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EFFECTS OF DIFFERENT NUTRITIONAL STRATEGIES ON INTESTINAL INFLAMMATION IN PIGS

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ABSTRACT

Intestinal health is essential for the health of the body since the gastro-intestinal mucosa is the main site of interaction with the external environment, as well as the major area colonized by the microbiota. Intestinal health relies on proper barrier function, epithelial integrity and related mechanisms of protection (mucous layer, tight junctions, immune and inflammatory system). In pigs, during the weaning transition, intestinal inflammation and barrier integrity play a crucial role in regulating intestinal health and, consequently, pig's health, growth and productivity. The aim of the project was to assess the impact of different nutritional strategies on the intestinal health of weaning piglets with reference to the inflammatory status and epithelial integrity. Therefore, *in vivo* trials were conducted to test the in-feed supplementation with zinc, tributyrin, or organic acids and nature-identical compounds (NIC) to weaning piglets. All the dietary interventions positively impacted the intestinal inflammatory status and, as a consequence, improved epithelial integrity by modulating tight junctions proteins (zinc or tributyrin) or by enhancing barrier properties measured with Ussing chambers (organic acids and NIC). These findings highlight that intestinal inflammation and barrier function are strictly linked, and that the control of inflammation is essential for adequate barrier function. In addition, in zinc trial and organic acids and NIC trial, better intestinal health could successfully result in better growth performance, as aimed for pig production improvement. To conclude, this work shows that dietary supplementation with bio-active substances such as zinc, tributyrin or organic acids and NIC may improve intestinal health of weaning piglets modulating intestinal inflammatory stress and barrier integrity and allowing better piglet's health, growth and productivity.

Keywords: weaning piglet, intestinal health, inflammatory cytokines, barrier integrity, tight junctions.

CHAPTER 1

INTRODUCTION

INTESTINAL BARRIER

Animals are inhabited by a vast number of microorganisms, such as bacteria, viruses, and unicellular eukaryotes, collectively known as "microbiota". The microbiota colonizes virtually every surface of the body that is exposed to the external environment, including the skin, and the mucosae of the airways, oral cavity, digestive tract and genitourinary tract. Among these multiple sites, the most heavily colonized organ is the gastro-intestinal tract (**GIT**), because of the huge surface area and the richness in substrates that can be used as nutrients by microbes. For example, it has been estimated that the human gut has a surface area of a tennis court (200 m^2) and that the colon alone hosts over 70% of all the microbes in the human body (Sekirov et al., 2010). Human gut microbiota comprises trillions of bacteria, consisting of more than 800 different bacterial species and 7000 strains, that make essential contributions to host metabolism while occupying a protected and nutrient rich environment (Hooper & Macpherson, 2010). Therefore, the gastro-intestinal tract represents the largest mucosal interface between the continuously changing external environment (dense microbial community, dietary antigens and food components) and the closely regulated internal milieu. Under normal conditions, the relationship between intestine and microbiota is defined "homeostatic", so that adverse health effects caused by intestinal microorganisms should be minimized even during environmental perturbations, including shifts in microbiota composition, changes in host diet or pathogenic challenges. According to Hooper and Macpherson, homeostasis is maintained by a hierarchy of three immunological barriers, aiming to limit the direct contact between intestinal bacteria and epithelial cells, to rapidly detect and kill bacteria that manage to penetrate epithelial cells, and to minimize the exposure of resident bacteria to the systemic immune system (Hooper & Macpherson, 2010). For these purposes, a set of immune mechanisms and anatomical and functional structures have been established at the intestinal level to form a – double tasks – selective intestinal barrier, allowing both digestion and absorption of food and protection from microbial challenges and toxic compounds. In this regard, the intestinal barrier function can be defined as the ability to control uptake across the mucosa and to protect the internal milieu from harmful substances present in the lumen (Barreau & Hugot, 2014). A schematic picture of the intestinal barrier structure is presented in Fig. 1 (adapted from Hooper & Macpherson, 2010).

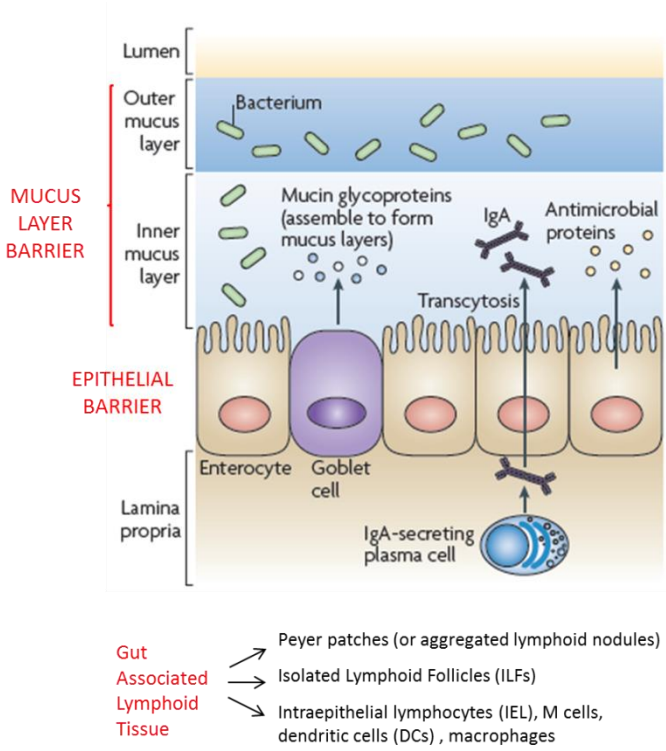

Fig. 1. Intestinal barrier structure

(adapted from Hooper & Macpherson, 2010)

The first line of defense is localized within the lumen itself and is represented by the mucous layer, the viscous gel-like layer that covers the intestinal epithelial cell surface. The mucous layer is composed by mucin glycoproteins secreted by epithelial goblet cells, and forms two structurally distinct strata. The outer –loosely adherent– mucous layer contains a great number of commensal bacteria that contribute to the host defense by secreting anti-microbial compounds and by competing for nutrients. The inner –firmly adherent– mucous layer is a protected zone at the apical epithelial surface, devoid of bacteria due to the abundance of anti-microbial proteins (defensins, cathelicidins, C-type lectins) produced by epithelial cells such as enterocytes, Paneth cells and goblet cells, and IgA secreted by plasma cells residing in the lamina propria and trancsytosed across the epithelium. In addition, epithelial cell surface is covered by membrane-bound mucins and other membrane glycoproteins that form the glycocalix. The actual physical barrier is represented by the single cohesive monolayer of polarized epithelial cells, with the apical surface facing the lumen and the baso-lateral side overlying the lamina propria and muscularis mucosa layer. Underneath the epithelium, there are several elements of the gut-associated lymphoid tissue (**GALT**) that is

regarded as the largest lymphoid organ in the body. GALT comprises both inductive sites (mesenteric lymph nodes, isolated lymphoid follicles (ILFs), and Peyer's patches in the ileum,) and effector sites (the epithelium itself and the underlying lamina propria), along with immune cells including intra-epithelial lymphocytes (IELs), IgA-secreting plasma cells, M cells, macrophages and dendritic cells (DCs), the latter populations being involved in antigen sampling and presentation, respectively (Kim & Ho, 2010; Garrett et al., 2010).

One of the two tasks of the intestinal epithelium is absorption and secretion, therefore, the flux of compounds across the epithelium can occur through both trans-cellular and para-cellular pathways. The trans-cellular transport is directional, energy dependent, and highly regulated by the cell-specific profile of transporters and channels localized on the apical and basolateral cell membranes. On the contrary, the para-cellular transport is passive, and involves movement through the space between adjacent cells (Anderson, 2001). The latter route can be potentially harmful for the penetration by pathogens or toxic compounds. Therefore, in order to regulate traffic through the para-cellular pathway, mammalian epithelial cells form a series of intercellular junctions along their lateral margins to seal the interspace: closest to the apical surface lies the apical junction complex, formed by three individual junctions, tight junctions (**TJ**), adherens junctions and desmosomes, while underneath are the gap junctions (Fig. 2).

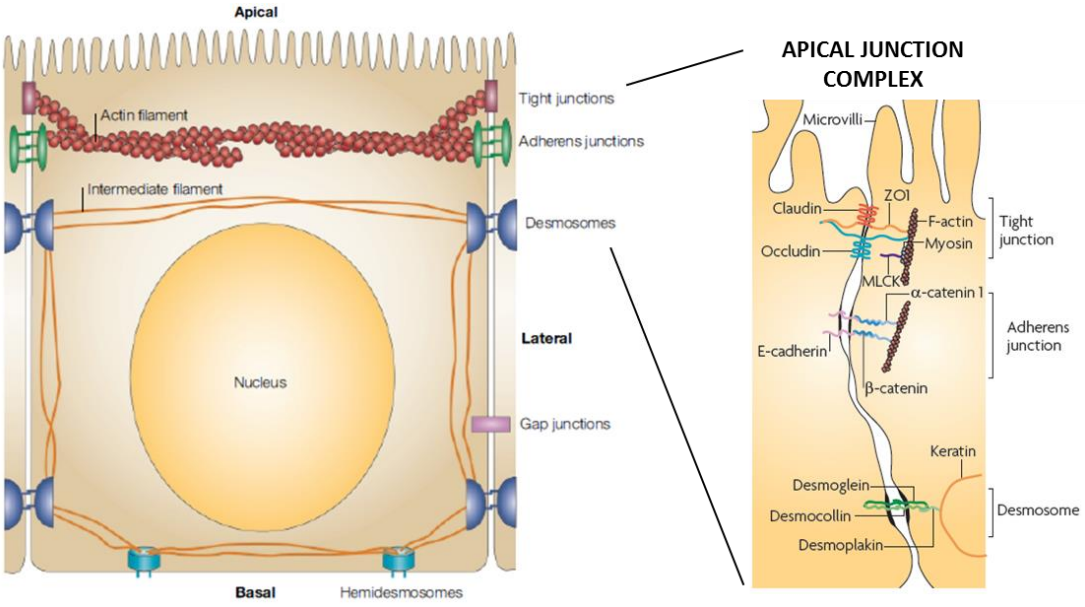

Fig. 2. Intercellular junctions linking epithelial cells

(adapted from Matter & Balda, 2003)

Components of the apical junction complex interact with cytoskeletal elements to strengthen their interactions. The adherens junctions, along with desmosomes, provide the strong adhesive force that keeps cellular proximity, though inter-space is still evident between the two adjoining cells. The adherens junctions are composed of E-cadherin, and catenins (α- and β-catenin), whereas desmosomes are formed by interactions between desmoglein, desmocollin, and desmoplakin. Both structures are linked to cytoskeleton filaments, of F-actin and keratin, respectively. The actual sealing point is represented by tight junctions, that are also considered "kiss-points" between two cells. Tight junctions are the most apically located intercellular junctions and are multi-protein complexes composed of three different elements: integral membrane proteins, junctional complex proteins and cell cytoskeleton structures (see Fig. 3). The integral membrane proteins organize into fibrils crossing the plasma membrane to interact with proteins from adjoining cell. These proteins are claudins, occludin, and JAMs (junctional adhesion molecules). Among them, the most important are members of the claudin family (from latin «claudere = to close»), a family of 20 proteins, that actually seal the intercellular space between adjacent cells. According to Van Itallie and Anderson, claudins are responsible for selective permeability of TJ to water, ions, and small molecules, since they form aqueous pores within the TJ, (Van Itallie & Anderson, 2004).

In addition, occludin is a tetra-spanning transmembrane protein that interacts with claudins and other cytoplasmic proteins, and JAMs (junctional adhesion molecules) are integral membrane proteins (single transmembrane domain) that belong to immunoglobulin superfamily. On the intracellular side of the membrane, the carboxy-terminal end of these proteins interacts with cytoplasmic junctional complex proteins belonging to the zonula occludens family (ZO-1, ZO-2, and ZO-3). Among them, the most important is zonula occludens 1 (**ZO-1**), a cytoplasmic protein that contains multiple protein interaction domains, and forms a scaffold for the transmembrane proteins (claudins and occludin) connecting them to the ring of actin microfilaments of the cell cytoskeleton. In addition, ZO-1 belongs to the membrane associated guanylate kinase superfamily and possesses an enzymatically inactive guanylate kinase-like domain, whose phosphorylation regulates TJ structure. Underneath the junctional complex lies a ring of actin microfilaments, linking TJ to cytoskeleton, whose contraction can separate TJ elements and has been proposed to regulate para-cellular permeability (Arrieta et al., 2006).

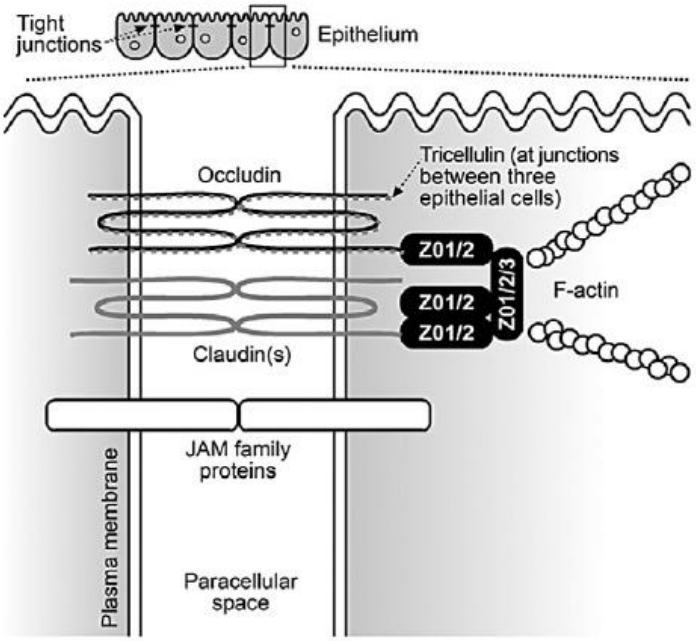

Fig. 3. Structure of tight junctions

(from Kotler et al., 2013)

Once considered static structures, TJ are actually extremely dynamic and the functional state is permanently modulated by various extracellular physiological, pharmacological, dietary, bacterial and inflammatory stimuli acting to activate intra-cellular signaling pathways and thus to regulate permeability. TJ permeability may be regulated directly through changes in TJ proteins or indirectly via the cytoskeleton, and theoretically all the TJ components described above can be considered as potential targets of TJ modulators. Deli and colleagues reported a series of regulatory mechanisms such as MLCK (myosin light chain kinase), Rho, PKC (protein kinase C), and MAPK (mitogen-activated protein kinase) pathways that modulate the cytoskeleton-TJ proteins interactions (mainly through phosphorylation / dephosphorylation reactions), resulting in altered epithelial permeability (Deli et al., 2009).

Proper and stable assembly of TJ is required for effective intestinal barrier function, and consequently for prevention of intestinal diseases. However, TJ formation has to be dynamic to allow intestinal epithelial cell turnover that occurs every 4-5 days. For this reason, under physiological conditions, TJ proteins are continuously internalized and recycled back to the plasma membrane via endocytosis (Guzman et al., 2013).

Intestinal Barrier and Inflammation

Among the mediators of the TJ status, inflammatory cytokines are major regulators of intestinal epithelial permeability (Lallès, 2010). At the intestinal level, cytokines can be produced by several types of mucosal cells, including macrophages, activated T cells, mast cell, and epithelial cells. Table 1 is taken from a review on gut barrier homeostasis by Lallès, and summarizes the effects of different cytokines on intestinal barrier function and TJ proteins. Barrier function is expressed in terms of trans-epithelial electrical resistance (**TEER**) and para-cellular permeability (**PCP**), both electrophysiological parameters obtained from *ex vivo* Ussing chambers analysis of the epithelium. Generally, a reduction in TEER and an increase in PCP are index of a reduced barrier function. The ability of cytokines, such as IFN-γ and TNF-α, to regulate the function of the TJ barrier was first described over 20 years ago (Madara & Stafford, 1989). Since then, various experiments conducted on epithelial cells, including intestinal Caco-2 cells, showed that both IFN-γ and TNF-α exert a TJ barrier disrupting effect (reduced TEER, enhanced PCP). According to Bruewer and colleagues, the pro-inflammatory–"leaky" action of IFN-γ is through the internalization of TJ proteins (mainly occludin) via macro-pinocytosis (Bruewer et al., 2005). Synergistically, TNF-α can increase intestinal permeability probably through the reduction in TJ strands mediated by the activation of NF-kB and the MLCK pathway (Al-Sadi et al., 2009). In addition, TNF-α has a pro-apoptotic action on epithelial cells and dying cell can cause a gap in the cellular lining, thus challenging barrier function (Gitter et al., 2000). However, anti-inflammatory cytokines such as IL-10 and TGF-β have been shown to counteract the disrupting effects of IFN-γ and TNF-α, allowing better barrier function.

Table 1. Effects of inflammatory cytokines on intestinal barrier (adapted from Lallès, 2010)

Cytokine	Source	Barrier function[1]	TJ changes	Intra-cellular signalling[2]
IFN-γ	Lymphocytes, dendritic cells, monocytes	TEER (-) PCP (+)	Pinocytosis of TJ proteins (occludin, claudin); claudin-2 (-)	Rho kinase, MLC phosphorylation
TNF-α	Activated lymphocytes and macrophages	TEER (-) PCP (+)	TJ strand numbers and depths (-)	NF-kB, MLCK, PKA
IL-1β	Various immune-modulatory cells	TEER (-) PCP (+)	Occludin (-)	NF-kB, MLCK
IL-10	T_{Reg} cells	TEER (+) PCP (-)	Protective against barrier and TJ changes induced by IFN- γ	
TGF-β	T_{Reg} cells, epithelial cells	TEER (+) PCP (-)	Claudin-1 (+)	ERK or PKC

[1] TEER = trans-epithelial electrical resistance; PCP = para-cellular permeability.
[2] ERK = extracellular signal-related kinase; MLC = myosin light chain; MLCK = MLC kinase; NF-kB = nuclear factor kappa B; PKA = protein kinase A; PKC = protein kinase C; Rho kinase = Ras homolog kinase.
(+) increased; (-) decreased

Figure 3 schematically shows the immunoregulatory responses that occur at the TJ level in order to maintain intestinal homeostasis (Turner, 2009). When a minor barrier defect allows bacterial products or dietary antigens to cross the epithelium and penetrate the lamina propria, it can lead to disease onset. If the luminal material is sensed by antigen-presenting cells (APCs) that drive the differentiation of effectors T helper cells (T_H1 and T_H2), disease can occur. In this case, APCs and T_H1 cells can secrete IFN-γ and TNF-α that – acting at the TJ level – can increase epithelial permeability and allow further flux of bacterial products and antigens, thus amplifying the inflammatory status. In addition, IL-13 released by T_H2 cells can enhance the flux of small solutes by altering the structure of claudin-2–assembled pores. On the contrary, homeostasis can prevail if APCs direct the differentiation of regulatory T cells (T_{Reg}), that can be stimulated by TGF-β and retinoic acid produced by epithelial cells. T_{Reg} cells can secrete IL-10 and TGF-β to switch-off pro-inflammatory action of T helper cells and restore mucosal homeostasis. Therefore, the final outcome is strictly dependent on the fine balance between pro-inflammatory and immunoregulatory responses. Excessive responses to pro-inflammatory signals, insufficient IL-10 production, or inadequate immune tolerance to luminal antigens or microbial compounds can favor the inflammatory process, thus underlining that – along to a

defective epithelial barrier – a susceptible host is required for homeostasis disruption and disease outbreak (Turner, 2009).

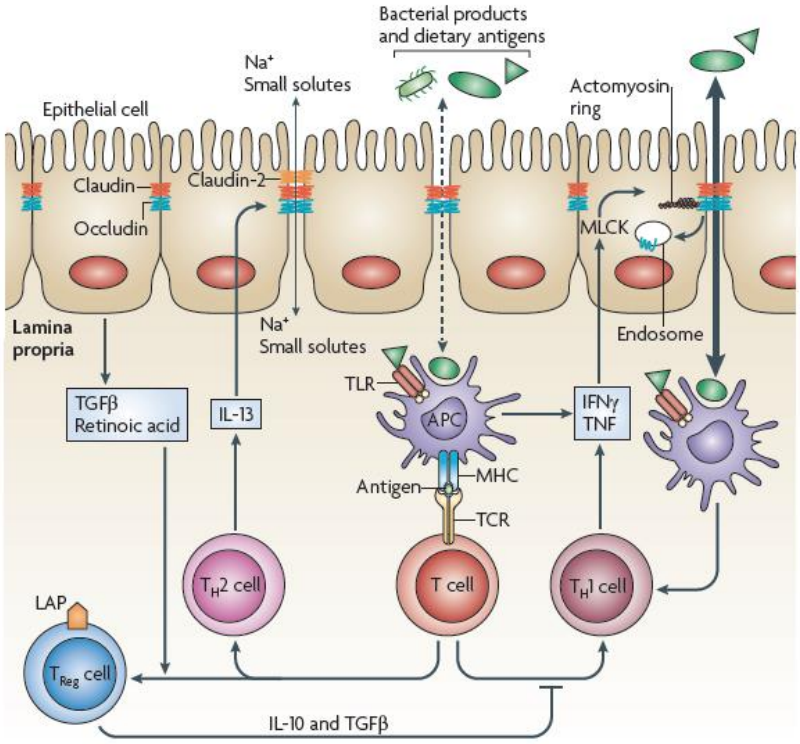

Fig. 3. Inflammatory cytokines and tight junctions regulate mucosal homeostasis (from Turner, 2009)

Defective barrier function and altered inflammatory responses are, for example, at the basis of several human intestinal inflammatory pathologies, such as inflammatory bowel disease (IBD), both Crohn's disease and ulcerative colitis, coeliac disease (CD), and irritable bowel syndrome (IBS).

