose itself. The ingested prebiotic stimulates the whole indigenous population of bifidobacteria to growth. The dose argument, as supported by scientific data can not be generalized because of the factors controlling the prebiotic effect are multiple (Roberfroid, 2005). One important question remaining unanswered is the effect of prebiotic, especially inulin-type fructans, not on the numbers of bacteria, especially bifidobacteria, but rather on activity associated with these bacteria. Inulin-type fructans classify as functional feed ingredients that target gastrointestinal functions but also, most likely, via their effects on the gut microflora, systematic functions that are known to be closely related to health and well-being. Nowadays gastrointestinal functions and especially colonic functions (control of the colonic environment, regulation of hormone-dependent metabolic processes, modulation of the brain-gut axis, systemic impact of gut fermentation products and activity of the immune system) deserve special attention. Indeed disturbances of colon functions may lead to dysfunctions not only in the gut but also in the whole body (**Table 3**).

Table 3 Experimental data that support the substantiate claims on inulin-type fructans

Property or target functions	Supportive evidence	Claims
Dietary fibre	Oligo/polysaccharides resistant to digestion, fermentation	**Inulin-type fructans are dietary fiber**
Caloric value	Non-digestible oligo/polysaccharides, colonic feed	**Inulin-type fructans are low calorie carbohydrates**
Bowel functions, stool production	Bulking effect, regulation of stool production, improved stool consistency	**Inulin-type fructans regulate bowel functions**
Colonic microbiota	Substrates for anaerobic saccharolytic fermentation, selective stimulation of growth of health-promoting bacteria	**Inulin-type fructans are prebiotic**
GI absorption Ca and Mg	Increased absorption of Ca and Mg	**Inulin-type fructans increase Ca and Mg absorption**
Lipid homeostasis	**Reduction of triglyceridaemia**	**Inulin-type fructans reduce triglyceridaemia in slightly hypertriglyceridaemic individuals**

1.1.7.1 Mannanoligosaccharides (MOS)

The mannanoligosaccharides are derived from the wall of yeasts and have mannose as a primary carbohydrate. The mannanoligosaccharides are not used as a substrate for microbial fermentation, but they carry out their action as growth promoters, increasing the resistance of the animal to enteric diseases. The MOS act as: preventing the adhesion of enteric bacterial pathogens to the mucosa, improving the immunity of the host, modulating the gut microflora, increasing the enteric mucosal barrier, reducing the red cell turnover, enhancing the integrity of the intestinal mucosa and inhibition of microbial colonization. Aim of this functional compound is offer a substrate to certain beneficial members of the gut flora and by this to achieve a shift in the composition of the gut bacteria in favour of a healthy flora. Studies showed that five of the seven strains of E. coli and seven of 10 strains of *Salmonella Typhimurium and S. Enteriditis* present in the intestines of chickens, are agglutinated by MOS (Spring et.al. 2000). Even strains of *Clostridium spp* do not bind to molecules of the MOS, but studies have shown a significant reduction in the number of clostridia in feces of

animals fed MOS (Lalles et al., 2009). This is probably due to the effects of MOS increasing the resistance of the barrier role of mucine or enhancing the immune function. It is well known that all animals reared under commercial conditions, are subjected to immunological stress caused by the presence of a high load of pathogens in their environment. The release of cytokines, associated with inflammatory processes and the immature immune system, lead to hyperthermia. The consequences are a decrease appetite, mobilization of the body reserves of glucose, amino acids, minerals, decrease of the intestinal nutrients absorption, loss of body fluid and the development of diarrhea. The use of MOS seems to reduce the production of pro-inflammatory cytokines by reducing hyperthermia, and the events mentioned, allowing an increase in growth performance. It has also been shown that MOS have a positive influence on immunity and humoral immune status. There is evidence that the administration of MOS, causes an increase in the number of immunoglobulins (IgA, IgG and IgM) in the intestine in both serum and blood. This increase in the antibody response is due to the immune system's ability to respond to exogenous antigens present on the molecule of MOS because of their microbial origin (Sauerwein et al., 2007). Although the cecum is the main intestinal site where occur fermentation process, the fermentation processes that occur in the jejunum have a greater influence on the digestion and absorption of nutrients (Lalles et al., 2009). The use of MOS can decrease the concentration of strains such as *Salmonella and E. coli* in the gastrointestinal tract increasing the growth of bacteria such as *Lactobacillus spp Enteric-coated* and *Bifidobacterium spp*. Thanks to the increase of volatile fatty acids production decreasing intestinal pH (Moore WEC et al., 1987). The benefits of MOS may be also associated with intestinal morphology. During the weaning period, the massive colonization by microorganisms of the intestinal lumen and the contribution of new types of food proteins is often associated with atrophy of the villi, which results in a decreasing ratio of villous height / crypt depth. This phenomenon is often due to malabsorption syndromes with subsequent onset of diarrhea (Pierce et al. 2006-2007). Studies in piglets, chickens and mice, have shown that mannano oligosaccharides have a major influence on the morphology of intestinal villi and their use in the diet after weaning increases the ratio villus height / crypt depth (Konstantinov et al. 2004a-2006). Maintaining a good relationship villus height / crypt depth is necessary for good absorption of nutrients and prevent the onset of diarrhea. The state of the lining of the intestinal lumen, is the first barrier that opposes the gut against enteric pathogens. Mucus production is proportional to the number of goblet cells in the gut and nutrition including MOS has been shown to be able to increase the number of these cells at the level of enteric villi (Bontempo et al. 2006). Thus, increased of prebiotics creates favourable lumen conditions for gut health but direct evidence for enhanced resistance to unfavourable conditions is still lacking (Lalles JP et al., 2007).

1.1.8 Plant Products

For thousands of years, herbs and spices have provided distinctive flavouring properties to foods and many have been proven as potent antimicrobial agents. The use of these substances was very common in both western and chinese culture. Some of the most common plant products (PP) known for their antimicrobial properties belong to the genus *Allium* as garlic, onion and leek; others are thyme, oregano, marjoram, basil and cumin. The natural plant antimicrobials are found in barks, roots, stems, leaves, flowers and fruits of many plants. In recent years, in vitro studies demonstrated the antibacterial activity of some of these products (Sen et al., 1998; Friedman et al., 2002), and more recently the supplementation of plant products to pigs diets has been proposed in order to counteract intestinal disorders, especially in the weaning period. However, the effects on growth performance and gut health are not so consistent which may depend on the plant product tested. One of the main issue concerning plant products is their characterization (Cowan, 1999). Plant products contain several different active compounds in different concentration and their composition is mainly affected by the method of extraction (solvent and extraction conditions) and the niche of the plant used related to plant variety and age, climatic conditions and geografic origin. Characterization is important for scientific as well as for legal purposes. All characteristics affecting plant product composition affect at the same time their biological effects. Hence, for scientific purpose it is better to work with pure active substances or with accurately controlled blends. In regards to legislation, traceability, and thus characterization, is one of the main requisites to register additives. It is, therefore important to accurately assess all the different compounds that the product contains.

1.1.8.1 Effect of plant products on microbiota

Plant products have intensively been used in the past for medical and food preservation purposes. The use is mostly motivated by the main characteristic of PP, their antimicrobial activity (Didry et al., 1994). This activity has been studied in several *in vitro* studies with promising results. This application is still too recent and there is too limited information available about the actual possibilities of these products. However, the legislative requirements and the great interest to this sector are motivating the appearance of the first studies using PP in vivo (Evans and Martin, 2000; Herman et al., 2003; Allan et al., 2005). It is difficult to define the antimicrobial or different active compounds present in a plant product. Usually, antimicrobial active substances in PP have a very different chemical structure, with high occurrence of phenolic rings, mostly hydrofobic and some of them with similar structure to important molecules from bacterial metabolism such as receptors or

enzyme substrates (Cowan, 1999). It also known that many of these substances are secondary metabolites that plants use against predators, or with different functions such as pigmentation, aromatizing or flavoring. In **Table 4** the principal chemical structures producing antimicrobial activity in plant products and the mechanism of action referenced are reported (Guen et al., 1991). Some of these effects need to be better investigated while some of them are more studied as the hydroxyl group (-OH) present in phenol compounds. The importance of this group on antimicrobial activity is well known (Cowan, 1999) and any variation in its position inside the mulecule, as it happens between carvacrol and thymol, produces marked differences in antimicrobial power (Dorman and Deans, 2000). Components with phenolic structures, such as carvacrol and thymol are highly active against test bacteria dispite their low capacity to dissolve in water. This has been known for centuries, but the importance of the hydroxyl group in the phenolic ring was confirmed in terms of activity when carvacrol is compared to its methyl ester. The high activity of the phenolic components may be further explaned in terms of the alkyl substitution into the phenolics nucleus, which is known to enhance the antimicrobial activity of phenols (Han et al. 2006). It was suggested that plant products act via two main mechanisms of action. The first is related to the general hydrophobicity of PP, which facilitates their adhesion to the bacterial surface inducing unstabilization (Tsuchiya et al., 1996). The second mechanism is the inactivation of different molecules of the bacteria (such as enzymes or receptors) through their adhesion to specific sites (Sharon and Ofek, 1986).

Table 4 Chemical structures implicated in antimicrobial effect of PP and relative mechanism of action (Cowan, 1999)

Class	Subclass	Mechanism of action
Phenol compounds	Simple phenols and phenolic acids	Enzyme inactivation (1) Membrane un-stabilizers (2)
	Quinones	Irreversible adhesion to adhesins, membrane polypeptides and enzymes that become inactive (3)
	Flavonoids, flavones and flavonols	
	Tannins	1,2,3 and metal chelators
	Coumarins	Interact with eukariote DNA
Terpenoids		Membrane un-stabilizers
Alkaloids		Insertion in cellular wall or in DNA structures
Lectines and polypeptides		Block viral fusion and adsorption Di-sulphur bridges formation

Some authors suggested a higher efficacy of plant products against gram negative organism but others did not find any difference between gram positive and negative bacteria and, sometimes even the opposite effect was proposed (Manzanilla et al., 2004; Han et al. 2006). Actually, it is possible that some PP have specific actions and other PP have diverse effects due to the different nature and composition. In fact, as it happens for antibiotics, the chemical structure will determine the mode of action and hence a possible selective effect of plant product. For instance, alkilic chains plus a phenol group seem to perform better activity against gram negative bacteria, due to the characteristic of their cellular wall. In any case, the specific effect of some PP could be interesting in therapeutic or preventive applications like it happens with antibiotics. Concerning growth promotion, it must be studied if a selective effect of PP could be useful, because of the risk of producing detrimental effects (Dorman and Deans, 2000). The effect of PP on different bacterial species has been determined in a high number of *in vitro* studies using spectrophotometry measurements or agar plate inhibition rings. Many of these studies have explored the real antimicrobial power of classic herbal products or spices (Dorman and Deans, 2000; Friedman et al., 2002). From these previous studies we can draw some conclusions. First of all, different bacteria have different sensitivity to different plant products. It can be also observed how some compounds as α-terpinen are highly effective against a very interesting target as Salmonella but not against the other microorganisms.

Table 5 Antibacterial activity of different essential oils against 25 different bacteria tested (from Lis-Balchin M, 2003)

Essential oil	***Antibacterial activity*** Number of bacteria
Peppermint	15-22
Petitgrain	21
Pine niddle	19
Rosewood	24
Rosemary	21
Sage, Dalmatian	16
Tea tree	24
Thyme	14-25
Verbena	18

In the application of PP *in vivo* it is important to consider the dosage. Compared to antibiotics, the *in vitro* dose of PP to obtain similar effect is normally 10- to 100-fold higher (Karaman et al., 2001). So far, we know that complex media as the chime in the digestive tract could affect the *in vitro* effective dosage.

1.1.8.2 Receptors analogs as anti-adhesive agents

There is evidence that receptor analogs as agents for anti-adhesion therapy would be practical primary against pathogens that bind to animal cells via carbohydrate-specific adhesins (i.e. lectins). In this case the receptor analogs are saccharides that are structurally similar to those of the glycoprotein and glycolipid receptors for the adhesin and, therefore, act by competitive inhibition. It was less than three decades ago that mannose was first shown to be a receptor for enterobacteria (Ofek et al., 1977). Since then, the sugar specifities of many bacteria have been determined, leading to the development of receptors-like carbohydrates, which inhibit the adhesion of pathogens to host cells and tissue (Ofek et al., 2003). The concentration of the carbohydrates required for effective inhibition of adhesion in vitro are usually high, in the millimolar range, because the affinity of the saccharides for the bacterial lectins is low. It can be increased several orders of magnitude by covalently linking a hydrophobic residue such as phenyl or methyl umbelliferyl to the saccharide (Firon et al., 1987). Affinity can be similarly increased by attaching many copies of the saccharide to a suitable carrier, yelding multivalent adhesin inhibitors, as demonstrated for type 1 fimbriated *Escherichia coli* (Lindhorst et al., 1998). The feasibility of using saccharides to protect against experimental infections by bacteria expressing adhesive lectins was first demonstrated more than two decades ago. Administration of methyl α-mannoside together with *E. coli* expressing the mannose-specific type 1 fimbrial lectin into the bladders of mice reduced the extent of bladder colonization by uropathogenic *E coli* by about two thirds compared to animals that had received the bacteria alone or with methyl α-glucoside, a sugar that does not inhibit the mannose-specific bacterial lectin. Subsequently many studies have confirmed the ability of saccharides to prevent experimental infections caused by different pathogenic bacteria in a variety of animals (Namkung et al., 2004) (**Table 6**).

Table 6 Carbohydrates preventing bacterial colonization and/or infection in vivo (modified from Ofek et al., 2003b)

Organism	Animal, site of action	Inhibitor
C. jejuni	Mouse intestine	Milk oligosaccharides
***E. coli*, type 1 fimbriated**	Mice GT	Mannose
***E. coli*, P fimbriated**	Mice UT	Globotetraose (Galα1,4Gal)-containing GP*
E. coli K99	Calf GT	Glycopeptides
H. pylori	Piglet GT	Sialyl-3'-LacNAc
***Shigella flexneri*, type 1 fimbriated**	Guinea pig eye	Mannose
S. pneumonia	Rabbit and rat lungs	Sialyl-3'-Galβ(1→4)LacNAc
S. sobrinus	Rat oral cavity	Oxidized α1,6 glucan
S. pyogenes	Mouse pharynx	Hyaluronan

*Gp: glycoprotein found in dove and pigeon egg white

1.1.8.3 Adhesins analogs as anti-adhesive agents

The strategy for using adhesins to prevent infections is based on the assumption that the isolated adhesin molecules, or an active synthetic or recombinant fragment, bind to the receptor and competitively block adhesion of the bacteria (Ofek et al., 2003). It has thus far been impratical to use analogs of adhesins for anti-adhesion therapy, because they are typically macromolecules that are not readly available and bacause they must be employed at relatively high concentrations. In addition, careful consideration must be given to their toxicity and immunogenecity. Novertheless, modern proteomics and recombinant biotechnology have permitted the development of unique types of relativelly small peptides for anti-adhesion therapy, as reported by Kelly et al. (1999). Non-proteinaceous adhesins may also be useful for anti-adhesion therapy, as shown in studies of the lipotheicoic acid (LTA)-mediated adhesion of group A and group B streptococci.

Table 7 Anti-adhesin activity of plant constituents (modified from Ofek et al.,2003)

Plant	Constituent	Bacterium affected
***Azadirachta indica* (neem stick)**	ND	*S sanguis*
***Camillia sinensis* (green tea)**	(-) epicatechin gallate, (-) gallocathechin gallate	*P gingivalis*
Oolong tea	Polyphenol	*S mutans; S sobranus*
***Gilanthus nivalis* (snowdrop)**	Mannose-sensitive lectin	*E coli*
***Gloipeltis furcata* and *Gigartina teldi* (seaweeds)**	Sulfated polisaccharides	*S sobrinus*
Hop bracht	Polyphenols (36-40 kDa)	*S mutans*
Melaphis chinensis	Gallotannin	*S sanguis*
***Persea americana* (avocado)**	Tannins	*S mutans*
Legume storage protein	Glycoprotein	*E coli*

ND: not determined

1.1.8.4 Dietary inhibitors of adhesion

Some of the most efficient anti-adhesion agents identified so far are present in feedstuffs. Feedstuffs containing either a mixture of inhibitors or an inhibitor with a broad spectrum of activity could be especially effective. While it may be possible to find suitable inhibitors for particular pathogens, it is unlikely that it will be possible to match every individual or group of pathogens with specific diets that contain complementary adhesins inhibitors (Ofek et al., 2003b). Empirical observations over the years have suggested that may be effective to counteract bacterial infections. However, caution should be used, because some dietary components may also be bactericidal and selective pressures imposed by such compounds are undesirable and should be avoided. Human milk and plant-derived constituents are rich in oligosaccharides and related compounds to which many bacteria bind.

1.1.8.5 Plant products as bacteria adhesion inhibitors

Because of their ready availability, plant materials possessing anti-adhesion activities are attractive candidates for antibacterial agents. There is, however a relative paucity of information regarding the anti-adhesive properties of most plant materials. Although plant lectins are well rappresented in the

diets, and many of these lectins are very characteristic, their application to anti-adhesion therapy is very limited. Theorically, these lecitins could interact with animal cell surface saccharides to block adhesion mediated by lectin-carrying bacteria and they may enhance clearance of bacteria from the host (Slifkin and Doyle, 1990). Feed lectins may have deleterious effects as well. They may bind to mucosal cells and thus function as receptors for bacterial glycans and enhance bacterial adhesion to the tissue. Moreover some dietary lectins may reach the GI tract in a faunctional form and may similarly enhance the binding of the bacteria to the different parts of the intestine. A pratical advantage in the search of dietary plant extracts for agents to use in the therapy of bacterial infections is that clinical trials are probably easier to perform, mainly because toxicity is usually not as much of an issue. Among the plant extract listed in **Table 8**, those obtained from *Vaccinium macrocarpon* (cranberry) are the most throughly studied with respect to their anti-adhesion activity *in vitro* and, so far, are the only one sow to be effective in *in vivo* trials. The initial in vitro experiments on the effects of cranberry extracts on bacterial adhesion were stimulated by the long known anecdotal evidence on the beneficial effects of cranberry juice consumption in therapy of urinary tract infections. Several lines of evidence implicate two different cranberry constituents as active anti-adhesive agents. One of these is a high molecular mass (> 15 kDa) material and the other is a protoanthocyanin. The high molecular mass material is devoid of proteins and carbohydrates and behaves in some respects like tannin (Ofek et al., 1996). It inhibited the adhesion to animals cells of uropathogenic *E. coli* including P fimbriae-, S fimbriae- and non-fimbrial.

Table 8 Anti-adhesion effects of juices or extracts of Vaccinium spp. (cranberry). From Ofek et al., 2003 (modified)

Bacterium	Disease	Adhesion assay	Effect
E. coli	UTI and pyelonephritis	HA UroEp.	I
E. coli	Diarrhea	HA	NI
E. coli	Meningitis	HA	I
Oral bacteria	Dental decay periodontitis	Coaggregation and buccal epithelial adhesion	I
H. pylori	Gastric ulcer	Human gastric mucus, TC cells	I

In addition, it inhibited the coaggregation of Gram-negative pairs of oral bacterial more often than it inhibited coaggregations between Gram positive bacteria. Extracts containing protoanthocyanins in their condensed form inhibited adhesion of P fimbriated *E. coli* to erythrocytes.

1.1.8.6 Plant products: antioxidant capacity

Antioxidative properties are well described for herbs and spices (Cuppett and Hall, 1998; Craig, 1999; Nakatani, 2000; Wei and Shibamoto, 2007). Among a variety of plants bearing antioxidative constituents, the volatile oils from the Labiatae family (mint plants) have been attracting the greatest interest, especially products from rosemary. Their antioxidant activity arises from phenolic terpenes, such as rosmarinic acid and rosmarol (Cuppett and Hall, 1998). Other Labiatae species with significant antioxidative properties are thyme and oregano, which contain large amounts of the monoterpenes thymol and carvacrol (Cuppett and Hall, 1998). Plant species from the families of Zingiberaceae (ginger and curcuma) and Umbelliferae (anise and coriander), as well as plants rich in flavonoids (green tea) and anthocyans (many fruits), are also described as exerting antioxidative properties (Nakatani,2000; Wei and Shibamoto, 2007). Furthermore,pepper (*Piper nigrum*), red pepper (*Capsicum annuum L.*), and chili (*Capsicum frutescene*) contain antioxidative components (Nakatani, 1994). In many of these plants, parts of the active substances are highly odorous or may taste hot or pungent, which may restrict their use for animal feeding purposes. The antioxidant property of many phytogenic compounds may be assumed to contribute to protection of feed lipids from oxidative damage, such as the antioxidants usually added to diets (α-tocopheryl acetate or butylated hydroxytoluene). Although this aspect has not been explicitly investigated for piglet and poultry feeds, there is a wide practice of successfully using essential oils, especially those from the Labiatae plant family, as natural antioxidants in human food (Cuppett and Hall, 1998), as well as in the feed of companion animals. The principal potential of feed additives from the Labiatae plant family containing herbal phenolic compounds to improve the oxidative stability of animalderived products has been demonstrated for poultry meat (Botsoglou et al., 2002, 2003a,b; Papageorgiou et al., 2003; Young et al., 2003; Basmacioglu et al., 2004; Govaris et al., 2004; Giannenas et al., 2005; Florou-Paneri et al., 2006), pork (Janz et al., 2007), rabbit meat (Botsoglou et al., 2004b), and eggs (Botsoglou et al.,2005). Oxidative stability was also shown to be improbe with other herbal products (Botsoglou et al.,2004a; Schiavone et al., 2007). Nevertheless, it remains unclear whether these phytogenic antioxidants are able to replace the antioxidants usually added to the feeds (α-tocopherols) to a quantitatively relevant extent under conditions of common feeding practice.

