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Influence of Dietary Zinc Oxide on Morphological and Immunological Characteristics in the Jejunum and Colon of Weaned Piglets

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List of Abbreviations

AMPs	Antimicrobial peptides
AB-PAS	Alcian blue-periodic acid Schiff
ANOVA	Analysis of variance
BW	Body weight
B2M	Beta-2 microglobulin
CD	Crypt depth
GIT	Gastrointestinal tract
HID-AB	High iron diamine-Alcian blue
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IL	Interleukin
LPS	Lipopolysaccharides
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MUC	Mucin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nucleotide-binding oligomerization domain receptors
PBS	Phosphate buffered saline
RPL13	60S ribosomal protein L13
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SEA	Sperm protein, enterokinase and agrin
TFFs	Trefoil factors
TGF-β	Transforming growth factor-β
VH	Villus height
wk	Week
ZnO	Zinc oxide

Chapter 1: Introduction

Weaning is a critical time in a pig's life, and results in high susceptibility to digestive disorders (Lallès et al., 2007). Diarrhea leads to intestinal integrity impairment (Hampson and Pluske, 2004). High mortality by post-weaning diarrhea causes a huge economic loss in pig production. Therefore, numerous management and dietary strategies have been studied for prophylaxis or to alleviate the response to weaning-associated intestinal disturbances in piglets (Kim et al., 2012).

The use of antibiotic growth promoters in the pig industry has raised concerns on multi-resistant pathogens being a threat for humans and animals. Since 2006 the European Union has passed legislation regarding a ban on the use of antibiotics as feed additives. Subsequently, various concepts were proposed as possible alternatives to improve animal health and growth performance in piglets. The trace element zinc has multiple effects on physiological and pathophysiological processes, including the modulation of diversity and metabolic activity of the intestinal microbiota (Katouli et al., 1999; Hojberg et al., 2005; Vahjen et al., 2010, 2011; Starke et al., 2013), the absorptive and secretory capacity (Feng et al., 2006; Carlson et al., 2007), and the intestinal associated immune response (Foster and Samman, 2012). So-called pharmacological levels of zinc oxide in pig diets have been frequently shown to prevent diarrhea and promote performance (Li et al., 2001; Martin et al., 2013b). The underlying mechanisms on alleviating intestinal disorders still need further studies.

Besides the maintenance of intestinal integrity and the digestive and absorptive functions during the weaning period, the ability of the immune system to cope with different challenges is significant (Bailey et al., 2001). Until now, the majority of previous studies focused on the adaptive immune system, whereas the knowledge on the innate immune system as a key component of intestinal barrier is still limited.

The mucus layer is the first defense barrier against pathogens throughout the gastrointestinal tract, and regulated by innate and adaptive immunity (Moncada et al., 2003b; McGuckin et al., 2011). It consists of various defense molecules produced by specialized secretory cells, such as mucins and antimicrobial peptides (Kim and Ho, 2010). It has been well documented that zinc plays an important role in the development of the immune system and helps to maintain the different functions (Keen and Gershwin, 1990; Shankar and Prasad, 1998; Rink and Kirchner, 2000). However, most studies were conducted in rodents and on the basis of zinc deficiency or zinc restriction, only few studies evaluated the effects of pharmacological levels of dietary zinc on intestinal mucosal immunity in weaned pigs (Roselli et al., 2003; Hu et al., 2012). The impact of dietary zinc on immune reactions in the gut mucosal surface and specifically the regulatory interaction between innate mucus layer and adaptive immune response during weaning period needs further characterization. In this study, the effects of different levels of dietary zinc oxide on gut immune response with special emphasis on intestinal innate and adaptive immunity were evaluated in weaned piglets during four weeks after weaning.

Chapter 2: Literature Review

2.1 Innate Barrier at Gastrointestinal Tract

The mucosal surface of gastrointestinal tract (GIT) is a complex organization of epithelial cells, immune cells, connective tissue, mucus and resident bacteria (McCracken and Lorenz, 2001). It is also the largest body surface in contact with external environment, such as feed compounds and microorganisms (Lievin-Le Moal and Servin, 2006). It has complex mechanisms for digestion, absorption and metabolism, as well as prevention of pathogen invasion. The functional integrity of intestinal mucosal epithelium depends on the coordinated regulation of the mucus formation, intercellular tight junctions, and host innate and adaptive immune response (Dharmani et al., 2009).

The intestinal mucus layer acts as the first defense line against pathogens and foreign substances and consists largely of mucins secreted by goblet cells (Gum et al., 1999). The mucus layer also contains other bioactive molecules such as trefoil factors and resistin-like molecule-β from goblet cells (Dharmani et al., 2009), antimicrobial peptides such as defensins secreted by specialized cells known as Paneth cells (Lievin-Le Moal and Servin, 2006), and high amounts of secretory IgA produced by plasma cells (Dharmani et al., 2009). In the GIT, the mucus layers can be divided into an inner firm layer and an outer loose layer. In the small intestine, the mucus gel layer is discontinuous and thin. The mucus thickness gradually increases from colon to rectum (Matsuo et al., 1997). The outer layer is a major site of host-microbial interactions, while luminal microbes are absent in the inner layer (Johansson et al., 2011). The mucus along the GIT lubricates the digesta, creates a permeable gel layer for the exchange of gases and nutrients, maintains a hydrated layer on the epithelium, and forms a physical barrier. It also plays an important role by binding bacteria, mainly through specific interactions with carbohydrate chains of mucins.

Commensal microorganisms coexist with host cells and there is a dynamic homeostasis between intestinal pathogens and mucosal immunity. Gut microbiota can regulate mucin

production by activating various signaling cascades and secretory elements (Dharmani et al., 2009). Commensal and pathogenic microorganisms derive significant benefits from their abilities to regulate mucin synthesis and secretion of host goblet cells. The resident bacteria are trapped in the outer mucus layer (Lievin-Le Moal and Servin, 2006). When foreign antigens and pathogenic bacteria get in contact with the epithelial barrier, specialized cells in the epithelium and the underlying mucosal tissues are the sites of intense immunological activity. Epithelial cells can sense pathogen-associated molecular patterns via pattern recognition receptors, such as toll-like receptors. Stimulation of this way induces production of inflammatory cytokines and chemokine signals, which can trigger immune responses in the lamina propria (Neutra and Kozlowski, 2006; McGuckin et al., 2009).

2.1.1 Mucins

2.1.1.1 Mucin Classification

Mucins can be categorized into membrane-bound and secreted mucins. The main difference between them is the presence of a hydrophobic C-terminal transmembrane domain in membrane-bound mucins (Andrianifahanana et al., 2006). Membrane-bound mucins are monomeric and located on the surface of epithelial cells. Membrane-bound mucins participate in cellular signaling and play an important role as sensor in response to pathogen invasion and intestinal inflammations (Carraway et al., 2003). Secreted mucins are characterized by high proportion of O-linked glycosylation patterns, and they are attributed to the formation of the mucus gel. Mucin monomers are encoded by various mucin genes (Roussel and Delmotte, 2004). The membrane-bound mucins mainly consist of MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20 (Hollingsworth and Swanson, 2004; Linden et al., 2008). The secreted mucins include MUC2, MUC5AC, MUC5B, MUC6, MUC7 and MUC19. The distribution of mucin genes in GIT is listed in **Table 2.1**.

Table 2.1: Distribution of mucin genes throughout the gastrointestinal tract[1]

Tissue	Oral cavity	Stomach	Small intestine	Large intestine
Secreted mucins	MUC5B MUC7 MUC19	MUC5AC MUC6	MUC2	MUC2 MUC5AC MUC6
Membrane-bound mucins	MUC1 MUC4 MUC16	MUC1 MUC16	MUC1 MUC3A MUC3B MUC4 MUC12 MUC13 MUC15 MUC16 MUC17	MUC1 MUC3A MUC3B MUC4 MUC12 MUC13 MUC15 MUC16 MUC17

[1]Mucin expression patterns in humans (McGuckin et al., 2011).

2.1.1.2 Mucin Structure

Mucins consist of a central protein core and carbohydrate side chains. The protein core of typical mucin comprises tandem repeats rich in proline, threonine and serine (Belley et al., 1999). The sequence of tandem repeats varies in different sites of the body. The abundance of threonine and serine residues provides attachment sites for the oligosaccharide chains (Herrmann et al., 1999). The carbohydrate side chains of a secretory mucin constitutes up to 80% of its dry weight, presenting a unique 'bottle-brush' appearance. The carbohydrates contribute to the rheological and viscoelastic properties of the mucus layer (Andrianifahanana et al., 2006). There are five primary oligosaccharides commonly found in mucins, namely N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose, and sialic acids. The densely packed oligosaccharides protect the protein core from proteases and preserve the integrity of the mucin polymer (Moncada et al., 2003b).

MUC2 mucin is the major secretory mucin synthesized and secreted by goblet cells in the small and large intestine. Other secreted mucins, such as MUC5AC, MUC5B and MUC6 expressed in gastric and respiratory glandular epithelium have similar structural and physicochemical properties as MUC2 mucin. MUC2 mucin has a protein core of approximately 5,179 amino acids (Perez-Vilar and Hill, 1999). The monomer consists of

central tandem repeat domains. The N- and C- terminal regions of mucins are rich in cysteine, which facilitates the formation of disulphide bridges and a filamentous network of multimers (Bell et al., 2001). MUC2 N-terminal region comprises three complete von Willebrand factor domains (D1-D3), while the C-terminal region consists of one such domain (D4), however, these poorly glycosylated regions are susceptible to proteolytic attack compared to other mucin domains (Khatri et al., 1998).

Intestinal membrane-bound mucins have similar structures, and MUC3 is the most abundantly expressed membrane mucin in small intestine. MUC3 involves extracellular and membrane-bound subunits. The extracellular subunit consists of highly O-glycosylated tandem repeat domains and two epidermal growth factor-like domains, which are separated by sperm protein, enterokinase and agrin (SEA) section. There is a proteolytic cleavage site between the two SEA sections formed in the endoplasmic reticulum during biosynthesis. The membrane-associated subunit contains a transmembrane domain, and a cytoplasmic tail with potential phosphorylation sites which are involved in signaling transduction (Gum et al., 1997; Gum et al., 2003).

2.1.1.3 Mucin Characteristics

Mucins are heavily glycosylated filamentous proteins and their concentration is around 2-5% in mucus gel (Cone, 1999). They have a high molecular mass, ranging in size from 0.5×10^6 to 25×10^6 Daltons (Roussel and Delmotte, 2004). Mucin oligosaccharide chains are often terminated with sialic acid or sulfated groups, which account for the polyanionic nature of mucins at a neutral pH and additional resistance to degradation by glycosidases (Corfield et al., 1992). Different glycosylation of mucins can be explained by the alteration of core proteins and assembled carbohydrate side chains (Jass and Walsh, 2001). Glycosylation patterns can vary among tissues and different goblet cell populations within tissues, and are related to their physical properties of rigidity, protease resistance and gel-forming capability (McGuckin et al., 2011). Glycosylation of mucins can be altered by infectious and inflammatory factors, and this is considered as a significant protective

mechanism against mucosal pathogens (Jentoft, 1990).

The basic functions of gastrointestinal mucins are protection and lubrication of epithelial surfaces (Corfield et al., 2001; Roussel and Delmotte, 2004). Mucins promote the elimination of gut contents and provide the first defense barrier against physical and chemical insults, such as ingested food, microbes and microbial products. Moreover, mucins are also involved in more complex biological processes, such as epithelial cell renewal and differentiation, cell signaling and cell adhesion (Andrianifahanana et al., 2006). The secretory mucins are stored in the vesicles of goblet cells and produced continuously in dynamic manner or as reaction to specific stimuli (Forstner, 1995). Abnormal changes in mucin glycan structures and mucin gene expression have been implicated in some diseases, such as intestinal infection, cystic fibrosis, inflammatory bowel disease and cancer (Van Klinken et al., 1995).

Mucins provide nutrients for growth of the resident microbiota but also of pathogenic bacteria (Aristoteli and Willcox, 2003). The indigenous enteric microbiota (Berg, 1996) and pathogens (Helander et al., 1997) can gain energy by degrading mucins. Secreted mucins have been reported to act as a barrier defense to bacteria and parasites, such as *Escherichia coli* (Metcalfe et al., 1991), *Yersinia enterocolitica* (Mantle et al., 1989) and *Entamoeba histolytica* (Moncada et al., 2003a). Resident bacteria inhibit the adhesion of pathogenic bacteria to intestinal epithelia cells, probably due to increased secretion of intestinal mucins (Lievin-Le Moal and Servin, 2006). Lipopolysaccharides up-regulate gene expression of MUC5AC and MUC5B (Smirnova et al., 2003). Moreover, lactobacilli increase the expression of MUC2 and MUC13 in intestinal epithelial cells *in vitro* (Mack et al., 1999; Mack et al., 2003). Mucin composition is also affected by the activity of glycosyltransferases and glycosidases presented in intestinal bacteria (Freeman et al., 1980).