WEANING IN PIGS

Weaning is widely accepted as the most significant and stressful phase in the pig's life because of marked intestinal, nutritional, immunological and behavioral changes that cause high incidence of intestinal disturbances and reduced growth performance. This "growth check" along with low voluntary feed intake after weaning are major limitations for enhanced efficiency in pig production (Pluske et al., 1997).

The weaning process is considered multi-factorial since pigs are subjected to several stressors (social, psychological, environmental, and nutritional) such as the separation from the sow, mixing with pigs from different litters, and social hierarchy stress, the different physical environment, the increased exposure to pathogens and dietary antigens, the nutritional change from highly digestible and palatable liquid milk to solid dry diet less digestible and palatable. In order to be productive and efficient, the piglet should rapidly adapt to the new conditions, otherwise poor growth and even mortality can occur (Campbell et al., 2013).

During weaning the gastro-intestinal tract undergoes a series of structural and functional changes that, together with the immunological immaturity, are responsible for higher susceptibility to infections and intestinal disorders, among all the so-called post-weaning diarrhea (**PWD**), usually associated with proliferation of enterotoxigenic *E.coli* (**ETEC**). Although the small intestine is the part of the GIT most affected by weaning, some relevant changes take place also in the stomach during weaning. In weaning piglets gastric pH has been shown to be higher compared to sow-reared pigs, probably because of a lower acid secretion capacity of the stomach mucosa along with a reduction in lactic acid production from milk-derived lactose. According to Heo and coworkers, this high gastric pH can, in part, contribute to the susceptibility of piglets to enteric infections because a higher number of bacteria can pass into the small intestine, being less effective the "gastric acid barrier" usually bactericidal for many pathogens (including *E.coli*). In addition, weaning also reduces gastric motility, and gastric stasis has been reported in early-weaned piglets as a contributing factor for PWD (Heo et al., 2013).

The main changes occur in the small intestine and involve both structure and function. The morphological transformations include a reduction in villous height (villous atrophy) and an increase in crypt depth (crypt hyperplasia), probably because of an increased rate of cell loss and cell production, and resulting in reduced villous height :

crypt depth ratio compared to unweaned pigs. These structural changes are more evident when pig are weaned earlier at 14 days of age rather than later at 28 days (Pluske et al., 1997). In addition, a modification in the shape of the villi occur, from the longer, finger-like villi of newborn and suckling piglets, to the wider tongue or leaf-like villi of weaned pigs. These structural changes are generally associated with a temporary decrease in the digestive and absorptive capacity of the small intestine. The activity of brush-border enzymes such as lactase and sucrase have been found to be reduced regardless of whether creep food was offered before weaning or not. On the contrary, increases in carbohydrases such as maltase and glycoamylase are likely due to a substrate induction of these enzymes by solid diet (Pluske et al., 1997). As a consequence of the reduced digestive capacity, along with low feed intake, at weaning there is a drop in the metabolisable energy (ME) intake and, as summarized by Le Dividich and Herpin, a period of 3-4 weeks after weaning is required for the pig to restore the same metabolisable energy intake of the milk diet (Le Dividich & Herpin, 1994). Moreover, weaning results in a reduction in the small intestine absorptive capacity of fluids, electrolytes, and malabsorption of nutrients that can contribute to the development of osmotic diarrhea because of the high amount of nutrients in the hindgut. In fact, in the large intestine weaning can cause a transient reduction in absorptive ability of the colon thus resulting in diarrhea since fluid loss exceeds fluid net absorption (Heo et al., 2013). In addition, such amount of undigested nutrients (particularly proteins) can favor microbial fermentations, overgrowth of bacteria such as coliforms and proteolytic clostridia, and consequent production of potentially harmful fermentation by-products.

It is common for pigs to develop the so-called PWD characterized by frequent loss of watery faeces during the first 2 weeks after weaning. PWD is generally associated with proliferation in the small intestine and faecal shedding of a great number of β-haemolitic enterotoxigenic *E.coli* (ETEC), so that it is also called post-weaning colibacillosis. Several serotypes of ETEC exist, with specific sets of virulence genes coding for fimbriae and toxins. ETEC fimbriae are required for adhesion and stable colonization of the small intestine mucosa, since they attach to glycoprotein receptors on the brush-border surface of villous enterocytes. Once attached to the host cell, bacteria can deliver the enterotoxins such as the heat-lable toxin and the heat-stable: these toxins activate cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) systems respectively, thus inducing an increased secretion of

sodium, chloride and hydrogen carbonate ions into the lumen, and a reduced absorption of salts and liquid, respectively (Heo et al., 2013). As a result of the hypersecretion of water and electrolytes in the small intestinal lumen that exceeds the absorptive capacity of the colon, diarrhea, dehydration, reduced feed intake, reduced nutrient digestibility, reduced growth and even death can occur. Several studies have been conducted in order to elucidate the mechanisms of adhesion to intestinal cells by ETEC and cellular responses (*in vitro* studies), and to investigate possible dietary interventions to minimize ETEC-induced PWD (*in vivo* trials with oral *E.coli* challenge models). In this regard, the major limitation of these *E.coli* challenges relies in the great discrepancy between the clean and hygienic environment of the research facilities where these studies are conducted and the commercial piggeries, where pigs are subjected to multiple stressors. In addition, many factors should be considered such as age, body weight and health status of animals, dose concentration and serotype of ETEC, animal susceptibility and receptors profile for ETEC adhesion. In addition to the hypersecretory effect, ETEC infection also affect the epithelial barrier function increasing para-cellular permeability and reducing trans-epithelial electrical resistance either through contraction of the actomyosin ring or occludin dephosphorylation and ZO-1 redistribution (Kim et al., 2012).

Other extremely important changes that occur during weaning are the immunological changes. At weaning, the intestinal immune system of the piglet is still immature and the withdrawal of the sow's milk deprives the piglet of IgA and bioactive compounds (as well as growth factors and hormones) thus increasing the susceptibility to enteric diseases. The immune immaturity impairs the ability of the piglet to develop an adequate response against pathogens and to tolerate dietary antigens. For example, a transient reduction in the response of intra-epithelial lymphocytes to mitogens, as well as a transient hypersensitivity to dietary soy protein have been reported in weaning piglets (Heo et al., 2013). Weaning has been described to induce intestinal inflammation in several species: increased number of T_H1 and T_H2 lymphocytes in mice; activation of the mucosal immune system, villous atrophy and cypt hyperplasia in rats. In pigs, McCracken and colleagues reported increased T helper and T cytotoxic lymphocytes in jejunal villi 2 days post-weaning and proposed post-weaning anorexia, along with consequent gut changes, as the major contributor to local intestinal inflammation, since pre-weaning values of inflammatory cells were achieved upon resumption of feed intake (McCracken et al., 1999). In addition, the intestinal

inflammatory response has been shown to be early and transient (occur within 2 days post-weaning and it is repaired by day 8), characterized by the up-regulation of pro-inflammatory cytokines genes, including TNF-α, IL-1β, and IL-6 (Pié et al., 2004). This hyper-activation of the intestinal inflammatory system can negatively affect TJ architecture and permeability, thus leading to impaired epithelial barrier function and increasing the risk of intestinal disorders. Therefore, an adequate modulation of the intestinal inflammation is essential to prevent weaning-associated intestinal diseases.

Moreover, as described by Smith and coworkers, the intestinal damage induced by weaning can have long-term consequences on the intestinal barrier function and intestinal health: comparing different weaning ages, the authors found that early weaning (from 15 to 21 days of age) resulted in huge impairment in the barrier function as suggested by reduced jejunal trans-epithelial electrical resistance and increased para-cellular fluxes across the mucosa, compared to the values of late-weaned piglets (from 23 to 28 days of age). This disrupting effect was observed at 5 weeks of age and was still appreciable at 9 weeks, thus indicating that stress occurring at weaning can alter the development of the mucosal barrier and have long-lasting effects on the intestinal health (Smith et al., 2010).

Nutritional strategies to control weaning

In order to assist the weaning-associated growth retard, antibiotics have long been used in pig industry as growth promoters, based on the modulatory effects on intestinal microbiota, including both commensal and pathogenic bacteria. However, increasing concerns about the risk of antibiotic resistance in humans, led the EU to ban the use of in-feed antibiotic growth promoters (**AGP**) in 2006. Since then, a large number of AGP alternatives, including feed additives and dietary interventions, have been studied and proposed.

The first goal of a nutritional strategy alternative to using in-feed antibiotic is to minimize intestinal dysfunction associated with intestinal changes, intestinal barrier disruption and enteric pathogens overgrowth. For this reason, the main targets of interventions are those factors that influence PWD through reducing pathogen proliferation and maintaining intestinal barrier function, such as dietary protein modulation and dietary carbohydrate modulation. Dietary protein level is critical

because at weaning piglet's capacity to digest proteins is low and dependent on the pancreatic and brush-border proteolytic enzymes not fully developed. Undigested proteins can be utilized as substrates for bacterial fermentations in the distal part of the small intestine and in the large intestine, leading to overgrowth of proteolytic bacteria (including *E.coli* and Clostridiaceae family) at the expense of saccharolytic and butyrate-producers microbes, pathogen proliferation, and production of potentially toxic compounds (ammonia, amines, volatile phenols and indoles) with negative consequences on the epithelial integrity (Piva et al., 1996). Therefore, low-protein diets are recommended rather than high-protein diets to maintain better intestinal health at weaning, provided that similar digestible energy is given and adequate levels of essential aminoacids are supplemented (Heo et al., 2013). The dietary non-starch polysaccharides (**NSPs**) modulation as source of carbohydrates in diets of weaning piglets is based on the different effect of soluble and insoluble NSPs. Soluble NSPs have been found to increase digesta viscosity, decrease digesta transit time and nutrient digestibility, consequently allowing the proliferation of ETEC, main responsible of PWD. On the contrary, insoluble NSPs are known to decrease digesta retention time and small intestinal pathogen proliferation, thus being recognized as a dietary ingredient to reduce post-weaning colibacillosis in weaned pigs (Kim et al., 2012).

Along with the abovementioned modulations of dietary components, other alternatives to antimicrobial compounds in diets for weaning piglets have been proposed, including supplementation with probiotics (lactic acid-bacteria and yeasts), prebiotics (such as non-digestible oligosaccharides, electively fermented by "positive" bacteria), exogenous enzymes (to improve digestive functions and reduce viscosity of the digesta), organic acids (with acidifying and anti-microbial properties against pathogens), and essential trace elements (zinc and copper).

CHAPTER 2
AIM OF THE STUDY

CHAPTER 2

Intestinal health is essential for the health of the body since the gastro-intestinal mucosa is the main site of interaction with the external environment, as well as the major area colonized by the microbiota. Intestinal health relies on proper barrier function, provided by epithelial integrity and related mechanisms of protection (mucous layer, tight junctions, immune and inflammatory system).

In pigs, during the weaning transition, intestinal inflammation and intestinal barrier integrity play a crucial role in regulating intestinal health and, consequently, pig's health, growth and productivity.

The aim of the project was to assess the impact of the diet on the intestinal health of weaning piglets with particular reference to the intestinal inflammatory status and epithelial integrity.

Therefore, a series of *in vivo* experimental studies were conducted in order to evaluate different nutritional strategies to control intestinal inflammation in weaning piglets:

- Zinc trial No. 1: the effect of zinc oxide, fed either free or microencapsulated, was investigated on the expression of inflammation markers and tight junctions proteins in the ileum of weaning piglets.
- Zinc trial No. 2: the effect of zinc oxide, fed either free or microencapsulated, was assessed on growth performance and ileal architecture of weaning piglets.
- Tributyrin trial: the effect of tributyrin, as dietary source of butyric acid, was evaluated on growth performance, intestinal architecture and inflammatory cytokines and tight junctions proteins expression in weaning piglets.
- Organic acids and nature-identical compounds trial: the effect of a mixture of organic acids and nature-identical compounds was investigated on intestinal barrier integrity and inflammatory status in early-weaned piglets.

CHAPTER 3

ZINC TRIAL No. 1

ABSTRACT

Aim of this study was to investigate the expression of inflammation markers and tight junction proteins in the ileum of weaning piglets fed with low doses of microencapsulated ZnO (**mZnO**) in comparison with a pharmacological dose of ZnO. Twenty-four piglets weaned at 28 d were divided in 4 groups: (1) control; (2) free ZnO, 2,850 mg Zn/kg diet; (3) mZnO-300, 140 mg Zn/kg diet; (4) mZnO-800, 380 mg Zn/kg diet. After 14 days, piglets were killed and ileal samples were collected for cytokines and tight junction components analysis at mRNA and protein level. Both groups receiving mZnO tended to have a reduced mRNA expression of IL-6 compared to both control and ZnO group (−25%, $P = 0.10$) and also a numerically lower level of tumor necrosis factor-α (**TNF-α**) mRNA (−25%, $P = 0.18$). In mZnO-800 group interferon-γ (**IFN-γ**) mRNA was the lowest ($P = 0.02$), and the protein tended to be lower than in ZnO group ($P = 0.08$). Both free ZnO and mZnO reduced TNF-α protein level ($P < 0.0001$). Compared to control and ZnO group, mZnO-800 increased occludin protein level (2–4 fold, respectively; $P < 0.001$), zonula occludens-1 protein level (1.3–1.5 fold, respectively; $P < 0.001$), and claudin-1 mRNA level (1.7–2.4, respectively; $P = 0.01$). The results suggest that ZnO released from a lipid matrix was available in the ileum of piglets where it positively impacted inflammatory status and, consequently, epithelial integrity via tight junction proteins. Therefore, microencapsulated ZnO is proposed as an effective and environmentally safe alternative to pharmacological ZnO.

Keywords: inflammatory cytokines, microencapsulated zinc oxide, piglets, tight junctions.

INTRODUCTION

The weaning phase represents a dramatic challenge to intestinal health due to various type of stressors (separation from sow, change of environment and feeding, etc.) and is accountable for several physiological, anatomical, and immunological changes that are responsible for a decrease in the absorptive surface of the mucosa, an impaired barrier and protection against environmental challenges, and, as a consequence, an increased susceptibility to infections. In particular, within the gut barrier components, the epithelial layer plays a central role in maintaining the integrity and health of the mucosa, as a leaky gut is the main cause of infections and reduced performance (Lallès,

2010). Mucosal integrity is essential for gut barrier function, as first line of protection against harmful exogenous agents, such as pathogens, bacterial products, dietary antigens and toxicants. Tight junctions (**TJ**) are the most important structures of the gut barrier, linking the cytoskeleton and actin filaments of two adjoining cells, and regulating the so-called para-cellular permeability. Several extra-cellular molecules continuously modulate TJ status, among which inflammatory cytokines are considered as major mediators of intestinal epithelial permeability (Lallès, 2010). Stress factors associated to weaning can compromise the intestinal barrier function through mucosal damage and alteration of TJ structure. These dramatic changes in barrier integrity and permeability occur mainly in the small intestine and are both acute and long-lasting alterations (Boudry et al., 2004; Smith et al., 2010).

In-feed zinc oxide (**ZnO**) has been used extensively to help reducing the symptoms of the post-weaning phase and to prevent diarrhea, after in the late 80's Poulsen described its beneficial properties (Poulsen, 1989). Due to ZnO low relative bioavailability and the low pig feed intake, the use of high supplemental ZnO, ie 2,500–3,000 mg/kg, appears to be essential to provide the amount of Zn necessary to exploit its beneficial effect, but increasing concerns on heavy metals excretion in manure in Europe, though, had imposed caution in the use of ZnO at high doses (EU, 2003).

Therefore, in this study we aimed to assess whether lipid microencapsulation could allow to reduce the inclusion of ZnO in the feed to levels substantially lower than the 2,500–3,000 mg/kg currently in use, and we did so by measuring the effects of microencapsulated ZnO on inflammatory cytokines and intestinal integrity markers such as tight junctions proteins.

MATERIALS AND METHODS

The study was conducted at the research facilities of the Research Centre for Animal Production and Environment (CERZOO), which is a Good Laboratory Practices-certified facility and operates according to the procedure of animal protection and welfare (Directive 86/609/EEC).

Experimental Design and Sample Collection

Twenty-four pigs (Landrace × Large White, barrows and gilts, 7.17 (SE 0.14) kg of BW) weaned at 28 days of age were divided into 4 experimental groups (6 animals per group) and fed *ad libitum* either the basal diet (control, providing Zn at 50 mg/kg), or the control diet added with ZnO at 3,000 mg/kg (fZnO, providing Zn at 2,850 mg/kg), or the control diet supplemented with microencapsulated ZnO (**mZnO**) providing Zn at 140 mg/kg or 380 mg/kg (mZnO-300 and mZnO-800, respectively). Basal diet was formulated to meet or exceed requirements recommended by the National Research Council (NRC, 1998), (Ingredients, as-fed basis: corn meal 40%, soybean meal (44%) 19.81%, barley meal 20.34%, wheat bran 5%, milk serum 8%, soybean oil 1.37%, calcium carbonate 1%, potato extract 2.5%, dicalcium phosphate 0.6%, Lys HCl 0.53%, NaCl 0.35%, L-Thr 0.21%, DL-Met 0.22%, L-Trp 0.07%). The health status of animals was monitored throughout the study. After 14 days pigs were killed and intestinal samples were collected. Ileal samples, 10 cm from the ileo-cecal valve, were cut longitudinally to expose the mucosa and washed with PBS to remove mucus and digesta. Then the mucosa was scraped gently with a glass slide, vacuum-packed, immediately frozen in liquid N_2 and stored at −80°C until gene and protein expression analysis was conducted.

Inflammatory Cytokines and TJ Components Gene Expression Profiling

Ileal scrapings were disrupted by grinding in liquid N_2 with mortar and pestle, then homogenized using TissueLyser (Qiagen, Hilden, Germany). Total RNA was isolated from ileal mucosa scraping using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (RNase-Free DNase Set, Qiagen, Hilden, Germany). RNA yield and quality were determined spectrophotometrically using A_{260} and A_{280} nm measurements. All samples showed A_{260}:A_{280} ratio of 1.8–2.0. A total of 1 µg of RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. Resulting cDNA was quantified with Quant-iT Oligreen ssDNA Assay (Life Technologies Italia, Monza, Italy) to normalize the quantity of cDNA template used for amplification by real-time PCR. Real-time PCR was performed using iCycler Thermal Cycler system and SybrGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA). Thermocycling protocol included initial denaturation for 1 min and 30 s at 95°C, 40

cycles of denaturation at 95°C for 15 s followed by 30 s of annealing and extension at 60°C. After amplification, all samples were subjected to a melt curve analysis, with a slow heating from 55 to 95°C with a rate of 0.5°C/s to validate absence of non-specific products.

Gene expression was normalized using two housekeeping genes coding for portions of porcine ribosomal subunit 60S, such as ribosomal protein L35 (**RPL35**) and ribosomal protein L4 (**RPL4**). Average threshold cycle ($\mathbf{C_T}$) was determined for each gene of interest (**GOI**), and geometric average was calculated for housekeeping genes (**HKs**), assuming C_T as number of cycles needed to reach a fixed arbitrary threshold. Delta C_T was calculated as C_T GOI−C_T HKs, then a modification of the $2^{-\Delta\Delta C}{}_T$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (Livak and Schmittgen, 2001).

The sequences, expected product length, accession number in the EMBL database/GenBank and references of porcine primers are shown in Table 1. Primer oligonucleotides for claudin-1, tumor necrosis factor-α (**TNF-α**), and interferon-γ (**IFN-γ**) were designed using PrimerQuest software (IDT, Integrated DNA Technologies, www.idtdna.com). Primers were obtained from Life Technologies (Life Technologies Italia, Monza, Italy).

ELISA Quantification of Inflammatory Cytokines

Inflammatory cytokines protein levels were analyzed using commercial ELISA kit specific for porcine cytokines (Quantikine ELISA, R&D Systems, Inc., Minneapolis, MN). Before analysis, ileal mucosa scrapings were disrupted by grinding in liquid N_2 with mortar and pestle, added with lysis buffer (10 m*M* Tris-HCl, 1 m*M*-EDTA, 0.5%-Triton X100) and homogenized using TissueLyser (Qiagen, Hilden, Germany). Analyses were performed according to manufacturer's instructions. Results refer to picograms of cytokine per 100 mg of tissue (pg/100 mg).