The tea plant, Camellina sinensis, is used in livestock as a feed additive. The major molecules that make up the tea and give the biological properties are: Methylxanthines: like caffeine or protein

(4%), smaller amounts of theobromine and theophylline, and traces of adenine and xanthine. They are partly related to tannins. Polyphenols: Catechins prevail, whose percentage ranged between 10 and 20% depending on the variety and age of the leaf. Were isolated several compounds such as, epicatechin, gallocatechin and other 4-catechin gallates; flavanil-3-ols and diols flavanil. Other: as oligomeric procyanidins, fenolcarbossilici acids, saponins, aldehydes, alcohols and monoterpenes (Fig. 4)

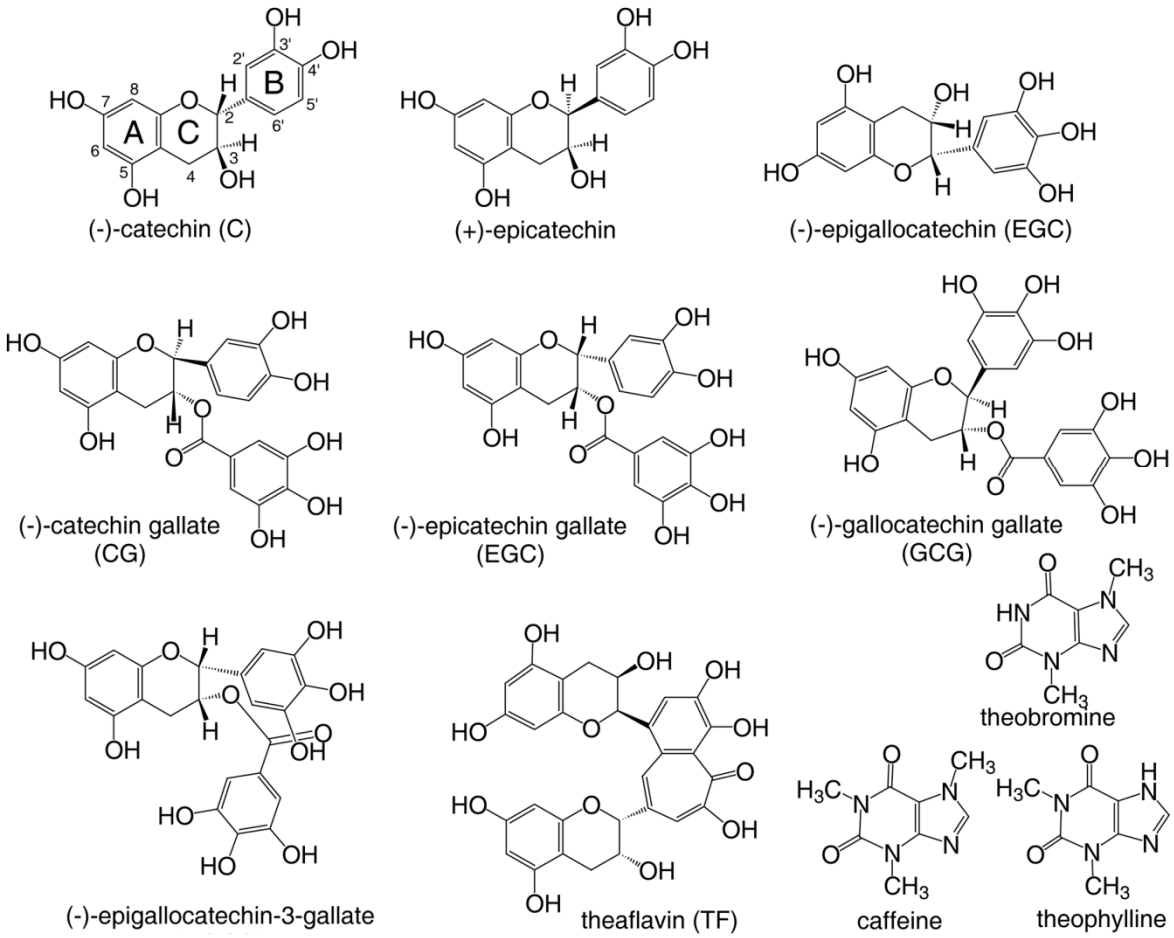

*Fig.*4 **Structure of molecole contained in Tea leaves (Friedman 2007)**

The catechins contained in tea leaves, have antibacterial and antitoxic effects on different microorganisms. Juneja et al (2007) have shown that tea extracts are able to inhibit the growth and sporulation of Cl. perfrigenes in land-based broth. Studies in vivo in mice, show how green tea extract, protect the animals from systemic and neurological symptoms caused by toxins of E. coli O157: H7 (Isogai et al., 2001). Alcoholic extracts of black tea, showed inhibitory to strains of Salmonella typhimurium (Ciraj, Sulaim., 2001). It is well documented the role of different antioxidant catechins as acceptors of H, are able to protect cells from free radicals. Human studies have demonstrated the ability of the polyphenols in green tea inhibit in vivo oxidation of LDL, thus

reducing the risk of atherogenesis. It also demonstrated a capacity of catechins antimicetica and antiviral (Weber et al, 2003). Grapes contain especially in the peel and seeds large percentage of the anthocyanins and proanthocyanidins. Anthocyanins are among the most important groups of pigments found in plants and belong to the family of flavonoids. These molecules are composed of a benzene molecule with a cast of Piran (heterocyclic ring containing oxygen), connected in turn with a phenyl group which may in turn be linked to various substituents. This complex molecule called a cation flavilio and is the basic structure of all anthocyanins. (Fig.5)

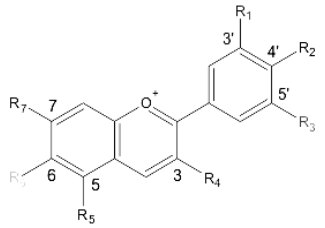

Fig.5 ***Flavilio cation is the basic structure of antiocians***

As part of the family of polyphenols, anthocyanins also have the same biological properties including a strong antioxidant properties. The Olive (Olea europaea.) Is a plant belonging to the family of Oleaceae. The essential oil of olive leaves is used in animal husbandry due to the presence of high concentrations of biologically active substances, including flavonoids, triterpenes, fatty acids, saturated and polyunsaturated fats (alpha-linolenic acid), monounsaturated (oleic), oleuropein, tannins and chlorophyll . This is known as oleuropein molecule that has a strong antioxidative action antibattericae, common to the family of polyphenols to which it belongs. Oleuropein metabolites, such as football elenolato derived from its hydrolysis, seems to be also responsible for antiviral action of the molecule.

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Chapter 2

Objectives

2.1 Objectives

Worldwide the swine industries are under pressure to reduce the use of antibiotics while maintaining animal health and performance. In 2006 all the AGP (antibiotic growth promoters) and some feed additives with growth promoting activities were banned (Cu, Zn). Since the first restrictive measures were taken, and due to the beginning of the negative consequences of the ban, numerous efforts have been done to find alternatives or replacement strategies to maintain pig growth performance and controlling enteric bacterial diseases. Therefore, the following studies aimed to find ways to control additive efficacy with due consideration to animal welfare and consumers requirements. The main objective of this thesis was to improve our knowledge on the properties of new additives as feeding strategy and oral vaccination to improve general and gut health in young pigs with the aim to substitute antibiotics growth promoters. To achieve these objectives, three different trials were designed to study different strategies to improve the gut health of the post-weaning piglets.

2.1.1 Abstract 1

I) Evaluation of the immune response against ovalbumin (OVA) in pigs orally vaccinated with OVA in tablets containing carboxymethyl high amylose starch (CM-HAS), flagellin and sow's milk.

This trial was designed with the aim to determine the effects of a new delivery system designed for oral vaccination or delivery of bioactive molecule in the gut. The trial was also designed to evaluate the potential of the sow's milk to modulate the systemic as well as local immune response against the antigen ovalbumin incorporated in the vaccine. Sixteen piglets of 28 days (d) of age were divided into 4 groups of 4 animals each. The first group was vaccinated with compressed tablets containing only ovalbumin (OVA); the second group was immunized with tablets containing ovalbumin and the B subunit of the flagellin from *Salmonella enterica* Typhimurium (OVA-FLA); the 3rd and 4th groups were immunized with tablets containing ovalbumin pre-incubated in sow milk with or without flagellin, respectively (OVA-LT or OVA-LT-FLA). The oral vaccination was performed in two periods of three days; the 1st period being from day 28 to day 30 of age (D1-D3 of the trial) and the 2nd from 42 d to 45 d of age (D14-D16). Blood samples were collected at days 1, 14, 21, 28 and 42 of the trial to determine, by ELISA, serum antibody titre against OVA. Feces samples were also collected before the first immunization and intestinal content from the ileum was collected at slaughtering to evaluate level of IgA anti-OVA by ELISA. All the animals were euthanized 42 days post-immunization to collect blood, mesenteric lymph nodes (MLN) and ileum content. The immune cells were isolated from the MLN and from blood and cultured with OVA to evaluate the lymphocyte proliferative response by flow cytometry (CFSE cell proliferation kit) and

production of the main cytokine produced in adaptative immune response such as Interleukine-2 (IL-2) , IL-4, IL-10 and interferon-gamma (IFN-γ). Experimental animal studies have indicated that oral administration of antigens targets the systemic T cell compartment, diminishes cell-mediated immune responses, and induces tolerance. This phenomenon might lead to the induction of cytokines such as Transforming Growth Factor beta (TGF–β) and IL-10, and consequently enhance antigen-specific antibodies such as IgA and IgG. While the humoral immune response is critical in the control of some mucosal pathogens, those effects might be inappropriate on other mucosal pathogens where cell mediated immune responses may play a larger role.

2.1.2 Abstract 2

II) Milk rice in piglet nutrition: benefits or not?

Rice milk has been shown to contain a high sugar level and antioxidants such as ϒ-Oryzanol. For this reason the effects on growth performance and health status of piglets have been studied on 36 litters from (Landrace x LargeWhite) x Penerland sows. At 10 days after birth 18 litters were supplemented with rice milk (T) until weaning while the others 18 received creep feed (C). At 21 days a total of 288 piglets were weaned. Half from each group (72 piglets) were randomly assigned to either control (C) or treated (T), so there were four experimental piglet groups: C-C, C-T, T-C and T-T. All the animals received a pre-starter (0-14 d post weaning) and a starter (14-42 d pw) diets. Piglets from C-T and T-T groups were also supplemented with rice milk from weaning to 14 days pw. Piglets of T-C group had significantly higher weight at 42 d pw ($P<0.01$), an higher average daily gain (ADG) and dry matter feed intake (DMFI) ($P< 0.01$). Rice milk supplementation had no influence in glucose, urea, total protein and lysozyme. Reactive oxygen metabolites (ROMs) levels resulted lower in T-T piglets than C-C and C-T groups at 14 d-post w. ($P<0.05$). Total antioxidant capacity was higher in C-C and C-T than TC piglets at 14 d after weaning ($P<0.05$). The data from this trial suggest that rice milk was associated with greater post weaning growth and health status when supplementation was given only during the nursing period.

2.1.3 Abstract 3

III) Effects of plant polyphenols and mannan oligosaccharide on growth performance, antioxidant defense system and gut health in Escherichia coli Challenged piglets

Objective of the current study was to evaluate the effects of plant polyphenols (PP) and/or, mannan oligosaccharide (MOS) on growth performance, plasma antioxidant capacity and health in *E. Coli-challenged* weaned piglets. Ninety-six piglets (7.43 $\pm$ 0.89 kg L.W., 21 d age) were randomly allotted into 4 dietary treatments: control (basal diet), 0.1% PP, 0.1 % MOS or PP+MOS in a 6 weeks study. At 21 and 25 d on trial half piglets of each group were orally inoculated with 4 ml of E.coli (1×10^9 cfu/ml) and half with the same amount of saline water.

No difference was observed in growth. Plasma urea was markedly increased in PP+MOS group compared to PP group ($P< 0.05$), and lysozyme content was significantly decreased in PP+MOS group compared to groups on d 7 ($P< 0.05$). PP+MOS dietary supplementation decreased intestinal lipase and trypsin compared to PP on d 21 ($P< 0.05$). Plasma malondialdehyde content (MAD) increased, while plasma total antioxidant capacity (TAOC) and catalase (CAT) activities decreased in piglets 6 days after infection. Challenged piglets fed PP and those fed MOS showed higher TAOC than challenged piglets fed PP in combination with MOS. CAT activity resulted higher in challenged piglets fed diet supplemented with PP or PP+MOS than control or MOS at d 13 after infection ($P< 0.05$). The results showed that dietary PP or MOS had the potential to improve enhance systemic antioxidant capacity. However no synergic effect was observed when PP and MOS were combined.

Chapter 3

First animal trial

Evaluation of the immune response against ovalbumin (OVA) in pigs orally vaccinated with OVA in tablets containing carboxymethyl high amylose starch (CM-HAS), flagellin and sow's milk.

3.1 Abstract

This trial was designed with the aim to determine the effects of a new delivery system designed for oral vaccination or delivery of bioactive molecule in the gut. The trial was also designed to evaluate the potential of the sow's milk to modulate the systemic as well as local immune response against the antigen ovalbumin incorporated in the vaccine. Sixteen piglets of 28 days (d) of age were divided into 4 groups of 4 animals each. The first group was vaccinated with compressed tablets containing only ovalbumin (OVA); the second group was immunized with tablets containing ovalbumin and the B subunit of the flagellin from *Salmonella enterica* Typhimurium (OVA-FLA); the 3rd and 4th groups were immunized with tablets containing ovalbumin pre-incubated in sow milk with or without flagellin, respectively (OVA-LT or OVA-LT-FLA). The oral vaccination was performed in two periods of three days; the 1st period being from day 28 to day 30 of age (D1-D3 of the trial) and the 2nd from 42 d to 45 d of age (D14-D16). Blood samples were collected at days 1, 14, 21, 28 and 42 of the trial to determine, by ELISA, serum antibody titre against OVA. Feces samples were also collected before the first immunization and intestinal content from the ileum was collected at slaughtering to evaluate level of IgA anti-OVA by ELISA. All the animals were euthanized 42 days post-immunization to collect blood, mesenteric lymph nodes (MLN) and ileum content. The immune cells were isolated from the MLN and from blood and cultured with OVA to evaluate the lymphocyte proliferative response by flow cytometry (CFSE cell proliferation kit) and production of the main cytokine produced in adaptative immune response such as Interleukine-2 (IL-2) , IL-4, IL-10 and interferon-gamma (IFN-γ). Experimental animal studies have indicated that oral administration of antigens targets the systemic T cell compartment, diminishes cell-mediated immune responses, and induces tolerance. This phenomenon might lead to the induction of cytokines such as Transforming Growth Factor beta (TGF–β) and IL-10, and consequently enhance antigen-specific antibodies such as IgA and IgG. While the humoral immune response is critical in the control of some mucosal pathogens, those effects might be inappropriate on other mucosal pathogens where cell mediated immune responses may play a larger role.

Key words: Piglets, gut health, ovalbumin, sow's milk , oral vaccination, cytokines

3.2 Introduction

Over the past few decades, research regarding the development of new dietary formulation for weanling pigs to improve performance and health has gained in interest. Since then, new pathogens emerge and it appears that new formulations with functional foods are needed to enhance immunity without the use of antibiotics with growth promoting action. Rather, nutritional approaches to enhance immune function should focus on supplying the nutrients at the appropriate times and in the appropriate amounts that complement the 'pool' associated with increased nutrient partitioning for immune function. As nutrients continue to be examined for their immunomodulatory properties,

it is important to carefully select criteria to be used to determine their effectiveness or response (Fulton, 2004). Specifically, it will be important for future studies in this area to select assays that are accurate in their estimation and are relevant and/or related to disease resistance (Cunningham-Rundles, 1998). It is not clear how changes in relative immune organ weights relate to disease resistance. Furthermore, it is assumed that *in vitro* lymphocyte proliferative response to mitogenic stimulation involve similar signalization pathways and cytokine production that triggers *in vivo* proliferation of lymphocytes after antigenic stimulations (Jun 2009). The gut is the largest lymphoid tissue in the body and therefore represents an attractive target for modulating immunity. Gut associated lymphoid tissue can be modulated by feeding supplements of live microorganisms, probiotics or prebiotics. Feeding probiotics and prebiotics results in improved gut health and increased enteric and systemic immune responses to specific pathogens (Erickson et al., 2000; Rastall et al., 2002). Drastic changes in the gut microbial ecology are detrimental and can result in opportunistic infections. The immune system in the gastrointestinal tract plays a crucial role in the control of infection, as it constitutes the first line of defense against mucosal pathogens. The attractive features of oral immunization have led to the exploration of a variety of oral delivery systems. However, none of these oral delivery systems have been applied to existing commercial vaccines. To overcome this, a new generation of oral vaccine delivery systems that target antigens to gut-associated lymphoid tissue is required. One promising approach is to exploit the potential of microfold (M) cells by mimicking the entry of pathogens into these cells. Targeting specific receptors on the apical surface of M cells might enhance the entry of antigens, initiating the immune response and consequently leading to protection against mucosal pathogens. The aim of this preliminary study was to determine the potential encapsulated ovalbumin (OVA) into monolithic tablets made of carboxymethyl high amylase starch (CM-HAS) to induce a gut mucosal immune response against OVA. In previous study, CM-HAS has been already used succesfully to protect probiotics bacteria against gastrointestinal conditions (Calineescu et Matescuu, 2008). The filament of the flagella, that gives the mobility to the bacteria, is a polymer constituted mainly by flagellin. In this study, the B subunit of the *Salmonella enterica* serovar Thyphimurium flagellin was chosen for is property to bind to toll-like receptor-5 and to pass through the M cells (Azizi et al., 2010) of the intestinal mucosa and so, deliver antigen to the Peyer patches which underlies the Microfold (M) cells. Through this oral vaccination approach, the immunological properties of sow's milk was evaluated by measuring the potential of milk to modulate the immune response against ovalbumin encapsulated in CM-HAS.

The experiment was subdivided in two animal trials.

3.3 Materials and methods

3.3.1 First animal trial

This first animal trial was designed to evaluate the potency of sow's milk to modulate humoral immune response following an intramuscular administration of ovalbumin with or without sow's milk.

3.3.1.2. Reagents and vaccine preparation

Ovalbumin and incomplete Freund adjuvant were purchased from Sigma-Aldrich. Sow's milk obtained at day 3 of lactation was pasteurized by heating the sample at 60 ºC for 30 minutes with gentle shaking. One hundred microliter of milk were plated on nutrient agar and MacConkey No.2 agar plates to verify the pasteurization process.

3.3.1.3. Animals, vaccine administration and blood samples

Twenty four Yorkshire X Landrace piglets weaned at 21 days were split in 3 groups ($n=8$). The piglets came from 3 different litters and were divided to ensure homogeneity between groups according to litter, weight (9.1± 0.95 kg) and sex in each group. The animals were housed in separate pens according to their groups. All the piglets were vaccinated at day 1 (28 days of age) and day 14 of the project. Group A was vaccinated with 1 mg ovalbumin that was emulsified with incomplete freund adjuvant. Group B was vaccinated with 1 mg ovalbumin that was mixed with 20 µL of pasteurized sow's milk. Group C was vaccinated with 1 mg ovalbumin that was mixed with 20 µL pasteurized sow's milk and was then emulsified in incomplete freund adjuvant. Blood samples were taken from the jugular vein at days 1, 14, 21, 28, 35 and 42 after the first vaccination.

3.3.1.4. Enzyme-linked immunosorbent assay against ovalbumin

After blood coagulation at room temperature and centrifugation, serum samples were stored at -20ºC until analyzed. Anti-OVA antibodies were measured by specific ELISA. Microtiter plates (Maxisorp, Nunc) were coated with a solution of 8 µg ovalbumin/mL 50 mM carbonate buffer pH 9.6. The plates were incubated overnight at 4ºC. The plate was blocked with 0.05% Tween 20 in phosphate buffered saline (PBST) pH 7.2 for 1 hour at 37ºC. Pre-titered serum used as standard and serum samples were diluted in PBST and then distributed in the plates. The plates were placed at room temperature for 2 hours. Horseradish-peroxydase (HRP) coupled anti-pig IgG (bethyl)

diluted in PBST was then added to the wells and incubated for 1 hour at 37ºC. The plates were revealed with 100 µL TMB 2-component system (Kirkegaard, Mandel) for 3 minutes and then stopped with 100 µL of 2M sulfuric acid. Plates were read on a SpectraMax 250 Microplate Reader at a wavelength of 450 nm. Between each step, the plates were washed 5 times with PBST. The titer was extrapolated from the standard curve.

3.3.1.5. Ovalbumin specific cell proliferation

On day 35 following the first immunization, heparinized blood was collected from the pigs of the 3 groups. Peripheral blood mononuclear cells (PBMCs) were obtained by layering 30 ml of blood on Ficoll-Paque PLUS density gradient and were processed as described by Lessard *et al*. (2005). The cells were counted with Countess® automated cell counter (Invitrogen). 1,5x 10^7 cells were then transferred to a 15ml culture tube and centrifuged at 750 x g for 5 minutes at room temperature. The cells were then resuspended in 750 µL of 0,5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) and then incubated for 15 minutes at 37ºC in a 5% CO_2 humidified atmosphere. The cells were then centrifuged 5 minutes at 750 x g at room temperature and placed in a quenching media (RPMI + 7,5% Donor calf serum) to remove excess of dye. After a 15 minutes incubation at room temperature, cells were centrifuge 5 minutes at 750 x g at room temperature and resuspended in complete media (RPMI + 10 % fetal bovine serum + 1X penicillin-streptomycin solution) and then put in 24-wells cell culture plate at a concentration of 2.5x 10^6cells per well. The cells were then left unstimulated (control) or stimulated with 5 µg OVA/mL of media. The cells were then incubated for 6 days at 37ºC in a 5% CO_2 humidified atmosphere. After de proliferation period, the cells were centrifuged 5 minutes at 750 x g at room temperature and resuspended in fixation buffer (1% formaldehyde diluted in ISOTON II (Beckman coulter))

3.3.1.6. Flow cytometry analysis of cell proliferation

The samples were analysed with a Becmand Coulter XL_MCL flow cytometer equipped with a 488nm argon ion laser. Cells were first gated according to forward scatter and side scatter to analyse the data from living lymphocytes. Then the fluorescence level was evaluated with FL1 channel. The voltage was set to obtain fluorescence of non-stimulated cells (control cells with no fluorescence) in the third log of FL1. A gate was place on the non stimulated cells sample of each animal with a percentage of gated cells of no more than 2%. The percentage of relative proliferating cells was then obtained from the OVA stimulated cells. For each animal, all samples were taken in duplicate.

3.3.2. Second animal trial

3.3.2.1. Cloning and expression of Salmonella enterica serovar Typhimurium's B subunit Flagellin

The purified FLA used for this study was kindly prepared by Dr. Archibault lab.

3.3.2.2. Tablets preparation

The monolithic tablets of carboxymethyl high amylose starch (CM-HAS) were provided by Dr. Mateescu. They were prepared as described by Calinescu & Mateescu (2008). The probiotic bacteria used in their experiment was replaced by 100 mg of ovalbumin (OVA) that was premixed, or not, for 1h with sow's milk (SM). Ten microgram of recombinant flagellin (FLA) prepared in Dr Denis Archambault's laboratory by Aurélie Girard (PhD student) and graciously given was added, or not, to the tablets. Hence, four types of tablets were produced: 1) CM-HAS + OVA; 2) CM-HAS + OVA + FLA; 3) CM-HAS + OVA + SM; 4) CM-HAS + OVA + FLA + SM.

3.3.2.3. Animals, vaccine administration, blood samples and feces samples.

Sixteen Yorkshire X Landrace piglets weaned at 21 days were split in 4 groups (*n=4*). The piglets came from 4 different litters and were divided to ensure homogeneity between groups according to litter, weight (10.74± 0.33 kg) and sex of the animals in each group. The animals were housed in separate pens according to their groups. All the piglets were vaccinated by tablets administration on 3 consecutive days on days 1 to 3 (28±1 to 30±1 days of age) and again, on 3 consecutive days from day 14 to 17 of the project. Blood samples were taken at days 1, 14, 21, 28, 35 and 42 after the first day of vaccination. Serum was stored at -20ºC until analysed. Feces were collected on day 1 and 42 of the project. Feces were diluted 10 times in PBS buffer, homogenized in stomacher 2 X 60 second and then centrifuged at 3000 x g for 20 minutes at 4ºC. The supernatant was stored at -20ºC until analysed. The animals were euthanized at day 42 and mesenteric lymph nodes were used for cell proliferation assay (as described in sections 2.1.4 and 2.1.4.1) and gene expression study.