2.1.2 Trefoil Factors

Trefoil factors (TFFs) are abundantly secreted on the mucosal surface by goblet cells

(Table 2.2). Three TFFs have been identified, TFF1, TFF2 and TFF3 (Suemori et al., 1991). Their structure and expression patterns vary in the GIT. TFF1 is a product of gastric mucus cells together with the MUC5AC, whereas TFF2 is expressed in gastric mucus neck cells and cells in the pyloric glands together with MUC6. TFF3 is generated in intestinal goblet cells along with MUC2 (Podolsky, 1999). TFFs were found to be upregulated in the surface of epithelial cells in gastric ulcer and inflammatory bowel disease, and they play an important role in the protection of epithelia and repair of intestinal injuries (Hauser et al., 1993; Poulsom et al., 1996; Podolsky, 1999). They also assist in the formation of mucus gels and maintain mucus stabilization by interacting or cross-linking with mucus (Kindon et al., 1995). It has been demonstrated that TFF3 and mucin glycoproteins together have a synergistic effect on protecting epithelial cells and for the healing of intestinal lesions (Kindon et al., 1995). For instance, TFF3-deficient mice indicated an increased susceptibility to intestinal injury (Beck et al., 2010).

Table 2.2: Innate defense mechanisms in the gastrointestinal tract

Molecule	Defensive role	Reference
Secretory mucins	Mucus gel formation; physical barrier; blocks chemical insults; binding sites for intestinal microflora	Moncada et al., 2003b; Lievin-Le Moal and Servin, 2006
Gastric acid	Antimicrobial	Moncada et al., 2003b
Antimicrobial peptides	Antimicrobial; immunomodulation	van der Marel, 2012
Lysozyme	Antibacterial	Moncada et al., 2003b
Trefoil factor proteins	Epithelial restitution; epithelial continuity	Podolsky et al., 1993
Tight junction proteins	Mechanical barrier	Hu et al., 2013a
Toll-like receptors	Innate recognition of pathogens	Takeda and Akira, 2004
Antibodies	Binding sites for intestinal flora	Ayabe et al.,2004; Kim, et al.,2010

2.1.3 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are a group of small endogenous antibiotic peptides, serving as a part of the barrier function in the first defense line against pathogens (Corrales-Garcia et al., 2011). As a component of the innate immune system they can

neutralize invading microorganisms (Corrales-Garcia et al., 2011). AMPs have cationic or amphipathic properties that allow them to interact with bacterial cell walls (Fernandes et al., 2010). The defensins are an important family of AMPs (Corrales-Garcia et al., 2011). Mammalian defensins are classified into 3 subfamilies based on the arrangement of the six canonical cysteine motifs and disulfide bridges that stabilize the β-sheet structure. In addition to their antiviral and toxin-neutralizing characteristics, β-defensins possess antimicrobial and immunomodulatory effects. Activation of β-defensins in epithelial cells is mediated by cell surface toll-like receptors or cytoplasmic peptidoglycan receptors that can recognize pathogen-associated molecular patterns (van der Marel. M, 2012). AMPs may affect mucin secretion and stimulate the secretion of chloride ions (Ouellette, 1999).

2.1.4 Toll-like Receptors

The innate immune system senses invading pathogens by recognizing pathogen-associated molecular patterns via pattern recognition receptors (PRRs). There are two classes of PRRs in the gut: toll-like receptors (TLRs) and NOD-like receptors (NLRs). TLRs are membrane-anchored proteins expressed either at the cell surface or associated with intracellular organelles, while NLRs are localized in the cell cytosol (Magalhaes et al., 2007). Thirteen members of mammalian TLRs have been discovered. They activate the NF-κB pathway and stimulate immune cells to produce inflammatory cytokines and chemokines. The expression of TLRs in intestinal cells (Abreu et al., 2002) and tissues of biopsy specimen (Cario and Podolsky, 2000) from inflammatory bowel disease indicates regulatory functions. The consensus finding in these studies is that the epithelium has low expression of these receptors, which is considered as normal mucosal immune response towards commensal antigens. However, the TLR receptors are activated under pathological conditions, leading to an amplified innate immune response characterized by secretion of proinflammatory cytokines with elimination of pathogens and probable mucosal impairment (Moncada et al., 2003b).

2.2 Characteristics Associated with Weaning in Piglets

2.2.1 Changes in Gut Morphology of Weaned Piglets

Weaning anorexia may contribute to intestinal inflammation and adverse morphological changes (McCracken et al., 1999). Adverse morphological changes occur in the gut of weaned piglets, including deceased villus height, increased villus width and crypt depth (Hampson, 1986; Sharma and Schumacher, 1995; Boonzaier et al., 2013). The villus height was reduced rapidly by 25-35% of pre-weaning values within 24 h in pigs weaned at 21 d of age, and continued to decrease until 5 d after weaning (Hampson, 1986). These morphological changes in villi and crypts affect the digestive and absorptive capacity during the weaning period (Pluske et al., 1997). Weaning was found to be accompanied by a temporary increase of goblet cell population in pigs (Brown et al., 1988).

2.2.2 Intestinal Immune Response of Weaned Piglets

Pigs are usually weaned from the sow at a point when innate immunity is declining and adaptive immunity is not fully developed. Lymphocyte proliferation decreased in pigs weaned at 2 to 3 weeks of age, indicating that early weaning is detrimental to cellular immune response (Klasing, 1988). Mucus composition is altered during weaning period, particularly the production of sulphated mucins is increased (Brown et al., 1988). Another immunological response during the weaning process is alteration in pro-inflammatory cytokines induced by intestinal inflammation (Moeser et al., 2007a). Associated with weaning, an upregulation of the majority of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, was observed in an early acute phase (Pie et al., 2004).

2.3 Role of the Trace Element Zinc in the Gastrointestinal Tract

The beneficial effects of the trace element zinc on the prophylaxis and treatment of diarrhea are well documented in pigs (Poulsen, 1995; Carlson et al., 2004; Sales, 2013). Zinc-deficient rats had reduced feed consumption, feed utilization and showed growth

retardation (Williams and Mills, 1970). Dietary zinc deficiency caused impairment of gut architecture and organelles, such as shorter and narrower jejunal villi, reduced absorptive surface area, decreased number of mitochondria, swelling of the endoplasmic reticulum and atrophic *Golgi* apparatus, accompanied by increased membrane permeability and declined cell mobility (Southon et al., 1986). Furthermore, zinc deficiency decreased the number of goblet cells and mucus thickness, inhibited mucin synthesis, which resulted in colonization by pathogenic microorganisms, penetration of mucus layer, and finally infectious diseases (Quarterman et al., 1976). In zinc deficient organs, a reduction of T-cell subsets may be the first response to the change of zinc status, even before the plasma zinc concentrations fall below the normal range (Fraker et al., 1977; Beach et al., 1980; Hansen et al., 1982). Zinc deficiency decreased $CD4^+$ T cells population (Miller and Strittmatter, 1992). It also affected cytokine secretion of T lymphocytes and macrophages, particularly decreased the production of IL-2 and INF-γ (Dowd et al., 1986; Salas and Kirchner, 1987).

Zinc oxide supplementation in starter diets improved growth rate and feed efficiency of newly weaned piglets (Carlson et al., 1999). Cell apoptosis and proliferation were also influenced by zinc as displayed by increased villus height and reduced crypt depth (Li et al., 2001). Zinc has been shown to increase the presence of mucus on the gastric surface (Esplugues et al., 1985). High levels of dietary zinc oxide fed to pigs resulted in altered chemical composition of mucins with a higher amount of neutral, acidic, and sulfated mucins in the cecum and colon (Hedemann et al., 2006). In recent studies, mRNA levels of TNF-α, IL-6 and IFN-γ decreased with increasing concentrations of dietary zinc in the jejunum of pigs (Hu et al., 2012; Hu et al., 2013b). TGF-β and IL-10 are anti-inflammatory cytokines and can thereby help to maintain intestinal barrier function. The mRNA levels of TGF-β and IL-10 were increased with high zinc oxide included in zeolite after weaning (Hu et al., 2013b). Zinc regulates the signaling pathways of TLRs, which may induce or inhibit the activation of NF-κB (Foster and Samman, 2012). It is yet unclear whether these changes are due to an alteration in intestinal microbial ecology or a direct effect of zinc on gene expression and regulatory processes in the intestinal epithelium.

Diarrhea can be associated with zinc deficiency (Bhandari et al., 1996; Bahl et al., 1998).

Zinc deficiency can induce diarrhea due to nutrient deficiency and cell dysfunction, including a decreased Na^+/K^+-ATPase activity and reduced sodium and water absorption. Diarrhea in the post-weaning period of piglets is often associated with pathogenic *Escherichia coli.* Zinc has counter-regulatory effects and controlled post-weaning diarrhea (Li et al., 2001; Case and Carlson, 2002). Furthermore, zinc treatment also reduced the response to 5-hydroxytryptamine and affected the secretion of vasoactive intestinal peptides in the intestinal mucosa (Wapnir, 2000; Carlson et al., 2008). This can decrease electrolyte secretion and post-weaning diarrhea (Feng et al., 2006).

In addition, high level of dietary zinc oxide affected the diversity of the intestinal microbial communities, and reduced bacterial translocation in piglets (Huang et al., 1999; Katouli et al., 1999; Hojberg et al., 2005; Broom et al., 2006; Vahjen et al., 2011). The equilibrium of the gastrointestinal microbiota plays a critical role in the development and maturation of the gut associated immune system in piglets. The effects of dietary zinc oxide in weaned piglets are summarized in **Table 2.3**.

Table 2.3: Summary of effects of dietary zinc oxide in weaned piglets

	Main findings	Reference
Growth performance	Incidence of post-weaning diarrhea ↓	Hu et al., 2012; Hu et al., 2013b; Martin et al., 2013b
	Growth rate, weight gain and feed intake ↑	Hill et al., 1996; Mavromichalis, et al., 2000; Case and Carlson, 2002; Hollis et al., 2005; Han and Thacker, 2009; Hu et al., 2012; Martin et al., 2013b; Hu et al., 2013b
Physiological parameters	Mucosal alkaline phosphatase activity ↑	Carlson et al., 2006; Martin et al., 2013b
	Amylase, carboxypeptidase A, chymotrypsin, trypsin, lipase activities in pancreatic tissue ↑	Hedemann et al., 2006
	Jejunal transepithelial electrical resistance ↑	Hu et al., 2013b
	Jejunal paracellular permeability ↓	Hu et al., 2012
Zinc absorption	Plasma zinc concentration ↑	Case and Carlson, 2002; Carlson et al., 2006; Martin et al., 2013b
	Zinc concentration in digesta ↑	Martin et al., 2013a
	Liver, spleen and pancreas zinc concentration ↑	Han and Thacker, 2009; Martin et al., 2013b
	Gene expression of ZnT1 ↑; ZIP4 ↓	Martin et al., 2013a,b
	Gene expression and protein abundance of MT ↑	Martin et al., 2013a
Morphological alteration	Mucosal thickness, villus height, villus width, the ratio of VH to CD ↑; crypt depth ↓	Li, et al., 2001; Hu et al., 2012
	Area of neutral, acidic and sulfomucin in the cecum and colon ↑	Hedemann et al., 2006
Bacterial growth	Viable counts of *Clostridium* spp and *Escherichia coli* ↓	Hu et al., 2013b
	Intestinal microflora and diversity of coliforms ↑	Katouli et al., 1999; Hojberg et al., 2005
	Bacterial translocation in small intestine ↓	Huang, et al., 1999
	Bacterial activity in digesta ↓	Hojberg et al., 2005
	Amounts of lactic acid bacteria and lactobacilli ↓; coliforms and enterococci ↑	Hojberg et al., 2005; Broom et al., 2006; Starke et al., 2013; Vahjen et al., 2011
	Bacterial metabolite concentrations ↓	Starke et al., 2013
	Ileal *Weissella spp*., *Leuconostoc spp*. and *Streptococcus spp*. ↑; *Sarcina spp*. and *Neisseria spp* ↓	Vahjen et al., 2010; Vahjen et al., 2011
Immune response	Intestinal IgA concentration ↑	Broom et al., 2006
	mRNA levels of TNF-α, IL-6 and IFN-γ ↓	Hu et al., 2012; Hu et al., 2013b
	TGF-β and IL-10 in jejunum ↑	Hu et al., 2013b

Chapter 3: Objectives

The thesis project was part of the DFG funded collaborative research center (SFB 852) – "Nutrition and intestinal microbiota – host interaction in the pig", taking an interdisciplinary approach in addressing the effect of nutrition on intestinal function and animal health. One focus of the collaborative work was to understand the mode of action of so-called pharmacological levels of dietary zinc from zinc oxide on intestinal health in young piglets. The underlying mechanisms involved in modulation of intestinal health need further characterization. In this thesis, the effects of pharmacological levels of dietary zinc oxide on gut immune response with special emphasis on intestinal innate and adaptive immunity were evaluated in weaned piglets over the first four weeks after weaning.

The hypotheses on dietary zinc oxide are as follows:

- Zinc oxide affects intestinal morphology.
- Zinc oxide influences the number of goblet cells.
- Zinc oxide alters mucin chemotypes.
- Zinc oxide enhances the diversity of intraepithelial lymphocytes.
- Zinc oxide impacts on the expression of genes related to immune defense mechanisms.

To test the above hypotheses, the following approaches were employed:

- Intestinal morphology, mucin chemotypes and goblet cell density were investigated by histological techniques.
- The population of intestinal intraepithelial lymphocytes was determined by flow cytometry.
- The gene expression of MUC, TFF3, β-defensin, TLR and cytokines was quantified by RT-qPCR.