TNF-α and TJ Components Western Blot Quantification

TNF-α, occludin and zonula occludens-1 (**ZO-1**) protein levels were analyzed by Western blot. Briefly, mucosal scraping of ileum (approximately 1 g) from each animal were homogenized in a 2 mL solution containing ice-cold lysis buffer (50 m*M* Tris-HCl, 150 m*M*-NaCl, 0.1%-SDS, 0.5%-sodium deoxycholate, 1%-NP-40, pH 7.4) supplemented with a protease inhibitor cocktail (EuroClone, S.p.A., Milano, Italy).

Table 1. Primers used for gene expression analysis by Real-Time PCR

Gene	Primer Sequence (F and R[1]; 5' → 3')	Product lenght (bp)	Product Tm[2] (°C)	Accession number	Reference
ZO-1[3]	F: GAGTTTGATAGTGGCGTT	298	87.5	XM_00335	Zhang and
	R: GTGGGAGGATGCTGTTGT			3439.2	Guo, 2009
Occludin	F: ATCAACAAAGGCAACTCT	157	83	NM_00116	Zhang and
	R: GCAGCAGCCATGTACTCT			3647.2	Guo, 2009
Claudin-1	F: TCCAGTGCAAAGTCTTCGACTCCT	121	85.5	NM_00124	Present
	R: ATGCCAACAGTGGCCACAAAGATG			4539.1	study
IL-6	F: AGCAAGGAGGTACTGGCAGAAAACAAC	110	83.5	NM_21439	Zannoni et
	R: GTGGTGATTCTCATCAAGCAGGTCTCC			9.1	al., 2012
IL-10	F: CGGCGCTGTCATCAATTTCTG	89	85	NM_21404	Duvigneau
	R: CCCCTCTCTTGGAGCTTGCTA			1.1	et al., 2005
TNF-α[4]	F: GCCCACGTTGTAGCCAATGTCAAA	99	87	NM_21402	Present
	R: GTTGTCTTTCAGCTTCACGCCGTT			2.1	study
IFN-γ[5]	F: GGCCATTCAAAGGAGCATGGATGT	149	83.5	NM_21394	Present
	R: TGAGTTCACTGATGGCTTTGCGCT			8.1	study
RPL35[6]	F: AACCAGACCCAGAAAGAGAAC	146	87.5	NM_21432	Alexander
	R: TTCCGCTGCTGCTTCTTG			6.2	et al., 2012
RPL4[7]	F: CAAGAGTAACTACAACCTTC	122	84	XM_00312	Alexander
	R: GAACTCTACGATGAATCTTC			1741.3	et al., 2012

[1] F = forward primer, R = reverse primer
[2] Tm = melting temperature
[3] ZO-1 = zonula occludens-1
[4] TNF-α = tumor necrosis factor-α
[5] IFN-γ = interferon-γ
[6] RPL35 = ribosomal protein L35
[7] RPL4 = ribosomal protein L4.

The homogenates were centrifuged at 10,000 × *g* at 4°C for 5 min to remove all insoluble material. The supernatant was collected and the protein concentration of each sample was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL). Total proteins (50 µg) were loaded either onto 10% SDS gels for occludin and TNF-α or onto 6% SDS gel for ZO-1, and subsequently electro-transferred onto nitrocellulose membranes. After blocking with

5% non-fat milk in Tris-buffered saline for 1 h at room temperature (**RT**), membranes were incubated for 2 h at RT with the following primary antibodies: anti-occludin (1:500; Life Technologies Italia, Monza, Italy), anti-ZO-1 (1:500; Life Technologies Italia, Monza, Italy), anti-TNF-α (1:200; Abcam, Cambridge, United Kingdom) and anti-actin (1:2000; Sigma-Aldrich S.r.l., Milan, Italy). Subsequently, they were washed and incubated for 1 h at RT with a horseradish peroxidase-conjugated secondary antibody (1:5000; Bio-Rad Laboratories Inc., Hercules, CA). The blots were developed using the ECL system (GE Healthcare Europe, GmbH, Milan, Italy): immunoreactivities were detected by chemiluminescence autoradiography according to the manufacturer's instructions. The optical densities of the protein bands of interest were determined densitometrically using the Scion Image software (Scion Corporation, Frederick, MD) and the values were normalized to actin levels.

Statistical Analysis

Animals were grouped in a completely randomized design and data were analyzed with one-way ANOVA, followed by Tukey post hoc test (Graph Pad Prism 5 version 5.03; GraphPad Software, Inc, San Diego, CA). The pig was the experimental unit. Differences were considered significant at $P < 0.05$ and trends for $0.05 < P \leq 0.1$.

RESULTS

Inflammatory Cytokines Profiling

The mRNA and protein levels of inflammatory cytokines in ileal mucosa are presented in fig. 1 and 2 respectively. Groups treated with microencapsulated ZnO tended to have reduced mRNA expression of IL-6, compared to both control and free ZnO groups (–25%, $P = 0.10$) and also had a numerically lower expression of TNF-α mRNA (–25%; $P = 0.18$). mZnO-800 group had the lowest expression of IFN-γ and significantly lower than mZnO-300 group ($P = 0.02$). No effects were observed in IL-10 gene expression.
IFN-γ and IL-10 protein content tended to be greater in fZnO group and mZnO-300 group than in control and mZnO-800 groups ($P = 0.08$). IL-6 protein content was not affected by the treatments. Both free ZnO and mZnO significantly reduced TNF-α expression compared to control group ($P < 0.0001$).

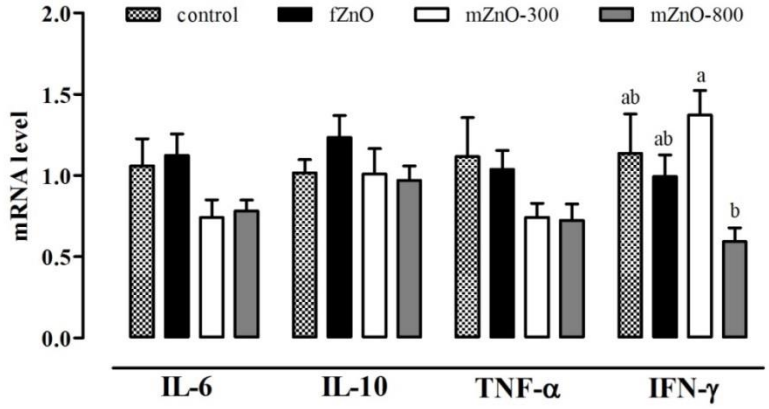

Fig. 1. Gene expression of inflammatory cytokines in ileal mucosa.

Data are expressed as means (n = 6) and SEM represented by vertical bars.
[a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$).
Control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2,850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg.
A modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).

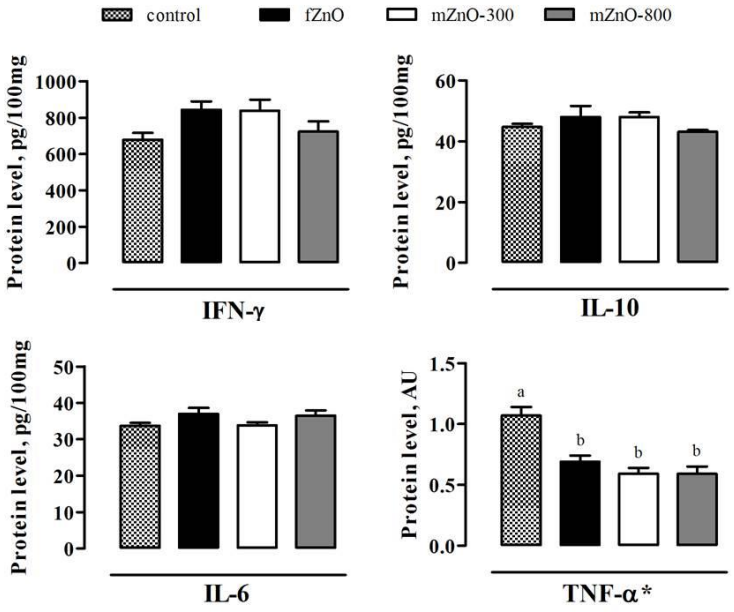

Fig. 2. Protein expression of inflammatory cytokines in ileal mucosa.

Data are expressed as means (n = 6) and SEM represented by vertical bars.
[a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$).
Control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2,850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg.
Data refer to picograms of cytokine per 100 milligrams of tissue (pg/100 mg) measured using ELISA method.
***** Tumor necrosis factor-α (**TNF-*α***) expression was measured by Western blot and data refer to arbitrary unit (**AU**) of protein expression.

TJ Components Profiling

Figure 3 summarizes the results from gene and protein expression analysis of TJ components. Occludin gene expression was the lowest in mZnO-800 group and significantly lower than in mZnO-300 group (2 fold lower; $P = 0.04$). ZO-1 expression was not affected by the treatments. Claudin-1 gene expression was 1.7–2.4 greater in mZnO-800 compared with control and fZnO, respectively ($P = 0.01$). Overall, mZnO-300 group tended to have intermediate values between fZnO and mZnO-800.

Both free ZnO and mZnO treatments had significantly higher occludin protein level than control group. Among the treatments, mZnO-800 group showed the highest level of occludin and was 2–4 fold greater than control and fZnO, respectively ($P < 0.001$). ZO-1 protein level was significantly greater in mZnO-800 group compared to both control and fZnO groups (1.5–1.3 fold respectively), whereas mZnO-300 had intermediate values ($P = 0.01$).

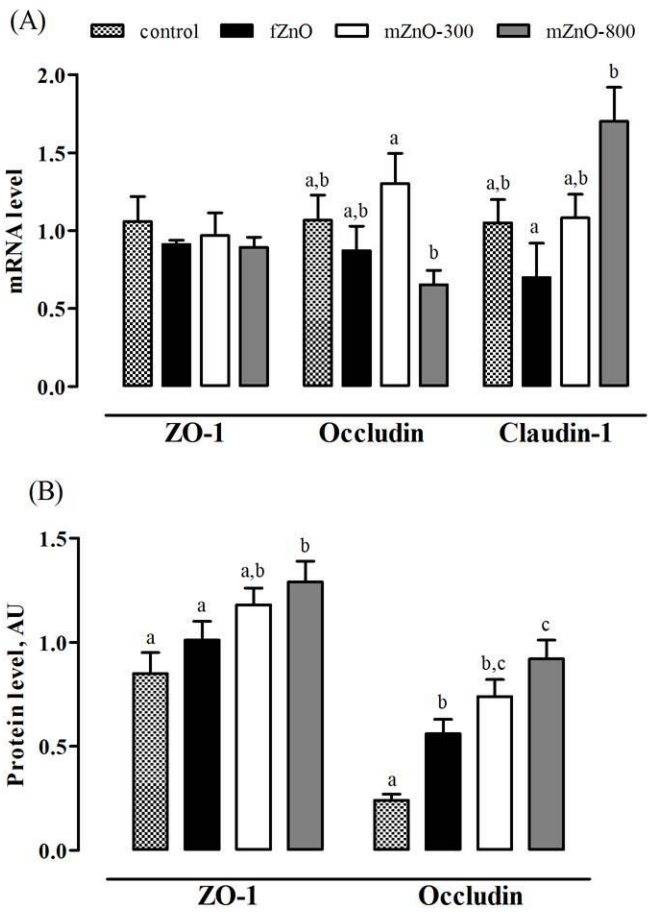

Fig. 3. Gene and protein expression of TJ components in ileal mucosa.

(A) and (B) show gene and protein expression, respectively. Data are expressed as means ($n = 6$) and SEM represented by vertical bars.

[a,b,c] Within the same gene or protein, mean values with unlike letters were significantly different ($P < 0.05$).

Control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2,850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg .

For gene expression, a modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).

For protein expression, data refer to arbitrary unit (**AU**) of protein expression measured using Western blot.

ZO-1 = zonula occludens-1; AU = arbitrary unit.

DISCUSSION

High levels of in-feed zinc oxide (2,500−3,000 mg/kg) are widely used to control post-weaning diarrhea and improve growth performance of pigs (Zhang and Guo, 2009; Pluske, 2013; Heo et al., 2013) and numerous mechanisms of action of ZnO have been proposed so far. Previous researches have reported that inorganic Zn as ZnO can increase the expression of antimicrobial peptides in the small intestine (Wang et al., 2004), exert positive effects on the stability and diversity of the microbiota (Katouli et al., 1999), prevent bacterial adhesion to the epithelium (Roselli et al., 2003), modulate the expression of genes encoding stress response proteins in enterocytes (Sargeant et al., 2011), improve intestinal growth through increasing insulin-like growth factor-I and insulin-like growth factor-I receptor expression in the small-intestinal mucosa (Li et al., 2006), and reduce electrolyte secretion from enterocytes *in vitro* (Carlson et al., 2006). Moreover, ZnO is supposed to positively act on mucosal barrier integrity by enhancing the expression of TJ proteins and reducing the intestinal permeability of weaning piglets (Zhang and Guo, 2009).

Zinc is essential to more than 300 enzymatic reactions among which it seems to be central in inhibiting the apoptotic process, and in protecting cells from death (Faa et al., 2008). Moreover, Zn is critical in the development and function of the immune system, including cytokine production, and it regulates the intestinal barrier integrity. Impairments in Zn homeostasis have in fact been observed in human patients suffering inflammatory diseases like inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and Crohn's disease where the altered permeability of the stressed gut mucosa is the cause of a malfunction at the basis of modifications in Zn homeostasis (Faa et al., 2008).

The central role of Zn as an anti-inflammatory agent is described by numerous researches. Roselli et al. reported ZnO (at concentration of 16.3 and 81.4 mg/L) to reduce and prevent enterotoxigenic *E. coli* adhesion and internalization, and reduce inflammatory cytokine expression on Caco-2 cells (Roselli et al., 2003). Similarly, Sargeant et al. showed that ZnO reduced the inflammatory response of porcine epithelial IPEC-J2 cells to enterotoxigenic *E.coli*, probably through up-regulated heat shock proteins and consequent inhibition of nuclear factor-kappa B (**NF-κB**) pathway (Sargeant et al., 2011). The reduction of inflammatory cytokines expression induced by

ZnO has been proposed as molecular basis for protection of epithelial integrity by preventing TJ disruption (Roselli et al., 2003).

Results from our study confirm previous findings on the anti-inflammatory properties of Zn. In fact, regardless of the treatment, ZnO impacted the expression of inflammatory cytokines and in particular of TNF-α and IFN-γ which in turn regulated the expression of epithelial integrity proteins. Both cytokines have in fact a preponderant role in altering the intestinal permeability by acting on the TJ complex. IFN-γ, which had the lowest expression in pigs fed with microencapsulated ZnO at 800 mg/kg (mZnO-800) affected occludin expression: occludin mRNA was the lowest in mZnO-800 group, but the protein amount in the same group was the highest, thereby indicating a negative feedback regulation of gene expression. The mechanism by which IFN-γ would affect epithelial integrity is by causing macro-pinocytosis of occludin, a trans-membrane protein whose expression is correlated with enhanced barrier properties (Bruewer et al., 2005), and the lower the level of IFN-γ, the higher the occludin amount.

TNF-α expression and protein content were numerically and significantly lower in all groups receiving supplemental ZnO compared to the control group. TNF-α has a dual effect on cell survival by either activating necrosis or apoptosis. While necrosis is induced by activation of NF-κB, the latter involves the caspase protein superfamily. It has been postulated that apoptosis of adjacent epithelial cells might contribute to the increase in permeability caused by the simultaneous action of TNF-α and IFN-γ (Al-Sadi et al., 2009), and Zn has been shown to inactivate casapase-3 and, therefore, inhibit apoptosis (Perry et al., 1997).

The role of other cytokines in modulating the intestinal permeability and integrity is still under investigation though it is known that IL-6, produced immediately after TNF-α and IL-1β in the inflammation cascade, contributes to the worsening of permeability and an increase in para-cellular flux whereas anti-inflammatory IL-10 prevents disarrangement of TJ structure (Al-Sadi et al., 2009). In our study IL-6 and IL-10 were marginally affected by ZnO, regardless of the dose or source, and appeared to have a minor role compared to TNF-α and IFN-γ on the regulation of the TJ structure.

Zonula occludens is a family of proteins of the TJ, and in particular ZO-1, along with occludin, has a pivotal role in maintaining the epithelial integrity. ZO-1, in fact, is a bridge connecting occludin and claudins to the β-actin filaments of the cell cytoskeleton (Fanning et al., 1998). In this study ZO-1 protein content was 1.5–2 fold greater in pigs

fed with the highest dose of mZnO compared to control and fZnO, respectively, whereas mZnO at 300 mg/kg had intermediate values. Also claudin-1 mRNA, another major protein of the TJ complex whose localization and expression is strictly connected to ZO-1, was up-regulated in pigs receiving the greatest dose of mZnO compared to the fZnO. These last results confirm the protective role of Zn from ZnO in preventing TNF-α and IFN-γ-mediated increase in para-cellular permeability and alteration of TJ functionality, but, more importantly, suggest that Zn from microencapsulated ZnO might be more available than Zn from free ZnO. In fact, despite the anti-inflammatory effect could be generally attributable to Zn from ZnO, regardless of the source, the TJ protein structure was clearly modulated in a dose-treatment dependent way. The abundance of both occludin and ZO-1, as shown in fig. 3, was mZnO-800 > mZnO-300 > fZnO > control, and it might be speculated that ZnO from microencapsulation was more available at ileal level than free ZnO. The relative bioavailability of Zn can vary substantially among different sources but, regardless of the source, pharmacological levels of ZnO seems to be critical to exert the beneficial and the growth promoting effect. Whether the efficacy is connected to a luminal or systemic effect remains unclear (Mavromichalis et al., 2000), but from this study it seems that an higher amount of Zn was available for the enterocyte in the ileum. Microencapsulation in a hydrogenated lipid matrix has been previously demonstrated to allow a slow release of active principles along the gut and prevent their complete disappearance upon entering the stomach (Piva et al., 2007; Grilli et al., 2010). ZnO in the stomach has a strong anti-buffering capacity that might decrease acidic secretion and impair the digestive capacities of young animals (Lawlor et al., 2005). Moreover, low acidic environments, such as the stomach, can cause ZnO to dissociate, liberate Zn ions which, once passing to the more neutral intestinal environment, can react with water molecules and create Zn dihydroxide, an insoluble compound that does not deliver adequate amount of Zn.

The encapsulation of ZnO in a lipid matrix allows to overcome these problems and delivers Zn to the ileum, the major site of intestinal immunological activity. Pharmacological levels of free ZnO had a milder effect on the expression of TJ proteins compared to microencapsulated ZnO, but we cannot exclude major effects upstream in the GI tract. In fact, Hu and colleagues showed that ZnO providing Zn at 2,250 mg/kg decreased the jejunal para-cellular permeability, decreased mRNA expression of pro-inflammatory cytokines, and up-regulated jejunal TJ protein expression (Hu et al., 2013).

In conclusion, lipid microencapsulation of ZnO allowed to reduce in-feed inclusion of ZnO by 6–15 times compared to pharmacological levels, allowing Zn to be released along the intestine and reach the ileum where it positively impacted the inflammatory status and ileal integrity. Therefore, microencapsulated zinc oxide is here proposed as an effective and environmentally safe alternative to pharmacological levels of ZnO.

CHAPTER 4

ZINC TRIAL No. 2

ABSTRACT

Aim of this study was to compare low doses of microencapsulated versus pharmacological level of ZnO in the diet of piglets on growth performance and ileal architecture. 144 weaned piglets, divided in 36 pens (n = 9), received a basal diet (control, Zn at 50 mg/kg) or the basal diet with ZnO at 3,000 mg/kg (free ZnO, fZnO), or with lipid microencapsulated ZnO providing Zn at 140 or 380 mg/kg (mZnO-300 and mZnO-800, respectively). After 15 and 42 d, 6 pigs per group per time were euthanized to collect ileal mucosa for immunohistochemistry, histomorphology, and Na-dependent glucose transporter (**SGLT-1**) gene expression. Pigs BW and feed intake were recorded at 0, 7, 14 and 42 d and ADG and G:F were calculated. Data were analyzed with 1 way ANOVA. At d 14 the fZnO group had a 32% and 7% higher ADG and BW, respectively, and +54% G:F compared with control (P < 0.05), whereas mZnO-300 and mZnO-800 had intermediate values. At 42d both groups receiving microencapsulated ZnO had 9% higher BW than control and did not differ from fZnO group (P = 0.01). ADG was on average 24% higher for fZnO and mZnO-800 than the control (P = 0.05) and G:F was numerically higher in all treated groups compared with control group (+26%; P = 0.11). At 14 d, villi length in mZnO-800 pigs was 11% and 8% higher than in control and fZnO, respectively (P < 0.01) and the villi:crypts ratio (V:C), as well as % of mitotic cells, were higher in all treated groups compared with control (P < 0.01). SGLT-1 gene expression was the lowest in mZnO-800 pigs. At 42d villi length and V:C ratio were the highest for fZnO compared with all of the other groups (+10% than control; P = 0.01). Mitotic cells were the highest in mZnO-800 group compared with other groups (+3% compared with control and fZnO; P < 0.01), whereas SGLT-1 expression tended to be lower in mZnO-300 and mZnO-800 groups compared with control and fZnO (P = 0.06). Pigs receiving low doses of microencapsulated ZnO had performance comparable to those receiving pharmacological level of ZnO overall the post-weaning phase. Moreover, in the first 2 weeks, microencapsulated ZnO improved the ileal architecture as reflected by the increased V:C ratio and the % of mitotic cells. The reduced SGLT-1 m-RNA abundance might suggest a reduced availability of glucose in the lumen of ileum, therefore suggesting a lower amount of undigested nutrients.