3.3.2.4 Gene expression study

Mesenteric lymph node were kept on ice in Hanks balanced salt solution without calcium and magnesium (HBSS) containing penicillin (200 I.U./mL) and streptomycin (200 µg/mL). The lymph nodes were grinded in small pieces and then passed several times into a 5 ml syringes to obtain a single cell suspension. Cell suspension was transferred into 15 ml conical tube. The debris were

allowed to decant for 5 minutes and the supernatant containing cell suspension was carefully layered over 4 mL of Ficoll Paque Plus. After 40 minutes of centrifugation at 750 x g, the mononuclear cells were removed and diluted in 10 ml of HBSS containing antibiotics. Cells were centrifuged and washed twice. Cells were then counted and $1x10^7$ cells were placed in a 6 well culture plate with 3 ml per well of RPMI containing antibiotics and 10% FBS. The cells were then stimulated with media (negative control) Concanevalin A at 0,5 µg/ml (positive control) or ovalbumin at 10 µg/ml. The supernatant was carefully removed after 24 hours of incubation in a humidified atmosphere with 5% CO_2 and cells were lysed adding 1 mL of Trizol reagent (Invitrogen). Cells were then mixed and transferred into tubes before freezing them at -80ºC. The RNA was extracted following the Trizol manufacturer's instructions. Six micrograms of RNA were digested with DNase from Ambion's DNA-free kit following the manufacturer's recommendations. For each sample 1 µg of total RNA was used for cDNA synthesis using Superscript reverse transcriptase (200U/reaction; Invitrogen) and anchored oligo-dT-primers (500 ng/reaction).The reaction was carried out in a final volume of 20 µL in an appropriate buffer (50 mM TrisHCl, pH: 8.3; 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT) and dNTPs (final concentration of 0.5 mM) for 1 h at 42°C. The cDNA samples were diluted 1:15 in ultrapure RNase DNase free water and frozen at -20°C till being used. Real-time PCR was performed with the 7500 Fast Real-time PCR system (Applied Biosystems, USA) to evaluate the expression of the cytokines IL-2, IL-4, IL-10, IFN-γ, and of the housekeeping genes cyclophillin and β-actin. Primers for cytokines as well as for two housekeeping genes, accession numbers of target gene sequences, concentration of primer used and amplicon sizes are described in Table I. The PCR mixture was composed of: 5 µl *POWER SYBR*® Green Master Mix (Applied Biosystems, USA), 0.1 µl of AmpErase®Uracil N-glycosylase (Applied Biosystems, USA), each set of primers (Applied Biosystems, USA) specific to molecules and housekeeping genes at the concentrations indicated in Table I, 3 µl of 1:15 diluted cDNA, and ultrapure water to a final volume of 10 µl. Primers were designed using the IDT SciTools PrimerQuest software program (http://www.idtdna.com/Scitools/Applications/Primerquest/) and selected using the following criteria: (1) forward and reverse primers were placed on two consecutive exons, (2) no more than two Gs or Cs within the last five nucleotides in the 3′ termini, (3) the calculated melting temperature of each primer, with 50mM Na+, had no more than 2.5ºC between the primer pair (4) primer length between 19 and 24 pb, (5) primer design insure minimal self annealing and primer dimers. The PCR cycling conditions used were in accordance with the manufacturer's protocol. an initial Taq-polymerase activating step at 50 ºC for 2 min followed by a denaturation step at 95 ºC for 10 minutes, then 45 cycles with a 3 sec at 95 ºC denaturation step, 0:30 min at 60 ºC annealing and extension step, during which data were collected. A melting curve

was also done to assess the amplification of only desired amplicon. Level of expression was determined using a standard curve of a dilution series of DNA plasmid for each gene tested (Pfaffl MW *et al*., 2001). These plasmid standards were created by cloning gene-specific cDNA PCR products using a TA Cloning kit according to the manufacturer's instructions (TOPO®TA cloning dual promoter kit, Invitrogen, Burlington, Ontario, Canada). The normalized expression level of the target gene was obtained as follows: target copy number/β-actin copy number. Amplification specificity was checked using a melting curve following the manufacturer's instructions. Each run included a no template control to detect DNA contamination of the reagents and each reaction was performed in quadriplicate.

3.3.2.5 ELISA and specific cell proliferation against OVA

The ELISA was performed on serum samples and on diluted feces supernatant as described in section 4.1.3. Anti-pig IgG and IgA coupled to HRP were used to measure both antibody isotypes in feces supernatant. The specific cell proliferation induced by ovalbumin was also measure in blood, as described in section 4.1.4 and 4.1.4.1.

3.4. Statistical analysis

A statistical analysis was carried out by using the GLM procedure of SAS (SAS institute v.9e) to evaluate the presence/absence of response of the different kind of vaccination for parameters such as PBMCs specific proliferation, feces IgA-IgG contents and cytokines (IL2, IL4, IL10, IFN-ɣ).

Statistical analysis was performed using the procedure PROC MIXED of SAS (SAS Institute v.9e) to calculate the presence/absence of response of the variable serum IgG to identify signifcant differences between groups ($P< 0.05$; $P<0.01$).

3.5. Results

3.5.1 First animal trial

3.5.1.1 Measure of circulating antibody against ovalbumin

A strong increase in OVA specific serum antibody (Fig.1) were observed 21 days after the vaccination for the animals vaccinated OVA+IFA and with a tendency to decrease during the last weeks of the trial with an effect on time ($P<0.01$). For the group vaccinated OVA+SM+IFA the peak of specific serum antibody (Fig.1) was observed 28 days after the inoculation of the vaccine and decreased progressively until 48 d with an effect on time ($P<0.01$). For the OVA+SM group the

test didn't find a high level of OVA-specific serum antibody (Fig.1). These results indicate that the immune response was higher in the animals vaccinated with the OVA in the presence of the Incomplete Freund Adjuvant (OVA+IFA) compared to animals of OVA+SM group from 21 to 42 d and OVA+IFA+SM group at 21 d ($P<0.01$). This result indicate that sow's milk use at our dosage was not as good as IFA to induce a strong immune response to OVA. Although antibody response was lower in OVA+SM the same decours of the animals vaccinated OVA+IFA with a peak at 21 days after the first vaccination with an homogeneous decrease until the end of the first animal trial. Adjuvant potential of the Sow's milk use at our dosage was not strong enough to determine a high presence of OVA specific serum antibody during the trial, although a low presence of the antibody was detected during the first animal trial and seems to have the same decours of the animals vaccinated OVA+IFA with a peak at 21 days after the first vaccination with an homogenous decrease until the end of the first animal trial ($P <0.01$).

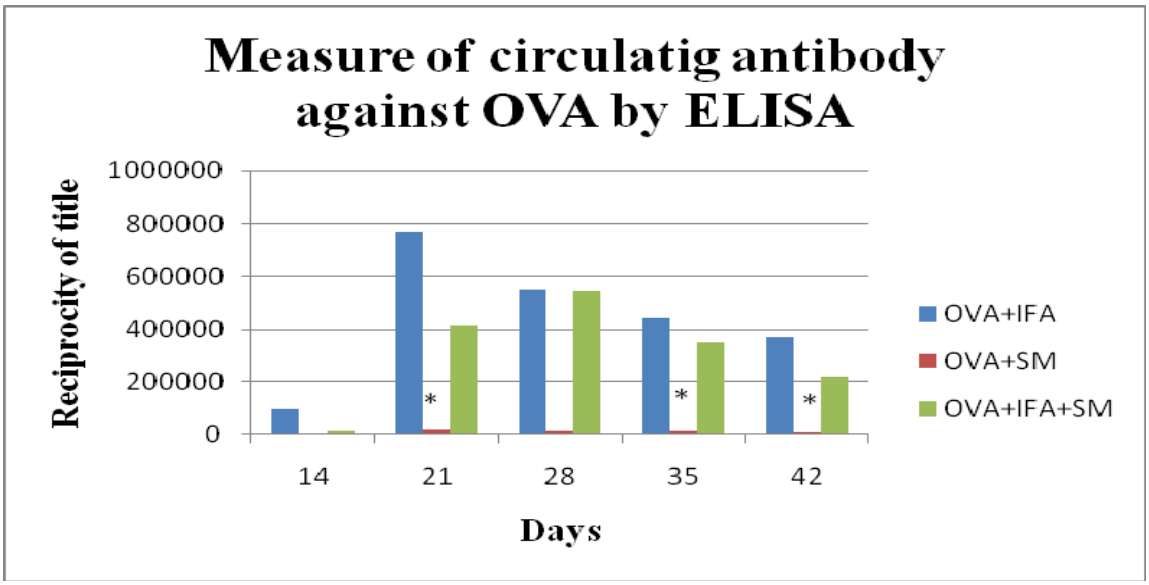


Fig 1. Measure of circulating antibody against ovalbumin. Piglets (28 days of age) coming from 3 different litters were vaccinated at day 1 and day 14 of the project. Group OVA+IFA (A) was vaccinated with 1 mg ovalbumin that was emulsified with incomplete freund adjuvant. Group OVA+SM (B) was vaccinated with 1mg ovalbumin that was mixed with 20 µL pasteurized sow's milk. Group OVA+SM+IFA (C) was vaccinated with 1 mg ovalbumin that was mixed with 20 µL pasteurized sow's milk and was then emulsified in incomplete freund adjuvant. Blood samples were taken at days 1, 14, 21, 28, 35 and 42 after the first vaccination.

*OVA specific antibody mean reciprocity of titre in piglets (n=8/treatment). Animals (28 days of age) were vaccinated 2 times at days 1 and 14 of the project. OVA+IFA→ Ovalbumin+ incomplete Freund adjuvant; OVA+SM → Ovalbumin + sow's milk; OVA+SM+IFA → Ovalbumin + sow's milk + incomplete Freund adjuvant.

3.5.1.2 Measure of specific in vitro cell proliferation of Peripheral blood mononuclear cells after antigen (ovalbumin) recall

Peripheral blood mononuclear cells proliferation marked with CFSE placed in the presence of OVA (Table 2 and Fig.2) was higher in the group vaccinated with OVA+IFA but not statistical differences were seen between the different groups. The animals vaccinated OVA+SM had tendencial higher proliferation percentage compared to the ones vaccinated OVA+SM+IFA. No significative difference in cell proliferation was seen and the level of circulating proliferating cells was shown.

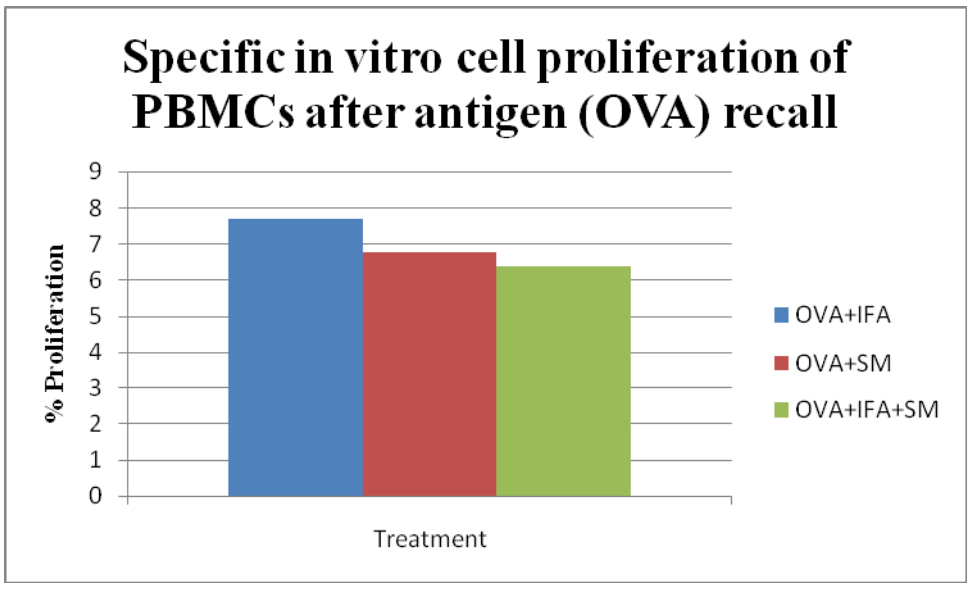


Fig.2 Specific peripheral blood mononuclear cells proliferation after in vitro antigen recall. On day 35 following the first immunization, heparinized blood was collected from the pigs of the 3 groups and isolated on density gradient.. The cells were then left unstimulated (control) or stimulated with 5 µg OVA /mL of media. After a 6 days incubation, the proliferation was measured by flow cytometry. Control cells were used to gate basal proliferation, wich was substracted from proliferation seen in OVA-stimulated wells.

3.5.2 Second animal trial

3.5.2.1 Measure of circulating antibody against ovalbumin

Higher serum IgG concentrations on day 21, 28, 35 and 42 ($P<0.05$) was observed in the group orally vaccinated with OVA+FLA+SM compared to other groups. The levels of serum IgG were significantly lower during all the trial period in the animal vaccinated orally with ovalbumin compared to the other groups.

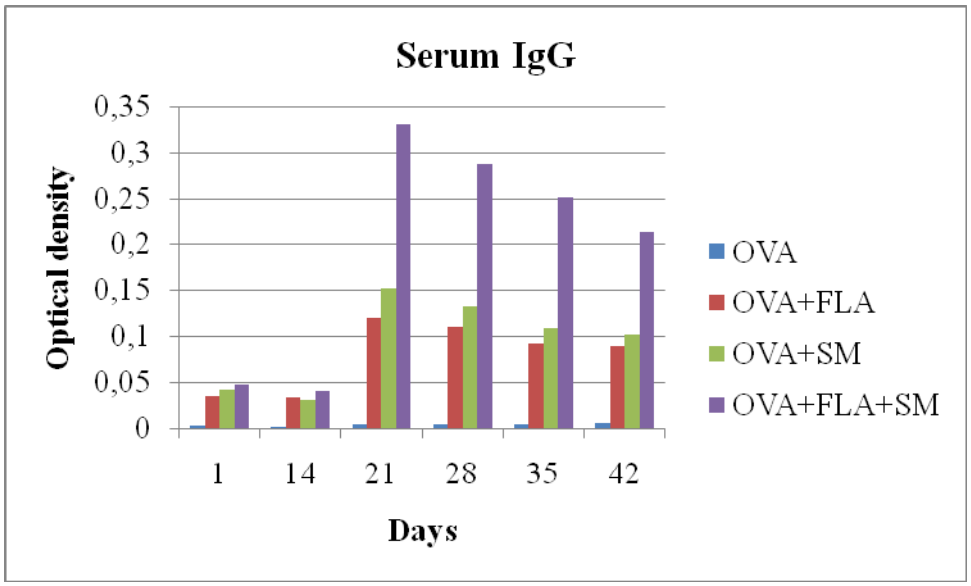


Fig.3 **Measure of IgG in the serum of animals vaccinated orally with ovalbumin by ELISA**

3.5.2.2 Measure of IgG and IgA in feces by ELISA

After oral administration of the four different kind of vaccine the IgA level were higher at day 42 compared to the IgA at day 1 of the trial ($P<0.05$). As shown in the Fig. 3 the level of the IgA at the beginning of the trial was homogeneous for all groups. Secretion and excretion of IgA into feces were remarkably higher in the group which received the oral vaccination with OVA+FLA+SM compared to the OVA+SM at 42 d after weaning ($P<0.05$). The amounts of IgA into feces in the OVA-oral vaccinated was lower at the end of the trial ($P<0.05$). At the beginning of the study also the level of IgG in the animals of the four groups were omogeneous. At the end of the trial the animals of the group vaccinated by OA+FLA+SM registered higher value of secreting and excreting IgG into feces.

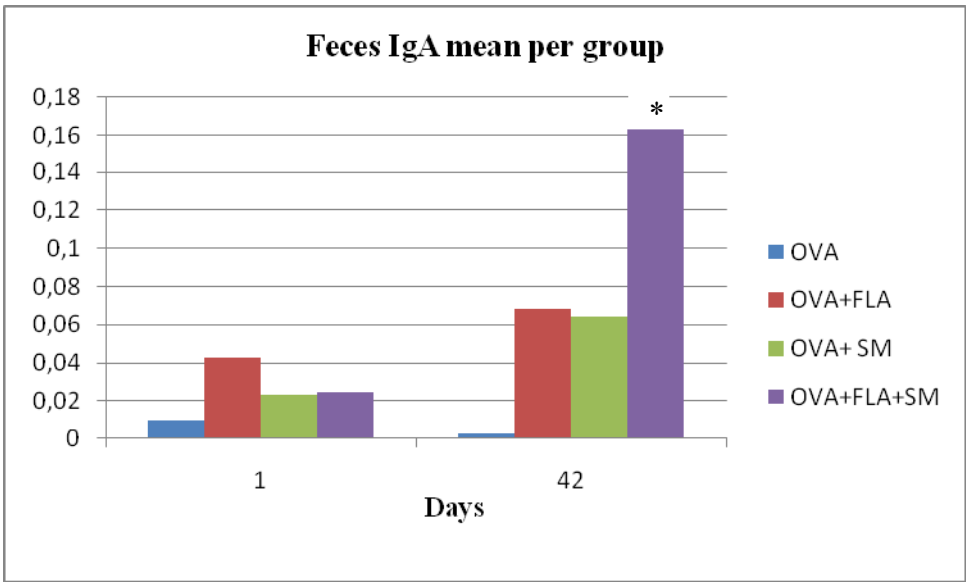


Fig.3 Measure of IgA in the feces of animals vaccinated orally with ovalbumin by ELISA. OVA: ovalbumin, OVA+FLA: ovalbumin+flagellin, OVA+SM : ovalbumin + sow's milk and OVA+FLA+SM: ovalbumin + flagellin + sow's milk

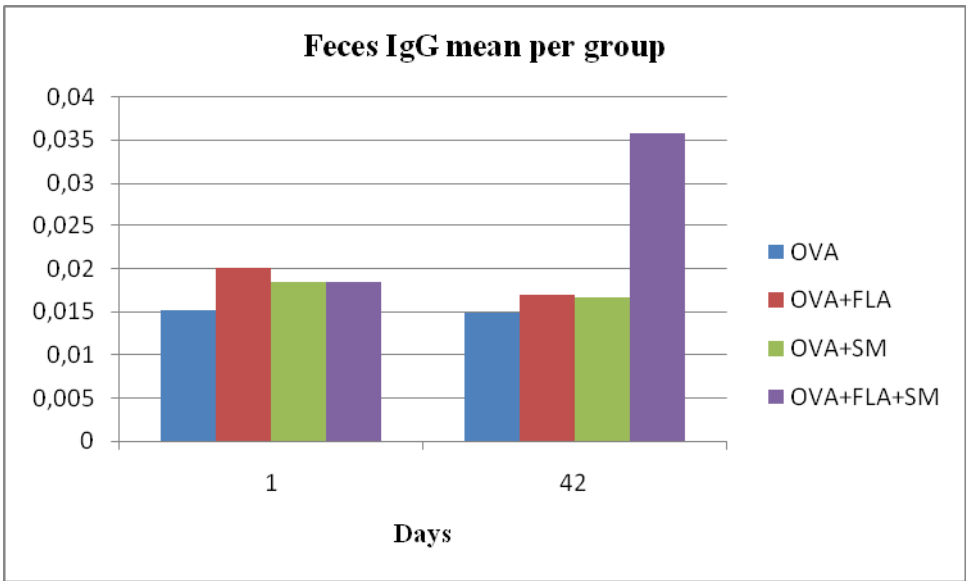


Fig.4 Effects of oral vaccination with OVA (ovalbumin), OVA+FLA (ovalbumin+flagellin),OVA+SM (ovalbumin + sow's milk) and OVA+FLA+SM (ovalbumin + flagellin + sow's milk) on IgG secretion and excretion into feces.

3.5.2.3 Measure of specific in vitro cell proliferation of PBMCs after antigen (OVA) recall

No statistical differences were shown for peripheral blood mononuclear specific cells proliferation marked with CFSE, in the presence of OVA, FLA or OVA+FLA between the four oral vaccinated groups (Fig.5). The animals vaccinated with OVA shown the lowest proliferation percentage

compared to the other three groups. The PBMCs cells of the group OVA+SM shown higher specific proliferation percentage compared to the OVA and OVA+FLA+SM groups when stimulated with OVA.

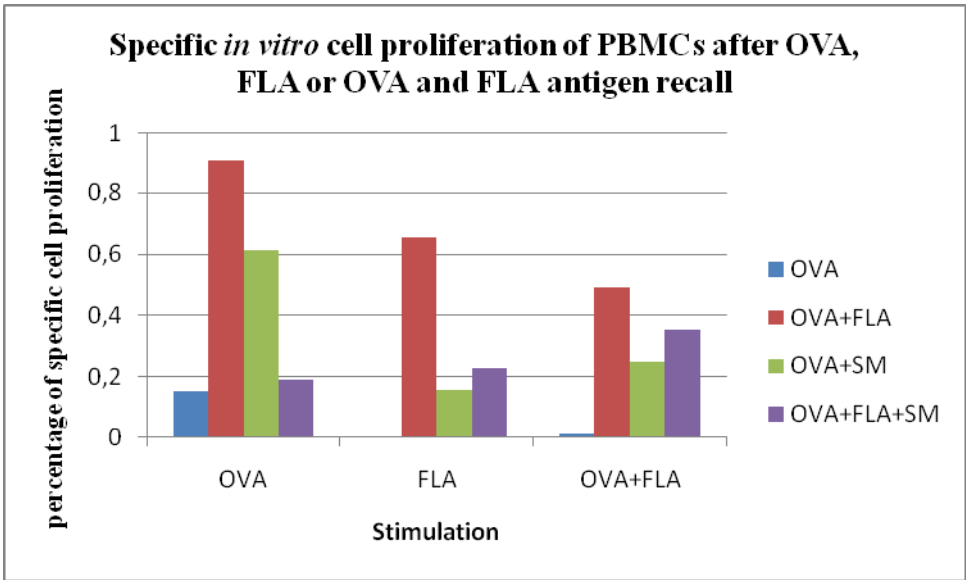
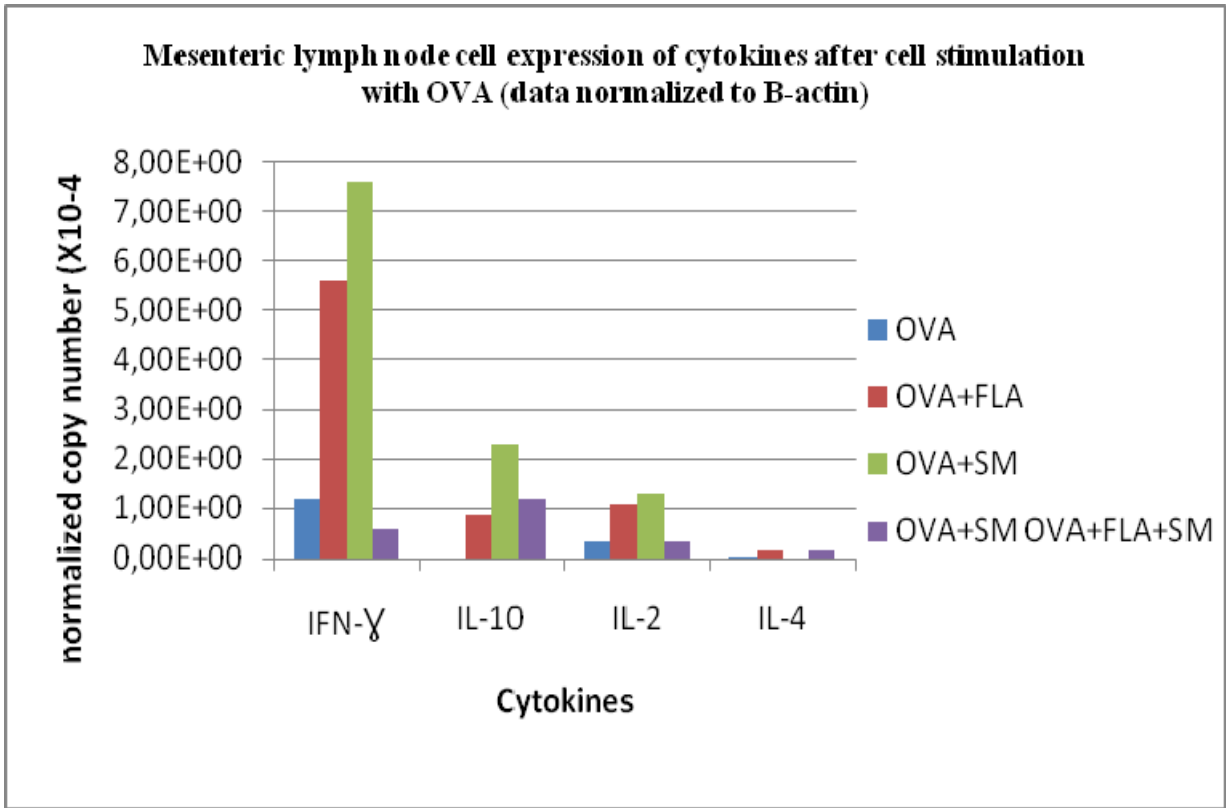


Fig.5 PBMCs (peripheral blood mononuclear cells) proliferative response to OVA (ovalbumin), FLA (flagellin) or OVA+FLA (ovalbumin+flagellin) after oral administration of four kind of vaccine: OVA, OVA+FLA, OVA+SM (ovalbumin+sow's milk) or OVA+FLA+SM.