Chapter 4: Effect of Dietary Zinc Oxide on Jejunal Morphological and Immunological Characteristics in Weaned Piglets

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ABSTRACT: The aim of this study was to evaluate age-related changes in morphological and immunological characteristics in the gastrointestinal tract of piglets and the influence of dietary Zn concentration. A total of 96 purebred Landrace piglets were weaned at the age of 26 ± 1 d, and randomly allocated into 3 treatment groups fed with low (57 mg Zn/kg DM), medium (164 mg Zn/kg DM), and high (2,425 mg Zn/kg DM) dietary Zn by addition of ZnO. Piglets (n =8, four male and four female per treatment group) were killed at 33 ± 1, 40 ± 1, 47 ± 1 and 54 ± 1 d of age, respectively. In the jejunum, villus height and crypt depth and the number of goblet cells producing neutral, acidic, sulfated and sialylated mucins was measured. Intraepithelial lymphocytes were characterized by flow cytometry and the gene expression of mucin 2 (MUC2), mucin 20 (MUC20), *β*-defensin 3 and trefoil factor 3 (TFF3) was determined by reverse transcription quantitative PCR. Villus height and crypt depth in the jejunum showed age related differences ($P < 0.01$), the dietary concentrations of Zn had no effect. The mucin types were modified mainly by age, dietary Zn had no effect in the proximal jejunum. In the distal jejunum, age and Zn had effects on the mucin types. The abundance of sulfomucins decreased ($P < 0.001$) and sialomucins increased with age ($P < 0.001$), while high dietary ZnO reduced the sulfomucins ($P < 0.001$) and increased the sialomucins ($P < 0.05$) in the crypts. The phenotypes of lymphocytes in the epithelium of the proximal jejunum showed relatively constant percentages of T cells as well as natural killer cells. High dietary Zn treatment led to a reduced abundance of $CD8^+$ γδ T cells ($P < 0.05$). The apportionment of different cytotoxic T cell was age dependent. While the percentage of $CD4^-CD8\beta^+$ increased ($P < 0.01$), the relative amount of $CD4^+CD8\beta^+$ decreased with age ($P < 0.05$). The expression of MUC2 and MUC20 was not influenced by age or dietary Zn concentration. High Zn intakes resulted in a reduced gene expression of *β*-defensin 3 ($P < 0.05$) and did not affect the expression of TFF3. It is concluded that Zn in the form of ZnO appears to have specific effects on the innate and adaptive gut associated immune system of piglets. These might contribute to the positive effects of Zn on the prevention of post-weaning diarrhea.

Key words: *β*-defensin 3, lymphocytes, morphology, mucin, trefoil factor 3, zinc oxide

4.1 Introduction

The development of the digestive tract as well as the associated innate and specific defense mechanisms is of outstanding importance for the development of optimized feeding concepts in pigs (Pluske, 2013). The gastrointestinal tract of piglets after birth undergoes a dynamic maturation, which includes morphological, resorptive and other functional aspects such as the mucosa-associated immune system (Simon, 2010). The mucus is particularly important because it is the first layer contact with the intestinal contents, i.e., digesta and intestinal microbiota (Corfield et al., 2000; Iniguez-Palomares et al., 2011). Furthermore, studies on the development of the gut-associated immune system in piglets showed that significant changes occur during early life (Brown et al., 2006; Scharek et al., 2007).

Diet plays an important role in maturation and modulation of the gut associated immune system. Several dietary factors have been shown to be essential for the development of gut function as well as the immune system (Castillo et al., 2008). In addition, ZnO supplementation has been shown to be associated with changes in the expression of genes that are responsible for maintaining nonspecific defense mechanisms (Sargeant et al., 2010). High dietary Zn intakes in pigs have been frequently shown to improve performance, change the composition of the intestinal microbial communities and reduce the incidence of intestinal disorders (Li et al., 2001; Hojberg et al., 2005). Whether this is also related to changes in the intestinal innate or adaptive immune response is yet not clear. Therefore, the objective of this study was to examine the effects of 3 different concentrations of dietary Zn on gut morphology, mucin composition and the development of the specific and non-specific gut associated immune system in weaned piglets.

4.2 Material and Methods

Pig handling and treatments were conducted in according with German law for the care and use of experimental animals. All procedures were approved by the ‘State Office of Health and Social Affairs Berlin’ (LaGeSo Reg. Nr. 0347/09).

4.2.1 Animals, Diets and Sampling

A total of 96 purebred Landrace piglets from 12 litters were weaned at the age of 26 ± 1 d (mean BW: 7.5 ± 1.2 kg) and randomly allocated into 3 treatment groups balancing for litter and sex. Piglets were housed in commercial flat-deck pens (2 piglets per pen) with stainless steel framings. Room temperature was maintained at 26˚C on the day of weaning and reduced at regular intervals to achieve 22˚C one week post-weaning. The humidity was kept constant and the lightning program was maintained at 16 h light and 8 h dark in each day. Feed was provided in dry form from stainless steel bowls, water was provided ad libitum from nipple drinkers. From 12 d of age, piglets were provided the non-medicated pre-starter diet. After weaning, piglets received a mash starter diet until 54 d of age. Piglets in each group received one of three experimental diets (**Table 4.5**) based on wheat, barley, and soybean meal. The analyzed concentration in the basal diet was 35 mg Zn/kg and the target Zn concentrations of the 3 diets were adjusted by replacing corn starch with analytical-grade ZnO. The final concentrations were adjusted to as low (57 mg Zn/kg DM), medium (164 mg Zn/kg DM), and high (2,425 mg Zn/kg DM) dietary Zn, respectively.

Piglets (n =8, four male and four female per treatment group) were killed at 33 ± 1, 40 ± 1, 47 ± 1 and 54 ± 1 d of age, respectively. Pigs were sedated with 20 mg/kg BW of ketamine hydrochloride (Ursotamin; Serumwerk Bernburg AG, Bernburg, Germany) and 2 mg/kg BW of azaperone (Stresnil; Jansen-Cilag, Neuss, Germany) prior to euthanization by intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61; Intervet, Unterschleißheim, Germany). The entire intestinal tract was removed and the small intestine was separated from the large intestine and the mesentery. Two sections from the proximal (2 cm from the pylorus) and distal (2 cm from the ileo-cecal junction) jejunum were fixed immediately in Bouin's solution for histological examinations. Approximately 20 cm of proximal jejunum was used for phenotypic analysis of intraepithelial lymphocytes by flow cytometry. Another 5 cm piece of jejunum was snap-frozen in liquid nitrogen and stored at -80°C until mRNA extraction and gene expression analysis.

4.2.2 Histochemistry

Samples from the proximal and distal jejunum were fixed in Bouin's solution for 3 days, subsequently rinsed several times in 70% ethanol to remove the remains of picric acid, dehydrated in a graded series of ethanol, and cleaned with xylol. The tissues were embedded in paraffin wax according to standard protocols (Romeis, 1989). Five micrometers sections were cut from the paraffin blocks using a rotary microtome for subsequent staining procedures. The sections were deparaffinized with xylene, rehydrated in a descending series of ethanol, and routinely stained with hematoxylin and eosin. They were examined with a microscope (Zeiss Photomicroscope Ⅲ; Zeiss, Oberkochen, Germany) with digital camera (Olympus DP72; Olympus, Tokyo, Japan) and analyzed with cellSens imaging software (Olympus, version 1.4). Ten villi and corresponding crypts were randomly chosen from different well-orientated parts of the sections, where most of the villi and crypts were cut longitudinally from the tip of the villi to the bottom of the crypts in the stained sections of each animal. Morphometric characteristics included villus height (from the tip of the villus to the villus-crypt junction), crypt depth (from villus-crypt junction to the base of the crypt), and villus height to crypt depth ratio.

In order to distinguish different mucin types characterized by the carbohydrates present, the following staining techniques were used. The Alcian blue pH 2.5-periodic acid Schiff (**AB-PAS**) staining procedure (Mowry, 1963) was carried out to distinguish neutral and acidic mucin in goblet cells. The neutral mucin containing in goblet cell stained magenta, and acidic mucin stained blue. The mixture of neutral-acidic mucins showed purple, magenta-purple or blue-purple colors in goblet cell. Meanwhile, to identify sialomucin and sulfomucin, sections were stained with the high iron diamine-Alcian blue pH 2.5 (**HID-AB**) technique, including counterstaining with nuclear fast red (Spicer, 1965). As a result, the sulfomucin stained black, sialomucin stained blue, and a mixture of sulfo-sialomucins resulted in black and blue colors. For the quantification of the different mucin chemotypes containing goblet cells, ten villi and corresponding crypts were randomly chosen from different well-orientated parts of the sections, where the goblet cells were showed clear staining characteristics in sections from each animal stained with AB-PAS and HID-AB

methods. Goblet cells containing the different mucin types were counted in villi and crypts. The corresponding basement membrane length was used as reference, and the number of the goblet cells with different mucin types was expressed as quantity in per 1mm of the basement membrane length.

4.2.3 Flow Cytometric Analysis

The isolation of the intraepithelial cells was performed as described previously (Solano-Aguilar et al., 2000) with slight modifications. To isolate the intraepithelial lymphocytes, the intestinal segment was inverted, tied at one end, filled with PBS and tied at the other end. The inverted sack was then incubated in 80 mL Hanks balanced salt solution containing dithiotreitol (HBSS-DTT; HBSS without Ca^{2+} and Mg^{2+}, 2 mM DTT, 0.01 mM HEPES) and gently shaken for 5 min at 37°C. The medium was discarded and replaced with 80 mL HBSS-EDTA (HBSS without Ca^{2+} and Mg^{2+}, 1 mM EDTA, 1 mM HEPES). A second incubation was carried out for 35 min at 37°C with gentle shaking. The cell suspension was sieved through a sterile 210 μm Nylon mesh, collected in 50 mL plastic tubes and centrifuged at 600 × g for 10 min at 4°C. The resulting cell pellets were re-suspended in 25 mL RPMI-1640 medium and kept at 8°C. This procedure was repeated with the original tissue section, and both cell suspensions were combined and centrifuged again. Sediments were re-suspended in 25 % Percoll diluted in HBSS and centrifuged at 600 × g for 30 min at room temperature. The top layer was aspirated and discarded, the Percoll solution was decanted, and the cell sediment was suspended in RPMI. Single and double cell-stainings were carried out as previously described (Mafamane et al., 2011). Monoclonal antibodies were used to identify surface molecules. The clone notations, the respective clusters of differentiation (**CD**), and the antibody distributors are given in **Table 4.6**. The resulting suspensions (containing leukocytes and epithelial cells) were subjected to flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). To analyze the proportion of the stained cells a lymphocyte gate was constituted following morphological criteria. Percentages of positive immune cells reflect the distribution of certain subpopulations within the relevant lymphocyte gate.

4.2.4 RNA Extraction and Gene Expression Analysis

Total RNA was extracted from 30 mg of jejunal tissue using the NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Then the concentration of extracts was determined by measuring absorbance at 260 and 280 nm (NanoDrop Technologies, Inc., Wilmington, DE). The quality and purity of mRNA was checked on a Bioanalyzer (Agilent 2100; Agilent Technologies, Waldbronn, Germany). All samples were diluted in RNA free water to 100 ng per μL for gene expression analysis. The expression of the following target genes was analyzed: mucin 2 **(MUC2)**, mucin 20 **(MUC20)**, trefoil factor 3 **(TFF3)** and *β*-defensin 3. Expression of 60S ribosomal protein L19 **(RPL19)**, 60S ribosomal protein L13 **(RPL13)** and *β*-2-microglobulin (**B2M**) were used as housekeeping genes for data normalization (**Table 4.7**). The reverse transcription quantitative PCR (**RT-qPCR**) was performed using the one-step QRT-PCR master mix kit (Brilliant II SYBR Green; Agilent Technologies, USA). Each reaction consisted of 1 μL diluted template RNA (100 ng/μl) and 25 μL master mix (10.5 μL RNase free H_2O, 12.5 μL SYBRII RT-qPCR reaction buffer with ROX and SYBR-Green, 0.5 μL each of 10 μmol/L gene-specific forward and reverse primers, 1.0 μL Reverse Transcriptase/RNase block enzyme mixture). The amplification was performed on a Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) with general cycling conditions as follows: one cycle of reverse transcription for cDNA synthesis at 50°C for 30 min, one cycle of denaturation at 95°C for 15 min, followed by 40 cycles with 30 s at annealing temperature given in Table 3 for each primer and 30 s extension at 72°C. PCR efficiency and melting curves were checked to ensure consistent amplification of a single PCR product. Standard curves were generated using serial dilutions of pooled RNA (within a range from 5 to 200 ng/μL) from 20 samples to convert Ct values into arbitrary values. These arbitrary values were then normalized using the mean values of the housekeeping genes and mean arbitrary values were used for statistical comparisons (Martin et al., 2012).

4.2.5 Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (Chicago, IL, USA). Data on jejunal

morphology, the differentiation of mucin chemotypes, intraepithelial lymphocytes and the gene expression of MUC2, MUC20, β-defensin 3 and TFF3 were analyzed using two-way ANOVA. The fixed factors were diet (three concentrations of 57, 164, and 2425 mg Zn/kg diet) and age (33, 40, 47 and 54 d); pen, litter and boar were considered as random factors. Diet effects were analyzed for each age group, and results were given as mean values. Differences were considered significant at $P < 0.05$.