Keywords: ileum, microencapsulation, piglet, SGLT-1, zinc oxide

INTRODUCTION

The post weaning syndrome is a multi-factorial disease causing important economic losses in terms of mortality and medication costs. Gut anatomical and physiological changes are at the basis of the massive damage to the intestinal mucosa resulting in decreased absorptive capacity and disruption of the barrier function. An increased host susceptibility to *E. coli* has been often associated to the onset of the post weaning syndrome and the inclusion of 2,500–3,000 mg/kg of ZnO to weaning diets has been generally accepted as a tool to prevent and smooth diarrhea, and improve feed intake and growth performance (Poulsen, 1989; Jensen-Waern et al., 1998; Sargeant et al., 2010). The mode of action of ZnO, though, is still debated, and despite Zn deficiencies are not involved in *E. coli* pathogenesis, the effective dose of ZnO is well above the requirements. Nevertheless, the lack of association between plasmatic Zn and incidence of diarrhea has suggested a pathway independent from absorption (Patel et al., 2010; Sargeant et al., 2011). Also, the fact that the major part of ingested ZnO is excreted with the feces implies a direct effect on the gut rather than a systemic effect (Mavromichalis et al., 2000; Sergeant et al., 2010). In order for ZnO to reach the lower gut, though, a portion is necessarily absorbed in the upper gut and this 'absorptive loss', due to the passage through the stomach and the upper gut, would justify the use of high doses of ZnO. Microencapsulation was previously demonstrated to overcome the stomach barrier and to allow a release of active principles along the gut by preventing a complete absorption (Piva et al 2007; Grilli et al. 2010; Grilli et al., 2013). Aim of this study was to investigate whether lipid microencapsulation could reduce ZnO in-feed inclusion by comparing the effects of feeding low doses of microencapsulated ZnO versus pharmacological dose of free ZnO on piglets growth performance, ileal architecture, and glucose transporter SGLT-1.

MATERIALS AND METHODS

The study was conducted at the facilities of the Research Centre for Animal Production and Environment (CERZOO), which is Good Laboratory Practices-certified and operates according to the procedure of animal protection and welfare (Directive No 86/609/EEC).

Animals and Diets

One hundred and forty-four Landrace × Large White male and female piglets, weaned at 28 days of age and 7.2 kg of BW, were divided in 36 pens and randomly assigned to one of the following experimental groups: the basal diet (control), providing Zn at 50 mg/kg; the basal diet added with free ZnO at 3,000 mg/kg (fZnO, providing Zn at 2,850 mg/kg); the basal diet supplemented with microencapsulated ZnO at 300 mg/kg or 800 mg/kg, providing Zn at 140 mg/kg or at 380 mg/kg (mZnO-300 and mZnO-800, respectively).

The basal feed was formulated in order to meet or exceed the nutritional requirements of pigs according to NRC (1998) but without supplemental Zn. Supplemental Zn sources were added to the basal diet in order to provide the amount of Zn necessary for each experimental diet. Feed and water were provided ad libitum and piglets were fed the experimental diets for 42 days. At the end of the first 2 weeks the diet was switched to meet the nutritional requirements of the pigs in the second phase (Table 1). The health status of animals was monitored throughout the study and any culling or mortality was registered.

Piglets were individually weighed at the beginning of the study (d0), after 7 days (d7), at the end of the first growing phase (d14), and at the end of the study (d42). Feed intake was measured per pen during the same time intervals. Average daily gain (**ADG**), average daily feed intake (**ADFI**) and gain to feed ratio (**G:F**) were calculated for each phase (0 to7 d, 7 to 14 d, 14 to 42 d) and for the overall period (0 to 42 d).

At d14 and d42 of the study, 6 pigs per treatment (one per 6 randomly selected pen per treatment) were euthanized to collect intestinal samples: the ileum was longitudinally cut 10 cm from the ileal-cecal valve to expose mucosa, washed with PBS to remove mucus and digesta, then scraped gently with a glass slide, vacuum-packed and immediately stored in liquid nitrogen until gene expression analysis. Histological samples were instead collected and fixed in formalin until staining.

At the time of sacrifice blood samples were also collected to perform plasma Zn analysis. Blood was collected by jugular venipuncture using 10 cc heparinized (lithium heparin) disposable syringes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at room temperature for 10 min at 3,000 × *g*; plasma was transferred with Pasteur pipette in a plastic test tube and frozen at –80°C until analysis.

Table 1. Composition of basal diets (as-fed basis)

	1st phase (0 to14 d)	2nd phase (14 to 42 d)
Ingredients, %		
Corn meal	40.00	40.00
Soybean meal, 44%	19.81	20.00
Barley meal	20.34	22.63
Sweet milk whey	8.00	4.00
Soft wheat bran	5.00	8.00
Potato proteins	2.50	1.00
Soybean oil	1.37	1.50
Calcium carbonate	1.00	1.12
Vitamin and mineral premix[1]	1.00	1.00
Calcium phosphate	0.60	0.54
L-Lys HCl	0.53	0.44
NaCl	0.35	0.40
L-Tre	0.21	0.17
DL-Met	0.22	0.15
L-Trp	0.07	0.05
Nutrients, % DM		
DM	89.92	89.82
CP	20.12	19.48
EE[2]	4.03	4.73
CF[3]	4.28	4,88
Ash	5.74	5.78
Starch	40.08	44.59
Ca	0.79	0.80
Total P	0.58	0.59
Zn mg/kg	50	45
DE[4], kcal/kg	3,839	3,836
NE[5], kcal/kg	2,862	2,868

[1]: Providing per kilogram of premix: vitamin A, 1,500,000 UI; vitamin D_3, 170,000 UI; vitamin E, 4,000 mg; vitamin B_1, 200 mg; vitamin B_2, 500 mg; vitamin B_6, 250 mg; vitamin B_{12}, 4 mg; vitamin H, 15 mg; vitamin K, 250 mg; vitamin PP, 3,000 mg; D-pantotenic acid, 1,500 mg; choline cloride, 38,500 mg; folic acid, 100 mg; Mn, 6,100 mg from manganese oxide; Fe, 15,000 mg from iron sulfate; Cu, 7,600 mg from copper sulfate; Co, 50 mg; I, 150 mg from potassium iodide; Se, 30 mg from sodium selenite.
[2]: EE = ether extract
[3]: CF = crude fiber
[4]: According to the equation proposed by Whittemore 1987
[5]: According to the equation proposed by Noblet 1994

Chemical Analysis

The DM, CP, ether extract, crude fiber, ash, and starch contents of the feed were determined according to the AOAC (2000) methods. Zn in feed and plasma were analyzed by Inductively Coupled Plasma Atomic Emission Spectrometry (EPA method 6010C, 2000) by using the Optima 2100 DV ICP/OES instrument (PerkinElmer, Inc. Shelton, CT).

Ileal Histometry and Immunohistochemistry

Haematoxylin and Eosin staining was carried out to assess the ileum micro-anatomical structure and perform histometry. For histometry, the following parameters were evaluated per section: villous height (**V**; 10 villi measured per section), crypt depth (**C**; 10 crypts measured per section), the villous height to crypt depth ratio (**V:C**). On other sections, the ileum mucin profile, and the respective histometry (number of mucous cells) were determined by staining sections with the Alcian blue 8GX pH 2.5-periodic acid Schiff (AB-PAS) sequence, which reveals neutral (PAS-reactive, purple stained) and acid (AB-reactive, azure stained) glycoconjugates. The number of mucous cells was determined in 5 villi and 5 crypts of each section.

Other ileal sections were processed by immunohistochemistry or immunofluorescence to reveal proliferating epithelial cells (**PCNA**; 1:1000; clone PC10, Sigma, Italy) and entero-endocrine serotonin secreting cells (1:100; AB938; Millipore Merck S.p.A., Milan, Italy). Proliferating cells were counted in 6 crypts of each section. The number of enterochromaffin serotonin-secreting cells (**EC**) was evaluated in 8 randomly selected visual fields of the mucosa (each field representing a tissue section area of 77,323.25 μm^2 at 400X) for each section, and the density was expressed as number of cells/mm^2 of mucosa.

For histology, histochemistry and immunohistochemistry, all the observations were made by a single investigator, blind to the pig groups, using an Olympus BX51 microscope (Olympus Italia Srl, Milan, Italy) equipped with a digital camera and DP software for computer-assisted image acquirement and management. Immunofluorescence reactions were observed using a Confocal Laser Scanning Microscope (FluoView FV300, Olympus Italia Srl, Milan, Italy).

Sodium-dependent Glucose Transporter (SGLT-1) Gene Expression

Ileal scraping samples were disrupted by grinding in liquid nitrogen with mortar and pestle, then homogenized using TissueLyser (Qiagen, Hilden, Germany). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (RNase-Free DNase Set, Qiagen, Hilden, Germany). RNA yield and quality were determined spectrophotometrically using A_{260} and A_{280} nm measurements. All samples showed A_{260}:A_{280} ratio of 1.8-2.0. A total of 1 µg of RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. Resulting cDNA was quantified with Quant-iT Oligreen ssDNA Assay (Life Technologies Italia, Monza, Italy) to normalize the quantity of cDNA template used for amplification by real-time PCR. Real-time PCR was performed using iCycler Thermal Cycler system and SybrGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA). Thermocycling protocol included initial denaturation for 1 min and 30 s at 95°C, 40 cycles of denaturation at 95°C for 15 s followed by 30 s of annealing and extension at 60°C. After amplification, all samples were subjected to a melt curve analysis, with a slow heating from 55 to 95°C with a rate of 0.5°C/s to validate absence of non-specific products.

Gene expression was normalized using two housekeeping genes coding for portions of porcine ribosomal subunit 60S, such as ribosomal protein L35 (**RPL35**) and ribosomal protein L4 (**RPL4**). Average threshold cycle ($\mathbf{C_T}$) was determined for each gene of interest (**GOI**), and geometric average was calculated for housekeeping genes (**HKs**), assuming C_T as number of cycles needed to reach a fixed arbitrary threshold. Delta C_T was calculated as C_T GOI – C_T HKs, then a modification of the $2^{-\Delta\Delta C}{}_T$ (Livak and Schmittgen, 2001) method was used to analyze the relative expression (fold changes), calculated relative to the control group.

The gene specific primers for SGLT-1, RPL35, and RPL4 were as follows: forward 5'-TGCTGTTTCCAGATGATGTGGGCT-3', reverse 5'-TGCTGCTGCTGTTAAAGATGGACG-3' for SGLT-1 (198 bp); forward 5'-AACCAGACCCAGAAAGAGAAC-3', reverse 5'-TTCCGCTGCTGCTTCTTG-3' for RPL35 (146 bp; Alexander et al., 2012); forward 5'-CAAGAGTAACTACAACCTTC-3', reverse 5'-GAACTCTACGATGAATCTTC-3' for RPL4 (122 bp; Alexander et al.,

2012). Primers were obtained from Life Technologies (Life Technologies Italia, Monza, Italy).

Statistical Analysis

Animals were blocked in a completely randomized design and data were analyzed using one-way ANOVA with GLM procedure of SAS followed by Tukey post hoc test to detect differences among treatments (SAS Institute Inc., release 9.2, Cary, NC). The pen was the experimental unit for growth performance, whereas the pig was the experimental unit for histometry and immunohistochemical and SGLT-1 analysis. Differences were considered significant at $P < 0.05$ and trends were defined at $0.05 < P \leq 0.1$.

RESULTS

Growth Performance and Plasmatic Zn

Animals maintained a good health status throughout the experiment and mortality was low and not affected by the treatments (overall 1.4%). Diarrhea scouring evaluated on a semi-objective scale was not significantly different among the groups. Growth performance is showed in Table 2. During the first week there were no differences among treatments, but starting at d14, at the end of the first feeding phase, animals receiving ZnO had better performance compared to control. In particular, in the second week of growth (7 to 14 d) the fZnO group had greater ADG and lower ADFI than the control group (+32% and -13%, respectively; $P = 0.002$ and $P = 0.004$, respectively) , which resulted in a better body weight (+7%) and G:F (+54%), respectively ($P = 0.02$ and $P < 0.001$, respectively).

Likewise, during the same week, the mZnO-800 group had an increased ADG than the control group (+18%) and a better G:F (+25%), whereas the group mZnO-300 had only a better G:F compared to the control group.

In the second feeding phase (14 to 42 d), though there were no significant differences among treatments, the ADG of all of the groups receiving ZnO, regardless of the source and dose, was greater than the control group (+25% on average; $P = 0.05$). Also the G:F tended to be better in treated animals compared to the control (+26% on average; $P = 0.06$).

Table 2. Growth performance

Item	Treatment[1]				SEM	*P*
	control	fZnO	mZnO-300	mZnO-800		
BW, kg						
d0	7.04	7.15	7.22	6.98	0.10	0.29
d7	8.35	8.58	8.58	8.40	0.13	0.45
d14	9.89[a]	10.61[b]	10.30[ab]	10.21[ab]	0.15	0.02
d42	20.49[a]	22.65[b]	22.11[b]	22.22[b]	0.46	0.01
0 to7 d						
ADG, g/d	187.7	204.0	193.7	203.2	9.85	0.60
ADFI, g/d	267.3	249.7	243.0	256.7	14.45	0.67
G:F	0.73	0.85	0.80	0.81	0.06	0.54
7 to 14d						
ADG, g/d	220.1[a]	290.5[c]	230.6[ab]	258.7[bc]	12.83	0.002
ADFI, g/d	456.2[b]	397.8[a]	396.4 [a]	435.5[b]	12.69	0.004
G:F	0.48[a]	0.74[b]	0.58[c]	0.60[c]	0.03	< 0.0001
14 to 42d						
ADG, g/d	282.6	347.2	357.6	351.4	20.67	0.05
ADFI, g/d	743.5	719.4	732.4	749.8	34.22	0.93
G:F	0.39	0.50	0.50	0.47	0.04	0.11
Overall						
ADG	230.1[a]	280.6[b]	247.8[ab]	271.1[b]	11.40	0.02
ADFI	489.0	455.6	457.3	480.6	16.38	0.39
G:F	0.48[a]	0.64[b]	0.60[b]	0.59[b]	0.03	0.002

[a-c] Within a row, means without a common superscript differ ($P < 0.05$).
[1] Treatments: control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2,850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg.

The overall performance (0 to 42 d) was in favor of the groups receiving ZnO either free or microencapsulated. In fact, between 0 to 42 d fZnO and mZnO-800 pigs had 20% higher ADG than the control ($P = 0.02$), whereas mZnO-300 had intermediate ADG. Nonetheless, at d42, all of the groups weighed on average 9% more than control group and G:F was on average 10 points higher ($P = 0.01$ and $P = 0.002$, respectively).

Zn source significantly affected Zn plasma levels at both stages, as ZnO at 3000 mg/kg had the greatest values ($P < 0.01$). In particular, plasma Zn concentration at d14 was 15, 23, 14.5, and 18 µmol/L for control, fZnO, mZnO-300 and mZnO-800 groups, , respectively. At d42 plasma Zn concentration was 10, 30, 10 and 12 µmol/L for control, fZnO, mZnO-300 and mZnO-800 groups, respectively.

Ileal Histometry and Immunohistochemistry

The histometrical results are summarized in Table 3. At d14 the villi were the longest in the ileum of mZnO-800 treated animals whereas they were the lowest in control piglets ($P < 0.0001$). The crypt depth did not reveal significant differences among the groups, but the V:C ratio was higher in fZnO, mZnO-300, and mZnO-800 than in control piglets ($P = 0.0002$). Zinc oxide treatments did not influence the goblet cells numbers.

The percentage of proliferating cells, which were counted in the ileal crypts, was lower in piglets of control group than in fZnO, mZnO-300, and mZnO-800 groups ($P = 0.0001$). The number of mucosal endocrine serotonin-secreting cells was not affected by dietary supplementations.

At d42 the villi length and V:C ratio were higher in the ileum of fZnO animals in comparison with the other groups ($P < 0.0001$). The crypt depth was not different among the groups. The percentage of proliferating cells, which were counted in ileal crypts, was greater in piglets of mZnO-800 group than in control, mZnO-300, and fZnO animals ($P < 0.0001$). Endocrine serotonin-secreting cells tended to be more numerous in ileum of mZnO-300 and mZnO-800 animals than in control and fZnO piglets ($P = 0.09$), whereas goblet cells number was not affected.

Table 3. Ileal histometry and immunohistochemistry

	Treatment[1]					
Item	control	fZnO	mZnO-300	mZnO-800	SEM	*P*
d14						
Villous height, µm	309.36[a]	316.98[ab]	329.81[bc]	342.74[c]	5.55	< 0.0001
Crypt depth, µm	331.69	322.74	321.57	335.63	5.36	0.12
V:C[2]	0.94[a]	0.99[b]	1.04[b]	1.03[b]	0.02	0.0002
Goblet cells / crypt	33.27	29.95	34.23	33.13	1.53	0.22
Goblet cells / villous	19.60	19.89	18.49	21.11	1.14	0.46
PCNA[3], %	86.02[a]	88.82[b]	90.93[b]	90.80[b]	0.85	0.0001
EC[4], % cells/mm^2	52.87	45.66	50.65	50.38	2.59	0.25
d42						
Villous height, µm	293.87[a]	327.01[b]	303.11[a]	294.13[a]	5.16	< 0.0001
Crypt depth, µm	325.11	332.50	333.81	325.22	4.93	0.42
V:C[2]	0.92[a]	0.99[b]	0.92[a]	0.91[a]	0.02	0.02
Goblet cells / crypt	23.08	26.09	25.43	24.03	0.94	0.12
PCNA[3], %	90.84[a]	89.83[a]	89.93[a]	93.78[b]	0.56	< 0.0001
EC[4], % cells/mm^2	42.30	43.38	47.96	49.94	2.47	0.09

[a-c] Within a row, means without a common superscript differ ($P < 0.05$).
[1] Treatments: control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2,850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg.
[2] V:C = villi height : crypts depth
[3] PCNA = proliferating cell nuclear antigen positive cells
[4] EC = enterochromaffin cells

SGLT-1

Figure 1 shows SGLT-1 gene expression in ileal mucosa at d14 and d42. At d14, SGLT-1 mRNA relative expression was the lowest in mZnO-800 pigs compared to mZnO-300, whereas the control and fZnO groups had intermediate values ($P = 0.02$). At d42, both groups receiving microencapsulated ZnO tended to have a reduced mRNA abundance than the control and fZnO groups ($P = 0.06$).

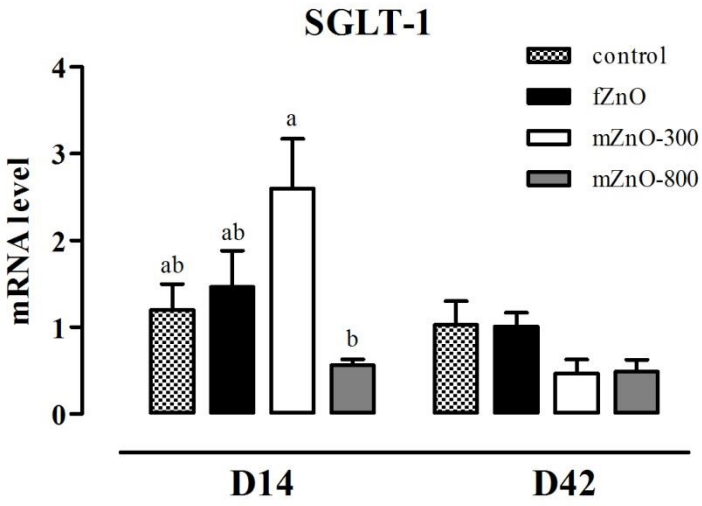

Fig. 1. Gene expression of SGLT-1 in ileal mucosa at d14 and d42.

Data are expressed as means (n = 6) and SEM represented by vertical bars.
[a,b] Within the same time point mean values with unlike letters were significantly different (P<0.05).
Control = basal diet providing Zn at 50 mg/kg; fZnO = basal diet + free ZnO providing Zn at 2850 mg/kg; mZnO-300 = basal diet + microencapsulated ZnO providing Zn at 140 mg/kg; mZnO-800 = basal diet + microencapsulated ZnO providing Zn at 380 mg/kg.
A modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).