3.5.2.4 Results of real-time PCR: cytokine expression of MLN (mesenteric lynph node) cells stimulated in vitro with OVA.

Cytokines produced by MLN cells were analyzed following *in vitro* culture in the presence of OVA. Fig.6 and 7 show the levels of expression in cell culture of IL-4, IL-10 and IFN- ɣ.. The MLN cytokine mRNA expression profiles from piglets after vaccination shows in Fig.6 that the expression of interferon gamma, the main TH1 cytokine driving cytotoxic response of $CD8^+$ T cells was expressed at least 5 times more, when compared to ova vaccination alone, in the groups vaccinated in the presence of flagellin or sow milk but not in the combination of the two product, showing an negative effect of the combination. The same profile was observed with the IL-2, showing that the two treatments gived better proliferation than OVA alone or the mix of the thwo component. The production of IL-10 in the groups receiving sow milk showed the potency of sow milk to odulate immune response toward a tolerance profile. The level of IL-4 production was surprisingly low for all groups, indicating a strong bias to the TH1 profile. The high presence of IL-2 was registered by the OVA group after the cells stimulation with the positive control of

proliferation: Concanavalin A (ConA) was expected since this lectin induce strong proliferation of T cells.



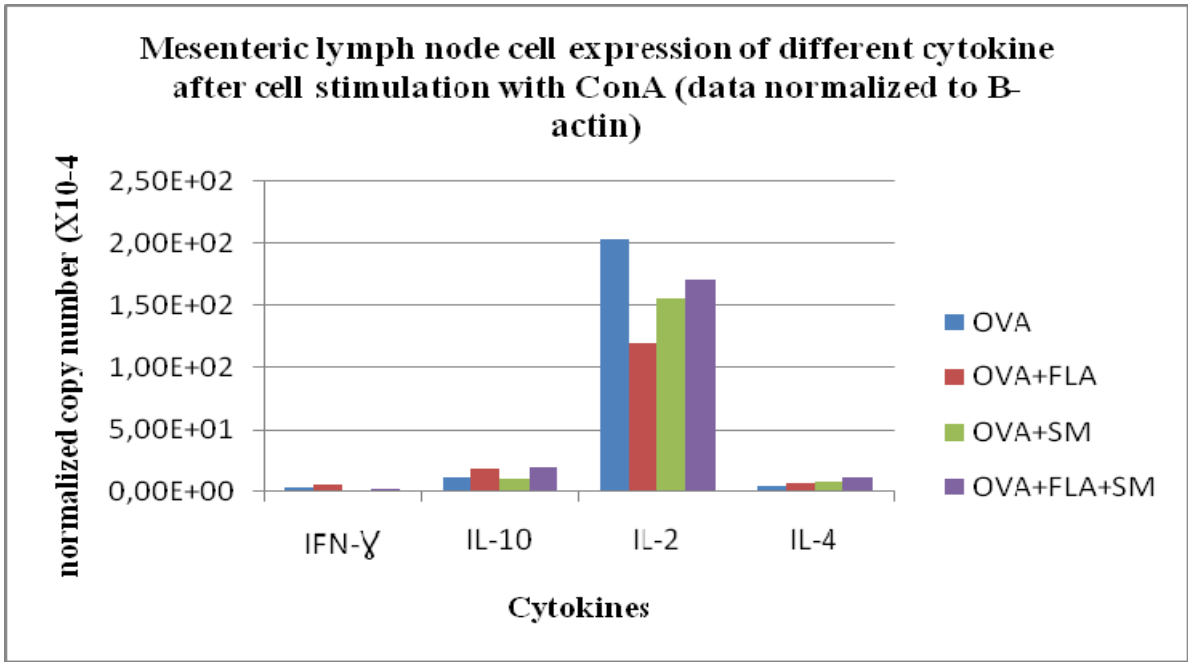


Fig.6-7 Cytokine mRNA expression in mesenteric lymph node after isolation and cell culture in the presence of the the vaccine antigen ovalbumin (OVA; Fig. 6) or a positive control of proliferation, concanavalin A (ConA; Fig. 7).

3.6 Discussion and conclusion

Over the past few decades, oral immunization has been extensively studied due to its many attractive features. The immunological potential, absorption, or limitation in the uptake of antigens, as well as the characteristic distribution of functional cell types in the gastrointestinal tract (GI) tract, have made it a vital target in the development of oral vaccines. The phenomenon of tolerance is a crucial challenge to overcome in the development of effective oral vaccines. Experimental animal studies have indicated that oral administration of antigen targets the systemic T cell compartment, diminishes cell-mediated immune responses, and induces tolerance. This phenomenon might lead to the induction of cytokines such as IFN-ɣ and IL-2, and consequently enhance antigen-specific antibody responses such as IgA and IgG (Azizi et al., 2010). While the humoral immune response is critical in the control of some mucosal pathogens, its effect might be questionable for other mucosal pathogens where cell-mediated immune responses may play a larger role. On the other hand the absence of a potent oral vaccine might be due to other challenges, including antigen degradation by proteolytic enzymes, the low dose of antigen absorbed, a lack of potent mucosal adjuvants, the absence of actively directed antigens to microfold cells (M cells). To overcome these issues, further investigations regarding oral vehicle delivery systems that protect antigens and specifically target M cells are required. Targeting M cells by mimicking the entry of mucosal pathogens such as pathogenic *E. coli*, *Salmonella*, and *Yersinia* may reflect the *in vivo* binding specificity required by orally administered antigens. Regarding this aspect, a number of studies showed that these pathogens bind to specific lectins expressed on the apical surface of M cells (Holmgren et al., 2005). The binding of orally administered vaccines to M cell lectins was further studied in murine models and indicated that a-L-fucose-lectin (UEA-1) is able to bind specifically to M cells and, to a lesser degree, enterocytes (Holmgren et al., 2005). However, the characterization of murine M cells by this lectin-binding pattern did not reflect the glycosylation patterns of human M cells. Unfortunately, pig M cell features, function, and differentiation from neighboring enterocytes are not well understood. Further investigations using nutritional strategies and oral vaccination might generate more specifically direct oral delivery of antigens to pig M cells. However, as these molecules are also expressed on neighboring enterocytes, it will likely be difficult to devise an ideal oral delivery system for targeting M cells. The understanding of M cell function, identification of more specific apical surface molecules, and the improvement of intestinal M cell–like models are crucial for the design and further development of M cell–targeted vaccines. Of course we have to remember that i*n vitro* assays are commonly used on standardized cell number as well as isolated cell populations, which does not reflect the cellular environment *in vivo*.

Until now, a small number of papers have been published to investigate the effect of oral vaccination with OVA, SM and FLA on humoral immunity in weaned piglets. IgG and IgA, the major serum immunoglobulins, are key components of the humoral immunity in all mammals (Li et al. 2007). Our results indicate, although the overall level of specific immunoglobulin was low, that the vaccination with OVA+FLA+SM can influence an immune response increasing feces IgA and IgG levels and was accompanied by enhanced serum IgG concentrations in weaned piglets. The results suggests that the presence of SM or FLA can influence the immune response against OVA and that there is a synergy between the two compounds (FLA+SM) but more knowledge will be neeed to understand exactly the way of action of these compounds. Politis et al. (2007) demonstrated the immune stimulatory action of milk to post weaned piglets and also in the current study the mechanism by which this vaccination enhanced immunoglobulin levels may be attributed to the immunostimulatory action of the peptide contained in the milk. Interestingly, introduction of these bioactive compounds to the immunological immature neonatal gastrointestinal tract may potentiate an inappropriate immune response. Immune stimulation accumulates in the partitioning of nutrients from normal growth to support components of the immune response improving also growth performance of the animals (data not shown) during a critical phase as the weaning. Cytokines are important molecules mediating antibody production and thus the immune response in the host. IL-2 and IL-6 are involved in immune regulation and host defense (Li et al. 2007). IL-10 can inhibit the synthesis of pro-inflammatory cytokines such as IFN-γ and can suppress the antigen presentation capacity of antigen presenting cells (ajouter référence). IL-2 regulates the differentiation and activation of T cells, B cells, natural killer and lymphocyte-activated killer cells, monocytes and macrophages that are involved in cellular and humoral immune responses (Wang 1991; Liu & Liu 1992). IL-4 plays a critical role in activation of B-cell and T-cell proliferation and is a key regulator in humoral and adaptive immunity. IFN-ɣ has potent antiviral properties that contribute to the control of acute viral infections and is an important mediator of cellular responses. Compelling evidence shows that many types of bioactive compounds can increase the production of cytokines (including IL-2 and IL-4), which play important roles in immune responses (Liu & Liu 1992). In the current study, the treatment with OVA+FLA or OVA+SM but not the combination of the two immunomodulator (OVA+FLA+SM) enhanced MLN mRNA expression of IFN-ɣ an IL-2. Thus, the current results suggest that the oral vaccination with components such as milk or flagellin has the potential to activate lymphocytes and to modulate cytokine response in weaned piglets. The gut is a major immune organ in mammals. Specifically, the T and B lymphocytes proliferate and mature in the gut-associated lymphoid tissue (GALT), mounting a successful immune response to

antigens. Many studies have shown that the GALT is composed of immune cells and lymph nodes, conferring both non-specific and specific immune functions (Poussier & Julius 1994). IFN-ɣ probably plays a key role in activation, proliferation and differentiation of T-cells, as well as improving both specific and non-specific immune responses in weaned piglets. The enhanced expression of IL-2, which is important for primary T-cell activation, could result in improved immunostimulation in pigs vaccinated with CMS tablets containing OVA with either FLA or SM. It is likely that stimulation of lymphocytes and modulation of cytokine production contributed to improve antibody response to OVA by the gut immune system. Further studies will explore the potential of these new diets strategies to prevent the negative impact of a pathogen challenge.

In conclusion, oral vaccination could represent the future of the nutrition thanks to its positive action directly on the gut health influencing humoral immune response in piglets, suggesting an enhancement of host defenses against presenting pathogens.

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Table I. List of genes and sequences of the primers used for real-time PCR.

mRNA target	Accession number	Primers (5' → 3')[a]	Product size (bp)	Final concentration (nM)[b]
IL-4	AY294020	F:GGTCTGCTTACTGGCATGTACC	117	F 150
		R:CTCCATGCACGAGTTCTTTCTC		R 150
IL-10	L20001	F:GATATCAAGGAGCACGTGAACTC	137	F 300
		R:GAGCTTGCTAAAGGCACTCTTC		R 300
IFN-γ	NM_213948	F:AGGTTCCTAAATGGTAGCTCTGGG	101	F 300
		R:AGTTCACTGATGGCTTTGCGCT		R 300
β-actin	U07786	F:CTCTTCCAGCCCTCCTTCCT	104	F 300
		R:GCGTAGAGGTCCTTCCTGATGT		R 300
PPIA	NM_214353	F:TGCAGACAAAGTTCCAAAGACAG	133	F 300
		R:GCCACCAGTGCCATTATGG		R 300

[a]F and R indicate forward and reverse primers, respectively.
[b]Final concentration of forward (F) and reverse (R) primers.

Chapter 4

Second animal trial

Milk rice in piglet nutrition: benefits or not?

4.1 Abstract

Rice milk has been shown to contain a high sugar level and antioxidants such as ɣ-Oryzanol. For this reason the effects on growth performance and health status of piglets have been studied on 36 litters from (Landrace x LargeWhite) x Penerland sows. At 10 days after birth 18 litters were supplemented with rice milk (T) until weaning while the others 18 received creep feed (C). At 21 days a total of 288 piglets were weaned. Half from each group (72 piglets) were randomly assigned to either control (C) or treated (T), so there were four experimental piglet groups: CC, CT, TC and TT. All the animals received a pre-starter (0-14 d post weaning) and a starter (14-42 d pw) diets. Piglets from CT and TT groups were also supplemented with rice milk from weaning to 14 days pw. Piglets of TC group had significantly higher weight at 42 d pw ($P<0.01$), an higher average daily gain (ADG) and dry matter feed intake (DMFI) ($P< 0.01$). Rice milk supplementation had no influence in glucose, urea, total protein and lysozyme. Reactive oxygen metabolites (ROMs) levels resulted lower in TT piglets than CC and C-T groups at 14 d-post w. ($P<0.05$). Total antioxidant capacity was higher in CC and CT than TC piglets at 14 d after weaning ($P<0.05$). The data from this trial suggest that rice milk was associated with greater post weaning growth and health status when supplementation was given only during the nursing period.

Key words: piglets, rice milk, growth performance, antioxidant capacity, ɣ-Oryzanol

4.2 Introduction

Rice milk is a kind of grain milk processed from rice. It is mostly made from brown rice and commonly unsweetened. The sweetness in most rice milk varieties is generated by a natural enzymatic process, cleaving the carbohydrates into sugars such as glucose and maltose. Compared to cow's milk, vegetable milks contain more carbohydrates, but do not contain significant amounts of calcium or protein, and no cholesterol or lactose. Commercial brands of vegetable milks such as rice milk, however, are often fortified with vitamins and minerals, including calcium, vitamin B12, vitamin B3, and iron. Characteristic of this product is beside the high level of glucose and maltose and high level of ɣ-Oryzanol, a mixture of ferulic acid, esters of sterol and triterpene alcohols

(Table 2-3-4). In addition to its outstanding antioxidant activity, it has been demonstrated that γ-Oryzanol has multiple neutraceutical functions, including reduction of total cholesterol, improvement of the plasma lipid pattern and inhibition of platelet aggregation (Juliano et al.,2005; Nam et al., 2008). Recent studies report that γ-Oryzanol acts as an active scavenger of diphenyl picrylhydrazyl (DPPH), hydroxyl, and superoxide radicals, and efficiently protects against lipid peroxidation (Xu et al., 2001). These studies suggest that OZ has high potential as a functional food ingredient and as a stabilizer of lipidic raw materials (Ismail et al., 2010). γ-oryzanol showed potential antioxidant *in vitro* activity similar to vitamin E in the regulation of antioxidants and oxidative stress gene markers (Xu et al., 1999).

Before and after weaning the piglets undergo a stress-related growth check, often associated with anorexia and under-nutrition, with predisposition to diarrhea and infection. Due to its richness in carbohydrates such as glucose and maltose rice milk may play an important role in nursery piglets thanks to its high palatability and sugars and antioxidant availability. High feed palatability with sweeteners may also improve feed intake before and immediately after weaning (Naranjo et al., 2010). Moreover carbohydrates present in rice milk may represent an important source of energy for the weaker piglets of litters which do not have enough strength to compete with the brothers for a correct consumption of maternal milk (Stephas et al., 1998). Carbohydrates sources have been evaluated as partial or total replacements for lactose in manufactured liquid diets for young pigs. Koreans researchers fed early weaned piglets (6,5 kg) with diets supplemented with lactose or maltose. No differences were shown on performance between the two treatments (Lee et al., 2000). The carbohydrate sources of rice milk (glucose and maltose) probably play an important role during the nursing phase determining more homogeneous weight at weaning of the piglets. In literature is reported that glucose can be a satisfactory energy source of carbohydrates such as lactose for the pig during its first week of life (Aherne et al., 1969). A study where lactose and glucose were compared as carbohydrate sources in piglets from 1 to 22 days of age shown that ADG, gain feed and the apparent digestion for crude protein, ether extract, nitrogen-free extract, ash, energy and dry matter were similar for the two groups. The weight of piglets at weaning has been shown to have a significant effect on their subsequent growth performance. For example, Kavanagh et al. (1997) and Mahan et al. (1998) demonstrated that compared to heavier pigs, lighter pigs at weaning had lower growth rates after weaning and required a greater number of days to reach a common slaughter weight. In addition, variation in piglet weights within a group at weaning can affect the productivity of commercial pig production systems, particularly those implementing all-in all-out animal

management (Hardy, 1998). Weaning weight has been shown to be closely related to birth weight (Wolter and Ellis, 2001) and to the amount of sow's milk consumed by the piglet during lactation (Lewis et al., 1978). Providing piglets with supplementary liquid milk replacer during lactation can increase weaning weights (Azain et al., 1996; King et al., 1998). Therefore, the objective of the current study was to evaluate the effects of dietary supplementation with rice milk on growth performance and health status of piglets during the nursing and nursery period.

4.3 Materials and methods

4.3.1Animals and diet

We studied 36 litters from (LxLW) sows (288 piglets LxLW)x Penerland matched for parity and assigned to 1 of 2 experimental groups (Control, C and Treated, T). There were two studies phases: (i) dietary treatments of litters from 10 days of age to weaning separated in two groups, and (ii) dietary treatment of the piglets from weaning (21 days of age) until 42 days after weaning. All animals were treated in accordance with European Community guidelines approved by the Italian Ministry of Health.

4.3.1.1 Nursing period

The litters were randomly allotted to control (C) or rice milk supplementation (T) groups of 18 replicates each standardized to 9-10 piglets per sow (excess piglets at 2 d post parturition were selected randomly and placed with other sows in the same treatment group in litters of similar age). Group C received creep feed beside sow's milk; group T received rice milk in addition to sow's milk (no creep feed). The two groups were housed in separate sheds (8.0 x 5.0 m) with slatted concrete floors. The animals had free access to the sow's milk and creep feed or rice milk from 10 d of age until weaning (21 d of age). Piglets body weight was determined at 10 d age and at weaning.

4.3.1.2 Post-weaning

At day 21, a total of 288 piglets were weaned by moving them to environmentally controlled pens, each replicate containing 12 piglets; males and females were separated and the animals were grouped by weight. Each pens was 2.69 m^2 (2,5 m x 1 m), had a slatted floor, and was equipped with two water nipples and six-hole self-feeder. The piglets were allowed an ad libitum access to feed and water. The house was lit by a combination of daylight (through skylights) and artificial light (non-programmable). Ventilation was archieved by single, variable-speed fans linked to

temperature sensors. The temperature inside the building was approximately 28-30°C at the start of the trial, adjusted weekly until a final temperature of 20°C. Half the litter in each sow group (C or T) were assigned to rice milk, the other half to control. So the piglets formed four experimental groups of 72 piglets each:

C-C: creep feed nursing; dry feed post weaning;

C-T: creep feed nursing; dry feed plus rice milk post weaning;

T-C: rice milk nursing; drey feed post weaning;

T-T: rice milk nursing; dry feed plus rice milk post weaning.

All piglets were fed a prestarter diet from weaning to 14 d-post weaning (pw) and a starter diet from 14 d to 42 d pw. In addition to basal diet, piglets from CT and TT groups had free access to milk rice from weaning to 14 d-post weaning. After 14 d from weaning all the animals were fed a starter diet (Table 1) and no more rice milk were supplemented. No antibiotics and other growth-promoting agents were included in the diets.

Table 1. Composition and calculated analyses of the experimental diets[1]		
Ingredients, %	**Prestarter 24-38 days of age**	**Starter 38-66 days of age**
Maize meal	20.9	30
Biscuits flaked	15	13.8
Maize flaked	13	---
Barley	8	11.9
Dried whey	7.5	5
Soybean meal 44 % CP	6.5	6.6
Probiolat[1]	5	5
Herring meal	5	5
Soy protein conc. (Soycomil)	5	3.5
Whey solubile	5	2.5
Wheat middlings	4	12
Calcium carbonate	1	1.15
Dicalcium phosphate	1	0.85
L-Lysine HCl 78	0.7	0.4
L-Threonine	0.6	0.15
Soy oil	0.5	1
Mineral Vitamin mixture[3]	0.5	0.5
Antibiotics[4]	0.4	0.4
DL-Methionine	0.25	0.1
Salt	0.15	0.15
Chemical analysis, % as fed		
ME, kcal/kg	3250	3150
Crude protein, %	18.82	18.13
Crude fibre, %	2.82	3.57
Ether extract, %	5.62	6.03
Ash, %	7.03	7.02
Lysine, %	1.47	1.21
Methionine, %	0.58	0.43
Met+Cys, %	0.81	0.67
Threonine, %	1.26	0.81
Tryptophane, %	0.18	0.19
Calcium, %	1.04	1.05
Available phosphorus, %	0.45	0.44

[1] Probiolat: Whey concentrate protein + soybean concentrate protein.
[2] Inclusion per kg in the diet: vitamin A, 3.600.000 IU; vitamin D_3, 360.000 IU; vitamin E (dl-α-tocopherol acetate), 24.000 mg; thiamine (vitamin B_1), 800 mg; riboflavin (vitamin B_2), 1200 μg; pyridoxine (vitamin B_6), 1000 mg; cyanocobalamin (vitamin B_{12}),8000 μg; vitamin K_3, 400 mg; niacin (nicotinic acid), 50 mg; pantothenic acid, 3.600 mg; folic acid, 400 mg; biotin, 60.000 μg; choline 90.000 mg; Mn (MnO), 8000 mg; Zn (ZnO), 29.000 mg; Fe ($FeSO_4 \cdot H_2O$), 46.000 mg; Cu ($CuSO_4 \cdot 5H_2O$), 30.000 mg; Co ($CoSO_4 \cdot 7H_2O$), 200 mg; I (KI), 300 mg; Se (Na_2SeO_3), 70 mg

Table 2-3-4 characteristics of rice milk

Table 2 Analitical analysis of rice milk g/ 100 g (DM)	Value
MOISTURE	86.12
ENERGY	53 kcal/100g
PROTEIN	8.28
FAT	7.63
FIBER	11.5
ASHES	2.59
CARBOHYDRATES	69.96
GLUCOSE	54,75
GALACTOSE	< 0.01
FRUCTOSE	< 0.01
LACTOSE	< 0.01
SACCHAROSE	< 0.01
MALTOSE	4.37
VITAMINES mg/kg (DM)	
VITAMINE B1	3.4
VITAMINE B2	0.32
VITAMINS PP	64
ELEMENTS mg/kg (DM)	
ZINC	5.96
CALCIUM	159
IRON (D.M)	7.77
PHOSPHORUS	1896
POTASSIUM	671
SELENIUM	< 0.05
SODIUM	2625
γ ORYZANOL (D.M)	31 mg / kg

Table 3 Aminoacidic composition of rice milk (mg/kg DM)	
ASPARTIC ACID	80
TREONINE	42
SERINE	61
ASPARAGINE	104
GLUTAMMIC ACID	167
GLUTAMMINE	28
PROLINE	39
GLICINE	50
ALANINE	130
VALINE	72
CISTINE	< 5
METHIONINE	38
ISOLEUCINE	76
LEUCINE	79
TYROSINE	43
PHENILANINE	48
ORNITINE	< 5
LYSINE	90
HISTIDINE	26
ARGININE	183

Table 4 Lipidic profile of rice milk % of total acids	
BUTIRRIC ACID	< 0.01
CAPRONIC ACID	0.62
ENANTIC ACID	< 0.01
CAPRILIC ACID	7.35
CAPRINIC ACID	5.49
CAPROLEIC ACID	< 0.01
LAURIC ACID	38.18
LAUROLEIC ACID	< 0.01
TRIDECANOIC ACID	< 0.01
TRIDOCENOIC ACID	< 0.01
MYRISTIC ACID	13.97
MYRISTOLEIC ACID	< 0.01
PENTADECANOIC AC.	< 0.01
PENTADECENOIC AC.	< 0.01
PALMITIC ACID	10.38
PALMITOLEIC ACID	< 0.01
EPTADECANOIC C:17:0	< 0.01
EPTADECANOIC C:17:1	< 0.01
STEARIC ACID	2.93
OLEIC ACID	12.6
LINOLEIC ACID	8.25
LINOLENIC ACID	0.25
ARACHIC ACID	< 0.01
EICOSENOIC ACID	< 0.01
BEENIC ACID	< 0.01
ERUCIC ACID	< 0.01
LIGNOCERIC ACID	< 0.01
SATURATED FATTY ACIDS	78.90
MONOINSATURATED FATTY ACIDS	12.60
POLINSATURATED FATT ACIDS	8.50

4.3.2 Recorded parameters

4.3.2.1 Performance:

Piglets were weighted individually at the beginning of the trial (10 d of age), weaning (21 d), at 14 and 42 d post weaning (end of trial). Feed intake was recorded per replicate at the same ages as the weight controls. Performance parameters, average daily gain (ADG), dry matter feed intake (DMFI) and feed convertio ratio (FCR) were therefore determined.