4.3 Results

4.3.1 Morphometric Data of Villus Height and Crypt Depth in the Jejunum

Villus height increased with age in both segments of the jejunum, while age affected crypt depth only in the distal jejunum ($P < 0.01$). The villus height to crypt depth ratio increased in age dependent manner in both segments of the jejunum ($P < 0.001$). Morphometric parameters were not influenced by the different dietary Zn intakes (**Table 4.8**).

4.3.2 Mucin Chemotypes in Jejunal Goblet Cells

The different mucin chemotypes were affected by age, but no diet-related effect was observed in the proximal jejunum (**Table 4.9**). In the villi of the proximal jejunum, older piglets had a greater abundance of goblet cells containing mixed neutral-acidic mucins and total number of mucins ($P < 0.001$). Very few goblet cells producing acidic mucins were detected. In the crypts, the number of acidic mucins in goblet cells decreased with age ($P < 0.05$), while the abundance of mixed neutral-acidic mucins and total mucins increased in the first two weeks post-weaning, then the cells were reduced after 40 d of age ($P < 0.05$). Moreover, in the villi, goblet cells produced mainly sulfomucins and their numbers increased with age ($P < 0.001$), but sialomucins and mixed sulfo-sialomucins were either absent or rarely found. In the crypt, the sulfomucins increased during the two weeks post-weaning, then decreased after 40 d of age ($P < 0.01$). In contrast, the number of goblet cells with sialomucins was increased with age ($P < 0.01$).

In the distal jejunum, the different mucin chemotypes were affected by age and dietary Zn concentration (**Table 4.10**). Neutral mucins in the goblet cells increased with age in both, villi and crypts ($P < 0.05$). In the villi, sialomucins and mixed sialo-sulfomucins were not detected or rarely found, the distribution was similar to that in the proximal jejunum. However, in the crypts, there were effects on cells producing sulfomucins and sialomucins by age and dietary Zn concentration. The abundance of goblet cells with sulfomucins decreased ($P < 0.001$), while sialomucin producing cells increased with age ($P < 0.001$). High Zn supplementation reduced the number of goblet cells containing sulfomucins ($P < 0.001$) and increased the sialomucins ($P < 0.05$). The cells with mixed sulfo-sialomucins were not affected by age or dietary Zn concentration in the crypts in proximal and distal jejunum.

4.3.3 Phenotyping of Intraepithelial Lymphocytes

The percentage of intraepithelial $CD3^+$ T cells and $CD2^+CD5^-$ natural killer cells was relatively constant and not affected by diet or age in the distal jejunum (**Table 4.11**). The piglets fed the high dietary ZnO had a decreased percentage of T cells with the $CD8^+$ γδ phenotype ($P < 0.05$), independent of the age. The percentage of several cytotoxic T cell clusters changed, the phenotypes $CD4^-CD8\beta^+$ increased ($P < 0.01$) and $CD4^+CD8\beta^+$ decreased ($P < 0.05$) depending on age.

4.3.4 Gene Expression in the Jejunum

The expression of MUC2 and MUC20 mRNA were not affected either by age or by zinc supplementation (**Table 4.12**). The expression level of *β*-defensin 3 was lower at high dietary zinc intakes ($P < 0.05$). The TFF3 expression was influenced by age ($P < 0.05$), having an increase during 3 weeks after weaning and then decreased.

4.4 Discussion

The growth performance was evaluated across 4 weeks after weaning. No differences and only low incidence of diarrhea was observed, the average daily gain and feed intake were increased with the high concentration of dietary Zn in the first two weeks but not over the 4 weeks period (Martin et al., 2013). Based on the clinical investigation, the influence of age and Zn concentration on morphological and immunological characteristics were further determined in the present study. Changes of villus height and crypt depth are considered as key parameters for the assessment of gut maturation and nutritional effects. The range of data collected here is well comparable with values reported for animals in the corresponding age (Miller et al., 1986; Cera et al., 1988). However, the dietary Zn concentrations did not affect intestinal morphology in the current study. Zn plays a major role in the regulation and differentiation of the intestinal tissues, likely mediated through molecular regulators including Zn finger proteins such as Krüppel-like factor 9 (Simmen et al., 2007). Severe Zn deficiency was shown to reduce cell influx into the villi causing morphological and functional changes in the small intestine of rats (Southon et al., 1985). Zn intake in the current study was slightly reduced in the low Zn treatment group compared to the estimated requirements of young pigs (GfE, 2006; NRC, 2012). However, studies in piglets showed an almost undisturbed architecture of the small intestinal tissue even with moderate Zn deficiency (Lalles et al., 2007). In a previous study, the administration of high concentration of dietary Zn moderately increased the villus height, and tended to decrease the crypt depth in the lower small intestine in piglets (Li et al., 2001), whereas no clear effects of high dietary Zn on intestinal morphology were observed by others (Hedemann et al., 2006). Thus, it seems that the impact of dietary Zn intake on the intestinal morphology is limited under normal physiological conditions. This could also be due to the generally good hygienic conditions in the current study. Positive effects of dietary Zn on intestinal morphometric characterizations might be more pronounced under suboptimal or on-farm conditions when intestinal health is more impaired and epithelial apoptosis is increased.

Mucins have multiple physiological roles, acting as diffusion barrier, microecological

habitat and natural defense mechanism (Ganz, 2002; Lievin-Le Moal and Servin, 2006). The ratio of neutral to acidic mucins in the gastrointestinal tract has a spatial pattern and pigs have a greater proportion of acidic mucins in the hind gut (Deplancke and Gaskins, 2001). Mucus secretion is known to be affected by weaning and the age of animals (Brown et al., 1988; Choi et al., 1991), the composition of the intestinal microbiota (Collinder et al., 2002) and dietary treatment (Hedemann et al., 2009). The composition of different mucins might be of importance with respect to binding of specialized bacteria and changing the intestinal microbial community composition during the post-weaning period. The histochemical approach to the study of mucin is highly informative (Corfield, 2000), and the previous studies used this method to determine mucin distribution and to quantify the number and the staining area of mucin-secreting goblet cells (Law et al., 2007; Boonzaier et al., 2013). In the present study, the number of goblet cells with different mucins changed depending on the age of the animals and to a lesser degree with dietary Zn concentration. Age effects on goblet cells containing mucins were described in other studies in piglets (Dunsford et al., 1991; Brown et al., 2006). In our study, the number of goblet cells producing acidic mucin was decreased with age in the crypts of proximal jejunum. Goblet cells with acidic mucins, sialomucins and mixed sialo-sulfomucins were either not observed or rarely found in the villi. The sialomucins and sulfomucins are sub-chemotypes of acidic mucins. The results showed that the goblet cells in the villi were mainly positive for sulfomucins. This staining characteristic was consistent with a previous study (Brown et al., 1988). Newly produced goblet cells seem to secret sialomucins preferentially and are mainly located in the lower crypt (Olubuyide et al., 1984), whereas mature goblet cells migrate up the crypts and villi in the small intestine with switching to sulfomucin production (Brown et al., 1988). The distribution of cells producing sialomucins and a mixture of sulfo-sialomucins in the villi of distal jejunum were similar to in the proximal segment, but the cells with acidic mucins were found more than in the proximal jejunum. Interestingly, in the crypt, sialomucins and sulfomucins showed similar age-dependent changes in both, proximal and distal jejunum. Generally, the sulfomucins decreased and sialomucins increased with age. The appearance of acidic mucins seems to be particularly important in the early development, acting in a protective role for the immature immune system (Cebra, 1999), and the transformation from predominately sulfated mucins to

sialylated mucins might be a defense strategy in young animals (Forder et al., 2007). Both sulfate and sialic acid groups have protective roles. Sialylated mucins, compared to sulfated mucins, have more acetyl groups that could inhibit enteric bacterial sialidases (Corfield et al., 1992). The colonic mucins are highly sulfated and sialylated in newborn piglets (Turck et al., 1993). Moreover, piglets fed the high dietary Zn level produced more sialomucins and less sulfomucins in the jejunal crypts suggesting an improved defense situation. Additionally, the mixed neutral-acidic mucins and the total mucins were increased with age in proximal jejunum. These changes of mucin profiles with age may attribute to the maturation of the barrier function of the intestinal mucosa in piglets.

In the present study, four major lymphocyte populations were monitored in the porcine jejunal epithelium of the piglets: shortly after weaning, the most frequent T cell type was $CD2^{+}CD5^{-}$. Based on prior studies with porcine blood immune cells, this population is most likely natural killer (**NK**) cells (Denyer et al., 2006; Gerner et al., 2009). Another frequent lymphocyte population was $CD4^{-}CD8\beta^{+}$. This phenotype represents the classical major histocompatibility complex (**MHC**)-class I restricted cytolytic T cells in the pig (Denyer et al., 2006). The percentage of these cells increased with age, which has also been observed in blood samples from pigs, and *in vitro* tests hint to an antigen dependent proliferation of the $CD4^{-}CD8\beta^{+}$ cells (Denyer et al., 2006). Furthermore, a minor population of $CD4^{+}CD8\beta^{+}$ cells was present in the jejunal epithelium of the piglets. Cells with this phenotype have been shown to express perforin, which was considered to be a marker for lymphocytes having active cytolytic function (Denyer et al., 2006). This intraepithelial lymphocyte (**IEL**) population was the smallest in the piglets and it decreased with age. Neither the relative numbers of these two cytolytic T cell populations nor the portions of NK cells were clearly affected by the supplementation of Zn. However, the relative numbers of the $CD8^{+}$ γδ T cells were significantly reduced due to high dietary Zn concentration. Since γδ T cells recognize unprocessed non-peptide antigen in an MHC-unrestricted manner (Tanaka et al., 1994), they are an important branch of the innate immune system. Although to date the expression of perforin has not been shown in porcine γδ T cells, the $CD8^{+}$ subtype is described to have cytotoxic capabilities (Pauly et al., 1996; de Bruin et al., 1997; Yang and Parkhouse, 1997). Interestingly, in contrast to all other

lymphocyte populations in the porcine intestinal epithelium, these lymphocytes also act as antigen presenting cells (Takamatsu et al., 2002, 2006), and even a memory function of these γδ T cells is under discussion (Tsuji et al., 1996). The T cells represent a link between natural and adaptive immunity. On stimulation with the mitogen phorbol myristate acetate porcine γδ T cells produce a wide variety of cytokines and chemokines (Denyer et al., 2006). Nanoparticles from ZnO activate antigen presenting cells and induce the expression of inflammatory signals to recruit neutrophils, macrophages and lymphocytes (Palomaki et al., 2010). However, since no increase in one or several immune cell populations was observed with the high dietary Zn supplementation, the observed relative decline in γδ T cells was unlikely due to an increase of the other monitored IEL populations. More likely, the decreased portion of γδ T cells could be a consequence of a lower pathogenic load or a reduced necessity to eliminate infected epithelial cells. This could set a link to the frequently observed changes in intestinal microbial ecology with high dietary ZnO levels (Hojberg et al., 2005; Starke et al., 2013).

The expression of MUC2, MUC20, *β*-defensin3 and TFF3 in jejunal tissue was studied in response to dietary Zn levels and age. MUC2 is the one major of secreted mucins expressed by both small intestine and colon (Einerhand et al., 2002; Dharmani et al., 2009). The mucus layer is organized by the highly glycosylated MUC2 mucin (Johansson et al., 2011) and MUC2 synthesis rate might be a potential parameter for intestinal barrier function (Schaart et al., 2009). Only a few studies determined the influence of nutritional factors on mucin gene expression in the intestinal tract of pigs. For example, high-protein diets increased the expression of MUC2 and MUC20 associated with increased mucosal cell turnover and pro-inflammatory markers (Pieper et al., 2012). However, no significant influence of age or Zn supplementation was observed on the expression of MUC2 and MUC20 in the present study. The *β*-defensins play a critical role in the mammalian innate immunity. The concentrations with 25 and 100 μmol/mL of Zn sulfate could induce expressions of *β*-defensins in porcine epithelial cells line (Mao et al., 2013). In our study, the expression of *β*-defensin 3 was down-regulated by high Zn supplementation in piglets, while the medium concentration of dietary Zn induced the greatest expression of *β*-defensin 3. Relative transcripts of TFF3 were significantly affected by bovine colostrum

supplementation in piglets (Huguet et al., 2007). However, the influence of Zn or other trace elements on the TFF3 gene was not reported until now. In the present study, the TFF3 mRNA expression altered depending on age, with an increase during 3 weeks after weaning. This may reflect the maturation of gut related immune mechanisms.

In conclusion, high concentration of dietary Zn had no effects on morphological characteristics in the jejunum of piglets, but the specific modulation of mucin chemotypes, intraepithelial lymphocytes and gene expression of the gut epithelium might be interesting for the positive effects of Zn on the prevention of post-weaning diarrhea.