DISCUSSION

Dietary ZnO at 2,500–3,000 mg/kg of feed is extensively used in worldwide pig production since in the late 80's Poulsen described it as an anti-diarrhea agent and a growth promoter (Poulsen, 1989). Many researches have in fact consistently reported that pigs benefit from supplemental ZnO at pharma levels in the first weeks post weaning, but increasing concerns on environmental pollution drove European legislators toward a more attentive policy on the excretion of heavy metals in manure (Carlson et al., 1999). With this perspective, the aim of this study was to investigate whether lipid microencapsulation could help reducing ZnO dietary inclusion by comparing the effects of low doses of microencapsulated ZnO to a pharmacological dose of free ZnO on piglets growth performance and intestinal architecture.

Numerous ZnO mechanisms of action have been reported over the years and it has been recently demonstrated that 2,000–3,000 mg/kg of Zn provided as ZnO increase the insulin-like growth factor-I and insulin-like growth factor-I receptor expression in the

intestinal mucosa and stimulate feed intake via ghrelin secretion from the stomach, thereby enhancing growth performance (Li et al., 2006; Yin et al., 2009). In our study, we measured a significant improvement of the average daily gain in the second week post-weaning due to the supplementation of ZnO, which was not supported by an increased feed intake, as reported also in other researches (Case et al., 2002; Li et al., 2006; Ou et al., 2007). The increased daily growth was dependent on the amount of ZnO fed rather than on the source and resulted in a higher body weight in pigs fed with ZnO at 3,000 mg/kg than in control animals and in a better feed conversion for all of the groups treated with ZnO compared to control after 14 days from weaning. During the second phase of growth all of the groups receiving ZnO tended to have better gain and feed efficiency compared to control, but again no impact on feed intake was measured due to ZnO. Beyond an increased feed intake, one of the most accounted hypothesis about ZnO mechanism of action is that Zn is anti-oxidant and anti-inflammatory, thereby implicating a host effect in the response to Zn. Sargeant and colleagues recently demonstrated a modulation of the innate immune response of IPEC cells and an inhibition of nuclear factor-kappa B following *E. coli* infection mediated by Zn as ZnO (Sargeant et al., 2011). Also Roselli et al. (2003) demonstrated a lower immune response of Caco-2 cells infected with ETEC K88 in presence of high levels of ZnO. This decreased intestinal inflammatory response during challenging insults would eventually improve the intestinal integrity and barrier function, and, as a consequence, piglets would convert feed in a more efficient way and result in a better growth (Faa et al., 2008; Prasad et al., 2008; Zhang & Guo, 2009). The hypothesis that a better growth performance is supported by a less inflamed and more absorptive intestine is here substantiated by the results on ileal morphometry. The trophic effect of ZnO on intestinal villi was also reported by other researchers, though generally at jejunal level (Li et al., 2006; Mavromichalis et al., 2000), and in our study 14 days after weaning piglets receiving ZnO, and in particular in the microencapsulated form, had an higher villi height and villi to crypts ratio compared to control. These parameters were also correlated to the number of cells positive to PCNA staining, indicating that the small intestine of ZnO treated animals was in a more trophic status than control animals. At weaning the intestinal mucosa undergoes several structural changes such as the reduction of villi height and an increase in crypts depth (Pluske et al., 1997); the shortening of villi and the consequent reduction of the intestinal absorptive surface help

to explain the increased susceptibility of the pig to infections and diarrhea. Because shorter villi and deeper crypts have fewer absorptive and more secretory cells, absorption might be poorer when villi are shorter therefore leading to an higher abundance of indigested nutrients in the distal part of the small intestine. SGLT-1 is a Na-dependent co-transporter of glucose whose expression can be reduced when glucose availability is reduced (Dyer et al., 1997; Woyengo et al., 2011) and in our data we did find the lowest relative abundance of SGLT-1 mRNA in pigs receiving microencapsulated ZnO at the highest dose. The lowest mRNA synthesis of SGLT-1 matched the highest villi length for the Zn800 group, thereby suggesting a down-regulation of SGLT-1 gene due to a lower availability of luminal glucose and to a lower amount of undigested nutrients at ileal level. This hypothesis is further confirmed by a work of Woodword and colleagues where ileal SGLT-1 expression was decreased by rapidly digestible starch in comparison with slowly digestible starch. They postulated that slowly digestible starch is digested more in the ileum than in the upper small intestine therefore increasing luminal glucose and SGLT-1 expression, whereas, by contrast, rapidly digestible starch would be digested more in the upper part of the GI tract (Woodword et al., 2012). A lower expression of SGLT-1 in pigs fed with microencapsulated ZnO was also still appreciable at the end of the study (d42).

In the second phase, though performance did not significantly differ, there was a strong trend in an improved growth and feed efficiency in all of the animals receiving ZnO, regardless of the dose, which resulted in about 2 kg heavier animals compared to control ones. Conversely to the first phase, the longest villi and the highest V:C was this time observed in animals fed with 3,000 mg/kg of free ZnO, whereas pigs fed with microencapsulated ZnO did not differ from the control. Pigs fed with microencapsulated ZnO at the highest dose (800 mg/kg) tended to have a higher number of EC cells than the fZnO and control groups. These cells are main mediators of intestinal peristalsis and secretory activity in response to chemical and mechanical stimuli via release of serotonin and have been reported to be mainly expressed during the restoration of the epithelial lining, either by the natural process of repair or by the presence of inflammatory mediators during inflammation (Linden et al., 2003). In light of these findings it might be therefore derived that pigs fed with microencapsulated ZnO at 800 mg/kg were undergoing a remodeling of the ileal mucosa as suggested also by the higher number of cells undergoing synthesis and positive to PCNA staining.

Plasmatic Zn levels of pigs fed with microencapsulated ZnO did not increase compared to the control, whereas plasmatic Zn in pigs supplemented with 3,000 mg/kg of ZnO were significantly higher compared to both control and the other groups. This latter finding, in addition to the effects observed on the ileal mucosa, would suggest a different availability of Zn from microencapsulated ZnO compared to free ZnO. Zn availability is strongly dependent on the source and ZnO has been univocally demonstrated to be less available of $ZnSO_4$ or other salts, despite the higher efficacy (Hahn & Baker, 1993; Wedekind et al., 1994; Shell & Kornegay, 1996). Hahn and Baker (1993) found that ZnO at pharmacological level was more effective than same level of $ZnSO_4$ in improving piglets feed intake and growth in the post-weaning phase despite a lower plasma Zn, and they suggested an inverse relationship between plasmatic Zn and Zn efficacy. This would imply that a lower rate of absorption is therefore necessary in order for Zn to reach the hindgut and exert its anti-inflammatory role in the ileum, where the major part of inflammatory response takes place. Hence, if Zn plasma concentration is a method to predict Zn efficacy, microencapsulation might have prevented the absorptive loss in the proximal jejunum while still allowing Zn to reach the ileum despite a 7-times lower dose compared to pharmacological ZnO.

In conclusion, from this study we can derive two sets of information. First, ZnO improved the growth of pigs by increasing ADG during the second week post weaning in a dose-dependent way, and the growth effect was also sustained by an improved absorptive surface at ileal level, especially when ZnO was fed microencapsulated. Second, lipid microencapsulation allowed to reduce ZnO inclusion dose by at least seven times by preventing ZnO immediate absorption in the upper gut and conveying Zn to the ileum in sufficient amount to promote pigs growth and a more mature ileal mucosa.

We here propose microencapsulated ZnO as an effective and environment-friendly tool to supplement pigs post weaning diets.

CHAPTER 5

TRIBUTYRIN TRIAL

ABSTRACT

Aim of this study was to evaluate growth performance, intestinal architecture and gene expression of inflammatory cytokines and tight junction proteins in weaning piglets fed with tributyrin, either free or microencapsulated, as dietary source of butyric acid. One hundred-eight weaned pigs (28 days) divided in 27 pens (n = 9), received either a basal diet (control, 0 mg/kg butyric acid) or the basal diet supplemented with tributyrin or its encapsulated form at 1,750 mg/kg (f-TB and m-TB, respectively), both providing 960 mg/kg of butyric acid equivalents. After 21 days, 7 pigs per group were euthanized to collect intestinal samples (duodenum, jejunum, ileum, colon) for histo-morphology, cytokines, and tight junction markers gene expression analysis. Pigs BW and feed intake were recorded at 0 and 21 d, and ADG and FCR were calculated. Data were analyzed with 1-way ANOVA. No significant effects were observed on growth performance parameters. Compared to control, m-TB induced deeper crypts in the duodenum (P = 0.09), numerically longer villi in the jejunum and significantly reduced villi:crypts ratio in the ileum (P = 0.04); goblet cells tended to be reduced by f-TB in duodenal villi (2.4 fold, P = 0.09), by both f-TB and m-TB in ileal villi (1.8–2.2 fold, respectively; P = 0.06), and by m-TB in colonic crypts (P = 0.08). Compared to control, f-TB showed higher TNF-α expression in duodenal and ileal mucosa (P = 0.06 and P = 0.10), and significantly higher IFN-γ level in the jejunum (P = 0.04), whereas in the colon m-TB down regulated IFN-γ (2.2 fold, P = 0.06) and IL-1β level (1.7 fold). Jejunal claudin-1 mRNA was significantly reduced in both groups receiving tributyrin compared to control (1.5–2.4 fold, for f-TB and m-TB; P = 0.05). Similarly, in the colon claudin-1 mRNA was numerically lower in m-TB (2.1 fold) than in control group and f-TB had intermediate value. Ileal occludin mRNA was significantly reduced in both groups receiving tributyrin compared to control (1.6–2.2 fold, for f-TB and m-TB; P < 0.01). In the colon occludin mRNA was downregulated both in f-TB and m-TB compared to control (1.4 fold both; P < 0.05). The supplementation of tributyrin in the diet modulated the intestinal architecture by reducing the mucous-secreting goblet cells in a tract-specific way, free tributyrin affecting the upper intestine while the microencapsulated form acting in the lower gut. Moreover, the reduced expression of pro-inflammatory cytokines, observed particularly in the colon, and the regulation of tight junctions proteins further substantiate the local anti-inflammatory role of butyric acid, released via tributyrin, in the large intestine.

Keywords: tributyrin, microencapsulation, weaning piglet.

INTRODUCTION

Weaning is the most critical period in the piglet's life because of a complex series of stressors (social, nutritional, environmental) associated with physiological, anatomical and immunological changes in the gastro-intestinal tract that impair the intestinal functions – digestion, absorption and barrier function – thus leading to reduced growth efficiency and higher incidence of infections and intestinal disturbances, such as post-weaning diarrhea (Pluske et al., 1997).

Among the strategies to help in overcoming this post- weaning growth retard, organic acids have been widely used due to their growth promoting effects, mainly based on their acidifying and anti-microbial properties. Among these acids, butyric acid seems particularly interesting because of its multi-functional role in the intestine: first, butyric acid is the primary energy source for the epithelial cells in the colon and in the terminal ileum (Roediger, 1980; Chapman et al., 1995); in addition, multiple beneficial effects on the intestinal cell homeostasis can be ascribed to butyric acid, such as anti-inflammatory, anti-oxidant, anti-apoptotic and proliferative, anti-carcinogenic, and protective action on the epithelial barrier integrity (by strengthening the tight junctions). In pigs, in-feed butyric acid, as sodium butyrate, has been shown to improve growth performance when fed in the first two weeks post-weaning (Piva et al., 2002a; Manzanilla et al., 2006) as well as in the fattening phase (Gálfi & Bokori, 1990). Tributyrin (**TB**) is a triglyceride containing three molecules of butyric acid esterified with glycerol. Since it is watersoluble, TB can be directly absorbed by the intestinal cell and, once inside, it is rapidly hydrolyzed into three molecules of butyric acid by specific cellular lipases (Leonel et al., 2013; Gaschott et al., 2001). Moreover, due to its not volatile and odorless nature, TB can be provided orally as a dietary source of butyric acid. Previous studies showed that tributyrin improved growth performance, intestinal morphology and enzyme activity in weaning piglets (Hou et al., 2006). In addition, Piva et al. reported that the synergistic effect of tributyrin and lactitol, as precursor and fermentable source of butyric acid, could improve the trophic status of the intestinal mucosa and control intestinal histamine in nursery piglets (Piva et al., 2002b). However, despite the above mentioned protective and anti-inflammatory

properties of butyric acid discovered *in vitro* in cellular models, no *in vivo* studies have been conducted focusing on the impact of tributyrin on the weaning-associated inflammation and intestinal barrier dysfunction occurring in the gut of weaning piglets. Therefore, the aim of the study was to investigate the effects of supplementation of tributyrin, either free or encapsulated in a lipid matrix, as dietary source of butyric acid, on growth performance, intestinal architecture and expression of inflammatory cytokines and tight junction proteins in weaning piglets.

MATERIALS AND METHODS

The study was conducted at the research facilities of the Research Centre for Animal Production and Environment (CERZOO), which is a Good Laboratory Practices-certified facility and operates according to the procedure of animal protection and welfare (Directive 86/609/EEC).

Experimental Design and Sample Collection

One hundred and eight pigs (Landrace × Large White, barrows and gilts, 6.67 (SE 0.07) kg of BW) weaned at 28 days of age were divided in 27 pens and randomly assigned to one of the following experimental groups (n = 9): the basal diet (control, providing butyric acid at 0 mg/kg); the basal diet added with 1,750 mg/kg of free tributyrin (f-TB); the basal diet supplemented with microencapsulated tributyrin (m-TB) at 3,800 mg/kg. Both f-TB and m-TB diets provided 960 mg/kg of butyric acid equivalents.

The basal diet was formulated to meet or exceed requirements for weaned piglets recommended by the National Research Council (2012). Chemical analysis of the diet was performed and DM, CP, ether extract, crude fiber, ash, and starch contents of the feed were determined according to the AOAC (2000) methods. The composition of basal diet is reported in Table 1.

The health status of animals was monitored throughout the study. Piglets were individually weighted at the beginning of the study (d0) and at the end of the study (d21). Feed was provided *ad libitum* and ADG, ADFI and FCR were calculated between 0 and 21 d. After 21 days 7 pigs per treatment were euthanized and intestinal samples were collected (duodenum, jejunum, ileum and colon). Intestinal samples were cut longitudinally to expose the mucosa and washed with PBS to remove mucus and digesta. Then the mucosa was scraped gently with a glass slide, vacuum-packed,

immediately frozen in liquid N_2 and stored at −80°C until gene expression analysis was conducted. Histological samples were instead collected and fixed in formalin until staining.

Table 1. Composition of basal diet (as-fed basis)

	Nursery diet (0 to 21 d)
Ingredients, %	
Corn meal	50.00
Soybean meal, 48%	20.53
Barley meal	13.84
Milk serum	8.00
Potato proteins	2.50
Soybean oil	1.72
Calcium carbonate	1.08
Vitamin and mineral premix[1]	0.50
Dicalcium phosphate	0.50
L-Lys HCl	0.51
DL-Met	0.21
L-Thr	0.19
L-Trp	0.08
NaCl	0.34
Nutrients, % DM	
DM	89.12
CP	18.87
EE[2]	4.19
CF[3]	3.64
Ash	4.64
Starch	38.50
DE[4], kcal/kg	3,427
NE[5], kcal/kg	2,545

[1]: Providing per kilogram of premix: vitamin A, 3,000,000 UI; vitamin D_3, 400,000 UI; vitamin E, 12,000 mg; vitamin B_1, 500 mg; vitamin B_2, 1,200 mg; vitamin B_6, 1,000 mg; vitamin B_{12}, 6 mg; vitamin C, 15,000 mg; vitamin K, 500 mg; vitamin PP, 5,000 mg; D-pantotenic acid, 4,000 mg; choline cloride, 69,200 mg; folic acid, 300 mg; biotin, 40 mg; Mn, 8,000 mg; Fe, 40,000 mg; Cu, 20,000 mg; Co, 80 mg; I, 300 mg; Zn, 24,000 mg; Se, 20 mg; sepiolite as anti-caking agent 50,000 mg; calcium carbonate, 38.61%; wheat midding 15%).
[2]: EE = ether extract
[3]: CF = crude fiber
[4]: According to the equation proposed by Whittemore 1987
[5]: According to the equation proposed by Noblet 1994

Intestinal Histo-morphometry

Small and large intestinal samples (duodenum, jejunum, ileum and colon) were formalin-fixed and paraffin-embedded and routinely stained (Hematoxilin-eosin). Slides were coded, and morphometric measures and goblet cells counting were carried out blindly by three skilled operators. All images were acquired with an optical microscope Leica DMLB, coupled to a Leica DFC camera (Leica Microsystems GmbH, Wetzlar, Germany). Acquired image were 2088×1550 pixel, Jpeg, The digital image analysis was conducted with ImageJ 1.46 (http://rsbweb.nih.gov/ij/download.html).

Hematoxilin-eosin stained slides were used for measurements of villous height/crypt depth by means of histo-morphometry of intestinal segments. Light microscope photographs were used to measure 10 of the tallest, best oriented villi from villous tip to crypt mouth, and 10 crypts from crypt mouth to base, adjacent to submucosa (Obj. 10X). The villi height to crypt depth ratio (V:C ratio) was calculated (Brown et al., 2006; Di Giancamillo et al., 2008; King et al., 2008).

Other Hematoxilin-eosin stained slides were used for goblet cells counting. In crypts, goblet cells were counted in 5 best oriented crypts / intestinal tract, from crypt mouth to base (adjacent to submucosa). Number of goblet cells are expressed as mean number of goblet cells / 100 μm of crypts epithelium (mean data of crypt length) in order to supply the number of goblet cells / unit length of epithelium (linear density), that is more comparable with bibliographic data (Obj. 40X), (King et al., 2008). The equation to determine the number of goblet cells / 100 μm was: goblet cells / 100 μm = number of goblet cells × 100 / (crypt depth × 2), (King et al., 2008). In villi, goblet cells were counted in 5 best oriented villi/intestinal tract, from villous tip to base (adjacent to crypts mouth). Number of goblet cells are expressed as mean number of goblet cells / 100 μm of villi epithelium (mean data of villous height) in order to supply the number of goblet cells / unit length of epithelium (linear density), that the is more comparable with bibliographic data (Obj. 40X), (King et al., 2008). The equation to determine the number of goblet cells / 100 μm was: Goblet cells / 100 μm = number of goblet cells × 100 / (villi height × 2), (King et al., 2008).

Inflammatory Cytokines and Tight Junctions (TJ) Components Gene Expression Profiling

Mucosa scrapings of duodenum, jejunum, ileum and colon were disrupted by grinding in liquid N_2 with mortar and pestle, then homogenized using TissueLyser (Qiagen). Total RNA was isolated from ileal mucosa scraping using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (RNase-Free DNase Set, Qiagen). RNA yield and quality were determined spectrophotometrically using A_{260} and A_{280} nm measurements. All samples showed A_{260}:A_{280} ratio of 1.8–2.0. A total of 1 µg of RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Resulting complementary DNA (cDNA) was quantified with Quant-iT Oligreen ssDNA Assay (Life Technologies) in order to normalize the quantity of cDNA template used for amplification by real-time PCR. Real-time PCR was performed using iCycler Thermal Cycler system and SybrGreen Supermix (Bio-Rad Laboratories). Thermocycling protocol included initial denaturation for 1 min and 30 s at 95°C, 40 cycles of denaturation at 95°C for 15 s followed by 30 s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis, with a slow heating from 55 to 95°C with a rate of 0.5°C/s in order to validate absence of non-specific products.

Gene expression was normalized using two housekeeping genes coding for portions of porcine ribosomal subunit 60S, such as ribosomal protein L35 (**RPL35**) and ribosomal protein L4 (**RPL4**). Average C_T was determined for each gene of interest (**GOI**), and geometric average was calculated for housekeeping genes (**HKs**), assuming C_T as number of cycles needed to reach a fixed arbitrary threshold. Delta C_T was calculated as C_T GOI−C_T HKs, then a modification of the $2^{-\Delta\Delta C}{}_T$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (Livak & Schmittgen, 2001).

The sequences, expected product length, accession number in the EMBL database/GenBank and references of porcine primers are shown in Table 2. Primer oligonucleotides for claudin-1, TNF-α, and IFN-γ were designed using PrimerQuest software (IDT, Integrated DNA Technologies). Primers were obtained from Life Technologies.