4.3.2.2. Health status

A daily health check was carried out during the whole trial, in order to record all possible incidents and implement suitable measures.

4.3.2.3. Sampling and analysis

Feed and rice milk samples were collected and analysed for nutritional homogeneity (AOAC, 2000). Blood samples were collected into a venoject tube from the jugular vein puncture, just before feeding, of 12 piglets of each before weaning at 10 days of age. At weaning half of the 12 piglets of the two nursing groups were transferred in one of the each of post weaning treatments and bleeded 14 d post weaning. After clotting at +4°C for 1 h, sera were separated by centrifugation (800 g, 10 min) on a Centrifuge Megafuge 10R (Haereus Italia, Rome, Italy) and immediately stored at -20°C until analysis. Serum samples were analysed for Urea and Glucose with Synchron CX5® of Beckman Coulter (Fullerton, CA, USA). ROMs and Oxy adsorbent test according to Brambilla et al.(2001 and 2002). Lysozyme and total serum protein according to Amadori et Archetti (2002).

4.4 Statistical analysis

The animals represented the unit for the statistical analysis on live body weight (LBW), ADG and serum metabolites data. Data relative to LBW, ADG and plasma metabolites were analysed by a mixed repeated procedure of SAS using the initial body weight as covariate (SAS System 9e; SAS, Cary,NC, USA). The replicates were retained as statistical unit for dry matter feed intake (DMFI) and feed conversion ratio (FCR) calculations. Data relatives to DMFI and FCR were also analysed by a Mixed procedure of SAS (SAS System 9e; SAS, Cary,NC, USA). Significance was declared for value of $P<0.05$ and $P<0.01$.

4.5 Results

4.5.1 Growth performance

The effects of rice milk on piglets growth performance are shown in Table 5. During nursing phase, rice milk supplementation increased piglets LW (P<0.01) and ADG (P< 0.01). Piglets fed milk rice during nursing phase and basal diet after weaning (TC) were heavier than CC and CT groups at 14 d pw (P<0.05) and than CC, CT, and TT piglets at 35 d pw (P<0.05). Piglets from TC group had also a higher ADG during postweaning with respect to CC (P< 0.05) and CT piglets (P<0.01).
Dry matter intake was higher in TC piglets than TT, but no difference was observed for CC e CT groups during postweaning. Non difference was detected for FCR in nursery phase.

Table 5: Effects of Milk rice dietary administration on piglets growth performances

Nursing Phase (10 d age – weaning)							
Item		**Control litter**		**Treated litter**		**STD. ERR.**	**P <**
Weight (Kg)	10 d age	4.51		4.53		8.07	0.967
	Weaning	6.24 ^{A}		6.91 ^{B}		8.08	0.01
ADG g/d	10d - weaning	180 ^{A}		237 ^{B}		3.92	0.01
Milk consumption g/d	10 d age-weaning	X		2.63		1.05	0.01
Creep feed consumption g /d	10 d age - weaning	9.80		X			
Nursery Phase (weaning – 42 d pw)							
		C-C	**C-T**	**T-C**	**T-T**	**STD.ERR.**	**P <**
Weight (Kg)	Weaning	6.23 ^{a}	6.26 ^{a}	6.90 ^{b}	6.91 ^{b}	0.23	**0.047**
	14 d pw	9.77 ^{A}	9.52 $^{A\,a}$	10.80 ^{B}	10.16 ^{b}	0.24	
	42 p.w	17.68 ^{A}	17.27 $^{A\,a}$	19.13 ^{B}	17.98 $^{A\,b}$	0.23	
ADG (g/d)	Weaning -14 d pw	277	255 ^{A}	299 ^{B}	254 ^{A}	27.36	**< 0.01**
	14 d pw- 42 pw	376	369 ^{a}	399 ^{b}	367 ^{a}	37.52	**0.041**
	Weaning – 42 d pw	301a	292 ^{A}	331B ^{b}	308	30.01	
DMFI (g/d)	Weaning -14 d pw	297 b	286	292	275 ^{a}	6.19	**0.013**
	14 d pw- 42 pw	758 ^{B}	758 ^{B}	773 ^{B}	700 ^{A}	6.19	**< 0.01**
	Weaning – 42 d pw	583 ^{B}	583 ^{B}	589 ^{B}	544 ^{A}	6.19	**< 0.01**
FCR	Weaning -14 d pw	1.15	1.30 ^{a}	1.06 ^{b}	1.18	0.07	**< 0.05**
	14 d pw- 42 pw	2.40	2.53 ^{a}	2.32 ^{b}	2.29 ^{b}	0.07	**< 0.05**
	Weaning – 42 d pw	1.85	1.94	1.76	1.78	0.07	**0.828**

$^{A, B}$ Means within row with different supscript differ at P<0.01; ^{a,b} Means within row with different supscript differ at P<0.05

4.5.2. Blood Parameters

The blood parameters examined in this study are presented in Table 6. No difference in glucose, urea, total protein and lysozyme serum concentration. Reactive oxygen metabolites levels (ROM's) resulted lower in piglets fed rice milk before weaning throughout 14 d post weaning (TT) ($P<0.05$). No difference were observed shown between the group TT and TC. Piglets fed rice milk only during nursing phase (TC) showed a lower total antioxidant capacity after weaning compared to the other groups ($P<0.05$).

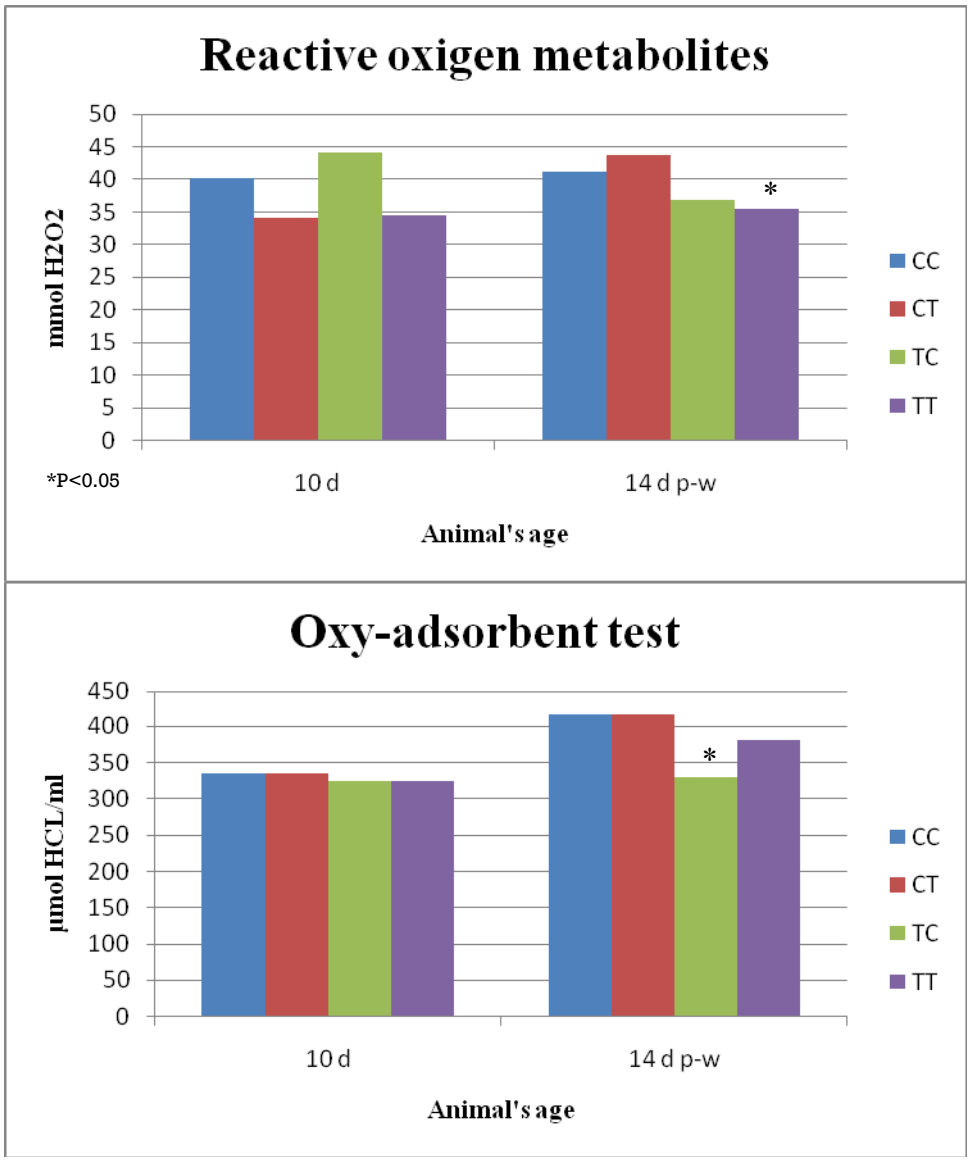

Fig.1-2 Shown the results obtained from serum ROMS and Oxy adsorbent test. Animals were bleeded at 10 d of age and at 14 d after weaning.

Table 6 Effects of rice milk supplementation on blood parameters

	Diet					
Age	**C-C**	**C-T**	**T-C**	**T-T**	**SEM**	**P <**
Glucose mmol/l						
10 d	6.95	7.05	6.88	6.52	0.306	**0.235**
14 d p.w	6.71	6.78	7.60	6.84	0.306	**0.095**
Lisozime microg/ml						
10 d	3.23	3.01	5.24	3.44	0.806	**0.853**
14 d p.w	4.36	3.56	4.51	4.16	0.806	**0.865**
Urea mmol/l						
10 d	2.55	2.48	2.18	2.20	0.309	**0.522**
14 d p.w	2.33	2.11	2.11	2.41	0.309	**0.503**
Oxy-adsorbent test µmol HCl 0/ml						
10 d	331	334.50	325.33	324.50	21.136	**0.851**
14 d p.w	410.17[b]	416.17[b]	330.67[a]	380.83	21.136	**0.05**
Rom's mmol H2O2						
10 d	40.1	34.15	44.03	34.5	9.096	**0.656**
14 d p.w	39.16[b]	44.48[b]	36.88	35.53[a]	8.67	**0.286**
Total seroprotein g/l						
10 d	52.67	55	54.83	53.67	2.364	**0.493**
14 d p.w	44.83	45.83	50.33	46.17	2.364	**0.115**
Albumine %						
10 d	44.83	48.35	51.55	49.13	1.437	**0.065**
14 d p.w	47.55	47.81	48.80	48.45	1.437	**0.758**
Alfa %						
10 d	20.58	20.60	21.60	20.53	1.017	**0.488**
14 d p.w	23.82	23.30	22.55	22.80	1.017	**0.508**
Beta %						
10 d	18.22	16.38	16.18	17.47	0.919	**0.414**
14 d p.w	17.75	17.38	18.23	17.03	0.919	**0.367**
Gamma %						
10 d	16.37	14.67	10.67	12.87	1.209	**0.067**
14 d p.w	10.88	11.50	10.42	11.71	1.209	**0.456**
A/G						
10 d	0.83	0.94	1.06	0.97	0.053	**0.061**
14 d p.w	**0.91**	**0.92**	**0.96**	**0.95**	**0.053**	**0.951**

[A, B] Means within row with different supscript differ at P<0.01
[a,b] Means within row with different supscript differ at P<0.05

4.5.3. Health status

With regard to digestive problems, no soft faeces were recorded during the whole trial in any treatment and only one piglet was treated for diarrhea.

4.6 Discussion and conclusion

At weaning piglets faces three challenges. First there are major changes to its food supply. Not only the piglets have to find the new food from a creep feeder but the characteristic of this feed are completely different from the sow's milk. The second major challenge at weaning is the change of the physical environment. At weaning litters are generally mixed together into weaner pools. The third challeng is the physiological stress that accompanies moving and mixing. When all these changes are taken into account it is little wonder tat the rate of growth of the piglet falls after weaning.

Special diets and management schemes have been developed with the goal to overcoming the post-weaning syndrome in piglets (Zehn-Ping et al., 2007). Systems which use milk-based liquid diets have demonstrated piglets growth performance comparable to that in suckling piglets (Kim et al., 2004; Lecce et al., 1969).

Aim of this trial was to determine the effects of dietary supplementation of rice milk on piglets growth and health status. In the present study we evaluated for the first time the use of an alternative milk in the nursing and nursery piglets. Piglets growth was higher when litters were fed rice milk until weaning. After weaning the positive effects of rice milk supplementation were reduced probably because the high palatability of the product favoured a high milk intake reducing dry feed intake. The higher growth of piglets supplemented with rice milk only during nursing may be attributed to the higher supply of sugars before the weaning leading treated litters to reach a more and higher homogeneous weight at the end of the suckling period compared to litters fed the creep feed. The richness of carbohydrates and its palatability make this product an interesting energy source rapidly available to evaluate during a critical period such as pre-weaning. The availability of these substances can homogenize the weight of the litters giving more available energy to the weakest animals that can't get enough energy from the sow's milk. The higher nutrients requirements and the absence of mother's milk reduce the effects of rice milk in the immediate postweaning.

However, it should be stated that rice milk is used in human nutrition since a long time, but it has a few chances to be used in animal nutrition due to the high production costs.

Beside the high sugar content, rice milk is also a great source of functional compounds such as γ - oryzanol so that it might also have beneficial effects on health. In this study, we evaluated some parameters related to oxidative stress as, it is well known, that piglets are subjected to a high level of stress during weaning. Reactive oxygen species (ROMs) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron such as a superoxide ion (O21 ±), nitrogen oxide (NO) and hydroxyl radical (HO±) (Aruoma, 1994). Even though naturally present in the organism, they are mainly connected to cell compartments and counterbalanced by natural anti-oxidant molecules, such as glutathione, glutathione peroxidase, superoxide dismutase, Q coenzyme, vitamin E and vitamin C, acting as free radical scavengers (Miller et al., 1993). When unbalanced, they lead to the oxidation of DNA, proteins and polyunsaturated fatty acids (PUFA) and to the formation of hydroxyl radicals (HR), which include lipid peroxides. Such a consequence on membranes is the first step in a cascade mechanism that leads to the systemic progressive release of ROMs from cell compartments, thus causing an increase of their concentration in the blood. Due to a high susceptibility, the early weaning pigs are quite exposed to oxidative stress which increase the amount of substrate for ROMs activity and thus the risk of lipid peroxidation (Nolan et al., 1995; Brambilla et al., 2002).

The early detection of oxidative imbalance by simple and reliable methods is important to prevent such consequences. The positive action on the parameters such ROMs and the oxi adsorbent test of the animals fed rice milk can be explained by the presence of the γ-oryzanol (OR). Infact is demonstrated that OR is one of the major compounds that contributes to antioxidant improvement and regulation of antioxidant and oxidative stress related genes of stressed rat liver (Hiramitsu et al., 1991). Other studies demonstrated that OR up-regulates the antioxidant genes while down-regulates the oxidative stress genes marker (Maznah et al., 2010).

In our study, a significant reduction in ROMs was observed only in piglets fed rice milk during nursing and nursery period (TT group) ($P< 0.05$). Our results are confirmed by a number of studies carried out in humans and mice on the health benefits of rice γ-oryzanol such as anti-inflammatory activity and inhibiting cholesterol oxidation (Rong et al., 1997; Akihisa et al., 2000; Xu et al., 2001; Rohrer and Siebenmorgen 2004; Imsanguan et al., 2007). γ-Oryzanol can inhibit or retard the oxidation of an oxiable substrate in a chain reaction protecting biolocal molecules and tissue from oxidative damage (Yoshida et al., 2003).

The animals of the control litters (CC; CT) shown higher value in the oxy-adsorbent test ($P< 0.05$) compared to the animals of the TT group. This data are not easy to explain because in the literature there are no studies on the use of this product in pig nutrition but seems to give a positive response that found confirm in the research on the activities of γ-Oryzanol. The supplementation of the rice

milk did not influence negatively the health parameters of the treated animals before and after weaning, and we demonstrate that also the serum glucose level are similar to all the groups; this means that rice milk is a great energetic source with high availability that can play an interesting role expecially in the nursing phase on the weakest animals homogenizing the weight at weaning. In conclusion, it can be postulate that rice milk supplementation might have positive effects in growth and feed intake during the immediate postweaning if administered from 10 d of age.

This study suggests that the dietary supplementation during nursery period can improve the growth performance in the piglets after weaning. Therefore this product may be considered a functional foods for its richness of bioactive compounds such as γ-oryzanol. More research will be needed to understand the potential of this functional food in piglets diet.

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Chapter 5

Third animal trial

Effects of plant polyphenols and mannan oligosaccharide on growth performance, antioxidant defense system and gut health in Escherichia coli Challenged piglets

5.1 Abstract

Objective of the current study was to evaluate the effects of plant polyphenols (PP) and/or, mannan oligosaccharide (MOS) on growth performance, plasma antioxidant capacity and health in *E. Coli-challenged* weaned piglets. Ninety-six piglets (7.43 $\pm$ 0.89 kg L.W., 21 d age) were randomly allotted into 4 dietary treatments: control (basal diet), 0.1% PP, 0.1 % MOS or PP+MOS in a 6 weeks study. At 21 and 25 d on trial half piglets of each group were orally inoculated with 4 ml of E.coli (1×10^9 cfu/ml) and half with the same amount of saline water.
No difference was observed in growth. Plasma urea was markedly increased in PP+MOS group compared to PP group ($P< 0.05$), and lysozyme content was significantly decreased in PP+MOS group compared to groups on d 7 ($P< 0.05$). PP+MOS dietary supplementation decreased intestinal lipase and trypsin compared to PP on d 21 ($P< 0.05$). Plasma malondialdehyde content (MAD) increased, while plasma total antioxidant capacity (TAOC) and catalase (CAT) activities decreased in piglets 6 days after infection. Challenged piglets fed PP and those fed MOS showed higher TAOC than challenged piglets fed PP in combination with MOS. CAT activity resulted higher in challenged piglets fed diet supplemented with PP or PP+MOS than control or MOS at d 13 after infection ($P< 0.05$). The results showed that dietary PP or MOS had the potential to improve enhance systemic antioxidant capacity. However no synergic effect was observed when PP and MOS were combined.

Key words: plant polyphenol(PP); mannan oligosaccharide (MOS), growth performance;

antioxidant capacity; piglets ; *E.coli* challenge

5.2 Introduction

The ban of antibiotics as feed additive requires alternatives to stabilize the performance and health particular of the young animals. The use of natural origin feed additive has gain credence due to the non-bacterial resistant, non-harmful and non residual property and the potential to promote the growth and health in animals. Plant polyphenols (PP) are a powerful kind of naturally bioactive compounds found in food such as fruits, wine and tea as well as wood waste like larch bark (Hammer et al. 1999; Heim et al. 2002). It is known for a long time that polyphenols like the flavanoid, epicatechin, are efficient scavengers of free radicals (Sichel et al. 1991). Polyphenols from grape seeds were reported to be responsible for the preventing oxidative damage to cellular DNA *in vitro* (Fan and Lou, 2004). Plant polyphenols (PP) had exhibited antimicrobial activity against bacteria causing food-borne disease (Abram and Donko, 1999; Taguri et al. 2004; Percival et al. 2006; Kim et al. 2009). In animal study, PP had also shown the ability to improve gut

microflora balance (Ishihara et al. 2001). More important, the British Pharmacopoeia (1996 Edition) reported that microorganisms do not build resistance to benzyl alcohol, phenols, polyphenols, and similar products. Thus, antioxidant and antimicrobial activity as well as gut modulation effects of plant polyphenols have been suggested (Windisch et al. 2008). Although massive studies have demonstrated antioxidative and antimicrobial efficacy of PP *in vitro*, evidences from animal trials are still quite scarce. Prebiotics is another solution for alternative of antibiotics. The common used prebiotics include mannanooligosaccharides (MOS), fructooligasccharides and galactoligosaccharides. MOS is derived from the outer cell wall of yeast. Meta-analyses of literature had shown that MOS supplementation improve body weight gain and feed efficiency in piglets (Jennifer et al. 2003, Eugeniusz et al, 2006). In addition, MOS were also reported to lower the level of total cholesterol and LDL-cholesterol in serum (Eugeniusz et al, 2006) and to mediate immunomodulatory effects in gut-associated lymphoid tissue (Janardhana et al. 2009). In modern swine production, the common practice of early weaning usually caused the post-weanling piglets suffering from microbially induced gastrointestinal pathologies or diarrhea .Enterotoxigenic *Escherichia coli* (ETEC), is reported to be one main etiological agent that cause piglet intestine disorder after weanling. ETEC is the most important and most common cause of piglet diarrhea identified by diagnostic laboratories and veterinary practitioners. It was estimated that ETEC infection accounted for 50% of mortality in post weanling diarrhea piglet (Gyles, 1994). It is worthwhile to explore the possible protective role of natural origin feed ingredient against the ETEC infection. Therefore the purpose of this study is to determine the efficacy of a prepared PP complex or MOS or both using post-weaning piglets in the presence of experimentally challenge of *E.coli*. Growth performance, haematological profile, antioxidant capacity, ileac inflammatory responses were investigated.

5.3 Materials and methods

5.3.1. Animals and diet

Ninety-six barrows crossbred weaned piglets (7.43 $\pm$ 0.89 kg L.W., 21 d of age) homogeneous for weight, age, and litter origin were used. Piglets were housed in 24 pens (4 piglets per pen) in an environmentally regulated room. Pigs had free access to feed and water in the pen equipped with a feeder and a nipple waterer. Room temperature was kept at 27°C during the first 3 days then decreased by 1°C for each subsequent week until 25°C.

After 1 week of adaptation to basal diet piglets were allotted to 4 dietary treatments: 1) Control (Basal Diet); 2) PP (1.0 g/kg diet); 3) MOS (1.0 g/kg diet); 4) PP+MOS (1.0 g/kg+1.0 g/kg diet). Each treatment consisted of 6 replicates.

The basal feed (Table 1) was formulated according to NRC nutrient requirements (1998). Feed was offered ad libitum in a mash form.

The main ingredients in PP mixture were from apple, grape seed, green tea and olive leaves polyphenols. PP mixture was prepared based on the results of *in vitro* trials (Zhang, not published data).

The study lasted 42 days and was divided in two phases: pre-challenge (0-21 d) and post challenge (25-42 d). At 21 and 25 d on trial, half piglets from each treatment (3 pens/12 piglet per treatment) were orally inoculated with 4 ml of 1×10^9 cfu/ml *Escherichia coli* while the other half received 4 ml of physiological saline solution. The challenge strain used was an *E. coli* O139:K82 LT⁻ isolated from a clinical case with PWD. Strains of O serogroup 139 have a well-established association with both OD and PWD.