Table 4.5: Composition of the experimental diets, as-fed basis

Item	Diet
Ingredient, g/kg	
Wheat	380
Barley	300
Soybean meal	232
Corn starch/ZnO[1]	10
Limestone	20
Monocalcium phosphate	20
Mineral-vitamin premix[2]	15
Soy oil	17.5
Salt	2.0
Lys HCl	2.5
Met	1.0
Analyzed composition	
DM, g/kg	879
CP, g/kg	194
Crude ash, g/kg	81
Crude fiber, g/kg	36
Ether extract, g/kg	34
Starch, g/kg	376
Lys[3], g/kg	11.7
Met, g/kg	4.0
Thr, g/kg	7.2
Trp, g/kg	2.4
Ca, g/kg	11.0
Total P, g/kg	8.0
Na, g/kg	3.1
Mg, g/kg	2.2
Zn[4], mg/kg	34
Fe, mg/kg	309
Mn, mg/kg	41
Cu, mg/kg	7
Metabolisable energy, MJ/kg	13.0

[1]Corn starch in the basal diet was partially replaced with analytical grade ZnO (Sigma Aldrich, Taufkirchen, Germany) to adjust for the Zn concentration.
[2]Mineral and Vitamin Premix (Spezialfutter Neuruppin Ltd., Neuruppin, Germany), providing per kilogram of diet: 1.95 g Na (sodium chloride), 0.83 g Mg (magnesium oxide), 10,500 IU vitamin A, 1,800 IU vitamin D_3, 120 mg vitamin E, 4.5 mg vitamin K_3, 3.75 mg thiamine, 3.75 mg riboflavin, 6.0 mg pyridoxine, 30 µg cobalamine, 37.5 mg nicotinic acid, 1.5 mg folic acid, 375 μg biotin, 15 mg pantothenic acid, 1,200 mg choline chloride, 75 mg Fe (iron-(II)-carbonate), 15 mg Cu (copper-(II)-sulfate), 90 mg Mn (manganese-(II)-oxide), 675 μg I (calcium-iodate), and 525 μg Se (sodium-selenite).
[3]All amino acids were given as total concentration.
[4]Analyzed concentration of Zn in the basal diet without ZnO supplementation. The diets as fed contained 57, 164, and 2,425 mg Zn/kg, adjusted by ZnO.

Table 4.6: List of antibodies (ab) used in the study for staining of intraepithelial lymphocytes

Specificity	Clone / host species of sec. ab[1]	Isotype	Cytochrome	Distributor
CD2	MSA4	IgG2a	none	VMRD[5]
CD3ε	PPT3	IgG1κ	none	Southern Biotech[6]
CD4α	74-12-4	IgG2b	FITC[3]	Southern Biotech[6]
CD5	9G12	IgG1	none	VMRD[5]
CD8α	76-2-11	IgG2a	PE[4]	Southern Biotech[6]
CD8β	PG164A	IgG2a	none	VMRD[5]
TcR1-N4 (δ)[2]	PGLBL22A	IgG1	none	VMRD[5]
IgG2a	pooled antisera from goats		PE[4]	Southern Biotech[6]
IgG1	pooled antisera from goats		FITC[3]	Southern Biotech[6]

[1]sec.ab = secondary antibodies.
[2]TcR1-N4 (δ) = δ chain of T cell receptor 1.
[3]FITC = fluorescein isothiocyanate.
[4]PE = phycoerythrin.
[5]VMRD, Pullman, WA.
[6]Southern Biotech, Birmingham, AL.

Table 4.7: List of primers used in this study

Target	Sequences of primers (5` to 3`)	A_T[1]	Reference
RPL19[2]	GCTTGCCTCCAGTGTCCTC	60	Pieper et al., 2012
	GCGTTGGCGATTTCATTAG		
RPL13[2]	CCGTCTCAAGGTGTTCGATG	60	This study
	GGATCTTGGCCTTCTCCTTC		
B2M[2]	CCCCGAAGGTTCAGGTTTAC	60	This study
	CGGCAGCTATACTGATCCAC		
MUC2[2]	CTGCTCCGGGTCCTGTGGGA	60	Pieper et al., 2012
	CCCGCTGGCTGGTGCGATAC		
MUC20[2]	GAAGGGGGCATCGCTGCCTG	60	Pieper et al., 2012
	GCCAGGGTCCCACTGCCATG		
β-defensin3	GTGCAGAAGGGGCAATGGTCG	60	This study
	GTTGCAGGTCTCATGCAGTAAGCA		
TFF3[2]	AGTGTGCCGTCCCTGCCAAG	60	This study
	GCAGCCCCGGTTGTTGCACT		

[1]A_T = Annealing temperature.
[2]RPL19 = 60S ribosomal protein L19; RPL13 = 60S ribosomal protein L13; B2M = Beta-2 microglobulin; MUC2 = mucin 2; MUC 20 = mucin 20; TFF3 = trefoil factor 3.

Table 4.8: Morphometric characteristics of the jejunal mucosa of weaned piglets[1]

	Dietary Zn concentration															
	57 mg Zn/kg				164 mg Zn/kg				2425 mg Zn/kg					*P*-value		
	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	SEM	Diet	Age	Diet×Age
Proximal Jejunum																
VH[2], µm	360	437	476	455	313	385	513	515	362	391	494	500	8	0.954	<0.001	0.346
CD[2], µm	224	248	246	250	215	215	239	250	226	218	225	237	4	0.215	0.117	0.762
VH:CD[2]	1.66	1.77	1.95	1.84	1.47	1.78	2.17	2.05	1.82	1.82	2.26	2.14	0.04	0.229	<0.001	0.627
Distal Jejunum																
VH[2], µm	341	358	485	458	307	397	470	497	367	396	488	455	9	0.777	<0.001	0.724
CD[2], µm	203	230	255	238	204	227	231	237	203	220	244	218	4	0.495	0.003	0.887
VH:CD	1.70	1.60	1.91	1.95	1.51	1.74	2.03	2.10	1.82	1.83	2.00	2.08	0.03	0.260	<0.001	0.697

[1]Ninety-six 26 d weaned purebred Landrace piglets were randomly allocated into 3 treatment groups with variation of 57, 164, and 2,425 mg Zn/kg diet from ZnO. Piglets (n = 8 per treatment group) were killed at 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d of age, respectively. The results were given as mean values.
[2]VH = villus height; CD = crypt depth; VH:CD = villus height to crypt depth ratio.

Table 4.9: Numbers of AB-PAS and HID-AB positive goblet cells in the proximal jejunum of weaned piglets[1]

		Dietary Zn concentration															
		57 mg Zn/kg				164 mg Zn/kg				2425 mg Zn/kg					*P*-value		
		33 d	40 d	47 d	54d	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	SEM	Diet	Age	Diet×Age
		AB-PAS staining															
Villus	Neu[2]	1.8	1.5	2.7	2.2	0.5	2.5	2.0	1.7	2.0	1.6	2.0	2.2	0.2	0.635	0.322	0.289
	Acid[2]	0.3	0.4	0.7	0.5	0.9	0.4	ND[3]	ND	0.2	1.0	0.2	0.4	–	–	–	–
	NA[2]	11.7	13.5	15.8	16.3	12.7	17.1	14.8	17.6	11.7	15.3	16.8	18.9	0.3	0.174	<0.001	0.389
	Total[2]	13.9	15.4	19.1	19.0	14.1	20.1	16.8	19.3	13.9	17.9	19.0	21.5	0.4	0.427	<0.001	0.252
Crypt	Neu[2]	0.6	1.9	2.8	3.1	1.3	2.7	1.4	0.9	2.1	1.1	0.9	2.5	0.2	0.594	0.590	0.127
	Acid[2]	16.9	12.6	12.9	14.3	16.2	12.6	10.1	8.1	11.9	14.9	10.4	10.8	0.5	0.099	0.016	0.180
	NA[2]	26.5	43.7	37.1	38.8	32.3	43.4	40.0	37.5	31.1	38.2	37.3	42.1	0.9	0.730	<0.001	0.597
	Total[2]	44.0	58.3	52.8	56.2	49.8	58.7	51.5	46.5	45.1	54.1	48.6	55.4	1.1	0.752	0.010	0.414
		HID-AB staining															
Villus	Sulfo[2]	12.3	20.0	20.1	17.3	12.7	21.3	20.8	22.6	13.3	19.7	21.5	21.8	0.5	0.286	<0.001	0.789
	Sialo[2]	0.1	ND	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–
	SS[2]	0.7	0.1	0.2	0.1	0.4	0.1	ND	0.4	0.1	ND	0.5	0.1	–	–	–	–
Crypt	Sulfo[2]	12.9	34.4	12.6	11.3	21.0	24.2	20.7	12.7	17.5	18.5	14.3	11.5	1.4	0.473	0.007	0.333
	Sialo[2]	15.8	21.5	23.8	19.6	12.6	19.3	20.7	24.0	16.5	19.7	24.0	23.4	0.8	0.680	0.004	0.815
	SS[2]	14.2	17.0	16.6	14.5	15.5	12.8	14.6	14.7	13.1	15.2	15.7	23.8	0.7	0.287	0.302	0.101

[1] Ninety-six 26 d weaned purebred Landrace piglets were randomly allocated into 3 treatment groups with variation of 57, 164, and 2,425 mg Zn/kg diet from ZnO. Piglets (n = 8 per treatment group) were killed at 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d of age, respectively. The results were given as mean numbers of goblet cells containing different mucins per 1 mm basement membrane.

[2]Neu = neutral mucin; Acid = acidic mucin; NA = mixed neutral and acidic mucins; Total = total number of AB-PAS positive goblet cells; Sulfo = sulfomucin; Sialo = sialomucin; SS = mixed sulfo-sialomucins.

[3] ND = not detected.

Table 4.10: Numbers of AB-PAS and HID-AB positive goblet cells in the distal jejunum of weaned piglets[1]

		Dietary Zn concentration															
		57 mg Zn/kg				164 mg Zn/kg				2425 mg Zn/kg					*P*-value		
		33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	SEM	Diet	Age	Diet×Age
		AB-PAS staining															
Villus	Neu[2]	0.6	1.7	2.7	2.4	1.1	3.0	2.1	2.0	1.0	1.8	1.4	1.7	0.2	0.334	0.018	0.482
	Acid[2]	2.7	2.1	1.1	1.9	3.6	2.2	1.8	1.5	2.8	2.6	1.5	0.5	0.3	0.849	0.191	0.978
	NA[2]	13.4	16.6	14.9	14.4	11.9	15.5	15.4	18.1	13.1	14.4	13.4	19.6	0.6	0.953	0.060	0.581
	Total[2]	16.7	20.3	18.7	18.6	16.6	20.7	19.3	21.6	17.0	18.8	16.3	21.8	0.6	0.753	0.138	0.902
Crypt	Neu[2]	1.7	4.7	5.9	5.5	3.4	5.0	5.1	2.1	3.0	3.8	4.3	3.4	0.3	0.505	0.040	0.305
	Acid[2]	18.1	15.5	11.9	15.4	15.7	14.9	12.2	13.9	17.2	16.3	17.2	17.0	0.6	0.224	0.349	0.878
	NA[2]	34.3	35.4	41.6	35.0	36.4	36.2	39.4	39.3	29.0	37.2	35.0	34.1	0.9	0.164	0.173	0.672
	Total[2]	54.1	55.7	59.4	55.9	55.6	56.0	56.7	55.3	49.2	57.2	56.4	54.5	0.8	0.545	0.190	0.798
		HID-AB staining															
Villus	Sulfo[2]	15.2	22.4	19.7	17.1	18.3	20.6	19.1	20.3	14.3	16.4	15.6	20.0	0.6	0.144	0.150	0.495
	Sialo[2]	ND[3]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–
	SS[2]	0.6	ND	0.1	0.6	0.2	0.1	0.1	0.5	0.4	0.5	0.5	1.7	–	–	–	–
Crypt	Sulfo[2]	35.1	40.8	31.7	26.4	42.9	39.0	37.6	22.9	26.4	27.4	26.3	20.2	1.2	<0.001	<0.001	0.450
	Sialo[2]	10.2	9.2	16.2	17.8	10.6	10.2	13.4	21.5	13.8	19.0	17.6	19.6	0.8	0.032	<0.001	0.304
	SS[2]	13.1	15.4	16.8	16.0	13.9	16.5	14.6	17.3	16.0	16.4	18.5	16.8	0.4	0.166	0.078	0.570

[1]Ninety-six 26 d weaned purebred Landrace piglets were randomly allocated into 3 treatment groups with variation of 57, 164, and 2,425 mg Zn/kg diet from ZnO. Piglets (n = 8 per treatment group) were killed at 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d of age, respectively. The results were given as mean numbers of goblet cells containing different mucins per 1 mm basement membrane.

[2]Neu = neutral mucin; Acid = acidic mucin; NA = mixed neutral and acidic mucins; Total = total number of AB-PAS positive goblet cells; Sulfo = sulfomucin; Sialo = sialomucin; SS = mixed sulfo-sialomucins.

[3]ND = not detected.

Table 4.11: Phenotypes of intraepithelial lymphocytes in the distal jejunum of weaned piglets[1]

Lymphocytes, % of total IEL[2]	Dietary Zn concentration															
	57 mg Zn/kg				164 mg Zn/kg				2425 mg Zn/kg				SEM	*P*-value		
	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	33d	40 d	47 d	54 d		Diet	Age	Diet×Age
$CD3^+$	42.3	53.2	52.3	60.5	53.1	54.5	61.9	58.5	46.0	55.3	51.5	60.3	3.5	0.482	0.103	0.897
$CD2^+CD5^-$	47.4	41.7	43.2	44.1	37.2	38.2	38.4	46.9	47.7	38.6	52.3	43.1	3.6	0.458	0.692	0.750
$CD8^+$ γδ	16.7	16.7	15.2	13.6	19.2	14.5	13.6	15.4	11.4	10.4	10.4	10.1	1.6	0.015	0.604	0.962
$CD4^-CD8\beta^+$	16.5	27.4	36.2	42.9	28.2	31.8	42.8	39.1	26.4	42.3	34.5	45.0	3.1	0.235	0.001	0.403
$CD4^+CD8\beta^+$	6.7	5.5	4.7	3.5	7.2	4.1	4.2	3.8	6.2	4.2	5.4	4.6	0.7	0.925	0.035	0.888

[1]Ninety-six 26 d weaned purebred Landrace piglets were randomly allocated into 3 treatment groups with variation of 57, 164, and 2,425 mg Zn/kg diet from ZnO. Piglets (n = 8 per treatment group) were killed at 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d of age, respectively. The results were given as mean values.
[2]IEL = Intraepithelial lymphocytes.