Table 2. Primers used for gene expression analysis by Real-Time PCR

Gene	Primer Sequence (F and R[1]; 5' → 3')	Product lenght (bp)	Product Tm[2] (°C)	Accession number	Reference
Occludin	F: ATCAACAAAGGCAACTCT R: GCAGCAGCCATGTACTCT	157	83	NM_00116 3647.2	Zhang and Guo, 2009
Claudin-1	F: TCCAGTGCAAAGTCTTCGACTCCT R: ATGCCAACAGTGGCCACAAAGATG	121	85.5	NM_00124 4539.1	Present study
TNF-α[3]	F: GCCCACGTTGTAGCCAATGTCAAA R: GTTGTCTTTCAGCTTCACGCCGTT	99	87	NM_21402 2.1	Present study
IFN-γ[4]	F: GGCCATTCAAAGGAGCATGGATGT R: TGAGTTCACTGATGGCTTTGCGCT	149	83.5	NM_21394 8.1	Present study
IL-1β	F: ACCTGAACCTGCCAAGGAAGTGATG R: TCCCAGAGGGCTGAGGTCAAGG	124	85	NM_00100 5149.1	Present study
RPL35[5]	F: AACCAGACCCAGAAAGAGAAC R: TTCCGCTGCTGCTTCTTG	146	87.5	NM_21432 6.2	Alexander et al., 2012
RPL4[6]	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122	84	XM_00312 1741.3	Alexander et al., 2012

[1] F = forward primer, R = reverse primer
[2] Tm = melting temperature
[3] TNF-α = tumor necrosis factor-α
[4] IFN-γ = interferon-γ
[5] RPL35 = ribosomal protein L35
[6] RPL4 = ribosomal protein L4

Statistical Analysis

Animals were grouped in a completely randomized design and data were analyzed with one-way ANOVA, followed by Tukey post hoc test (Graph Pad Prism 5 version 5.03; GraphPad Software, Inc., San Diego, CA). The pen was the experimental unit for growth performance, whereas the pig was the experimental unit for histomorphometry and for gene expression analysis. Differences were considered significant at $P < 0.05$ and trends for $0.05 < P \leq 0.1$.

RESULTS

Growth Performance

Treatments with free or microencapsulated tributyrin did not affect growth performance parameters compared to control group (Table 3).

Table 3. Growth performance

	Treatment[1]				
Item	control	f-TB	m-TB	SEM	*P*
BW, kg					
d0	6.66	6.69	6.66	0.07	0.92
d21	11.92	11.34	11.75	0.35	0.50
0 to 21 d					
ADG, g/d	262.9	232.1	254.5	17.6	0.45
ADFI, g/d	438.8	416.4	441.8	21.9	0.68
FCR	1.68	1.85	1.76	0.07	0.23

[1] Treatments: control = basal diet (control, 0 mg/kg of butyric acid); f-TB = basal diet + free tributyrin at 1,750 mg/kg (providing 960 mg/kg of butyric acid equivalents); m-TB = basal diet + microencapsulated tributyrin at 3,800 mg/kg (providing 960 mg/kg of butyric acid equivalents).
BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

Intestinal Histo-morphometry

Table 4 summarizes histometrical results and goblet cells counts in the intestinal tracts analyzed. In the duodenum, the villi length was not affected by the treatments, whereas the crypts tended to be deeper in pigs fed microencapsulated tributyrin compared to controls and free tributyrin treated (P = 0.09). However, no differences in V:C ratio were observed. In the jejunum, the villi were numerically longer in m-TB and numerically shorter in f-TB compared to control group, but no effects on crypt depth and V:C ratio were evident. In the ileum, villi and crypts were not affected by treatments, but V:C ratio was significantly lower in m-TB group compared to f-TB (P = 0.04). The crypt depth in the colon was not influenced by the treatments.

The goblet cells were counted in the villi and crypts of the intestinal tracts analyzed and are presented as numbers of goblet cells per 100 µm of villous or crypt. In the

duodenum, goblet cells numbers were not different in the crypts but in the villi tended to be reduced in the f-TB group compared both to control and m-TB (2.4 fold less, P = 0.09). In the jejunum, no effects were observed in villi or crypts. In the ileum, goblet cells tended to be less numerous in the villi of both f-TB and m-TB groups compared to control (1.8 and 2.2 fold less, respectively; P = 0.06). In the crypts of the colon, goblet cells number tended to be reduced in m-TB group (P = 0.08).

Table 4. Intestinal histo-morphometry

	Treatment[1]				
Item	control	f-TB	m-TB	SEM	*P*
Duodenum					
Villous height, µm	234.1	252.2	286.7	28.3	0.43
Crypt depth, µm	356.7	376.8	427.8	20.8	0.09
V:C[2]	0.66	0.69	0.62	0.08	0.83
Goblet cells / 100µm villous	1.02	0.43	1.22	0.25	0.09
Goblet cells / 100µm crypt	2.92	2.40	2.18	0.29	0.35
Jejunum					
Villous height, µm	279.5	231.1	294.1	24.6	0.17
Crypt depth, µm	253.9	257.3	276.1	25.9	0.83
V:C[2]	1.12	0.93	0.99	0.09	0.36
Goblet cells / 100µm villous	0.15	0.28	0.55	0.18	0.62
Goblet cells / 100µm crypt	2.49	2.15	2.69	0.33	0.53
Ileum					
Villous height, µm	213.6	240.2	226.7	19.6	0.64
Crypt depth, µm	221.3	223.2	267	24.4	0.34
V:C[2]	0.97[ab]	1.19[a]	0.89[b]	0.09	0.04
Goblet cells / 100µm villous	2.25	1.24	1.03	0.34	0.06
Goblet cells / 100µm crypt	4.77	5.39	4.24	0.56	0.37
Colon					
Crypt depth, µm	349.5	347.1	370	27.2	0.82
Goblet cells / 100µm crypt	4.42	4.09	3.08	0.28	0.08

[a-b] Within a row, means without a common superscript differ (P < 0.05).
[1] Treatments: control = basal diet (control, 0 mg/kg of butyric acid); f-TB = basal diet + free tributyrin at 1,750 mg/kg (providing 960 mg/kg of butyric acid equivalents); m-TB = basal diet + microencapsulated tributyrin at 3,800 mg/kg (providing 960 mg/kg of butyric acid equivalents).
[2] V:C = villi height to crypts depth ratio

Inflammatory Cytokines and TJ Markers Gene Expression Analysis

Inflammatory cytokines mRNA levels in ileum, duodenum, jejunum, and colon are summarized in Fig. 1. In the duodenum, IFN-γ expression was not influenced by the treatments while TNF-α tended to be higher in f-TB group compared both to control and m-TB group (P = 0.06); IL-1β was not detected. In the jejunum, IFN-γ mRNA level was significantly higher in f-TB compared to both control and m-TB (P = 0.04); TNF-α was not different; IL-1β was not detected. In the ileum, no effects were observed on IFN-γ and IL-1β, while TNF-α expression tended to be higher in f-TB compared to m-TB group (P = 0.10). In the colon, IFN-γ level tended to be lower in m-TB group compared to control (2.2 fold less, P = 0.06) and IL-1β level was numerically lower in f-TB group and m-TB (1.5 and 1.7 fold, respectively). TNF-α expression in the colon was not affected by treatments.

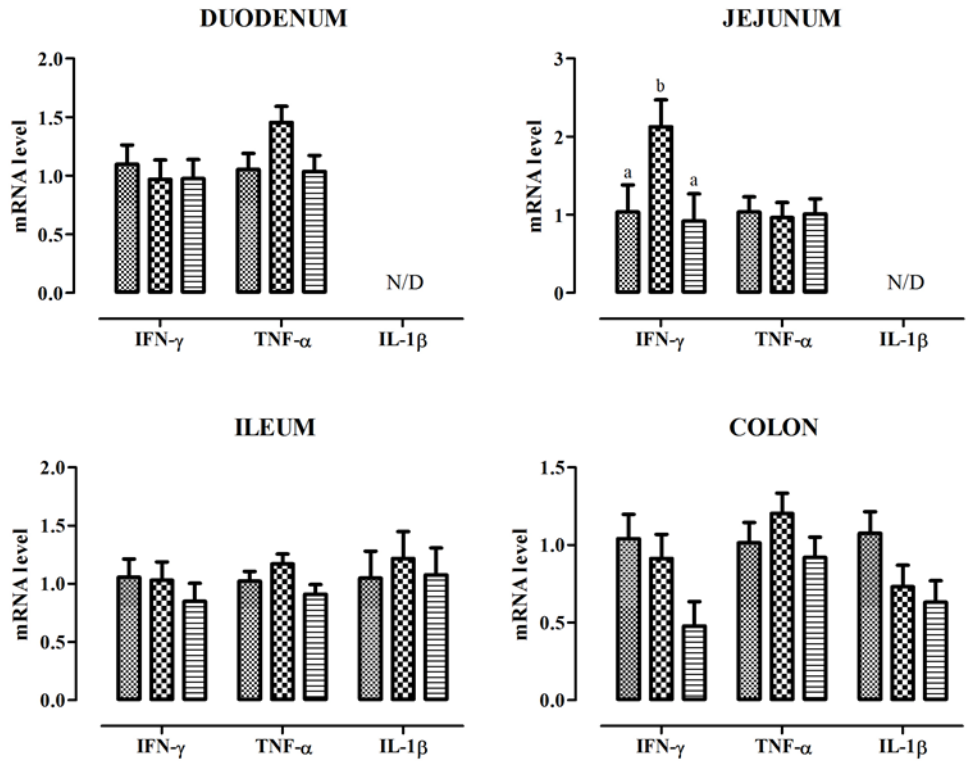

Fig. 1. Gene expression of inflammatory cytokines in intestinal mucosa.

Data are expressed as means (n = 7) and SEM represented by vertical bars.

[a,b] Within a gene, mean values with unlike letters were significantly different (P < 0.05).

A modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).

, control; , f-TB (free tributyrin at 1,750 mg/kg providing 960 mg/kg of butyric acid equivalents); , m-TB (microencapsulated tributyrin at 3,800 mg/kg providing 960 mg/kg of butyric acid equivalents).

Fig. 2 shows TJ components mRNA expression. Claudin-1 gene expression was not affected by treatments in the ileum. In the jejunum, claudin-1 mRNA level was significantly reduced in m-TB group compared to control (2.4 fold; $P < 0.05$), whereas f-TB group had intermediate value (1.5 fold less than control). Similarly, in the colon claudin-1 was numerically lower in m-TB group compared to control group (2.1 fold). No effects were evident on occludin gene expression in the jejunum. In the ileum, occludin mRNA level was significantly reduced in both groups receiving tributyrin compared to control (1.6 and 2.2 fold, for f-TB and m-TB, respectively; $P < 0.01$). In the colon, m-TB had significantly lower occludin level than control (1.4 fold, $P < 0.05$), whereas f-TB had intermediate value.

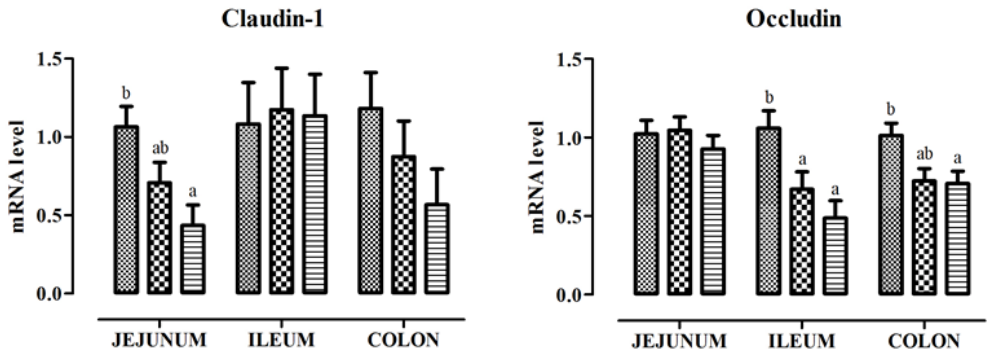

Fig. 2. Relative gene expression of TJ components in intestinal mucosa.

Data are expressed as means ($n = 7$) and SEM represented by vertical bars.
[a,b] Within an intestinal tract, mean values with unlike letters were significantly different ($P < 0.05$).
A modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).
, control; , f-TB (free tributyrin at 1,750 mg/kg providing 960 mg/kg of butyric acid equivalents); , m-TB (microencapsulated tributyrin at 3,800 mg/kg providing 960 mg/kg of butyric acid equivalents).

DISCUSSION

Butyric acid has been proposed as a nutritional tool to control weaning-associated intestinal dysfunction and subsequent growth failure in pig production, as a result of its well-known beneficial properties on the intestinal mucosa, mainly trophic effect on colonocyte and enterocyte, as well as anti-inflammatory, anti-oxidant, barrier-

protective, anti-apoptotic and proliferative effect (Roediger, 1980; Chapman et al., 1995; Leonel & Alvarez-Leite, 2012). Butyric acid is usually provided as salt, mainly sodium butyrate, due to its odor less strong than the acid, but alternative approaches have been documented such as butyric acid ester (tributyrin) or substrates to enhance microbial butyrogenic fermentations (lactitol, gluconic acid) (Piva et al., 2002b; Biagi et al., 2006). However, growth and intestinal health promoting effects have been shown to be dependent on several factors, above all, dose, supplementation duration, intestinal tract observed, and age of piglets (Lallès et al., 2009).

The purpose of the present study was to assess the impact of the in-feed supplementation with tributyrin in weaning piglets and to compare free tributyrin and its microencapsulated form, as dietary source of butyric acid, with particular reference to small and large intestine architecture and inflammatory status, as key-elements for intestinal health and pig production.

At weaning the intestinal mucosa undergoes several structural and functional changes (reduction of villi height, increase in crypts depth, impairment of enzymatic activity) that reduce the intestinal absorptive, digestive, and barrier function (Pluske et al., 1997). In our study, dietary supplementation with tributyrin impacted intestinal morphology: compared to control animals, piglets receiving microencapsulated tributyrin tended to have deeper crypts in the duodenum, whereas piglets fed free tributyrin had higher villi to crypt ratio in the ileal mucosa. These results appear partly in contrast with previous works. Piva et al. reported no influence on jejunal villous length and drastic reduction in cecal crypt depth induced by tributyrin 2 weeks post-weaning (Piva et al., 2002b). Again, Hou and colleagues in a four-weeks post-weaning trial showed decreased crypt depth both in the duodenum and in the ileum of tributyrin-treated animals compared to controls (Hou et al., 2006). The discrepancy may rely on the different dose of tributyrin tested, 10,000 and 5,000 mg/kg (for Piva et al., and Hou et al., respectively), thus 3-6 times higher than our concentration. However, consistently to our findings, Mentschel and Claus, feeding pigs with resistant potato starch to increase microbial butyrate formation in the colon, found a decrease in the colonic crypt depth as a result of the anti-apoptotic action of butyrate, suggested by increased anti-apoptotic factor Blc-2 (Mentschel & Claus, 2003).

Our most intriguing result was observed in the mucous-secreting goblet cells number where the dietary supplementation with tributyrin induced a reduction in the cells counts in a tract-specific way: free tributyrin affected the upper intestine while the

microencapsulated form impacted the lower gut. In this regard, microencapsulation seemed to allow an higher amount of tributyrin to be gradually released along the gastro-intestinal tract and reach the large intestine, whereas the free tributyrin appeared to be sooner available in the small intestine. This result is consistent with previous findings where slow release and targeted delivery of bio-active compounds was obtained with lipid microencapsulation (Piva et al., 2007; Grilli et al., 2010). The reduction in the mucous-secreting cells number may be explained by a beneficial and protective effect of tributyrin on the intestinal epithelium. Indeed, goblet cells number, induction and mucins secretion have been shown to be induced by a wide series of bioactive compounds, including hormones, neuropeptides, inflammatory cytokines, bacterial and parasitic products. Particularly, goblet cells hyperplasia and mucins hyper-secretion have been observed in many intestinal infections, caused by bacteria, parasites and viruses. For example, in *Salmonella* infections, mucins production and mucous release into the gut lumen is mediated by IFN-γ signaling (Kim & Khan, 2013). Thus, in our study, a protective action of tributyrin on the intestinal epithelium – maybe through an anti-inflammatory effect – may lead to reduced mucous-secreting goblet cells counts.

Weaning represents a dramatic challenge to intestinal health and is known to be associated with up-regulation of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Pié et al., 2004). As a consequence, excessive inflammatory response can lead to tissue damage and intestinal dysfunction. Particularly, pro-inflammatory cytokines can affect intestinal barrier function and permeability through modulation of epithelial tight junctions as main regulators of the para-cellular route (Al-Sadi et al., 2009). To investigate the impact of tributyrin, either free and encapsulated, on small and large intestine inflammatory status and epithelial integrity, we analyzed a set of inflammatory cytokines and tight junctions components by gene expression.

The main effect was observed in the colon where microencapsulated tributyrin allowed a trend for IFN-γ reduction and a numerical reduction of IL-1β, compared to control while free tributyrin had intermediate values. The reduction in pro-inflammatory cytokines levels induced by tributyrin, mostly when released from lipid encapsulation, may provide a protection for the colonic epithelium and affect its mucous-secreting cells set: less inflamed and damaged colon, less numerous goblet cells. This anti-inflammatory action of tributyrin is consistent with the protective effect previously

described in experimentally induced colitis both in mice and pigs. In a murine model of dextran sodium sulfate (DSS)-induced colitis, tributyrin reduced the mucosal damage and the neutrophils infiltration induced by DSS in the colon, stimulating immune-regulatory T_{Reg} cells and anti-inflammatory TGF-β and IL-10 cytokines (Leonel et al., 2013). In addition, Hou et al. found that dietary tributyrin supplementation could alleviate intestinal injury in the acetic acid (ACA)-induced porcine model of colitis, by reducing lymphocytes counts both in plasma and in colonic mucosa (Hou et al., 2014). Moreover, an anti-inflammatory activity is accounted also for butyric acid that has been shown to reduce pro-inflammatory IFN-γ, TNF-α, IL-1β, IL-6, induce IL-10 and TGF-β expression, induce nitric oxide synthase (iNOS) and metalloproteinases and reduce lymphocytes proliferation and activation in several *in vitro* studies using cell models. The proposed mechanism of action involves the inhibition of the nuclear factor-kappa B (NF-κB), that controls the expression of genes coding for inflammatory cytokines, adhesion molecules, and inflammation-inducing enzymes (Leonel & Alvarez-Leite, 2012). In addition, in piglets aged 40 days, Le Gall and colleagues (2009) observed a tendency by sodium butyrate to a reduced expression of IL-18 in the intestine (Le Gall et al., 2009). Despite the anti-inflammatory action of microencapsulated and – to a smaller extent – free tributyrin in the colon, a stimulation of inflammatory cytokines was induced by free tributyrin in the upper gut. Particularly, in piglets fed free tributyrin, TNF-α mRNA tended to be hyper-expressed in the duodenum and in the ileum, while IFN-γ was significantly higher in the jejunum compared to both controls and microencapsulated tributyrin treated animals. We do not have a clear explanation for this surprising result as, to our knowledge, no immune-stimulatory or toxic effects have been described for butyric acid and tributyrin, in human or animal species, neither in pig trials testing butyric acid or tributyrin doses significantly higher than the dose we used in the current study.

Butyric acid has also been shown to play an important role in maintaining the epithelial barrier function. Several *in vitro* studies, using both Caco-2 and IPEC-J2 cell models, revealed that butyric acid, as sodium butyrate, can improve epithelial integrity by strengthening inter-cellular tight junctions likely via activation of AMP-activated protein kinase (Ohata, 2005; Peng et al., 2009; Ma, 2012). A tighter epithelium can allow more efficient intestinal barrier function. In our experiment, dietary treatment with tributyrin modulated gene expression of tight junction components along the gastro-intestinal tract: particularly, in comparison to control, microencapsulated

tributyrin induced a significant reduction of occludin (in the ileum and in the colon) and claudin-1 (in the jejunum) mRNA levels, while free tributyrin showed intermediate values. Both occludin and claudin-1 are essential components of tight junctions, as they are trans-membrane proteins that anchor two epithelial cells and seal the inter-cellular space, thus regulating the para-cellular permeability (Turner, 2009). We hypothesize that the reduction in the gene expression of tight junctions components may be a result of a general improvement in the intestinal health induced by tributyrin: tributyrin may reduce the intestinal damage occurring in weaning and, therefore, less damaged epithelium could require less transcription of new tight junctions proteins. Particularly in the colon, this effect on tight junctions may be explained by the concomitant reduction in the expression of pro-inflammatory cytokines such as IFN-γ and IL-1β. In fact, IFN-γ has been described to exert a "leaky" action on the epithelium through the internalization of tight junctions proteins (mainly occludin) via macro-pinocytosis (Bruewer et al., 2005). In addition, IL-1β has been shown to increase intestinal epithelial permeability and to be markedly elevated in human intestinal inflammatory diseases such as IBD (Al-Sadi & Ma, 2007). Thus in the colon, tributyrin, reducing the pro-inflammatory milieu, may reduce the epithelial disruption and, as a consequence, reduce the necessity to express new tight junctions proteins. However, taking into account the unusual hyper-expression of inflammatory cytokines induced by free tributyrin in the upper gut, in addition to the proposed anti-inflammatory effect, we should also consider a cyto-protective action of tributyrin on the intestinal epithelial cells, likely by improving the trophic status. Tributyrin, as source of butyric acid, may in fact enhance cellular metabolism both directly, by increasing β-oxidation of butyric acid, and via an indirect pathway through the stimulation of trophic factors, including glucagon-like peptide-1 (GLP-1) and, even more, GLP-2. Moreover, in addition to the well known trophic effect, GLP-2 has been shown to exert a protective action on the epithelial integrity: in a Caco-2 cell model, GLP-2 enhanced the barrier formation (allowing higher TER) and attenuated TNF-α induced disruption of the TJ integrity (Moran et al., 2012).