Fig1. Weaning Room of University of Milan facilities

Table 1 Composition and nutrients composition of the basal diets		
Ingredients, %	**Prestarter 22-36 days of age**	**Starter 36-64 days of age**
Maize meal	31.3	30.4
Biscuits flaked	15	13.8
Maize flaked	13	---
Barley	8	11.9
Dried whey	7.5	5
Soybean meal 44 % CP	6.5	6.6
Probiolat[2]	5	5
Herring meal	5	5
Soy protein conc. (Soycomil)	5	3.5
Whey solubles	5	2.5
Wheat middlings	4	12
Calcium carbonate	1	1.15
Dicalcium phosphate	1	0.85
L-Lysine HCl 78	0.7	0.4
L-Threonine	0.6	0.15
Soy oil	0.5	1
Mineral Vitamin mixture[2]	0.5	0.5
DL-Methionine	0.25	0.1
Salt	0.15	0.15
Chemical analysis, % as fed		
ME, kcal/kg	3250	3150
Crude protein, %	18.82	18.13
Crude fibre, %	2.82	3.57
Ether extract, %	5.62	6.03
Ash, %	7.03	7.02
Lysine, %	1.47	1.21
Methionine, %	0.58	0.43
Met+Cys, %	0.81	0.67
Threonine, %	1.26	0.81
Tryptophane, %	0.18	0.19
Calcium, %	1.04	1.05
Available phosphorus, %	0.45	0.44

[1] Mos and/or Poliphenols included in T at 100 g/tonne (1 x 10^9 CFU/g feed); in T2 at 4 kg/tonne (1 x 10^9 CFU/g feed).
[2] Probiolat: Whey concentrate protein + soybean concentrate protein.
[3] Inclusion per kg in the diet: vitamin A, 3.600.000 IU; vitamin D_3, 360.000 IU; vitamin E (dl-α-tocopherol acetate), 24.000 mg; thiamine (vitamin B_1), 800 mg; riboflavin (vitamin B_2), 1200 μg; pyridoxine (vitamin B_6), 1000 mg; cyanocobalamin (vitamin B_{12}),8000 μg; vitamin K_3, 400 mg; niacin (nicotinic acid), 50 mg; pantothenic acid, 3.600 mg; folic acid, 400 mg; biotin, 60.000 μg; choline 90.000 mg; Mn (MnO), 8000 mg; Zn (ZnO), 29.000 mg; Fe ($FeSO_4 \cdot H_2O$), 46.000 mg; Cu ($CuSO_4 \cdot 5H_2O$), 30.000 mg; Co ($CoSO_4 \cdot 7H_2O$), 200 mg; I (KI), 300 mg; Se (Na_2SeO_3), 70 mg.

5.3.2. Growth performance and samples collection

The piglets were weighted weekly and feed intake per pen was recorded accordingly. At days 7, 21, 25, 28, 35 on trial, 1 piglet from each pen was selected and blood sample was taken from the jugular vein in heparinized centrifuge tube. Plasma was proceed by centrifugation at 4°C 1,800 × g for 10 min. The resulting plasma sample was stored at –20°C pending analysis.

At days 21 (before E. Coli challenge), 25 and 28 on trial, one piglet/treatment/d, homogeneous for weight was selected and slaughtered via exsanguinations in a standard commercial slaughter house. Digesta samples from distal duodenum were collected and immediately stored at - 80°C pending analysis.

5.3.3 Chemical.analysis

5.3.3.1 Blood parameters

Blood samples were analysed by using the automated Sysmex XT-2000iV hematology analyzer and the following biochemical parameters were determined: Hemoglobin (HGB) and Hematocrit (HCT), Red Blood Cells Count (RBC), Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Cell Hemoglobin Cconcentration (MCHC), Red distribution width (RDW), Platelet Count (PLT), Mean Platelet Volume (MPV), Platelet distribution width (PDW), White Blood Cells Count (WBC) as well as White Blood Cell Pprofiles, including counts and percentages of neutrophil granulocytes (Neu), lymphocytes (Lym), monocytes (Mono), eosinophil (Eos) and basophil (Baso) granulocytes.

5.3.3.2. Plasma parameters

Plasma urea nitrogen and lysozyme content

Plasma was analyzed for urea nitrogen concentration (PUN) by using a commercial kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Plasma lysozyme activity was measured according the method of Kreukniet et al. (1994) by using micrococcus lysodeikticus cells as substrate.

Total protein and lipid profile

The plasma protein and lipid profile analysis were carried out according to Amadori et Archetti (2002).

Assay of plasma antioxidant indexes and ceruloplasmin

Total antioxidant capacity (T-AOC) was measured according to Benzie and Strain (1996). The marker of lipid peroxidation MDA was quantified by measuring the thiobarbituric acid (TBA) reactive substances with spectrophotometer at 535 nm (Wills, 1966). TBA-material is described as nM of malondialdehyde (MDA) per mg protein or per mL. Glutatione peroxidase activity (GSH-Px) was assayed by the method of Lawrence and Burk (1976) using hydrogen peroxide as the substrate. One unit of GSH-Px is expressed as the amount of GSH-Px needed to oxidized 1μmol of NADPH per min. Total superoxido dismutase activity (SOD) was assayed according to the methods of Spitz and Oberly (1989). Units of SOD activity were defined by the amount of the enzyme required to inhibit the rate of formazan dye formation by 50% under defined conditions. The catalse (CAT) activity was estimated by the decomposition of H_2O_2 to yield H_2O and O^2, and monitored changes in absorbance at 240nm for 2 min (Cohen, 1970). One unit of activity was expressed as the amount of enzyme catalyzing the decomposition of one μM of H_2O_2 per min at 25°C and pH 7.0. The specific activity of all enzymes assayed was expressed as activity per mL. Plasma ceruloplasmin concentration was determined by the procedure of Schosinsky et al. (1974) using o-dianisidine dihydrochloride as substrate.

Plasma hydroxyl radical inhibiting capacity was determined according to Yu et al. (2008) using the Fenton reaction as the hydroxyl radical generation system and Rhodamine B as a spectrophotometric indicator. To define the hydroxyl radical oxidation one unit of plasma hydroxyl radical inhibiting capacity was defined by the capacity to reduce the concentration of H2O2 by 1 mmol/L at 37°C for 1 minute in 1 mL of plasma under defined conditions.

Superoxide anion and hydroxyl radical inhibiting capacity was estimated by using cytochrome c reduction method by capturing the superoxide anion produced from reaction system of xanthine oxidase plus hypoxanthine (or xanthine) at pH 7.4 at 550 nm (Babior et al. 1970). One unit of plasma superoxide anion inhibiting capacity was expressed correspondence to capacity of one mg of Vitamin C to inhibit superoxide anion production in one liter of plasma at 37°C for 40 min in the reaction system.

5.3.3.3 Intestinal parameters

Inducible nitric oxide synthase activity and Nitrite Synthesis assay.

Ileal mucosa samples were homogenized using a tissue homogenizer with 9 volume ice cold saline (9 g sodium chloride/l L of distilled water). The homogenates were centrifuged at 1,800×g for 10 min. The inducible nitric oxide synthase (iNOS) activity was measured according to the method of Devaux et al. (2001). Protein concentration in mucosal homogenates was measured by the method

of Lowry et al (1951) using bovine serum albumin as standard.The nitrate in the supernatant of mucosa homogenate was measured by the Griess assay (Devaux et al. 2001).

Myeloperoxidase (MPO) activity

MPO activity was measured using the O-dianisidine MPO (OD-MPO) assay according to Salter et al. (2001). MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute and per gram of wet weight of tissue

Digestive enzymes activity

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

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

Pepsin content of the small intestine digesta was assayed by the method of Anson (1938). One unit of pepsin activity was equivalent to an increase in extinction at 280 nm of 0.001/min at 37℃.

Trypsin activity was estimated in digesta of small intestine as described by Hummel (1959).

5.4 Statistical analysis

The data were analyzed using General Linear Model procedure of SPSS (15.0) software as a 4×2 factorial arrangement with dietary treatments and challenge types (E.coli or saline) as main factors. The pooled growth performance was analyzed using ONE-WAY ANOVA procedure. The significance of differences among different treatments was evaluated by Least Significant Difference (LSD) post-hoc multiple comparisons test. Statistical significance was set at $P < 0.05$.

5.5 Results

5.5.1 Growth Performance

Table 3 shows the results on growth performance of piglets during the trial. There were no significant difference among treatments. During the first week FCR was better in Ctr and PP than PP + MOS but not compared to those fed MOS. During the second week of the trial FCR was better

in piglets fed only MOS or PP than those fed diet PP + MOS. Throughout the pre-challenge period (0-21 d) piglets fed Ctr or PP diets showed FCR values lower than those fed on PP+MOS ($P<0.05$). No effect of challenge and interactions between diet and challenge on growth performance were observed (Table 3). During the 4 days after the first challenge, there was a significant decrease in feed intake in PP and MOS groups compared to Control and PP+MOS groups, and feed efficiency in PP and MOS groups were higher than PP+MOS group ($P<0.05$). Two days after the E. Coli challenge, piglets fed MOS diet grew faster than those in Control and PP+MOS group ($P<0.05$). Body gain and FCR were lower in Control group compared to other treatment groups. There was a sharp decrease of FCR in piglets during 25-28 days than 21-25 days. There were no differences of growth performance of piglets during 7 days after second challenge among all groups. Piglets fed PP and MOS diets had improved FCR during 7 days post primary challenge, FCR was higher in MOS group than Control group during 12 days post challenge ($P<0.05$). Since there were no challenge effect on growth performance. There were no significant difference among groups during 35-42 d, dietary PP and MOS had improved feed efficiency compared with PP+MOS group during the period of whole 35 and 42 days ($P<0.05$) (Table 3). PP dietary supplementation had higher FCR than others during the whole experiment period.

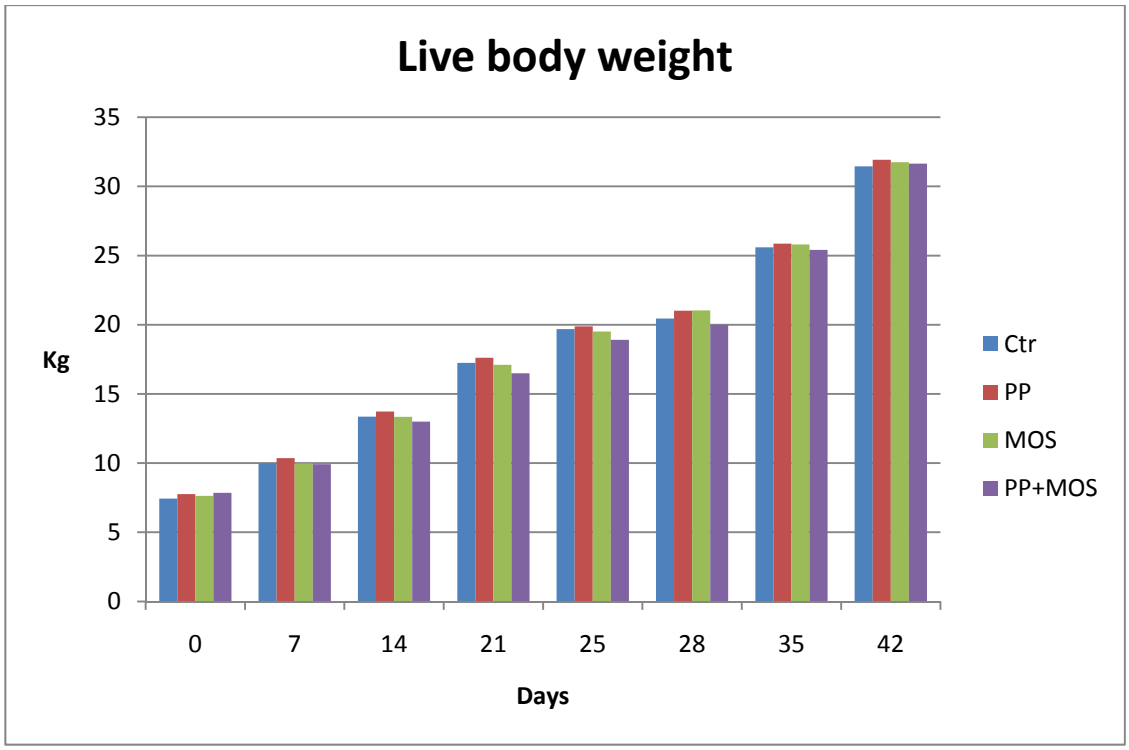

Fig.2

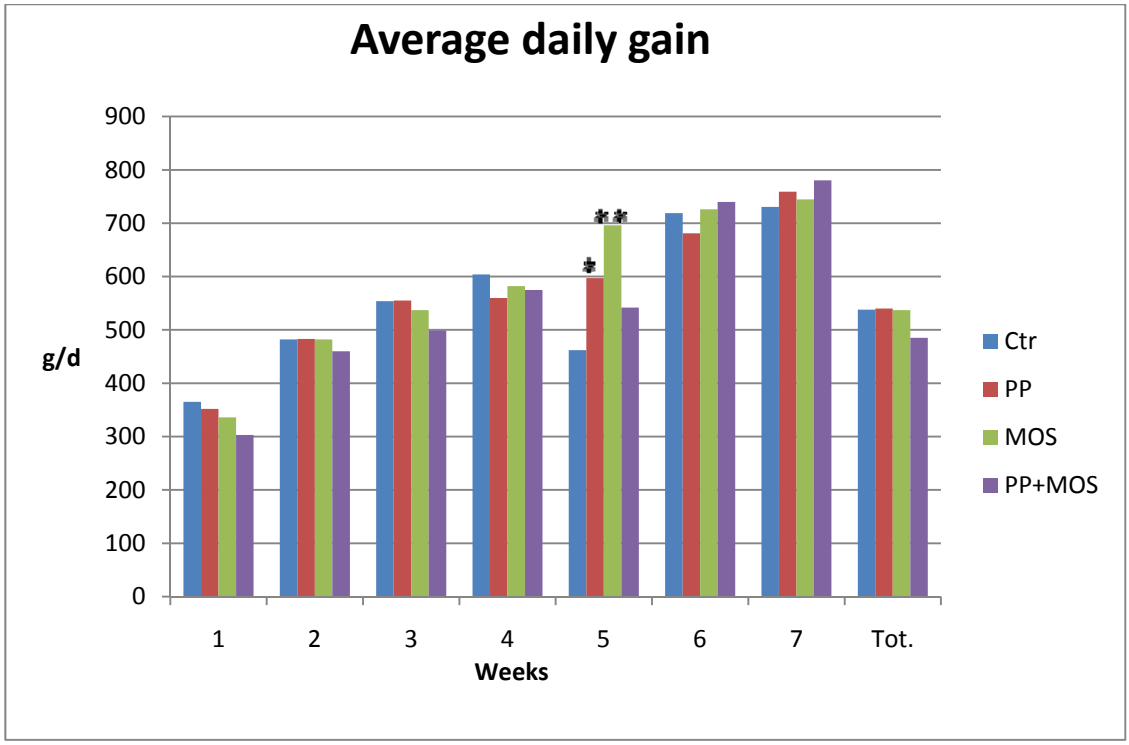

*Fig. 3 (* P< 0.05) ; (** P <0.05 *)*

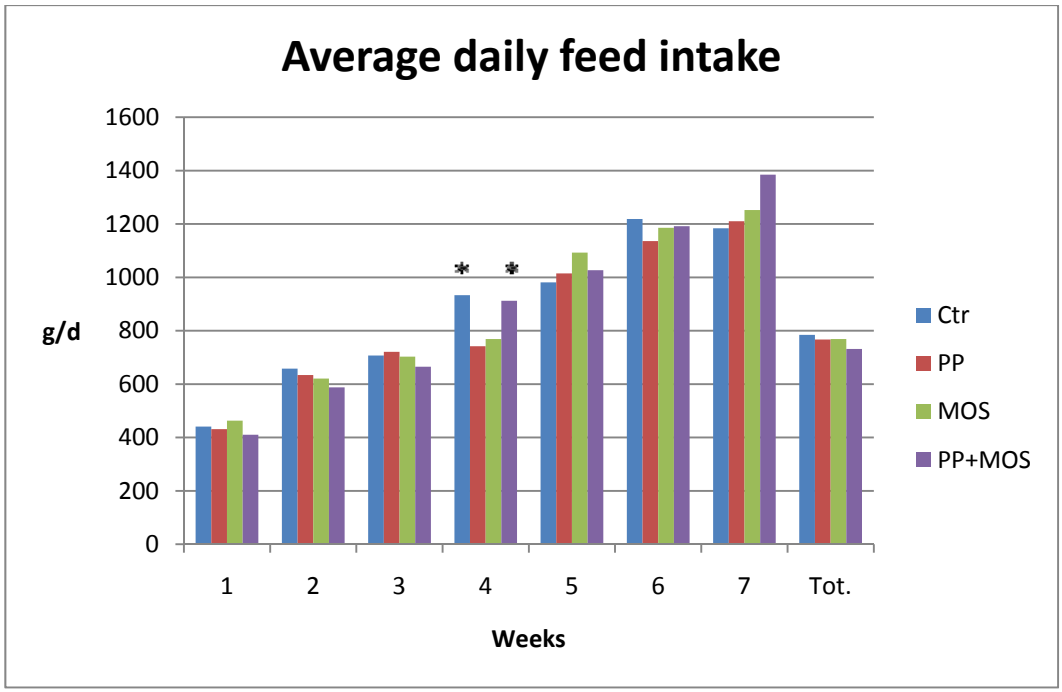

*Fig.4(* P< 0.05)*

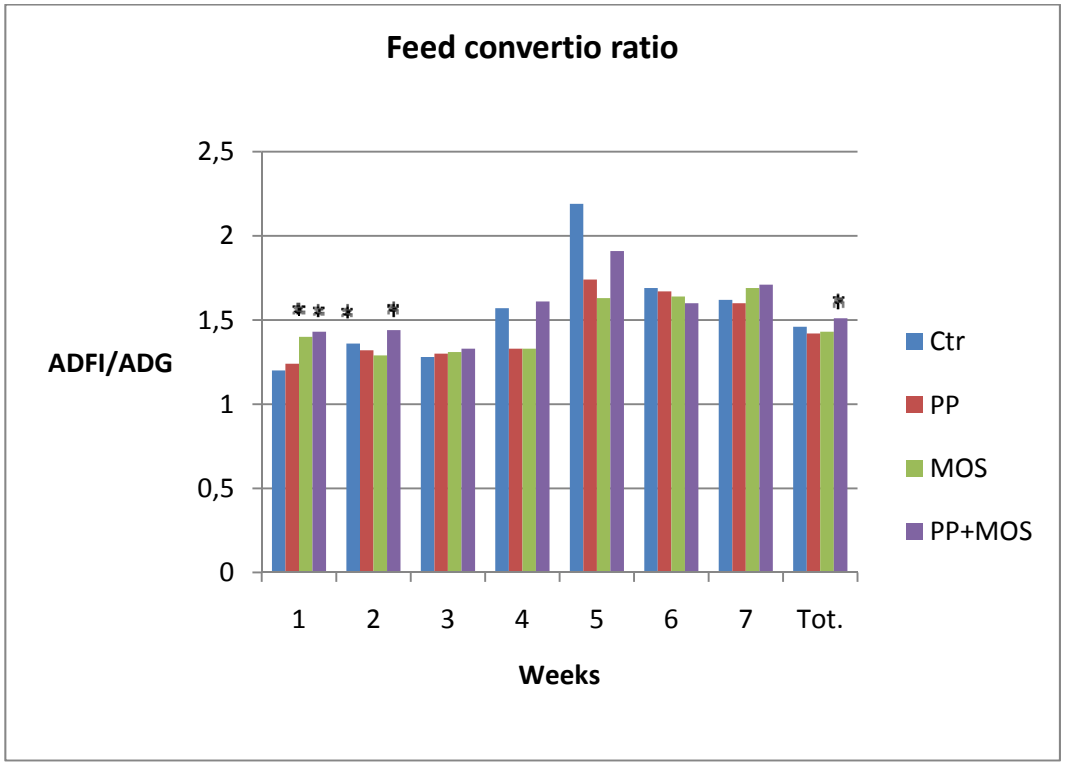

*Fig.5 (*P<0.05)*

5.5.2. Blood parameters

On d 25, no challenge effects and interaction of challenge and diet on plasma parameters were observed (Table 5). Dietary MOS enhanced peripheral blood RBC count, HGB content and PCV value compared with Control or PP+MOS diet ($P< 0.05$).

On d 28, E. Coli challenge markedly increased blood PLT counts ($P< 0.05$). Dietary PP induced an increase of blood HGB content and PCV value compared with MOS or PP+MOS diet ($P< 0.05$).

On d 35, challenge partially increased peripheral blood PLT counts ($P= 0.081$). Dietary PP induced a significant reduction of blood PLT content compared with control or MOS diet ($P< 0.05$). There were no interactions of diet and challenge on haematological profile.

Table 5 haematological profile in piglets post infection

Diet	CT		PP		MOS		PP+MOS		SEM	Diet				Challenge		P value		
Challenge	Saline	challenge	Saline	challenge	saline	challenge	Saline	challenge		CT	PP	MOS	PP+MOS	saline	challenge	diet	challenge	Interaction
d 25																		
WBC 10^6/mL	13.77	13.67	11.65	13.25	12.64	12.34	14.16	11.15	0.575	13.72	12.45	12.49	12.65	13.06	12.60	0.844	0.699	0.573
RBC (10^9/mL)	5.92	6.01	5.81	6.63	6.71	6.79	6.18	5.84	0.094	5.97b	6.22ab	6.75a	6.01b	6.16	6.32	0.034	0.399	0.217
HGB(g/dL)	9.50	9.87	9.37	10.73	10.80	11.07	9.93	9.30	0.178	9.68b	10.05ab	10.93 a	9.62b	9.90	10.24	0.067	0.351	0.301
PCV (%)	32.00	32.93	31.33	35.90	35.67	36.63	33.90	31.23	0.550	32.47b	33.62ab	36.15a	32.57b	33.23	34.18	0.103	0.400	0.187
PLT	408.33	440.67	330.33	330.33	251.67	277.67	344.67	280.33	23.883	424.50	330.33	264.67	312.50	333.75	332.25	0.16	0.975	0.885
d 28																		
WBC 10^6/mL	12.36	12.20	20.11	12.78	11.75	13.14	13.09	14.94	0.879	12.28	16.45	12.45	14.20	14.44	13.27	0.316	0.554	0.251
RBC (10^9/mL)	6.39	6.41	8.75	7.13	6.50	6.21	6.08	6.53	0.271	6.40	7.94	6.36	6.35	7.01	6.57	0.124	0.515	0.578
HGB(g/dL)	10.33	10.07	13.50	10.90	9.53	9.33	9.35	9.97	0.389	10.20ab	12.20a	9.43b	9.72b	10.80	10.07	0.081	0.443	0.516
PCV (%)	34.07	33.67	44.23	35.50	31.93	31.67	30.80	33.77	1.008	33.87ab	39.87a	31.80b	32.58b	35.66	33.65	0.070	0.516	0.391
PLT	302.67	564.33	231	454	224	450	346.50	441.67	32.922	433.50	342.5	337	403.60	269.64	477.50	0.683	0.008	0.840
Mono %	54	64	52.67	61.67	62.67	72.67	52	62.67	1.991	59	57.17	67.67	58.40	55.64	65.25	0.226	0.025	0.999
d 35																		
WBC 10^6/mL	12.56	13.67	19.47	16.19	11.95	14.95	14.97	18.25	0.86	13.11	17.83	13.45	16.37	14.75	15.76	0.180	0.555	0.522
RBC (10^9/mL)	6.10	5.87	6.34	6.67	5.66	6.03	6.28	6.33	0.11	5.99	6.51	5.85	6.30	6.11	6.23	0.187	0.562	0.765
HGB(g/dL)	10.13	10.00	9.67	10.17	9.37	9.97	9.50	10.07	0.15	10.07	9.92	9.67	9.74	9.65	10.05	0.814	0.224	0.810
PCV (%)	34.20	33.90	33.33	33.63	31.90	33.50	32.23	34.07	0.49	34.05	33.48	32.70	33.01	32.86	33.78	0.805	0.393	0.841
PLT	365.00	480.67	212.00	287.67	273.67	340.00	363.75	500.33	26.58	422.83a	249.83b	306.83ab	422.29a	308.23	402.17	0.069	0.081	0.959

*Note: Data in the same line with different superscripts differ significantly (P<0.05).

5.5.3 Antioxidants

Plasma antioxidant activities

At d 21 (before challenge), piglets fed PP diet had decreased plasma MDA level ($P< 0.05$) and higher TAOC compared to Control and PP+MOS ($P< 0.05$) (Table 6). There were no other difference with regard to plasma antioxidant enzymes among groups.