Table 4.12: Relative gene expression of different MUC2 and 20, *ß*-defensin and TFF3 in the distal jejunum of weaned piglets[1]

	Dietary Zn concentration															
	57 mg Zn/kg				164 mg Zn/kg				2425 mg Zn/kg					*P*-value		
	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	33 d	40 d	47 d	54 d	SEM	Diet	Age	Diet×Age
MUC2[2]	0.77	1.00	0.73	0.58	0.60	0.95	1.60	1.16	0.79	1.27	1.21	0.94	0.39	0.336	0.315	0.657
MUC20[2]	1.05	1.16	0.92	0.47	0.69	1.07	0.69	1.02	0.91	0.80	0.62	0.57	0.28	0.616	0.442	0.651
β-defensin	1.52	1.95	2.17	2.05	0.68	2.17	1.53	3.49	1.05	0.59	0.60	0.32	0.51	0.022	0.543	0.345
TFF3[2]	0.88	1.32	1.57	0.59	0.89	1.27	1.80	1.36	0.65	1.39	1.10	0.80	2.34	0.332	0.049	0.766

[1]Ninety-six 26 d weaned purebred Landrace piglets were randomly allocated into 3 treatment groups with variation of 57, 164, and 2,425 mg Zn/kg diet from ZnO. Piglets (n = 8 per treatment group) were killed at 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d of age, respectively. The data were given as mean arbitrary values.
[2]MUC2 = mucin 2; MUC20 = mucin 20; TFF3 = trefoil factor 3.

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Chapter 5: Effect of Dietary Zinc Oxide on Morphological Characteristics, Mucin Composition and Gene Expression in the Colon of Weaned Piglets

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Abstract

The trace element zinc is often used in the diet of weaned piglets, as high doses have resulted in positive effects on intestinal health. However, the majority of previous studies evaluated zinc supplementations for a short period only and focused on the small intestine. The hypothesis of the present study was, that low, medium and high levels of dietary zinc (57, 164 and 2425 mg Zn/kg from zinc oxide) would affect colonic morphology and innate host defense mechanisms across 4 weeks post-weaning. Histological examinations were conducted regarding the colonic morphology and neutral, acidic, sialylated and sulphated mucins. The mRNA expression levels of mucin (MUC) 1, 2, 13, 20, toll-like receptor (TLR) 2, 4, interleukin (IL)-1β, 8, 10, interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were also measured. The colonic crypt area increased in an age-depending manner, and the greatest area was found with medium concentration of dietary zinc. With the high concentration of dietary zinc, the number of goblet cells containing mixed neutral-acidic mucins and total mucins increased. Sialomucin containing goblet cells increased age-dependently. The expression of MUC2 increased with age and reached the highest level at 47 days of age. The expression levels of TLR2 and 4 decreased with age. The mRNA expression of TLR4 and the pro-inflammatory cytokine IL-8 were down-regulated with high dietary zinc treatment, while piglets fed with medium dietary zinc had the highest expression. It is concluded that dietary zinc level had a clear impact on colonic morphology, mucin profiles and immunological traits in piglets after weaning. Those changes might support local defense mechanisms and affect colonic physiology and contribute to the reported reduction of post-weaning diarrhea.

5.1 Introduction

For reasons of health prophylaxis and growth-promoting purpose, so-called pharmacological levels of zinc oxide are often used in the feeding of weaning piglets. The beneficial effects of the trace element zinc on the prophylaxis and treatment of diarrhea are well documented both in pigs (Carlson et al., 2004; Fairbrother et al., 2005) and in other species including humans (Prasad, 2012). Zinc has been shown to have a positive impact

on daily gain in the post-weaning period of piglets when administered at so-called pharmacological concentrations (Sales, 2013). Zinc has multiple effects on physiological and pathophysiological processes, including the diversity and metabolic activity of the intestinal microbiota (Katouli et al., 1999; Hojberg et al., 2005; Vahjen et al., 2010, 2011; Starke et al., 2013), the gut associated immune system (Kreuzer et al., 2013) and absorptive and secretory processes (Carlson et al., 2004; Feng et al., 2006; Carlson et al., 2007).

Previous studies showed that high alimentary zinc intakes lead to significantly increasing concentrations of zinc in the digesta of the gastrointestinal tract, particularly in the colon. Pigs fed 2000 mg additional zinc as ZnO per kg complete diet were shown to have 600 mg zinc per kg digesta retained in the ileum, which enriched to 2141 mg zinc per kg digesta in the colon (Davin et al., 2012). Such high concentrations of zinc may induce a broad spectrum of reactions in the gut tissue. Despite well-established counter-regulatory mechanisms of the zinc transporters in the intestine in response to high dietary intakes (Martin et al., 2013a), there is a time-dependent accumulation of high quantities of zinc in a variety of tissues (Castellano et al., 2013; Martin et al., 2013b). The intestinal mucosa is of specific interest. It is the primary contact site between digesta and the host organism and might react in a specific manner. It is yet unclear if and to which extent elevated concentrations of zinc in the digesta induce morphological changes or affect inflammatory parameters in the colon of piglets. Pigs that have been fed diets with high zinc levels for different lengths of time might have different reaction patterns concerning pro-and anti-inflammatory cytokines, but this has not been studied in detail.

Therefore, the aim of the present study was to evaluate the impact of three concentrations of dietary zinc over four weeks after weaning on morphological parameters, mucin composition and gene expression related to innate immunity and inflammatory response in colonic tissue of young piglets.

5.2 Materials and Methods

This study involving pig handling and treatments was carried out in accordance with German animal welfare law. The protocol was approved by the State Office of Health and Social Affairs Berlin (LaGeSo Reg. Nr. 0347/09).

5.2.1 Animals, Housing and Diets

A total of 96 purebred Landrace piglets was weaned at the age of 26 ± 1 d (mean BW: 7.5 ± 1.2 kg) and randomly allocated into three groups balancing for litter and gender. Piglets were housed in commercial flat-deck pens (2 piglets per pen) with stainless steel framings. Room temperature was maintained at 26˚C on the day of weaning and reduced at regular intervals to achieve 22˚C one week post-weaning. The humidity was kept constant and the lightning program was maintained at 16 h light and 8 h dark per day. Water and feed were provided ad libitum. From 12 days of age, piglets were provided a non-medicated pre-starter diet. After weaning, piglets received a mash starter diet until 54 days of age. Piglets in each group received one of three experimental diets (**Table 5.9**) based on wheat, barley, and soybean meal. The analyzed zinc concentration in the basal diet was 35 mg/kg and the target zinc concentrations of the three diets were adjusted by replacing corn starch with analytical-grade zinc oxide (Sigma). The final zinc levels were adjusted to low (57mg/kg zinc), medium (164mg/kg zinc), and high (2425 mg/kg zinc) dietary zinc, respectively.

5.2.2 Sampling

Randomly selected piglets (n = 8 per dietary treatment and time point, respectively) were euthanized at 33 ± 1, 40 ± 1, 47 ± 1 and 54 ± 1 d of age balancing for litter and gender, resulting in a duration of dietary treatment of 1, 2, 3, and 4 weeks, respectively. Piglets were anesthetized with 20 mg/kg BW of ketamine hydrochloride (Ursotamin®, SerumwerkBernburg AG, Germany) and 2 mg/kg BW of azaperone (Stresnil®,

Jansen-Cilag, Neuss, Germany) prior to euthanization by intracardial injection of 10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany). The gastrointestinal tract was removed and the small intestine was separated from the large intestine and the mesentery. A 3 cm long segment of ascending colon was longitudinally cut along mesenteric attachment and rinsed with PBS buffer, and then the tissue was pinned on cork and fixed immediately in Bouin's solution for histological examinations. Another 5 cm segment of colonic tissue was snap-frozen in liquid nitrogen and stored at -80°C until mRNA extraction and gene expression analysis performed.

5.2.3 Histochemistry of the Colon

Samples from ascending colon were fixed in Bouin's solution for three days, and then rinsed several times in 70% ethanol to remove the remaining picric acid, dehydrated with a graded series of ethanol, and cleaned with xylol. The samples were embedded in paraffin wax according to standard protocols (Romeis, 1989). Five μm sections were cut from the paraffin blocks using a rotary microtome, and sections dried at 37°C in an incubator. Afterwards the slides with sections were deparaffinized with xylene, and rehydrated in a series of descending ethanol for subsequent staining procedures.

Different staining methods were applied in order to distinguish different mucin chemotypes characterized by the specific carbohydrates present on their surfaces. The Alcian blue pH 2.5-periodic acid Schiff (AB-PAS) (AB-8GX, Sigma; Schiff`s reagent, Merck, Darmstadt, Germany) staining procedure (Mowry, 1963) was carried out to distinguish neutral and acidic mucin in goblet cells. The neutral and acidic mucins were stained in magenta and blue colors, respectively. The mixture of neutral-acidic mucins showed purple, magenta-purple or blue-purple colors in goblet cells. To identify sialomucins and sulfomucins, tissues were stained with the high iron diamine-Alcian blue pH 2.5 (HID-AB) technique, including counterstaining with nuclear fast red (Spicer, 1965). As a result, sulfomucins were stained black, sialomucins were stained blue, and a mixture of sulfo-sialomucins resulted in black and blue colors. For the quantification of the cells

producing different mucin chemotypes, ten well-defined colonic crypts were randomly selected from different sections in each animal stained with AB-PAS and HID-AB methods, respectively. Goblet cells producing various mucins were classified and quantified in crypts. The corresponding basement membrane length in the colonic crypts was used as reference, and the number of the goblet cells with different mucins was expressed as quantity in per 1 mm basement membrane length.

The colonic tissues stained with AB-PAS were further used for the morphological measurements. Crypt depth and area were measured in ten well-orientated crypts in each animal. Crypt depth was measured as the distance from the crypt base at the basement membrane to the crypt mouth. The area was determined on the same crypts as the area encircled by the basement membrane and crypt mouth including the crypt lumen (Brunsgaard, 1998). All measurements were carried out using a light microscope (Zeiss Photomicroscope III, Oberkochen, Germany) connected with a digital camera (Olympus DP72, Japan) and analyzed for morphometric characteristics with cellSens standard software (Olympus, version 1.4).

5.2.4 RNA Extraction and Gene Expression in Colon by Real-Time PCR

RNA extraction and analysis of colonic tissue gene expression were accomplished as described (Martin et al., 2012). The quality and quantity of mRNA was determined with a Bioanalyzer (Agilent 2100, Waldbronn, Germany). The expression of the following target genes was analyzed: mucin (MUC) 1, 2 13, 20, toll-like receptor (TLR) 2, 4, interleukin (IL)-1β, 8, 10, interferon-γ (IFN-γ), transforming growth factor-β (TGF-β). The 60S ribosomal protein of L19 (RPL19), RPL13 and *β*-2-microglobulin were used as housekeeping genes for data normalization (**Table 5.10**). The Ct values were normalized using the mean values of the housekeeping genes and arbitrary values were calculated and used for statistical comparisons. Melting curves and PCR efficiency were used as standard quality criteria for each RT-PCR run.

5.2.5 Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (Chicago, IL, USA). The data on the colonic morphology, mucin chemotypes in goblet cells and gene expression related to innate immunity and inflammatory cytokines were tested for normal distribution by Shapiro Wilk test. They were analyzed using two-way analysis of variance (ANOVA) followed by Tukey post hoc test with diet and age as fixed factors, and data were given as mean values. Differences were considered significant at $P < 0.05$.

5.3 Results

5.3.1 Morphometric Measurements

The colonic crypt depth was not affected by the dietary zinc concentration or age (**Table 5.11**), whereas the crypt area increased age-dependently ($P = 0.001$). The highest values were observed in the medium dietary zinc group ($P = 0.003$).

5.3.2 Mucin Chemotypes in Goblet Cells

With AB-PAS staining, goblet cells with neutral mucins were found to be spread close to the epithelial surface and in the upper crypt, while acidic mucins dominated in the lower crypt area of the colon. The mixture of neutral-acidic mucins was mainly found along the crypt (**Figure 5.7**). HID-positive cells with sulfomucins dominated in the epithelial surface and the upper crypt, whereas goblet cells with sialomucins and mixed sulfo-sialomucins were located in the lower crypt of the colon (**Figure 5.8**).

The chemotypes of colonic mucins changed mainly depending on zinc intake, but also on age (**Table 5.12**). High dietary zinc increased the amount of mixed neutral-acidic mucins ($P = 0.019$) and the total number of mucin producing goblet cells ($P = 0.001$). In addition, the older piglets had more goblet cells containing sialomucins ($P = 0.043$). There was no age or diet related effect on sulfomucins and mixed sulfo-sialomucins.