The improvement in the epithelial cellular health induced by tributyrin may be therefore reflected by reduced mucous-secreting goblet cells number and reduced expression of new tight junctions components, as healthier epithelium may not require additional defensive mechanisms.

Our growth performance data showed that, though modulating the intestinal architecture and health, tributyrin, either free or microencapsulated, did not result in improved pig growth. One possible explanation may rely in the doses tested: our concentrations (1,750 and 3,800 mg/kg for free and microencapsulated tributyrin, respectively) appear to be lower compared to those tested in previous studies, though results are conflicting. For example, tributyrin at 5,000 mg/kg has been shown to allow greater weight gain and better feed efficiency when fed for 4 weeks post-weaning (Hou et al., 2006). Conversely, Piva and colleagues (2002b) reported adverse effect on growth performance for tributyrin fed at 10,000 mg/kg for 2 weeks post-weaning, and attributed the negative effects to the high dose of tributyrin tested. For next studies it should be accurately evaluated the dose of tributyrin to be tested, however taking into account the great potential of the microencapsulated form that can be derived from the present study. Microencapsulation of tributyrin in a lipid matrix likely allowed an higher amount of tributyrin to be available in the colon, compared to free tributyrin, as reflected by the above-described tract-specific modulation of the intestinal architecture.

Overall, tributyrin, both free and microencapsulated, has been shown to impact intestinal health by modulating intestinal architecture and expression of inflammatory cytokines and tight junctions components, thus being a promising tool to provide butyric acid to the intestine of weaning piglets.

CHAPTER 6

ORGANIC ACIDS AND NATURE-IDENTICAL COMPOUNDS TRIAL

ABSTRACT

Dietary inclusion of organic acids and botanicals has been shown to improve pig performance, but the mechanism behind their efficacy has yet to be elucidated. The aim of this study was to assess the impact of dietary inclusion of a microencapsulated blend of organic acids (**OA**) and nature-identical compounds (**NIC**) on measures of intestinal health in early-weaned pigs. At the research facilities of the North Carolina State University, twenty pigs weaned at 18 d of age and placed into one of two pens (n = 10) received either a basal diet or the basal diet supplemented with OA+NIC mixture (5,000 mg/kg) for 14 d. Individual body weight were recorded initially and every 7d, and ADG was calculated. Blood samples were collected at d0, d7 and d14 for protein expression of inflammatory cytokines in serum. At the completion of the study, within each group the 6 pigs closest to their group mean ADG were selected for tissue collection. Ileal and jejunal samples were collected for Ussing chambers analysis of trans-epithelial electrical resistance (**TER**), intermittent short-circuit curret ($\boldsymbol{I_{SC}}$), and dextran flux. Scrapings of ileal mucosa were collected for analysis of cytokines (IL-6, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β) at mRNA and protein level. Growth performance results showed that pigs fed the OA+NIC mixture had greater ADG during both the second week of the study and overall ($P < 0.05$). Pigs fed the OA+NIC mixture also had reduced jejunal dextran flux and I_{SC} in the ileum ($P < 0.12$ and $P < 0.07$, respectively). The ileal gene expression of several cytokines (IL-12, TGF-β, IL-6) were downregulated in OA+NIC pigs and the corresponding proteins followed similar patterns. At d7 OA+NIC group showed higher serum levels of IL-12, IFN-γ and TNF-α compared to controls, while at d14 control group tended to have elevated IL-6.

Overall, it appeared that dietary inclusion of the OA+NIC mixture improved intestinal health by allowing for tighter junctions in the jejunum (reduced dextran flux), and by possibly reducing the secretory activity of ileal mucosa (reduced I_{SC}) via reduction in inflammation (reduced gene and protein expression of cytokines). In addition, the increment in circulating inflammatory cytokines levels at day 7 (IL-12, IFN-γ, and TNF-α) could support a role for the mixture of OA and NIC in stimulating a more rapid immune maturity in weaning piglets.

Keywords: organic acids, nature-identical compounds, weaning piglet, inflammatory cytokines, intestinal health.

INTRODUCTION

Organic acids and botanicals have long been used in pig husbandry as alternative to conventional antibiotic growth promoters. The efficacy of these products has been linked to a reduced bacterial pressure in the gut and the proposed mode of action is the reduction of pathogenic bacteria in the GI tract. According to the "anion model" proposed by Russel and Diez-Gonzalez (1998) organic acids, including those produced as a result of intestinal fermentation, can diffuse across the bacterial cell membrane in the uncharged, protonated form and dissociate inside the cell, lowering the internal pH. To overcome the pH reduction, several ATP-dependent systems are induced, finally impairing bacterial protein synthesis; furthermore, as the internal pH remains high, the differential pH becomes larger allowing a logarithmic intracellular accumulation of anions, which is believed to be the real toxicity mechanism (Russell & Diez-Gonzalez, 1998). Botanicals and nature-identical compounds (i.e. chemically synthesized botanicals), instead, are proposed as membrane permeabilizers or pore-forming agents that would allow ion leakage and membrane potential disruption (Lambert et al., 2001; Nazer et al., 2005). The consistent improvement of growth performance and feed efficiency observed over 25 years of usage of these products in different farms with different genetics of animals and different sanitary pressures (Mroz, 2003; Tung & Pettigrew, 2006) makes unlikely that it is just a reduction of pathogenic bacteria at the basis of improved growth performance or feed efficiency but rather it would implicate a host-mediated response.

Intestinal health is strictly connected to the barrier function and the ability to keep out pathogens and toxins while allowing for very efficient uptake of water and nutrients. The intestinal barrier is composed by a single layer of enterocytes and connecting inter-epithelial tight junctions which are responsible of regulating the paracellular flux of solutes and macromolecules (Blikslager et al., 2007). When inflammation occurs, like at weaning, the epithelial barrier and tight junctions functionality are impaired and the ability of the enterocyte to absorb nutrients as well. As a result, animal growth performance are decreased and susceptibility to infections is increased. Therefore, maintaining the barrier function is a key-point in order to maintain a correct growth and development of the young animal.

Aim of this study was to try to elucidate an alternative mechanism – beyond the antibacterial effect – behind organic acids and botanicals efficacy in improving growth

performance of pigs. We therefore assessed the impact of dietary inclusion of a microencapsulated mixture of sorbic, citric acid, thymol and vanillin, on measures of intestinal health in pigs weaned at an early age and in a relatively clean environment, in order to exclude a major interference of pathogenic microflora.

MATERIALS AND METHODS

Experimental Procedure

The North Carolina State University Institutional Animal Care and Use Commitee approved all studies. Twenty piglets (commercial hybrids) were weaned at 18 days of age and placed into one of two groups (n = 10) receiving either a basal diet (control group) or the basal diet supplemented with a mixture of organic acids and nature identical compounds (OA+NIC, 5,000 mg/kg of feed, providing sorbic acid, citric acid, thymol and vanillin) for 2 weeks. The control diet contained 2.5% plasma protein (APC, Ankeny, IA), 35.5% SBM (48% CP), 58.7% corn (US #2), 1.65% dicalcium phosphate, 1% Limestone, and 1% corn oil, along with a vitamin and mineral premix to meet or exceed the nutrient requirements for this size/age of pig (NRC, 1998). No antibiotics were added to this diet. Individual body weights were recorded initially (d0), at day 7 (d7) and at day 14 (d14), and ADG was calculated. Blood samples were collected initially at every 7 days by venipuncture using tubes without anticoagulant, and sera samples were stored at –20°C until analysis of inflammatory cytokines.

At the completion of the study 6 pigs per group were selected for sacrifice, sample collection and analysis. Sacrifice and tissue collection were spread over three days (n = 2 piglets / group /day). Piglets were killed by penetrating captive bolt followed by exsanguination. Segments of jejunum and ileum were collected and immediately prepared for Ussing chambers analysis. Ileal mucosa scrapings were collected, snap-frozen in liquid N_2 and stored at –80°C until gene and protein expression analysis.

Ussing Chamber Analysis

Ussing chamber experiments were conducted according to Smith et al. (2010). Segments of jejunum and ileum were collected from the pig, and the mucosa was stripped from the seromuscular layer in oxygenated (95% O_2-5% CO_2) Ringer solution

(in mmol/l: 154 Na^+, 6.3 K^+, 137 Cl^-, 0.3 H_2PO_4, 1.2 Ca^{2+}, 0.7 Mg^{2+}, 24 HCO_3^-; pH 7.4). Tissues were then mounted in 1.13-cm^2 aperture Ussing chambers and bathed on the serosal and mucosal sides with 10 ml of Ringer solution. The serosal bathing solution contained 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O_2-5% CO_2) and circulated in water-jacketed reservoirs maintained at 37°C. The spontaneous potential difference (**PD**) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. Tissues were maintained in the short-circuited state, except for short intervals to record the open-circuit PD. Trans-epithelial electrical resistance (**TER**; $\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current ($\boldsymbol{I_{SC}}$), as previously described (Argenzio & Liacos, 1990). After a 30-min equilibration period on Ussing chambers, TER was recorded at 15-min intervals over a 1-h period and then averaged to derive the basal TER values for a given animal.

Mucosal permeability was assessed measuring mucosal-to-serosal fluxes of dextran, according to Moeser et al. (2012). FITC-dextran 4 kDa (FD4) was added to the serosal bathing reservoir of Ussing chambers at a concentration of 100 mg/ml and the flux to the serosal compartment was measured. After a 30-min equilibration period, standards were taken from the serosal side of each chamber. Samples (0.5 mL) were collected from the serosal chambers at 30-min intervals over a 90-min period. The quantity of FD4 was established by measuring the fluorescence in serosal reservoir fluid samples in a fluorescence plate reader at 540 nm. Data are presented as the rate of FD4 flux (mg/cm^2/min) over a 90-min period.

ELISA Quantification of Inflammatory Cytokines

Protein levels of IFN-γ, TGF-β, TNF-α, IL-6, IL-10, IL-12 were determined both in sera samples and in ileal samples using commercial ELISA kits specific for porcine cytokines (R&D Systems, Minneapolis, MN). Sera samples were prepared according to manufacturer's instructions. Ileal mucosa scrapings were lysed in lysis buffer (10 m*M*-Tris, 1 m*M*-EDTA, 0.5%-Triton X100) and homogenized using a handheld rotor-stator homogenizer before ELISA analysis. Analyses were performed according to manufacturer's instructions. Results refer to picograms of cytokine per 100 mg of tissue (pg/100 mg).

Inflammatory Cytokines, SGLT-1 and CFTR Gene Expression Analysis

Ileal mucosa scrapings were disrupted by grinding in liquid N_2 with mortar and pestle, then homogenized using a handheld rotor-stator homogenizer. Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (Ambion DNA-free kit, Life Technologies, Grand Island, NY). RNA yield and quality were determined spectrophotometrically using A_{260} and A_{280} nm measurements. All samples showed A_{260}:A_{280} ratio of 1.9–2.0. A total of 1 µg of RNA was reverse transcribed with Superscript III (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen Life Technologies, Grand Island, NY) to ensure the removal of residual RNA, then cDNA was quantified with Quant-iT Oligreen ssDNA Assay (Life Technologies) in order to normalize the quantity of cDNA template used for amplification by real-time PCR. Relative quantities of the transcripts of interest were determined by semi-quantitative real-time PCR (My iQ Single Color Real-Time PCR Detection System and SybrGreen Supermix, Bio-Rad Laboratories, Hercules, CA). Thermocycling protocol included initial denaturation for 3 min at 95°C, then 40 cycles of 20 s of denaturation at 95°C followed by 20 s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis to exclude presence of non-specific products. Gene expression was normalized using two housekeeping genes coding for portions of porcine ribosomal subunit 60S, such as ribosomal protein L35 (**RPL35**) and ribosomal protein L4 (**RPL4**). Average C_T was determined for each gene of interest (**GOI**), and geometric average was calculated for housekeeping genes (**HKs**), assuming C_T as number of cycles needed to reach a fixed arbitrary threshold. Delta C_T was calculated as C_T GOI−C_T HKs, then a modification of the $2^{-\Delta\Delta C}{}_T$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (Livak & Schmittgen, 2001).

The sequences, expected product length, accession number in the EMBL database/GenBank and references of porcine primers are shown in Table 1. Primer oligonucleotides for IL-12, tumor necrosis factor-α (**TNF-α**), transforming growth factor-β (**TGF-β**), sodium/glucose co-transporter 1 (**SGLT-1**), and cystic fibrosis transmembrane conductance regulator (**CFTR**) were designed using PrimerQuest software (IDT, Integrated DNA Technologies, www.idtdna.com). Primers were obtained from IDT, Integrated DNA Technologies (www.idtdna.com).

Table 1. Primers used for gene expression analysis

Gene	Primer Sequence (F and R; 5' → 3')	Product lenght (bp)	Product Tm (°C)	Accession No.	Reference
IL-6	F: AGCAAGGAGGTACTGGCAGAAAACAAC R: GTGGTGATTCTCATCAAGCAGGTCTCC	110	83.5	NM_214399.1	Zannoni et al., 2011
IL-10	F: CGGCGCTGTCATCAATTTCTG R: CCCCTCTCTTGGAGCTTGCTA	89	85	NM_214041.1	Duvigneau et al., 2005
IL-12	F: GCCCAGGAATGTTCAAATGCCTCA R: AGGCAACTCTCATTCGTGGCTAGT	199	85.5	NM_213993.1	Present study
TNF-α	F: GCCCACGTTGTAGCCAATGTCAAA R: GTTGTCTTTCAGCTTCACGCCGTT	99	87	NM_214022.1	Present study
TGF-β	F: AGGCCGTACTGGCTCTTTACAACA R: TTGGTTGCCGCTTTCCACCATTAG	134	83.5	NM_214015.1	Present study
IFN-γ	F: GGCCATTCAAAGGAGCATGGATGT R: TGAGTTCACTGATGGCTTTGCGCT	149	83.5	NM_213948.1	Present study
SGLT-1	F: TGCTGTTTCCAGATGATGTGGGCT R: TGCTGCTGCTGTTAAAGATGGACG	198	90.5	NM_001012297.1	Present study
CFTR	F: GAAGAACTGGATCAGGGAAGAG R: GAATCCCAAGACACACCATCTA	97	84.5	NM_001104950.1	Present study
RPL35	F: AACCAGACCCAGAAAGAGAAC R: TTCCGCTGCTGCTTCTTG	146	87.5	NM_214326.2	Alexander et al., 2012
RPL4	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122	84	XM_003121741.3	Alexander et al., 2012

F, forward primer; R, reverse primer; Tm, melting temperature; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; SGLT-1, sodium/glucose co-transporter-1; CFTR, cystic fibrosis transmembrane conductance regulator; RPL35, ribosomal protein L35; RPL4, ribosomal protein L4.

Statistical Analysis

Data were analyzed using a two-tailed Student's t-test with the GLM procedure of SAS (SAS Institute Inc., release 9.2, Cary, NC). Gene expression and cytokine data were analyzed without covariate. Collection day was used as a covariate for the Ussing chamber data. Initial BW was used as a covariate for growth performance data. Differences were considered significant at $P < 0.05$, trends at $0.05 < P \leq 0.10$.

RESULTS

Table 2 summarizes growth performance results. Feeding the mixture of organic acids and nature-identical compounds improved growth performance. Treated pigs had higher BW compared to controls at d14 (9.13 kg versus 8.45 kg; $P < 0.05$) and greater ADG both during the second week of the study and overall ($P < 0.05$).

Table 2. Growth performance

Item	Treatment[1]		SEM	*P*
	control	OA+NIC		
BW, kg				
d0	7.08	7.15	0.07	0.91
d7	8.08	8.01	0.08	0.57
d14	8.46	9.13	0.21	0.049
ADG, g/d				
0 to 7d	136.77	127.40	11.72	0.59
7 to 14d	191.63	286.87	29.57	0.049
0 to 14d	95.89	143.60	14.78	0.049

[1] Treatments: control = basal diet; OA+NIC = basal diet + mixture of organic acids and nature-identical compounds at 5,000 mg/kg.

BW, body weight; ADG, average daily gain.

Results from Ussing chambers analysis are reported in Fig. 1. In OA+NIC group intermittent short-circuit current (I_{SC}) tended to be reduced in the ileum ($P < 0.10$) and was numerically reduced in the jejunum. In addition, dextran flux in the jejunum was numerically lower in treated pigs compared to controls.

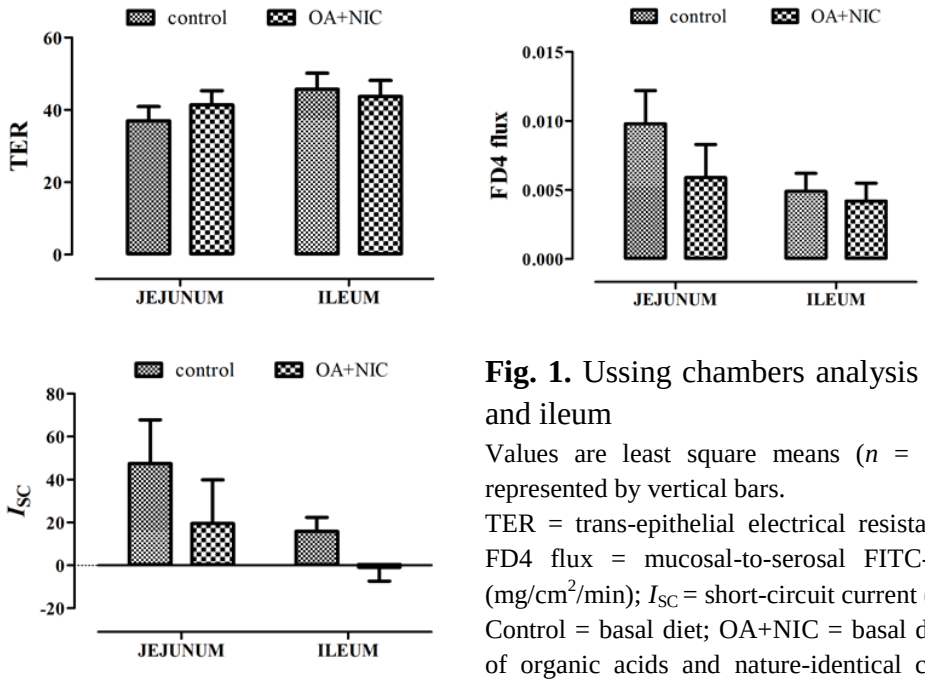

Fig. 1. Ussing chambers analysis of jejunum and ileum

Values are least square means (n = 6) and SEM represented by vertical bars.
TER = trans-epithelial electrical resistance ($\Omega \cdot cm^2$); FD4 flux = mucosal-to-serosal FITC-dextran flux ($mg/cm^2/min$); I_{SC} = short-circuit current ($\mu A/ cm^2$).
Control = basal diet; OA+NIC = basal diet + mixture of organic acids and nature-identical compounds at 5,000 mg/kg.

Figure 2 shows mRNA levels of inflammatory cytokines, SGLT-1 and CFTR in ileal mucosa. The group treated with OA+NIC mixture had significantly lower mRNA level of TGF-β and IL-12 ($P = 0.04$ and $P = 0.003$, respectively), and tended to have reduced mRNA level of IL-6 and IFN-γ ($P < 0.10$ both). SGLT-1 mRNA level was numerically lower in treated group compared to control, whereas CFTR gene expression was not affected by the treatment with OA+NIC.

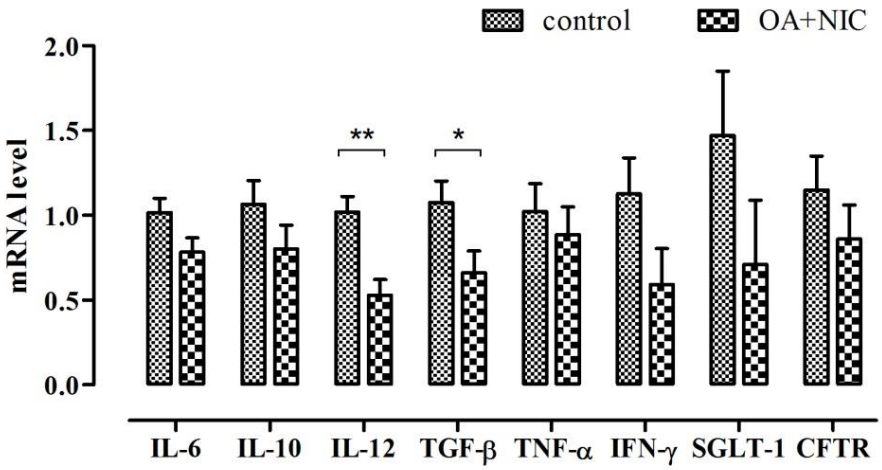

Fig. 2. Gene expression of inflammatory cytokines, SGLT-1 and CFTR in ileal mucosa

Values are least square means and SEM ($n = 6$).
Within a gene, columns with symbols are different: * = $P < 0.05$; ** = $P < 0.01$.
A modification of the $2^{-\Delta\Delta C}{}_{T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control), (Livak & Schmittgen, 2001).
Control = basal diet; OA+NIC = basal diet + mixture of organic acids and nature-identical compounds at 5,000 mg/kg.