Challenge did not induce any effects on plasma antioxidant properties on d 4 post infection (Table 5). Dietary PP or MOS induced a significant increase of GPx activity compared to Control or PP+MOS treatments ($P= 0.003$). No interactions between diet and challenge were observed.

Challenge tended to increase plasma MDA content ($P= 0.078$), and decreased plasma TAOC ($P< 0.05$) and CAT activity ($P< 0.05$) at 7 d post infection. There was no difference due to dietary treatments on plasma antioxidant activity. No interactions between diet and challenge were also found.

At d 35 on trial there was a reduction in plasma TAOC ($P< 0.01$). Dietary PP or MOS increased TAOC with respect to PP+MOS treatment ($P< 0.05$). PP or PP+MOS dietary supplementation markedly increased CAT activity compared with Control or MOS groups ($P< 0.01$). No interactions between diet and challenge was also found.

E. Coli challenge tended to increase at d 25 ($P= 0.077$) or increased at d 28 ($P= 0.01$) plasma ceruloplasmin values (Table 7). At d 28 dietary supplementation with PP, MOS and PP+MOS did not inhibit the increase of ceruloplasmin expected from challenge, but comparatively decreased ceruloplasmin contents were observed in dietary PP ($P= 0.066$) and PP+MOS ($P= 0.066$) treatments with regard to Control.

Table 6 Antioxidant properties (activity/mL)

	Diet				Challenge		P	
	Ctr	**PP**	**MOS**	**PP+MOS**	**NaCl.**	**E.coli**	**Diet**	**Challenge**
7 d								
MDA	5.08	3.64	3.71	3.81	/	/	0.305	/
TAOC	18.84	17.67	18.06	19.32	/	/	0.537	/
CAT	16.34	18.89	18.09	12.17	/	/	0.565	/
GPx	403.75	380.97	414.06	458.27	/	/	0.377	/
TSOD	102.75	94.42	75.75	103.29	/	/	0.420	/
21 d								
MDA	3.64	1.93	3.64	3.79	/	/	0.076	/
TAOC	17.73 ^{b}	22.08 ^{a}	19.91 ^{ab}	18.18 ^{b}	/	/	0.060	/
CAT	16.37	27.41	17.22	32.25	/	/	0.252	/
GPx	396.87	410.04	384.4	452.89	/	/	0.458	/
TSOD	96.96	102.73	102.89	90.86	/	/	0.383	/
25 d								
MDA	3.68	2.33	4.09	2.73	2.69	3.73	0.177	0.102
TAOC	19.55	21.39	20.28	21.64	20.90	20.55	0.684	0.799
CAT	9.11	17.98	7.53	18.22	15.26	11.08	0.169	0.276
GPx	348.21^{B}	449.09^{A}	485.56^{A}	364.45^{B}	405.19	418.47	0.003	0.601
TSOD	97.34	101.65	97.79	94.73	100.86	94.89	0.712	0.175
28 d								
MDA	3.64	2.93	4.24	3.27	2.93	4.11	0.509	0.078
TAOC	24.77	23.75	27.02	22.58	27.59^{a}	21.4 7^{b}	0.678	0.047
CAT	11.43	12.83	14.74	17.33	17.94^{a}	10.23^{b}	0.581	0.025
GPx	494.83	467.78	394.14	486.75	446.97	474.78	0.215	0.446
TSOD	97.21	100.23	99.38	95.46	97.92	98.22	0.586	0.911
35 d								
MDA	8.95	6.33	8.09	7.91	7.76	7.88	0.571	0.933
TAOC	20.78	22.78	22.80	18.76	22.71^{a}	19.84^{b}	0.091	0.031
CAT	10.61^{B}	18.01^{A}	7.71^{B}	18.96^{A}	15.09	12.73	0.003	0.238
GPx	630.64	621.58	623.05	642.27	640.86	617.91	0.922	0.347
TSOD	89.88	92.34	89.78	94.55	91.06	92.31	0.738	0.737

^{a,b} = P< 0.05
A,B = P< 0.01

Table 7 Plasma ceruloplasmin content (mg/L) post challenge

Diet	CT		PP		MOS		PP+MOS		SEM	Diet				Challenge		P value		
Challenge	NaCL	Chal.	NaCL	Chal.	NaCL	Chal.	NaCL	Chal.		CT	PP	MOS	PP+MOS	NaCL	Chal.	diet	Chal.	Int.
d 28																		
Ceruloplasmin	62.59	75.97	51.07	66.25	55.43	64.88	55.05	62.04	1.626	69.28	58.65	60.16	58.55	56.03	67.29	0.10	0.01	0.87
d 35																		
Ceruloplasmin	85.09	71.05	75.75	72.86	75.97	76.02	64.39	72.09	3.146	78.07	74.30	75.99	68.24	75.30	73.01	0.72	0.72	0.60

*Note: Data in the same line with different superscripts differ significantly (P<0.05).

Plasma superoxide anion and hydroxyl radical inhibiting capacity

No main effects of diet, challenge and their interaction on plasma superoxide anion and hydroxyl radical inhibiting capacity were observed on d 4 post infection (Table 8). On d 7 post challenge, challenge decreased plasma superoxide anion ($P< 0.05$) and hydroxyl radical inhibiting capacity ($P< 0.05$). Dietary PP and MOS increased and plasma superoxide anion inhibiting capacity compared to Control group ($P<0.05$).

Plasma superoxide anion inhibiting capacity was not influenced at d 13 post infection, diet, challenge and their interactions did not affect; piglets fed PP dietary supplementation had higher plasma hydroxyl radical inhibiting capacity than Control ($P<0.05$). No interaction between diet and challenge on plasma hydroxyl radical inhibiting capacity were observed.

Table 8 Plasma superoxide anion and hydroxyl radical inhibiting capacity (U/L)

		Superoxide anion inhibiting ability			**Hydroxyl radical inhibiting ability**		
Diet	Challenge	d 25	d 27	d 34	d 25	d 27	d 34
CT	Saline	187.38	183.43	181.90	595.85	683.55	543.05
	Challenge	185.49	170.87	173.69	657.15	529.50	565.94
PP	Saline	195.04	188.53	171.78	897.92	684.46	697.24
	Challenge	183.19	179.10	162.80	634.54	571.55	740.56
MOS	Saline	181.65	191.77	162.20	725.05	693.31	656.80
	Challenge	186.88	184.81	170.04	792.38	683.20	638.61
PP+MOS	Saline	185.92	178.45	174.60	620.49	658.20	617.12
	Challenge	187.20	172.04	171.12	761.77	562.01	686.85
SEM		1.714	1.160	2.217	39.85	18.55	20.46
Diet	CT	186.43	177.15b	177.80	626.50	606.52	554.49 [b]
	PP	189.12	183.82 [a]	168.19	766.23	628.01	718.90 [a]
	MOS	184.26	188.29 [a]	166.12	758.71	688.25	647.70 [ab]
	PP+MOS	186.56	175.88 [b]	172.86	691.13	610.10	651.99 [ab]
Challenge	Saline	187.50	185.74	172.62	709.83	682.71	628.55
	Challenge	185.69	177.13	170.01	711.46	583.74	657.99
				P Value			
Diet		0.800	0.005	0.250	0.580	0.394	0.079
Challenge		0.605	0.002	0.481	0.984	0.016	0.482
Interaction		0.365	0.795	0.523	0.315	0.708	0.891

*Note: Data in the same column with different superscripts differ significantly ($P<0.05$).

5.5.4 Plasma metabolites

No dietary treatment effects on plasma total cholesterol were observed. Dietary MOS decreased plasma total triglyceride content at d 7 of the experiment, and there were no difference at d 21 among treatments (Table 9).

There were no differences in plasma total protein in piglets fed treated diets during pre-challenge phase (Table 9). At 7 days, piglets from PP showed a lower PUN value than piglets fed PP+MOS ($P< 0.05$). With regard to plasma lysozyme activity, piglets fed PP+MOS diet had a decreased lysozyme content compared to other piglets ($P< 0.05$). During challenge there were no main effects of diet, challenge and their interactions on plasma urea nitrogen content in piglets (Table 10).

Challenge did not affect plasma lysozyme content. Dietary PP increased plasma lysozyme activity compared to PP+MOS supplementation on d 28 ($P< 0.05$). No interactions between diet and challenge on plasma lysozyme content existed ($P>0.05$).

Table 9 Plasma metabolites (mg/L) pre challenge

Item	CT	PP	MOS	PP+MOS	P value
Total cholesterol					
d 7	66.22±8.73	56.74±14.09	63.44±7.16	52.32±25.95	0.432
d 21	69.02±7.88	85.01±11.27	81.81±15.03	75.18±8.60	0.120
d 7	43.69±23.68 ^{a}	32.41±10.9 ^{ab}	15.36±9.11 ^{b}	43.51±19.12 ^{a}	0.031
d 21	25.78±5.74	37.72±25.44	39.26±17.58	24.23±12.42	0.031
Total protein (mg/mL)					
d 7	35.45±5.40	37.91±4.23	40.78±7.30	37.58±4.35	0.428
d 21	37.69±1.48	38.58±3.24	41.66±4.53	38.69±3.35	0.219
Urea nitrogen (mg/L)					
d 7	68.00±18.59 ^{ab}	53.18±17.96 ^{b}	80.70±25.20 ^{ab}	92.39±31.81 ^{a}	0.058
d 21	43.57±14.90	34.25±17.25	52.84±14.08	49.52±13.89	0.247
Lysozyme (μg/mL)					
d 7	6.53±0.58 ^{a}	6.69±0.46 ^{a}	6.40±0.48 ^{a}	4.87±1.89 ^{b}	0.027
d 21	6.66±0.51	6.33±0.71	6.81±0.33	5.66±1.75	0.222

*Note: Data in the same line with different superscripts differ significantly ($P<0.05$).

Table 10 Plasma urea nitrogen (mg/L) and lysozyme content (μg/mL) post infection

Diet	CT		PP		MOS		PP+MOS		SEM	Diet				Challenge		P value		
Challenge	Saline	Challenge	Saline	challenge	Saline	challenge	Saline	Challenge		CT	PP	MOS	PP+MOS	saline	challenge	diet	challenge	Interaction
d 25																		
Urea nitrogen	46.99	51.72	41.92	58.10	65.06	50.60	50.04	49.98	3.48	48.88	50.01	57.83	50.01	51.00	52.68	0.791	0.822	0.473
Lysozyme	6.50	6.87	7.07	6.84	6.60	6.74	6.42	6.73	0.098	6.68	6.95	6.67	6.57	6.64	6.80	0.567	0.449	0.707
d 28																		
Urea nitrogen	42.79	58.29	39.35	40.67	63.71	50.94	64.08	45.55	3.01	50.54	40.02	57.32	54.81	52.48	48.87	0.226	0.556	0.228
Lysozyme	6.23	6.63	7.05	6.50	5.70	5.84	5.28	4.97	0.213	6.43^{ab}	6.72 ^{a}	5.77 ^{ab}	5.09 b	6.05	5.986	0.083	0.854	0.864
d 35																		
Urea nitrogen	91.20	61.88	64.78	69.26	84.94	71.55	75.83	74.68	4.881	76.53	67.02	78.24	75.25	79.19	69.34	0.853	0.328	0.632
Lysozyme	6.71	5.60	6.72	6.59	6.63	6.40	6.21	5.97	0.144	6.15	6.67	6.51	6.09	6.57	6.14	0.461	0.156	0.600

*Note: Data in the same line with different superscripts differ significantly (P<0.05).

5.5.5 Enzyme activities

Ileal inflammatory enzyme activity and nitric oxide production

On d 25, challenge led to a comparative enhancement of iNOS activity (P= 0.052) and NO production (P= 0.063), and significant increase of ileal activity of MPO (P= 0.001). No dietary effects and interactions between diet and challenge were observed (table 11). On d 28, challenge significantly increased ileal activities of iNOS, MPO, and NO production (P< 0.05). Dietary PP or PP+MOS significantly inhibit the increase of iNOS activity and NO production following challenge compared to control (P< 0.05). Dietary PP or MOS markedly suppressed the elevation of MPO activity post infection (P< 0.05). No interactions on the enzyme activity and NO production were observed.

Table11 . Enzymatic activity and iNOS production (activity/mL)

	Diet				Challenge		P	
	Ctr	**PP**	**MOS**	**PP+MOS**	**NaCL**	**E.coli**	**Diet**	**Challenge**
25 d								
MPO	0.38	0.33	0.33	0.37	0.25^{B}	0.46^{A}	0.799	0.001
iNOS	1.02	0.95	1.00	0.99	0.84	1.14	0.983	0.052
NO	4.60	3.21	5.85	2.25	2.45	5.51	0.360	0.063
35 d								
MPO	0.36^{a}	0.22^{b}	0.24^{b}	0.30^{ab}	0.23^{b}	0.33^{a}	0.045	0.014
iNOS	0.98	0.81	0.93	0.94	0.73^{b}	1.09^{a}	0.077	0.021
NO	3.92	2.79	3.12	2.99	2.43^{B}	3.99^{A}	0.085	0.001

^{a,b} = P< 0.05
A,B = P< 0.01

Digestive enzymes activity

There were no significant difference in pH in stomach and small intestine digesta among treatments (Table 12). Dietary PP enhanced lipase activity compared to MOS and PP+MOS treatments (P<0.05). Trypsin activity in CT and PP groups was markedly higher than that in PP+MOS group (P<0.05). No difference was observed with regard to amylase and pepsin activity among treatments.

Table 12 Digestive tract pH and enzyme activity

Item	CT	PP	MOS	PP+MOS	P value
pH					
stomach	4.84±1.27	5.02±0.55	5.40±0.95	5.60±0.45	0.667
Small intestine	6.07±0.76	5.91±0.52	6.23±0.29	6.06±0.23	0.885
Digestive enzyme activity (U/mg prot)					
Amylase	226.88±93.48	393.93±169.87	370.27±88.10	403.17±157.06	0.272
Lipase	3.95±1.49 ^{ab}	5.97±1.91 ^{a}	3.39±0.75b	3.04±0.87 ^{b}	0.039
Trypsin	241.96±53.61 ^{a}	243.47±40.92 ^{a}	181.20±23.11 ^{ab}	148.27±34.74 ^{b}	0.013
Pepsin	3.97±1.74	4.34±1.77	4.71±1.55	4.54±0.83	0.910

*Note: Data in the same column with different superscripts differ significantly (P<0.05).

5.6 Discussion and conclusion

Although some oligosaccharides and polyphenols are considered to be non-nutritive, interest in these compounds has arisen due to their possible beneficial effects on health. The ability of MOS to improve growth performance in piglets has been reported by several authors (Miguel et al. 2004; Rozeboom et al. 2005). It was observed that plant polyphenols supplemented in the diet at low dosage did not negatively affect the growth in chickens (Brenes et al. 2008) and pigs (Sehm et al. 2007). In the current study, feeding diets containing PP or MOS did not affect animal performance. Similar to our observations, Orban et al. (1997) did not observe changes of growth performance in piglets fed control diet or diet supplemented with either an antibiotic or an oligosaccharide, and a recently study in early weaned rabbits demonstrated that supplementation of the starter diet with MOS or inulin had no effect on growth of during the whole fattening period (Volek et al. 2007). Miguel et al. (2004) reported that the growth promotion effect of a MOS (Bio-Mos) seemed to be pronounced in piglets that had a slow growth rate (< 180 grams per day) during the first 2 weeks postweaning. In the current study, Piglets gained more than 300 grams per day during the second week post weaning, which demonstrated the diets were in high quality and piglets grew rapidly and healthily. The unexpected results of this study were that the dietary combination of PP and MOS impaired piglets' growth performance, which suggested an unclear interaction between PP and MOS pending for further research. Our results indicated that *E.coli* challenge did not influenced negatively growth

performance in piglets. Oral inoculation of *E.coli* only partially reduced feed intake during 21-35 d (P=0.117), 25-35 d (P=0.103), 28-35 d (P=0.104), suggesting a mediocre and chronic infection. The reason may be attributed to several factors, including diet quality, environmental conditions, health status within herd. In our research, the diets were in high quality, facilities and sanitary conditions were excellent. Thus the piglets were in good health status (with an average of around 5 log CFU E.coli and 5 log CFU clostridium in every gram of caecum content post infection, data not shown) and no piglets suffered from diarrhoea before the challenge and only one piglet undergone one day of slight diarrhoea after challenge in control group. And hence the infection resistance in the herd to *E.coli* challenge was higher. Similar to our results, no growth performance changes were observed in lambs after parenteral administration of E. coli O157:H7 (Demirel et al. 2007). Dietary PP or MOS decreased feed intake while improved feed efficiency during d 21-25, and improved feed efficiency during d 21-28. Furthermore, dietary MOS increased ADG during d 25-28, improved feed efficiency during d 21-35. Unexpectedly, dietary PP+MOS didn't exert effects on growth performance. There is little literature on dietary PP or MOS on growth performance in challenged piglets. It was reported by Demirel et al. (2007) that MOS fed lambs did not modify the growth after *E.coli* administration. While improved infection resistance effects of MOS were observed by Torrecillas et al. (2007) in *Vibrio alginolyticus*--infected European sea bass. Similarly, reduced gut associated lymphoid tissue (GALT) activation via the Peyer's patches in the ileum and improved health in piglets fed polyphenol rich apple pomace or red-wine pomace diet was reported by Sehm et al. (2007). These results suggested that dietary MOS or PP may increase the animal's ability to cope with stress. In the current research, no detectable changes occurred in the levels of organisms in faeces and caecum content throughout the experimental period. Similar no impact results of dietary oligosaccharide on microbe population were also observed in other previous studies in piglets (Orban et al, 1996; van der Peet-Schwering et al. 2007). The lack of response of dietary supplements on microbial population would probably be ascribed to the nutritional balanced diet and the excellent facility or the health status of the piglets. No piglets suffered from diarrhoea in this study, suggesting that there were few problems with intestine health. It was possible that dietary treatments may affect other bacterial populations beyond those enumerated in this study. Dietary PP partially increased plasma total cholesterol content at 21 d in the present study (P<0.10). Less

accumulation of cholesterol in the aorta was observed in guinea pigs fed the grape diet (Zern et al. 2003). Apple polyphenols significantly decreased total cholesterol level in moderately obese adults (Nagasako-Akazome et al. 2007). Polyphenols decreased cholesterol in familial hypercholesterolemic swine (Metzger et al. 2009). Above studies were all performed in adult animals with comparatively higher basic cholesterol level compared with the current study using the growing piglets. More investigations are required to elucidate the effect of PP on cholesterol metabolism in growing animals. Effects of oligosaccharides on piglet blood lipid profile are not well defined. Results from Grela et al. (2006) showed that Fructooligosaccharides (FOS) and MOS addition lowered the level of total cholesterol distinctly and decrease triglyceride with tendency in piglets. Yalcinkaya et al. (2008) did not observed effects of MOS on serum triglyceride in broilers. MOS additive induced a transitory decrease of plasma triglyceride in the current study, different from the results of Santoso et al. (1995) and Kannan et al. (2005) in which MOS decreased serum triglyceride in broiler. The lower level of triglyceride was said to be due to increased level of lactic acid producing bacterial in the gut. 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 platelet(PLT) counts and monocyte percentage on d 7 post infection, and comparative increase of PLT counts on d 14 post infection (P=0.081), indicating the duration of transitional effects of infection. Limited information is available in the literature regarding the haematological changes in E.coli infected piglets. The increased platelet counts may be mediated by the activation of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria such as E.coli or salmonella (Saluk-Juszcazk et al. 1999; Zielinski et al. 2001). Monocytes are the main cellular players responsible for the inflammatory response. Upon challenge, the activation of peripheral blood monocytes may enhance their attachment and or migration into infection sites and their various secretory and other functions such as phagocytic capacity of monocyte-derived macrophages, thus influencing the inflammatory reaction and pathogenesis (Kelley et al. 1998). Values of PCV, Hb and RBC generally fluctuate together, which make it advantageous in clinical practice that we could predict changes of Hb and RBC from the known value of PCV. Modern pigs are raised in confined house and have no access to obtain iron from soil and are susceptible to anemia. It was reported that pigs with access to an outdoor area had a 30% higher hemoglobin content in blood

(Chmielowiec-Korzeniowska et al. 2008). Increased blood HGB and RBC in piglets fed MOS diet on d 25 and increased HGB and PCV in PP fed piglets on d 28 demonstrated that the two supplements exerted favorable modulation in peripheral blood profile.

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), peroxidase (GSH-Px) and catalase (CAT) play most important role in the antioxidant protective system. First, SOD converts superoxide anion to hydrogen peroxide in a cellular antioxidant reaction. Thereafter, GSH-Px and CAT independently detoxify produced hydrogen peroxide (Joeschke, 1995). In the present work, decreased systemic TAOC and CAT activity, increased MDA content were seen on 6 post infection, which indicated that the excessive release of ROS or free radical were beyond the scavenging capacity of antioxidant defense system. Increased TAOC and CAT activity in PP-fed piglets was observed on 14 d post infection, which suggested that dietary PP could enhance the capacity of scavenging free radicals and decrease the damage of tissues or cells due to infection. And the challenge effects on antioxidant defense system seemed to diminish thence. In the current study, challenge led to a significant higher production of superoxide anion and hydroxyl radical generation in plasma due to challenge on d 28 which was perhaps due to increase of blood PLT counts. It is reported that bacterial LPS are able to activate blood platelet, and the activated platelets produce thiobarbituric acid-reactive substances, free radicals, and cause the secretion of the substances stored in platelet granules (Saluk-Juszcazk et al. 1999). Thus, the increased PLT counts may contribute the higher superoxide anion and hydroxyl radical production. Decreased plasma superoxide anion and hydroxyl radical inhibiting capacity on 7 d post infection were indicative of excess of ROS production in the inflammatory process. Excessive production of active forms of oxygen would damage the tissues of the macroorganism (Korkina et al. 1992). Enhanced plasma superoxide anion

and hydroxyl radical inhibiting capacity were seen in PP and MOS fed piglets on 7 d post infection and in PP fed piglets on 14 d post infection, which demonstrated that dietary supplement favorably affected the ability of animal to quench the ROS during challenge. Roles of potent scavengers of oxygen free radicals have been observed in polyphenols from apple (Lu and Foo, 2000), green tea (Salah et al. 1995; Unno et al. 2000), grape seed (Bagchi et al. 1997) and Olive leaves (Benavente-Garcia et al. 2000; Lee et al. 2009) in vitro. The present work confirmed the effects of plant polyphenosl mixture on superoxide anion inhibition and lipid peroxidation level reduction in animal challenge model. The current study indicated that dietary MOS may be also positively affect the antioxidant capacity of piglets since increased plasma GPx activity on d 25 and superoxide anion inhibiting capacity on d 28. Coincided with our results, previous studies had reported higher antioxidant activity in chickens and piglets fed MOS-supplemented diets (Zhou et al., 1999; Shao et al., 2000). Furthermore, oligosaccharides from agar were also shown to exert the in vitro and in vivo hepatoprotective effect through scavenging oxidative damage induced by ROS (Chen et al. 2006). As an acute-phase reactant, ceruloplasmin participates in inflammatory responses. An elevation of plasma ceruloplasmin concentration commonly accompanies pathological states include infection, trauma, surgery, tissue infarction and advanced cancer (Gabay and Kushner, 1999). Bacterial infection induced the increase of plasma ceruloplasimin concentration as part of the acute phase response, which is the natural response of body to extrinsic stimulation. However, excessive and long-term existence of ceruloplasmin is associated with injury of body. Comparable reduction in plasma ceruloplasmin in piglets fed PP and PP+MOS diets on 7 d post infection observed in the present study indicated that dietary PP was probably beneficial to shorten or overcome acute phase response. The ileum is a frequent site of involvement in necrotizing enterocolitis, a disease probably related to bacterial endotoxin (Hsueh et al. 1987). Due to its vulnerability to develop injury during bacterial challenge, we selected the ileum segment of intestine to examine the inflammatory response. During the early phase of infection, endotoxin and other bacterial by-products activated the primed neutrophiles, monocyte and macrophages, initiating a signal transduction cascade leading to the release of a number of pro-inflammatory cytokines and inducible enzymes such as: iNOS, inducible phospholipase A2 and the inducible cyclooxygenase (Lazarov et al. 2000), which would lead to a release of massive amounts of oxidants and cause further tissue damage. In

response to microbial invasion, MPO is released from the cytoplasmic granules of neutrophils and activated monocytes into the extracellular space and phagosome, where it generates hypocephalous acids that have a strong microbicidal effect. As an important component in degranulation material of leukocytes, MPO exert critical in animal innate host defenses and is used as a marker of inflammation caused by disease such as asthma, systemic vasculitis or environment irritants (Sugiyama et al. 2001). The increased MPO activity post infection on d 28 may partly due to the secretion of monocyte in the peripheral blood. The inhibition on the elevation of MPO activity due to *E.coli* infection in PP and MOS fed piglets suggested that dietary PP and MOS have the potential to alleviate the inflammatory response of the intestine. Although there was a marked elevation of ileac MPO activity on d 25, we didn't observe the corresponding change of neutrophile or monocyte in peripheral blood. This may be related to the fact that the hematological and biochemical indices of pigs are affected by a broad range of environmental and physiological factors including nutrition, age, sex and zootechnical conditions. During the phase of infection, challenge induced significant elevation of MPO activity, suggesting the infiltration of neutrophils. On d 28, although the main effect of challenge on MPO activity was significant, the elevation of MPO activity was mainly from the Control group, and the MPO activity in challenged piglets fed PP and MOS diets retrieved to the normalcy. Nitric oxide produced by iNOS is a central effector molecule in the innate immune system, and its primary function in host defense appears to be to damage and destroy pathogens (Nathan et al. 2000). Thus, nitric oxide is produced by the body to fight infections, whereas the excess production of NO in the subsequent or lasting phase is involved in cellular damage, tissue and .organ dysfunction (Liang et al. 1999).Activated neutrophils and macrophages express iNOS, and produce excessive amounts of NO, which play key roles in infection pathogenesis (Tsukahara et al. 2001). In the present study, challenge induced elevations of iNOS activity and subsequent NO production, indicating the involvement of inflammatory response. The reduced elevation of iNOS activity and NO production due to challenge on d 28 in PP fed piglets suggested that dietary PP might be beneficial to the local gut immunity and inflammatory response of the body. Our results showed that dietary PP had the potential to reduce the duration of enteric infection. On the 14th day post-infection, judged by the antioxidant property and haematological profile, the effects of E.coli infection started to diminish. Only blood PLT counts and TAOC were negatively

affected in challenged piglets than the controls ($P<0.05$). Other parameters were not significantly affected. The results suggested that by the 14th day post infection, the systemic antioxidant defense capacity was beginning to recover. This was consistent with the similar growth performance subsequently. In this study, dietary PP inhibited the elevation of MPO and iNOS activity and NO production on 7 d post infection, suggesting the potential to reduce the duration of ileac inflammatory response. This was in agreement with the comparatively enhanced growth performance during the period d 35 to 42.