5.3.3 mRNA Expression in the Colonic Tissue

The genes related to innate immunity and inflammatory cytokines were influenced by the dietary zinc concentration and age (**Table 5.13**). The expression of MUC2 increased after 40 days of age, having the highest level at 47days of age (P = 0.040). The mRNA level of TLR2 decreased until 47 days of age, and increased in the fourth week post-weaning (P = 0.031). The mRNA level of TLR4 was affected by age and dietary zinc concentration. The expression of TLR4 was down-regulated with age (P = 0.002), while its mRNA level was higher with medium dietary zinc treatment compared to the low dietary zinc group (P = 0.012). Moreover, an obvious dietary effect was found on the expression of IL-8 (P = 0.015). Piglets fed high dietary zinc treatment had the lowest level of IL-8 compared to those fed low and medium zinc supplementations, and the medium dietary zinc group had the highest expression.

No interaction was observed between age of piglets and zinc concentration on parameters related to morphometric characteristics, mucin composition and gene expression.

5.4 Discussion

The influence of age and dietary zinc level on the morphology, composition of mucin and immunological traits in the colon of weaned piglets has not been fully elucidated. The present study shows that three different dietary zinc levels, covering a broad range from marginally low to pharmacologically high concentrations, have led to significant effects on various parameters in colonic tissue. All diets contained enough zinc to cover the recommended dietary levels (NRC, 2012). Feed intake, feed conversion and growth rate of the piglets were not affected by the different dietary zinc concentrations in the first two weeks of the experiment, however, weight development of the high dietary zinc group were reduced after three weeks of the experiment (Martin et al., 2013b). Amongst dietary effects, a substantial part of the observed changes in the measurements was also age-dependent. Interestingly, in addition to the morphometric measurements that were

affected by dietary zinc concentration and age of piglets, there were also significant effects regarding the expression of biomarkers relevant for the innate immune system and inflammatory response.

Crypt depth was affected neither by the dietary zinc intake nor age, but the crypt area increased between 33 and 54 days of age. Interestingly, the highest dimensions were obtained with the medium dietary zinc group. So far, only few data are available on the morphology of the colon in pigs in response to dietary factors. The lack of dietary effects on the crypt depth may be partly explained by the fact that even the low dietary zinc diet (57 mg/kg diet) was sufficient for the constant renewal of the colonic crypts. Generally, the lowest dosage of zinc in this study practically corresponds to a marginal-adequate supply situation, according to NRC recommendation (NRC, 2012). Due to the expected high priority of nutrient partition for intestinal cell growth and renewal, it can be assumed that the proliferative activity of colonocytes was maintained even at suboptimal supply situations. In rats, it was shown that very low dietary zinc intakes induced a lower proliferative activity of mucosal epithelial cells (Southon et al., 1985) and colonocytes (Lawson et al., 1988). Regarding the crypt area, an age effect became apparent, older animals having greater crypt areas. This could probably be explained by the growth in body size and ongoing maturation of the intestinal tract during the post-weaning period. The highest surface areas were observed in the medium dietary zinc group, while the high dietary zinc intake resulted in the lowest surface area and the low concentration of dietary zinc resulted in intermediate values. This suggests that there was a relevant physiological effect on the tissue structure between the low and medium dietary zinc concentration, while the high dietary zinc treatment did not lead to further reactions. This could, in view of the high zinc content in the digesta, indicate that very high zinc supplement in the diet lead to a reversal effect on cell growth in the colon. With regard to the morphological data of the colonic tissue, there is relatively little comparative information on the impact of zinc or other trace elements in pigs. High dietary zinc intakes were shown to have no significant effect on crypt depth in the large intestine of pigs (Hedemann et al., 2006), confirming our data. Other dietary factors, such as protein intake (Dobesh and Clemens, 1987), hydrolyzed straw meal (Munchow and Berg, 1989), butyrate (Mentschel and Claus, 2003),

fructooligosaccharides (Tsukahara et al., 2003), potato protein, and cellulose (Swiech et al., 2010) were shown to increase crypt depth in the colon of pigs. A study focusing on the effects of different fractions of grain kernels found only minor changes on crypt depth in the large intestine of pigs across treatments (Glitso et al., 1998). Spray dried porcine plasma had an impact on immune cells in the tissue, however, it did not affect colonic crypt depth (Nofrarias et al., 2006). Coarse ground corn, sugar beet pulp and wheat bran also had no impact on this parameter (Anguita et al., 2007). The data on crypt depth in the referenced studies were similar to data in the present study. If and how the colonic surface area can affect the absorptive capacity of the large intestine for electrolytes and water (Dobesh and Clemens, 1987), needs further characterization in piglets.

Mucins are an important protective barrier within the gastrointestinal tract. The mucin layer safeguards the intestine and its structural integrity. A considerable part of the formed mucins is secreted into the intestinal tract and used as nutrient source for the resident microbial communities (Barnett et al., 2012). Furthermore, the intestinal lining serves as binding site for various gut wall-associated microorganisms, namely lactobacilli (Rojas and Conway, 1996; Roos and Jonsson, 2002) but also pathogens such as salmonellae (Hedemann et al., 2005). Mucins can be broadly categorized into neutral and acidic chemotypes, and the latter are further divided into sialomucins and sulfomucins based on the presence of the respective terminal acids in the oligosaccharide chain (Filipe, 1979). It has been well documented that sulfomucin abundance was greater than sialomucin production in colonic mucosa, both in humans (Croix et al., 2011) and pigs (Brown et al., 1988). This staining characteristic was also observed in the present study. The goblet cells with sulfomucins were mainly distributed in the upper crypt and surface epithelium, whereas sialomucins were presented in the lower crypt area of the ascending colon. Reverse distributions have been shown in humans (Croix et al., 2011). This may be due to species differences, but also due to age effects and the studied segment of the large intestine. Moreover, the distribution of neutral, acidic and mixed neutral-acidic mucins was similar in a previous study in pigs (Brown et al., 1988). Mucin chemotypes in the colonic goblet cells were affected by both, age and zinc intake in our study. Older piglets had a higher density of sialomucin producing goblet cells, indicating a qualitative maturation

process in the 4-week experimental period. Therefore, age seems to have a strong impact on mucin composition in piglets. The colonic mucins are highly sulfated and sialylated in newly born pigs (Turck et al., 1993). In the colon, no major changes of mucin chemotypes occurred in piglets 1-13 d after weaning on a soy based diet (Brown et al., 1988). High dietary zinc treatment increased the amount of neutral-acidic mucins and the total number of mucin containing goblet cells in this study. This confirms previous studies, where high dietary zinc enlarged the total area of mucin staining in the cecum and in the colon of young piglets (Hedemann et al., 2006). Speculatively, this might result in a better protection against diarrhea and pathogen invasion in the post-weaning period of piglets. It also has been shown that several other feed ingredients influenced the formation and composition of mucins in the colonic mucosa (Hedemann et al., 2009). In particular, physical factors of the diet are considered as important. A coarser particle structure and a change in the viscosity with diets containing carboxymethylcellulose changed the number of goblet cells and mucins and these effects were considered as indicators of gut maturation (Hedemann et al., 2005; Piel et al., 2005).

The interaction between age and dietary factors and the expression of various mucin types are considered increasingly relevant, as mucins are important as first barrier against invading bacteria. In the present study it was shown that the expression of the MUC2 gene increased age-dependently. Apparently, no effect was caused by the varying zinc intake. MUC2 is mainly responsible for the formation of the gel properties of the intestinal mucus (Johansson et al., 2011). A lack of mucin 2 or changes in the tissue specific glycosylation lead to a predisposition of diseases in humans such as colitis and colon cancer (Corfield et al., 2001; Kawashima, 2012). A fault in the corresponding mucus formation and also a change in the glycosylation of mucins can lead to disorders of colonic function and health impairment. Zinc supply is related to mucin formation, as MUC2 expression is regulated by the zinc-finger GATA-4 transcription factor in intestinal cells (van der Sluis et al., 2004) and ZIP4 knockout mice exhibited abnormalities in mucin accumulation in *Paneth* cells (Geiser et al., 2012). Obviously, all diets in the present study provided sufficient zinc for the MUC2 gene expression. In pigs, preterm conditions have been shown to impair MUC2 synthesis, predisposing young piglets to develop necrotizing enterocolitis (Puiman et al.,

2011). Colonic mucin gene expression was influenced by the inclusion of laminarin in the diet (Smith et al., 2011) while there was no effect of low digestible dietary protein and fermentable carbohydrates (Pieper et al., 2012). MUC13 belongs to the most abundant MUC genes in the gastrointestinal tract. Piglets fed a diet with faba beans had a higher MUC13 expression in the intestinal segments perfused with a faba bean suspension (Jansman et al., 2012). MUC1 and MUC20 are as well important for the intestinal mucus formation, however, also here only few studies determined the effects of nutritional factors on mucin gene expression in the intestinal tract of pigs. MUC1 is a large transmembrane glycoprotein expressed on the apical surface of the majority of reproductive tract epithelia (Gendler and Spicer, 1995), whereas the porcine MUC20 gene is associated with susceptibility to enterotoxigenic *Escherichia coli* F4ab/ac (Ji et al., 2011).

The luminal mucin forms the first barrier against pathogen invasion, the epithelium and the lamina propria are the second line of host defense. They sense the invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), and TLRs are one group of PRRs in innate immunity. TLRs can activate a common signaling pathway leading to the activation of mitogen-activated protein kinase and nuclear translocation of transcription factor NF-κB, which activate immune cell response and lead to production of inflammatory cytokines and co-stimulatory molecules (Moncada et al., 2003b). There are 13 known members of mammalian TLRs. TLR2 and 4 are expressed in various lymphoid tissues of the porcine intestinal tract and play an important role in innate immunity in the young pigs (Tohno et al., 2005; Tohno et al., 2006). However, only limited data showed that dietary factors, such as yeast extracts (Badia et al., 2012) and a fish oil supplement (Liu et al., 2012) reduced the expressions of TLR2 and TLR4 in pig intestine. In the present study, compared to medium dietary zinc supplementation, sub-optimal levels of dietary zinc treatments down-regulated the expression level of TLR4. Moreover, we also found that the expressions of TLR2 and TLR4 were down-regulated in our piglets with age, which is in accordance with previous findings indicating that TLRs were regulated by age of pigs (Bering et al., 2012; Uddin et al., 2013). Weaning induces transient gut inflammation in piglets (Lalles et al., 2007) and the expression levels of some pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α

were up-regulated in newly weaned piglets (Pie et al., 2004). Over-production of pro-inflammatory cytokines usually results in impaired intestinal integrity and epithelial function (McKay and Baird, 1999). Controlling the expression level of pro-inflammatory cytokines may have potential benefits in alleviating gut mucosal inflammation and reducing the incidence of diarrhea (Liu et al., 2008). Zinc has been well documented to show a beneficial role on inflammatory lesions (Dreno et al., 2001). In some recent studies, mRNA levels of TNF-α, IL-6 and IFN-γ decreased with increasing concentrations of dietary zinc in pigs (Hu et al., 2012; Hu et al., 2013b). IL-8 is a signal protein, which is essential for neutrophil recruitment and seems to be of importance in establishing protective immunity (Kelly and Conway, 2005). Normally barely detectable in healthy tissues, it is rapidly induced by 10 to100-fold in response to pro-inflammatory cytokines and the variation of expression is one of the remarkable properties of IL-8. *In vitro*, zinc oxide counteracted the expression of the inflammatory IL-8 level caused by enterotoxigenic *Escherichia coli* (Roselli et al., 2003). Accordingly, we also found that the mRNA level of IL-8 was down-regulated with the high concentration of dietary zinc treatment. TGF-β and IL-10 are anti-inflammatory cytokines and can protect the intestinal barrier function. The mRNA levels of TGF-β and IL-10 were increased with high zinc oxide supported on zeolite on day 7 post-weaning (Hu et al., 2013b). However, weaning induced transient increase of inflammatory cytokines for 2 days, then most of cytokines rapidly decreased to the pre-weaning levels after day 9 post-weaning (Pie et al., 2004).

5.5 Conclusions

The present study revealed that high levels of dietary zinc oxide had specific effects on the colonic morphology, mucin composition and expression of genes related to innate immunity and inflammatory processes in weaned piglets. The findings suggest a positive impact on the maturation of the barrier function of the colonic mucosa, displayed by an increase in mucin producing goblet cells and a down-regulation of IL-8 and TLR-4. The observed changes suggest that high dietary zinc dosages stimulate protective mechanisms in colonic function which may help to understand the protective mode of action of very high dietary levels of zinc oxide against the commonly occurring post-weaning diarrhea in

piglets.