Protein levels of inflammatory cytokines in ileal mucosa are presented in Fig. 3. Compared to control group, OA+NIC group had numerically lower TGF-β level, whereas no effects were observed in IL-6, IL-10, IL-12, IFN-γ or TNF-α.

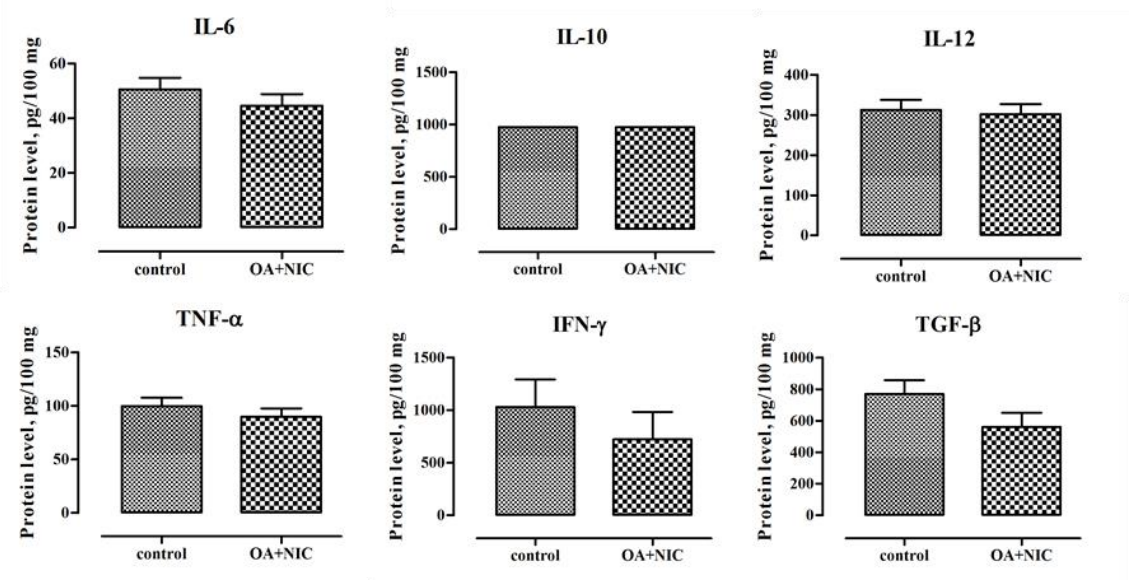

Fig. 3. Protein levels of inflammatory cytokines in ileal mucosa

Values are least square means (n = 6) and SEM represented by vertical bars.
Data refer to picograms of cytokine per 100 milligrams of tissue (pg/ 100mg) measured using ELISA method.
Control = basal diet; OA+NIC = basal diet + mixture of organic acids and nature-identical compounds at 5,000 mg/kg.

Figure 4 shows protein levels of inflammatory cytokines in sera samples measured at d0, d7 and d14 of the study. At d0 no differences were observed. At d7 pigs fed with the mixture of organic acids and nature-identical compounds had significantly higher TNF-α ($P < 0.01$) and numerically higher IL-12, while IFN-γ tended to be higher than in controls ($P = 0.08$). At d14 IL-6 level was numerically lower in treated group compared to control, whereas no effects were observed on other cytokines.

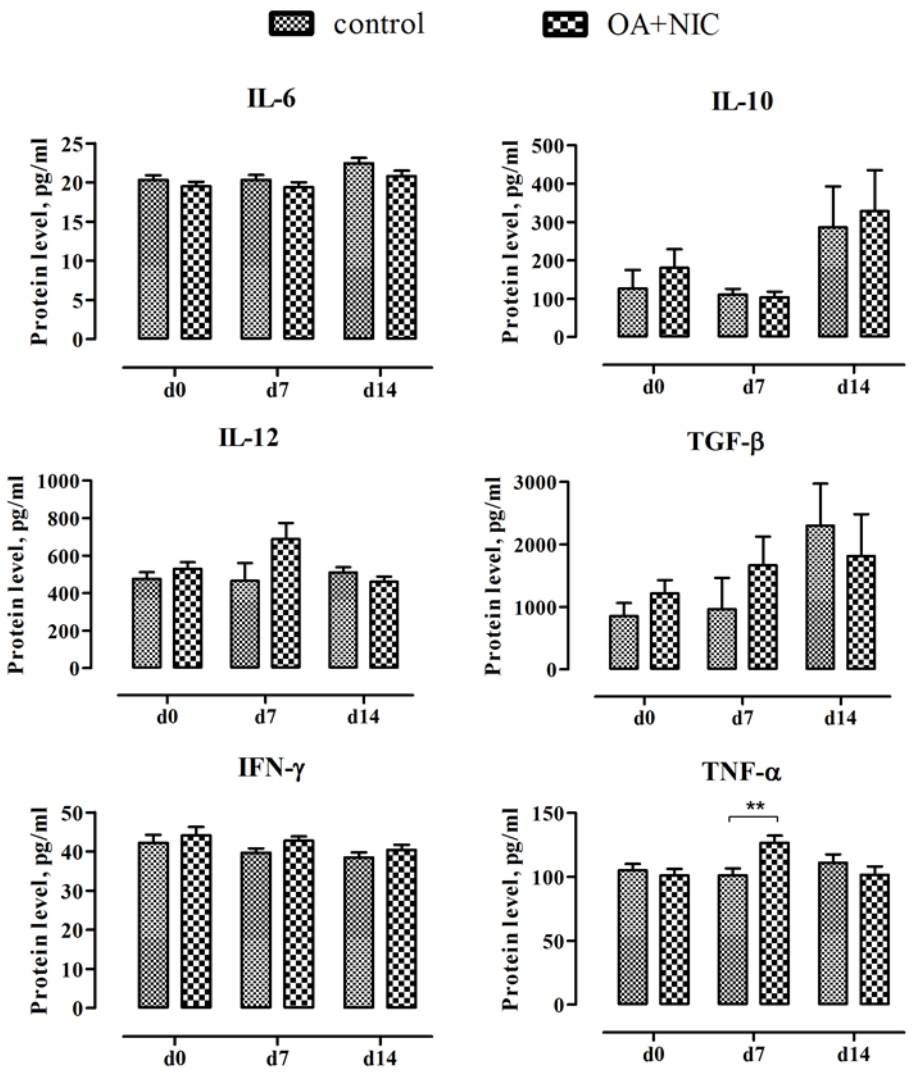

Fig. 4. Protein levels of inflammatory cytokines in serum at d0, d7, and d14

Values are least square means (n = 6) and SEM represented by vertical bars.
Within a time-point (d0, d7, or d14) columns with symbols are different: * = $P < 0.05$; ** = $P < 0.01$.
Data refer to picograms of cytokine per milliliter of serum (pg/ml) measured using ELISA method.
Control = basal diet; OA+NIC = basal diet + mixture of organic acids and nature-identical compounds at 5,000 mg/kg.

DISCUSSION

In pig feeding, the use of organic acids and botanicals has been widely accepted as tool to improve growth performance and control weaning-associated intestinal disorders, due to their acidifying and anti-microbial properties largely demonstrated and reviewed (Partanen & Mroz, 1999; Kroismayr et al., 2008; Suryanarayana et al., 2012). Previous studies from our research group showed that microencapsulation of organic acids and

natural identical flavors (i.e. chemically synthesized botanicals) in a lipid matrix allowed those compounds to be slowly released along the gut of the pig and reach the distal part of the intestine, where they can modulate microbial ecosystem, thus preventing the rapid disappearance upon exiting the stomach (Piva et al., 2007). In addition, when fed to weaning piglets for a 41-days post-weaning study, the microencapsulated mixture of organic acids and nature identical compounds improved growth performance as showed by higher feed intake, higher average daily gain and final body weight, along with better intestinal metabolism (Grilli et al., 2010).

Our hypothesis was that, beyond the anti-microbial properties and the ability to reduce pathogenic bacteria in the gastro-intestinal tract, organic acids and nature-identical compounds may exert a direct effect on the intestinal health in weaning piglets. Therefore, the aim of the present study was to establish a possible mode of action of the mixture of organic acids and nature identical compounds on the intestinal health, by evaluating the effects on barrier integrity and inflammatory status in early-weaned piglets.

In order to focus on the health changes induced by OA and NIC without bacterial health challenges, we used healthy piglets in a clean research facility, subjected to early weaning stress (18 days of age) to highlight the spontaneous inflammation naturally occurring in the intestine at weaning. In fact, in addition to the morphological and functional changes that impair intestinal functions, weaning is known to be associated with up-regulation of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Pié et al., 2004). Moreover, previous studies have shown that early weaning (15-18 days of age) resulted in consistent impairment of the development of the intestinal barrier function and of the mucosal immune response to pathogenic challenges with long-term consequences (Smith et al., 2010; McLamb et al., 2013).

In our study, we measured the effects of the treatment with OA and NIC on the intestinal integrity by Ussing chamber analysis. The treatment influenced the net electrogenic ion transport across the intestinal epithelium in terms of intermittent short-circuit current (I_{SC}). Compared to control, in treated group I_{SC} tended to be reduced in the jejunum and was significantly lower in the ileum. I_{SC} is reflective of ions movement across the epithelium, particularly Cl^- and HCO_3^- secretion, which drives fluid flux into the intestinal lumen, likely leading to diarrhea (McLamb et a., 2013). The reduction in the I_{SC} value induced by OA and NIC particularly in the ileum (where I_{SC} became negative compared to control value) may be due to a reduced secretion of Cl^- and

HCO_3^- ions. Therefore, we analyzed the gene expression in the ileal mucosa of the cystic fibrosis transmembrane conductance regulator (CFTR), which is an apical anion channel responsible for the cAMP-dependent efflux of Cl^- ions into the lumen (Field, 2003). However, CFTR mRNA level in the ileal mucosa was not different between control and treated animals, thus suggesting that the effect of OA and NIC on ions and fluids flux across the epithelium may be related not only to the trans-cellular route, but also to the para-cellular route. The para-cellular route involves the flux of solutes and compounds across the lateral inter-spaces between two adjacent cells and it is regulated by the tight junctions (TJ), multi-proteins complexes, that close the inter-cellular space and modulate para-cellular permeability (Arrieta et al., 2006). In our experiment, para-cellular permeability was assessed by measuring the flux of dextran, an high-molecular weight compound that can be adsorbed only via para-cellular way. In the jejunum, the dextran flux was numerically lower in treated animals compared to controls. Thus, OA and NIC may allow tighter epithelium, reducing the leakage of ions, fluids and consequent risk of diarrhea. This protective action on the epithelial integrity exerted by OA and NIC may be explained by the effects on the inflammatory status in the ileal mucosa. In fact, epithelial permeability and, particularly, tight junctions status, have been shown to be continuously regulated by several physiological, bacterial, dietary and pharmacological stimuli, among which inflammatory cytokines are regarded as major mediators (Lallès, 2010). Especially, pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-12 and IL-1β have been demonstrated to exert a "leaky" action on the epithelium, inducing the opening of the tight junctions and, consequently, allowing para-cellular permeation to dangerous luminal compounds. The mechanisms of action involve the disruption of the multi-protein structure of the tight junctions. However, other cytokines, including IL-10 and TGF-β, have been shown to play an anti-inflammatory action protective of the tight junctions structure and the epithelial integrity (Al-Sadi et al., 2009). In our experiment, the treatment with OA and NIC modulated the inflammatory status in the ileal mucosa at both gene and protein expression level: compared to control group, mRNA levels tended to be reduced for IFN- γ and IL-6 and was significantly reduced for IL-12 and TGF-β; similar pattern were observed in protein levels. The reduction of pro-inflammatory "leaky" cytokines induced by OA and NIC may be supportive for a tighter and more resistant epithelium, as described from the Ussing chamber analysis of intestinal barrier integrity. It seems, therefore, that OA and NIC positively modulated the inflammatory stress in the intestinal epithelium

directly by reducing pro-inflammatory stimulus, rather than enhancing the anti-inflammatory response. In this view, the reduction both in gene and protein expression of the anti-inflammatory cytokine TGF-β can be considered as a consequence of the reduced pro-inflammatory milieu in the intestinal mucosa. The immuno-modulatory action of the OA and NIC mixture that we observed in the present study is consistent with the anti-inflammatory properties ascribed to botanicals in previous works both *in vitro* and *in vivo*. Several plant extracts have been described to exert anti-inflammatory activity in cell models: carvacrol reduced the expression of pro-inflammatory cytokines (TNF-α, IL-1β) in porcine alveolar macrophages stimulated with LPS (Liu et al., 2012); thymol reduced the *in vitro* activation of ovine neutrophils by down-regulating the expression of adhesion molecules and ROS production (Farinacci et al., 2008). In addition, botanicals have been tested as therapeutic agents in murine models of inflammatory bowel disease. In rats suffering DSS-induced colitis, essential oil of thyme was able to reduce the inflammatory response in the colon by down-regulating the pro-inflammatory transcription factor NF-kB, while enhancing anti-inflammatory IL-10 and tight junction protein claudin-3 (Mueller et al., 2013). Moreover, vanillin is known to be a potent inhibitor of NF-kB and it has been tested in a murine model of TNBS-colitis by Wu and colleagues (2009): vanillin improved macroscopic and histological features of colitis by reducing NF-kB activation and the colonic expression of IL-1 β, TNF- α, IL-6 and IFN-γ (Wu et al., 2009). Therefore, we can hypothesize that, in the mixture of OA and NIC, thymol and vanillin might be responsible for the down-regulation of the intestinal inflammatory stress and consequent improvement of the barrier integrity. In addition, sorbic and citric acid, the organic acids in the mixture, may have improved the trophic status of the epithelium, supplying energy to intestinal cells. In fact, as reviewed by Suryanarayana and colleagues, organic acids act as direct energy source in the gut of pigs as they are intermediary products of tricarboxylic acids (Suryanarayana et al., 2012). Also, in-feed supplementation of sorbic acid to weaning piglets has been shown to improve growth performance and the proposed mechanism for the growth promoting effect of sorbic acid was the stimulation of the insulin-like growth factor (IGF) system, by increasing the hepatocyte-mediated IGF-1 gene expression and secretion (Luo et al., 2011). So, in our study, organic acids might have improved the energy status of the intestinal epithelium, while thymol and vanillin reducing the inflammatory stress, thus resulting in a healthier and tighter epithelium.

Treatment with OA and NIC also influenced the systemic immune response of the weaning piglets as indicated by the inflammatory cytokines levels measured in the serum. After the first week of treatment, the OA+NIC group had increased circulating levels of TNF-α and IFN-γ, as well as numerically higher IL-12, compared to control group. This result might appear surprising as these three cytokines are pro-inflammatory but no adverse effects on the piglets health were observed in the present study, neither immuno-stimulatory responses have been ascribed to organic acids and botanicals in previous studies. One possible explanation may depend on the increment of IL-12: in fact, IL-12 is a pro-inflammatory cytokine whose expression plays a crucial role in coordinating innate and adaptive immune responses. Particularly, IL-12 stimulates the production of IFN-γ by natural killer cells, T cells, macrophages and dendritic cells, as well as the differentiation of IFN-γ-producing T helper 1 cells, therefore regulating the development of the adaptive immunity (Watford et al., 2003). In this view, in piglets fed OA and NIC after the first week, IL-12 may have driven the transient over-expression of pro-inflammatory cytokines (IFN-γ and TNF-α), thus suggesting a more rapid development of the immune response induced by OA and NIC. This IL-12-mediated immuno-stimulation induced by OA and NIC was transient, as shown by the results at the end of the study, when no differences in the inflammatory cytokines levels were observed, while, conversely, IL-6 was numerically higher in control group compared to treated group.

Our growth performance results showed that the supplementation with the OA and NIC mixture positively influenced piglets growth. Although the small number of animals used in the present study, the treatment with OA and NIC allowed significantly higher BW at the end of the study as a result of increased ADG both in the second week of treatment and overall in the 2-weeks experimental period. This result is consistent with previous findings from our research group in a large-scale feeding trial: in a 170-weaning piglets study, feeding the same microencapsulated mixture of OA and NIC improved growth performance and intestinal metabolism, compared to control diet (Grilli et al., 2010). Despite the low number of animals, the present study confirms the growth promoting effect of the OA and NIC mixture and gives evidence for a possible mechanism of action directly on the intestinal health, beyond the well-known microbial-modulating action. The microencapsulation of OA and NIC in a lipid matrix allowed the bioactive compounds to be released in the small intestine where positively impacted the epithelial integrity probably by reducing the mucosal inflammatory stress.

CHAPTER 7

CONCLUSIONS

Intestinal health plays a crucial role for the health of the body as the gastro-intestinal mucosa represents the main interface with the external environment and the microbiota residing along the gastro-intestinal tract. Aiming to maintain a homeostatic relationship between intestine and microbiota, intestinal health depends on proper barrier function, epithelial integrity and related mechanisms of protection (tight junctions, mucous layer, inflammatory and immune system).

In pigs, weaning is associated with low growth and high incidence of intestinal disturbances due to several stressors that impair intestinal health. Particularly, epithelial barrier integrity and intestinal inflammation are essential for intestinal health and piglet's health and growth.

Therefore, the aim of the study was to evaluate the impact of different nutritional strategies on the intestinal health of the piglet at weaning, with special reference to the intestinal inflammation and the epithelial barrier integrity. *In vivo* studies were conducted to assess the potential use of zinc, tributyrin and a mixture of organic acids and nature-identical compounds as tools to control the inflammatory stress naturally occurring in the intestine of the piglet at weaning and, consequently, intestinal disorders and growth retard.

Our results showed that all the dietary interventions tested were able to positively impact the intestinal inflammatory status and, as a consequence, improve the epithelial integrity by modulating tight junctions protein expression (zinc and tributyrin trials) or by enhancing barrier properties measured with Ussing chambers (organic acids and nature-identical compounds). These findings confirm the central role for intestinal inflammation in regulating the intestinal health not only by providing the first immune response against dietary or microbial challenges, but also by modulating the epithelial barrier structure and function. In fact, inflammatory molecules such as cytokines have been shown to be major mediators of the functional status of the tight junctions, structures that seal the inter-spaces between two epithelial cells therefore regulating the para-cellular permeability across the epithelium. In our trials, the dietary treatment with zinc, tributyrin and organic acids and nature-identical compounds showed effectiveness in reducing the intestinal inflammatory stress, as suggested by reduced expression of inflammatory cytokines. This reduction of inflammatory stress attenuated the risk of

cytokine-mediated epithelial disruption and intestinal damage. Tighter epithelium may, therefore, provide more adequate barrier function and help to maintain intestinal health.

Compared to studies in literature about the dietary supplementation of weaning diets with zinc oxide or organic acids and nature-identical compounds, our work gives evidence that microencapsulation allowed to deliver those bio-active compounds precisely to the small intestine of the piglet as critical target-site for the control of weaning-associated disorders. Consistently with previous results from or research group, microencapsulation of compounds in a lipid matrix provided gastro-protection and targeted release of them in the small intestine where they could modulate inflammatory stress and epithelial integrity. Similarly, concerning tributyrin trial, microencapsulation of tributyrin appeared a valid tool to increase the amount of butyric acid reaching the colonic epithelium, as major site for exerting its beneficial actions.
With reference to the zinc trials, in addition to the above-described advantages, microencapsulation of zinc oxide resulted a valid approach to reduce the usual high dosage of zinc oxide. In fact, zinc oxide is widely used at high – pharmacological – dosage (2000–3000 mg/kg) in weaning diets to control diarrhea and improve growth, but increasing concerns on heavy metals excretion in manure in Europe had imposed caution to its use. In our experiments, low doses of microencapsulated zinc oxide were able to positively impact ileal architecture, inflammatory status and epithelial integrity, therefore microencapsulated zinc oxide has been proposed as an effective and environmentally safe alternative to pharmacological zinc oxide.

Along to the improved intestinal inflammatory status and epithelial integrity, zinc trial and organic acid and nature-identical compounds trial showed better intestinal health induced by supplements successfully resulted in improved growth performance. This result fulfills the main goal of pig production as weaning-associated growth failure is one of the most important problems for pig productivity.

To conclude, this work shows that dietary supplementation with bio-active substances such as zinc, tributyrin and organic acids and nature-identical compounds may improve intestinal health of weaning piglets by modulating intestinal inflammatory stress and barrier integrity and allowing better piglet’s health, growth and productivity.

CHAPTER 8

REFERENCES

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