Plant extracts and prebiotics represented typically natural origin components that favorably affect the growth of animals and are being widely used in EU. Combination with synergistically acting components of natural origin seems to be a promising way for the efficient replacement of antibiotic growth promoter from the viewpoint of practice (Bomba et al. 2006). Synergistic effects were observed in the constituents of green tea extract on protection against experimentally induced cytotoxicity in renal cells (Hisamura et al. 2008).In co-culture studies, a synergistic effect of the probiotic strains and the green tea extract was observed against both *Staph. aureus* and *Strep. pyogenes* (Su et al. 2008). However, in the present study, administration of PP and MOS together failed to exhibit the cooperative or synergistic action in piglets. Information about the combination of plant extracts and oligosaccharide in animal trial is little. Reasons for the unexpected results were not clear, and the following possibility may affect. First, in our study, the formulation for combination PP and MOS was obtained from *in vitro* results based on optimization of antioxidant and antimicrobial activity. It was possible that the results of *in vitro* vary a lot with animal trial. Second, metabolites of PP and MOS in the animal body may react and form some deleterious substances which may impair or burden the innate immune system, since the decreased plasma lysozyme was observed on d 28. Third, the diet and the dosage may also affect the response of piglets to dietary manipulation. Our results may remind of the caution of the specious synergistic action between feed additives with their respectively beneficial effects in practice. Further studies are needed to elucidate the interaction of plant polyphenols and MOS in the piglet diet. In conclusion, this study demonstrated that dietary supplementation of PP or MOS did not substantially improved piglets' growth performance. Dietary PP reduced systemic lipid peroxidation, enhanced the antioxidant defence system. Piglets fed diet with addition of PP in conjunction with MOS were characterized by the lower activity of digestive enzyme and lysozyme, higher plasma urea

nitrogen, and the inferior feed efficiency. The unfavourable responses of combination of PP and MOS might be indicative of some unclear interactive effects between the two additives. Growth performance was not a better indicator of degree of challenge than plasma antioxidant capacity and inflammatory enzymes. Administration of plant polyphenols and mannan oligosaccharide had the potential to improve feed efficiency during infection, while combination of PP and MOS had no effect. Dietary PP and MOS favourably affected the systemic antioxidant capacity by enhancing the GPx activity and TAOC. Dietary PP shortened the ileac mucosa inflammatory response via inhibition the elevation of MPO and iNOS activity and NO production. Further studies are warranted to clarify the mechanisms underlying the role of dietary PP and MOS on antioxidant capacity and gut health modulation as well as the interaction of PP and MOS in weanling piglets.

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Chapter 6

General discussion

6.1 General discussion

Weaning period is 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 hypothesis is that the reduction of feed intake following weaning is critical in determining subsequent gut development, growth, maturation of gut-associated lymphoid tissue and related disease susceptibility (Pluske et al., 1997; Van Beers-Schreurs et al.,1998; Sprewenberg et al., 2001; Vente Spreuwenberg et al. 2003). A relationship between feed intake (FI) after weaning and villus height has been reported and it was speculated that this relationship may affect the overall efficiency of nutrient capture and utilisation (Pluske et al. 1997). It is also recognised that feed intake in pigs is highly variable following weaning. Our data confirmed that growth performance are clearly affected by weaning. In the trial that had the aim to determine the effects of dietary supplementation of rice milk on piglets growth and health status we evaluated for the use of a product rich in sugars and antioxidants in the nursing and nursery piglets and we saw that piglets growth was higher when litters were fed rice milk until weaning. The higher growth of piglets supplemented with rice milk only during nursing period may be attributed to the higher supply of sugars before the weaning leading treated litters to reach a more and higher homogeneous weight at the end of the suckling period compared to litters fed the creep feed.

Also dairy products are known to have a beneficial effect on the gut-lymphoid associated tissue of the weaning piglets (Politis et al., 2007) and this was one of the principle of the first trial. This trial was designed with the aim to determine the effects of a new delivery system designed for oral vaccination or delivery of bioactive molecule in the gut. The trial was also designed to evaluate the potential of the sow's milk to modulate the systemic as well as local immune response against the antigen ovalbumin incorporated in the vaccine. Until now, a small number of papers have been published to investigate the effect of oral vaccination with Ovalbumin (OVA), Sow's milk (SM) and Flagelline (FLA) on humoral immunity in weaned piglets. Our results indicate, although the overall level of specific immunoglobulin was low, that the vaccination with OVA+FLA+SM influenced feces IgA-G levels and was accompanied by enhanced serum IgG concentrations in weaned piglets. The action of these three compounds together determined higher response of the piglets

humoral immune system compared to other treatments. Others authors demonstrated the immune stimulatory action of milk to post weaned piglets and also in the current study the mechanism by which this vaccination enhanced immunoglobulin levels may be attributed to the immunostimulatory action of the peptide contained in the milk Politis et al. (2007). Interestingly, introduction of these bioactive compounds to the immunological immature neonatal gastrointestinal tract may potentiate an inappropriate immune response. Immune stimulation accumulates in the partitioning of nutrients from normal growth to support components of the immune response improving also growth performance of the animals (data not shown) during a critical phase as the weaning. Others author also reported the positive effects of these bioactive compounds such Spray dried plasma on the growth performance of the weaned piglets, modulating also the microflora increasing the number of lactobacilli in ileum and caecum (Torrallardona et al. 2003).

In the last trial of this work attention was focused to the stress that piglets face off at weaning in association with a challenge with *E. coli* during a dietary treatment of mannanooligosaccharides (MOS) and a mixture of Plant Polyphenols (PP). The use of no fermentable carbohydrates could enhance colonic microbial stability and diversity, with concomitant stimulation of the growth of *Lactobacillus sobrius*, a novel and beneficial member of the porcine commensal microflora (Konstantinov et al., 2004a). Nutritional supplementation (PP or MOS) improved feed efficiency in presence of *E.coli* challenge. Dietary PP or MOS had the potential to improve the antioxidant property, and dietary PP favorably modulated gut mucosa inflammatory response induced by *E.coli* infection. (Konstantinov et al. 2004 a; 2006 b). In the study presented no positive effect were shown on the microflora of the treated animals. Increased intake of prebiotics creates more favourable lumen conditions for gut health but direct evidence for enhanced resistance to unfavourable conditions is still lacking. Empirical evidence suggests that plant extracts may offer benefits in boosting the immune system; thus preventing disease in production animals and plant derived products have gained increased interest as possible as feed additives for non-ruminant species (Wenk 2003; Windish et al. 2008). Plants, and where they have been indentified their bioactive components, are very different and their potential to enhance pig health and immunity has only been scarcely evaluated in vivo. The study we conducted with or without MOS included the treatment with or without mixture of polyphenols from plant extract which does not allow the investigation of the immune

properties of a specific bioactive component; in fact this mixture was derived from a study in vitro using a large number of plant extracts to understand which could have more interesting results on the oxidative stress. The overall results demonstrated that dietary PP had the potential to enhance systemic antioxidant capacity, while dietary PP+MOS was adverse to growth probably due to some unclear interactions.

To conclude, weaning period in pigs is complex and its nutritional control is not easy to achieve. However important progress has been made in understanding this complexity at the GIT level, with reference to the physiology, microbiota and local immune system. By contrast there is still the need for a basic knowledge on the functional foods to understand exactly their mechanism before to use these substances in commercial diets.

Chapter 7

Summary

7.1 Summary

At weaning piglets faces three challenges. First there are major changes to its food supply. Not only the piglets have to find the new food from a creep feeder but the characteristic of this feed are completely different from the sow's milk. The second major challenge at weaning is the change of the physical environment. At weaning litters are generally mixed together into weaner pools. The third challenge is the physiological stress that accompanies moving and mixing. When all these changes are taken into account it is little wonder tat the rate of growth of the piglet falls after weaning (Pluske et al., 2000).

Special diets and management schemes have been developed with the goal to overcoming the post-weaning syndrome in piglets (Zehn-Ping et al., 2007). Systems which use milk-based liquid diets have demonstrated piglets growth performance comparable to that in suckling piglets (Kim et al., 2004; Lecce et al., 1969).

Economic interests have a large impact on modern commercial pig production, and by decreasing the weaning age, the number of weaned pigs produced annually per sow have increased dramatically. Early weaning may have negative consequences, manifested as poor growth and PWD, because the pig's GI tract is not fully developed at this young age.

Related to the recent ban on in-feed antibiotics, many efforts have been made in the past years for optimizing further diet composition in terms of protein, AA and energy. Additional substances including bioactive molecules of milk, prebiotics, and compounds of plant origins with known antimicrobial properties have also been evaluated. It has become clear that diet supplementation with functional foods is probably the best way for preventing post-weaning gut disorders. Mainly of these compounds have also proven successful, acting on gut ecology and preventing outgrowth of pathogenic bacteria (Bontempo et al., 2007). However when these compounds play positive effects *in vivo*, their mechanisms of actions are still unclear, thus calling for additional work. Investigations *in vitro*, on the other hand, clearly show antimicrobial activity of the essential oils. They are supported by one or two mayor molecules or by more subtle synergistic interactions within the extract mixture. However, mayor discrepancies do exist between *in vitro* and *in vivo* results, the latter being variable and rather inconsistent. It appears that the diet itself may neutralize the antimicrobial activity of essential oils *in vivo*.

Finally the bioavailability and pharmakocinetics of these substances in pigs are mostly unknown. This will undoubtedly constitute an area for future investigation.

The aim of this PhD thesis was to evaluate the inclusion of different functional foods and strategy of oral vaccination in weaned piglets by different experimental approaches on growth performance, oxidative status, blood metabolites and gastrointestinal immune status. During past years research with animal models has identified different nutritional approaches that may modulate the production of key factors involved in the activation and regulation of innate and adaptive immunity. Development of new feeding strategies such as functional foods in the diet may influence the regulation of immune response decreasing the production of inflammatory factors, reducing the immunosuppressive effect caused by weaning improving the recovery of immune functions against pathogens. Nowadays there is much interest in the potential health benefits of functional foods such bioactive milk molecules and plant extracts as included in the diet of livestock.

In this chapter, due to the peculiarity of the three trials that were carried out during the last years, the results will be discussed separately.

In the first study the aim was to evaluate the potential of the sow's milk to stimulate the systemic as well as local immune response against the antigen ovalbumin incorporated in the vaccine in 4 groups of 4 animals each. Ovalbumin (OVA); Ovalbumin and flagellin (OVA-FLA); OVA and sow's milk (OVA-SM); (OVA-SM-FLA) to dosage the serum antibodies against OVA, IgA anti-OVA in feces, immunitary cells cultivated with OVA to evaluate the response to the proliferation for cytometrie and the cytokines production Interleukine-2 (IL-2) and IL-4. Experimental animal studies have indicated that oral administration of antigens targets the systemic T cell compartment, diminishes cell-mediated immune responses, and induces tolerance. This phenomenon might lead to the induction of cytokines such as TGF β and IL-10, and consequently enhance antigen-specific antibodies such as IgA and IgG. While the humoral immune response is critical in the control of some mucosal pathogens, its effect might be questionable on other mucosal pathogens where cell mediated immune responses may play a larger role. Until now, a small number of papers have been published to investigate the effect of oral vaccination with OVA, SM and FLA on humoral immunity in weaned piglets. IgG and IgA, the major serum immunoglobulins, are key components of the humoral immunity in all mammals (Li

et al. 2007). Our results indicate that the vaccination with OVA+FLA+SM influenced feces IgA-G levels and was accompanied by enhanced serum IgG concentrations in weaned piglets. The action of these three compounds together determined higher response of the piglets humoral immune system compared to other treatments. In the literature is demonstrated the immune stimulatory action of milk to post weaned piglets and also in the current study the mechanism by which this vaccination enhanced immunoglobulin levels may be attributed to the immunostimulatory action of the peptide contained in the milk (Politis et al.,2007). Interestingly, introduction of these bioactive compounds to the immunological immature neonatal gastrointestinal tract may potentiate an inappropriate immune response. Immune stimulation accumulates in the partitioning of nutrients from normal growth to support components of the immune response improving also growth performance of the animals (data not shown) during a critical phase as the weaning. In the current study, the treatment with OVA+FLA or OVA+SM but not the combination of the two immunomodulator (OVA+FLA+SM) was able to enhanced MLN mRNA expression of IFN-ɣ and IL-2, two important cytokines on the regulation of host immune response. Thus, the current results suggest that the oral vaccination with component such as milk or flagellin provokes a modulation of cytokine response in weaned piglets. The gut is a major immune organ in mammals. Specifically, the T and B lymphocytes proliferate and mature in the gut-associated lymphoid tissue (GALT), mounting a successful immune response to antigens. Many studies have shown that the GALT is composed of immune cells and lymph nodes, conferring both non-specific and specific immune functions (Poussier & Julius 1994). IFN-ɣ probably plays a key role in activation, proliferation and differentiation of T-cells, as well as improving both specific and non-specific immune responses in weaned piglets. The enhanced expression of IL-2, which is important for primary T-cell activation, could result in a high immunostimulation following the treatment with the vaccine OVA+SM. It is likely that the increase of this cytokine production associated with the vaccination observed in this study contributed to higher antibody response improving the gut immune system. Further studies will explore the potential of these new diets strategies to prevent the negative impact of a pathogen challenge. In conclusion, oral vaccination could represent the future of the nutrition thanks

to its positive action directly on the gut health influencing humoral immune response in piglets, suggesting an enhancement of host defenses against presenting pathogens.

The aim of the second trial was determine the effects of dietary supplementation of rice milk on piglets growth and health status. In the present study we evaluated for the first time the use of an alternative vegetable milk such rice in the nursing and nursery piglets. Characteristic of this product is beside the high level of glucose and maltose and high level of ɣ-Oryzanol. In addition to its outstanding antioxidant activity, it has been demonstrated that ɣ-Oryzanol has multiple neutraceutical functions, including reduction of total cholesterol, improvement of the plasma lipid pattern and inhibition of platelet aggregation (Juliano et al.,2005; Nam et al., 2008). Before and after weaning the piglets undergo a stress-related growth check, often associated with anorexia and under-nutrition, with predisposition to diarrhea and infection. Due to its richness in carbohydrates such as glucose and maltose rice milk may play an important role in nursery piglets thanks to its high palatability and sugars and antioxidant availability. Moreover carbohydrates present in rice milk may represent an important source of energy for the weaker piglets of litters which do not have enough strength to compete with the brothers for a correct consumption of maternal milk (Stephas et al., 1998). Piglets growth was higher when litters were fed rice milk until weaning. After weaning the positive effects of rice milk supplementation were reduced probably because the high palatability of the product favoured a high milk intake reducing dry feed intake. The higher growth of piglets supplemented with rice milk only during nursing may be attributed to the higher supply of sugars before the weaning leading treated litters to reach a more and higher homogeneous weight at the end of the suckling period compared to litters fed the creep feed. The richness of carbohydrates and its palatability make this product an interesting energy source rapidly available to evaluate during a critical period such as pre-weaning. In literature is reported that glucose can be a satisfactory energy source of carbohydrates such as lactose for the pig during its first week of life (Aherne et al., 1969). The availability of these substances can homogenize the weight of the litters giving more available energy to the weakest animals that can’t get enough energy from the sow’s milk.

However, it should be stated that rice milk is used in human nutrition since a long time, but it has a few chances to be used in animal nutrition due to the high production costs.

In this study, also parameters related to oxidative stress were considered as it is well known that piglets are subjected to a high level of stress during weaning.

Reactive oxygen species (ROMs) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron (Aruoma, 1994) such as a superoxide ion (O21 ±), nitrogen oxide (NO) and hydroxyl radical (HO±). Even though naturally present in the organism, they are mainly connected to cell compartments and counterbalanced by natural anti-oxidant molecules, such as glutathione, glutathione peroxidase, superoxide dismutase, Q coenzyme, vitamin E and vitamin C, acting as free radical scavengers (Miller et al., 1993). Due to a genetic susceptibility, the early weaning pigs are quite exposed to oxidative stress which increase the amount of substrate for ROMs activity and thus the risk of lipid peroxidation (Nolan et al., 1995; Brambilla et al., 2002). The early detection of oxidative imbalance by simple and reliable methods is important to prevent such consequences. The positive action on the parameters such ROMs and the oxi adsorbent test of the animals fed rice milk can be explained by the presence of the ɣ-Oryzanol. Infact is demonstrated that ɣ-Oryzanol is one of the major compounds that contributes to antioxidant improvement and regulation of antioxidant and oxidative stress related genes of stressed rat liver (Hiramitsu et al., 1991). Other studies demonstrated that ɣ-Oryzanol up-regulates the antioxidant genes while down-regulates the oxidative stress genes marker (Maznah et al., 2010). The test to evaluate the antioxidant capacity of the piglets before and after a challenge such the weaning shown interesting data. The animals before weaning presented similar antioxidant capacity (evaluated by the oxy-adsorbent test and by ROMs) and after weaning the animals of the group that received the dietary supplementation with rice milk before and after weaning (TT) shown lower levels of ROMs compared to the animals of the animals that received the control diet in the nursing period (CC, CT)

$P < 0.05$ while no differences where shown between the piglets of TC and TT groups. This confirm our hypotesis about the functionality of the rice milk supplementation in the pre-weaning period.

The animals of the control litters (CC; CT) shown higher value in the oxy-adsorbent test ($P<0.05$) compared to the animals of the TT group. This data are not easy to explain

because in the literature there are no studies on the use of this product in pig nutrition but seems to give a positive response that found confirm in the research on the activities of ɣ-Oryzanol. In conclusion, it can be postulate that rice milk supplementation might have positive effects in growth and feed intake during the immediate postweaning if administered from 10 d of age.

This study suggests that the dietary supplementation during nursery period can improve the growth performance in the piglets after weaning. Therefore this product may be considered a functional foods for its richness of bioactive compounds such as γ-oryzanol. More research will be needed to understand the potential of this functional food in piglets diet.

Therefore the purpose of the third study was to determine the efficacy of plant poliphenols and/or mannanoligosaccharides on growth performance, haematological profile, antioxidant capacity and ileac inflammatory responses of post-weaning *E.coli challenged* piglets. Although some oligosaccharides and polyphenols are considered to be non-nutritive, interest in these compounds has arisen due to their possible beneficial effects on health. Plant extracts and prebiotics represented typically natural origin components that favourably affect the growth of animals and are being widely used in EU. Combination with synergistically acting components of natural origin seems to be a promising way for the efficient replacement of antibiotic growth promoter from the viewpoint of practice (Bomba et al. 2006). Synergistic effects were observed in the constituents of green tea extract on protection against experimentally induced cytotoxicity in renal cells (Hisamura et al. 2008). In co-culture studies, a synergistic effect of prebiotic and the green tea extract was observed against both *Staphilococcus aureus* and *Streptococcus pyogenes* (Su et al. 2008). However, in the present study, administration of PP and MOS together failed to exhibit the cooperative or synergistic action in piglets. Information about the combination of plant extracts and oligosaccharide in animal trial is little. Reasons for the unexpected results were not clear, and the following possibility may affect: first, in our study, the formulation for combination PP and MOS was obtained from *in vitro* results based on optimization of antioxidant and antimicrobial activity. It was possible that the results of *in vitro* vary a lot with animal trial. Second, metabolites of PP and MOS in the animal body may react and form some deleterious substances which may impair or burden the innate

immune system, since the decreased plasma lysozyme was observed on d 28. Third, the diet and the dosage may also affect the response of piglets to dietary manipulation. Our results may remind of the caution of the specific synergistic action between feed additives with their respectively beneficial effects in practice. Further studies are needed to elucidate the interaction of plant polyphenols and MOS in the piglet diet. In conclusion, this study demonstrated that dietary supplementation of PP or MOS did not substantially improved piglets' growth performance. Dietary PP reduced systemic lipid peroxidation, enhanced the antioxidant defence system. Piglets fed diet with addition of PP in conjunction with MOS were characterized by the lower activity of digestive enzyme and lysozyme, higher plasma urea nitrogen, and the inferior feed efficiency. The unfavourable responses of combination of PP and MOS might be indicative of some unclear interactive effects between the two additives. Growth performance was not a better indicator of degree of challenge than plasma antioxidant capacity and inflammatory enzymes. Administration of plant polyphenols and mannanooligosaccharide had the potential to improve feed efficiency during infection, while combination of PP and MOS had no effect. Dietary PP and MOS favourably affected the systemic antioxidant capacity by enhancing the GPx activity and TAOC. Dietary PP shortened the ileac mucosa inflammatory response via inhibition the elevation of MPO and iNOS activity and NO production. Further studies are warranted to clarify the mechanisms underlying the role of dietary PP and MOS on antioxidant capacity and gut health modulation as well as the interaction of PP and MOS in weanling piglets.

Chapter 8

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