5.6 Acknowledgements

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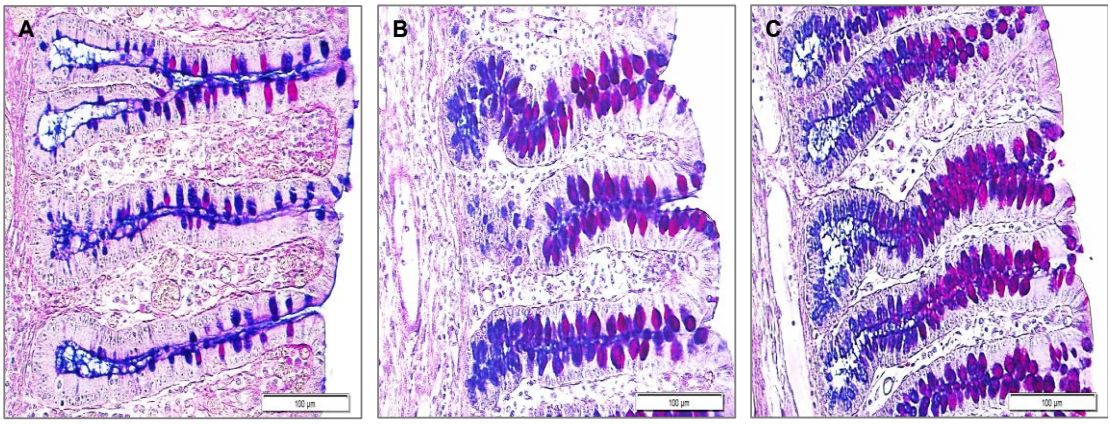

Figure 5.7: Alcian blue (pH 2.5)-periodic acid schiff stained section in the ascending colon of weaned piglets. Mucin distribution and characteristics with three concentrations of dietary zinc treatments on 33 days of age in piglets. A. Low dietary zinc treatment (57 mg/kg zinc); B. Medium dietary zinc treatment (164 mg/kg zinc); C. High dietary zinc treatment (2425 mg/kg zinc), magnification X160. Neutral mucins (magenta) were found to be spread over the epithelial surface and the upper crypt, while acidic mucins (blue) dominated in the lower crypt area of the colon. The mixture of neutral-acidic mucins (magenta-purple or blue-purple colors) was mainly found along the crypt.

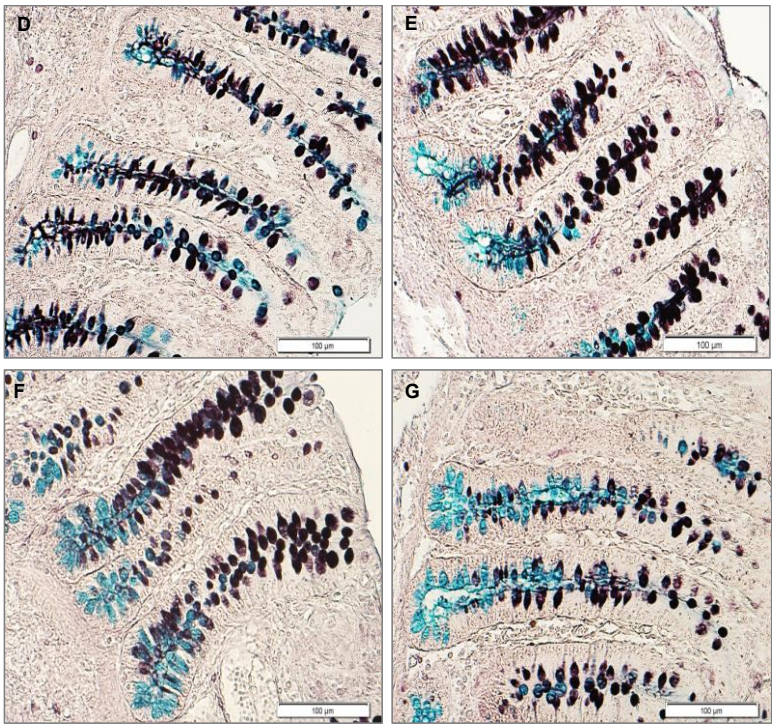

Figure 5.8: High iron diamine-Alcian blue (pH 2.5) stained section in the ascending colon of weaned piglets. Acidic mucins distribution and characteristics over 4 weeks post-weaning in piglets fed with medium dietary zinc treatment (164 mg/kg zinc). D. At 33 d of age; E. At 40 d of age; F. At 47 d of age; G. At 54 d of age; magnification X160. Secreting goblet cells with sulfomucins (black) dominated in the epithelial surface and the upper crypt, whereas goblet cells with sialomucins (blue) and mixed sulfo-sialomucins (black and blue colors) were located in the lower crypt of the colon.

Table 5.9: Ingredients and analyzed chemical composition of diets

Ingredients (g/kg as feed)		Analyzed chemical composition	
Wheat	380	Dry matter (g/kg fresh matter)	879
Barley	300	Crude ash (g/kg DM)	81
Soybean meal	232	Crudeprotein (g/kg DM)	194
Corn starch/ zinc oxide[1]	10	Crudefiber (g/kg DM)	36
Limestone	20	Ether extract (g/kg DM)	34
Monocalcium phosphate	20	Starch (g/kg DM)	376
Mineral & Vitamin Premix[2]	15	Lysine (g/kg DM)	11.7
Soy oil	17.5	Methionine (g/kg DM)	4.0
Salt	2.0	Threonine (g/kg DM)	7.2
Lysine HCl	2.5	Tryptophan (g/kg DM)	2.4
Methionine	1.0	Calcium (g/kg DM)	11.0
		Phosphorus (g/kg DM)	8.0
		Sodium (g/kg DM)	3.1
		Magnesium (g/kg DM)	2.2
		Zinc[3] (mg/kg DM)	34
		Iron (mg/kg DM)	309
		Manganese (mg/kg DM)	41
		Copper (mg/kg DM)	7
		Metabolisable energy (MJ/kg)	13.0

[1]Corn starch in the basal diet was partially replaced with analytical grade zinc oxide (Sigma Aldrich, Taufkirchen, Germany) to adjust for the zinc concentration.
[2]Mineral and Vitamin Premix (SpezialfutterNeuruppin Ltd., Neuruppin, Germany), providing per kg feed: 1.95 g Na (sodium chloride), 0.83 g Mg (magnesium oxide), 10,500 IU vitamin A, 1,800 IU vitamin D3, 120 mg vitamin E, 4.5 mg Vitamin K_3, 3.75 mg thiamine, 3.75 mg riboflavin, 6.0 mg pyridoxine, 30 µg cobalamine, 37.5 mg nicotinic acid, 1.5 mg folic acid, 375 µg biotin, 15 mg pantothenic acid, 1,200 mg choline chloride, 75 mg Fe (iron-(II)-carbonate), 15 mg Cu (copper-(II)-sulfate), 90 mg Mn (manganese-(II)-oxide), 675 µg I (calcium-iodate), 525 µg Se (sodium-selenite).
[3]Analyzed concentration of zinc in the basal diet without ZnO supplementation. The diets as fed contained 57, 164, and 2425 mg zinc/kg, adjusted by zinc oxide.

Table 5.10: PCR primers used for gene expression analysis

Target	Sequences of primers (5` to 3`)	A_T	Reference
RPL19	GCTTGCCTCCAGTGTCCTC	60	Pieper et al., 2012
	GCGTTGGCGATTTCATTAG		
RPL13	CCGTCTCAAGGTGTTCGATG	60	This study
	GGATCTTGGCCTTCTCCTTC		
B2M	CCCCGAAGGTTCAGGTTTAC	60	Martin et al., 2013
	CGGCAGCTATACTGATCCAC		
MUC1	GGTACCCGGCTGGGGCATTG	60	Pieper et al., 2012
	GGTAGGCATCCCGGGTCGGA		
MUC2	CTGCTCCGGGTCCTGTGGGA	60	Pieper et al., 2012
	CCCGCTGGCTGGTGCGATAC		
MUC13	GCTACAGTGGAGTTGGCTGT	60	This study
	GACGAATGCAATCACCAGGC		
MUC20	GAAGGGGGCATCGCTGCCTG	60	Pieper et al., 2012
	GCCAGGGTCCCACTGCCATG		
TLR2	CACGTGCTGATGGAGGGGCAT	60	This study
	GCCCAATGACGCCTCGGTGAT		
TLR4	AGGCCGTCATTAGTGCGTCAGT	60	This study
	AGCCCACAAAAAGCAACAAGTGGA		
IL-1β	TGAAGTGCCGCACCCAAAACCT	60	Pieper et al., 2012
	CGGCTCCTCCTTTGCCACAATCA		
IL-8	GGTCTGCCTGGACCCCAAGGAA	60	This study
	TGGGAGCCACGGAGAATGGGTT		
IL-10	GTCCGACTCAACGAAGAAGG	60	Pieper et al., 2012
	GCCAGGAAGATCAGGCAATA		
IFN-γ	TCCAGCGCAAAGCCATCAGTG	58	This study
	ATGCTCTCTGGCCTTGGAACATAGT		
TGF-β	AGAAGCAGAGGGTGGGAAAT	60	Pieper et al., 2012
	AAGAAGGCGAGAGGAGGAAC		

A_T: annealing temperature; RPL19: 60S ribosomal protein L19; RPL13: 60S ribosomal protein L13; B2M: beta-2 microglobulin; MUC1: mucin 1; MUC2: mucin 2; MUC13: mucin 13; MUC20: mucin 20; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; IL-1β: interleukin 1β; IL-8: interleukin 8; IL-10: interleukin 10; IFN-γ: interferon-γ; TGF-β: transforming growth factor-β.

Table 5.11: Morphometric characteristics in the ascending colon of weaned piglets[1]

Age	33d			40d			47d			54d				*P*-value		
Diet	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	SEM	Age	Diet	Age×Diet
CD (μm)	338	341	320	357	347	344	361	362	335	358	379	347	4.01	0.102	0.089	0.927
CA (μm^2)	18267^{ab}	19892^{ab}	16609^{b}	20577^{ab}	18242^{ab}	18152^{ab}	20700^{ab}	22270^{a}	19317^{ab}	20672^{ab}	21811^{a}	19729^{ab}	252	0.001	0.003	0.255

[1]Ninety-six 26 day weaned Landrace piglets were randomly allocated into three diets with low, medium and high dietary zinc (57, 164, and 2425 mg/kg diet from ZnO). Eight piglets in each group were killed at the age of 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d. Data were analyzed by two-way ANOVA followed by Tukey post hoc test. The data were given as mean values.

LZn: low dietary zinc; MZn: medium dietary zinc; HZn: high dietary zinc; CD: crypt depth; CA: crypt area.

[ab]Means with different superscripts within a row indicate significant differences between groups ($P < 0.05$).

Table 5.12: Numbers of AB-PAS and HID-AB positive goblet cells per 1 mm basement membrane in crypts in the ascending colon of weaned piglets[1]

Age	33d			40d			47d			54d				*P*-value		
Diet	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	SEM	Age	Diet	Age×Diet
AB-PAS staining																
Neu	4.8	3.1	3.0	2.6	3.1	2.8	4.7	2.8	3.7	3.1	3.4	4.3	0.24	0.535	0.490	0.444
Acid	22.1	20.9	24.0	20.8	21.1	23.1	22.0	24.7	26.5	21.9	28.0	25.4	0.64	0.196	0.155	0.725
NA	55.2	58.2	65.8	50.1	57.3	62.5	53.5	54.3	68.1	60.1	61.0	55.2	1.18	0.823	0.019	0.176
Total	62.1^{ab}	61.0^{ab}	68.9^{a}	55.3^{b}	60.8^{ab}	65.7^{ab}	60.8^{ab}	62.1^{ab}	73.6^{a}	63.2^{ab}	70.1^{a}	64.9^{ab}	0.82	0.088	0.001	0.099
HID-AB staining																
Sulfo	41.1	50.1	51.4	41.7	43.4	43.8	45.7	48.5	52.3	40.0	43.7	45.2	1.37	0.296	0.183	0.983
Sialo	3.6^{ab}	1.1^{b}	4.6^{ab}	4.4^{ab}	3.7^{ab}	3.8^{ab}	5.4^{ab}	3.6^{ab}	3.5^{ab}	7.2^{a}	4.9^{ab}	6.0^{ab}	0.37	0.043	0.130	0.813
Mixed	18.2	15.5	14.2	12.4	13.5	16.2	12.4	12.8	10.0	20.2	15.8	15.4	0.78	0.087	0.617	0.717

[1]Ninety-six 26 day weaned Landrace piglets were randomly allocated into three diets with low, medium and high dietary zinc (57, 164, and 2425 mg/kg diet from ZnO). Eight piglets in each group were killed at the age of 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d. Data were analyzed by two-way ANOVA followed by Tukey post hoc test. The data were given as mean values.

LZn: low dietary zinc; MZn: medium dietary zinc; HZn: high dietary zinc; AB-PAS: Alcian blue pH 2.5-periodic acid Schiff staining; HID-AB: high iron diamine-Alcian blue pH 2.5 staining; Neu: neutral mucin; Acid: acidic mucin; NA: mixed neutral and acidic mucins; Total: total number of AB-PAS positive goblet cells; Sulfo: sulfomucin; Sialo: sialomucin; Mixed: mixed sulfo-sialomucins.

[ab]Means with different superscripts within a row indicate significant differences between groups ($P < 0.05$).

Table 5.13: Relative gene expression (arbitrary values) of mucins and cytokines in the ascending colon of weaned piglets[1]

Age	33d			40d			47d			54d				*P*-value		
Diet	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	LZn	MZn	HZn	SEM	Age	Diet	Age×Diet
MUC1	1.07	1.17	1.20	1.36	1.29	1.16	1.22	1.29	1.30	0.82	0.90	1.08	0.07	0.351	0.923	0.985
MUC2	0.55	0.49	0.61	0.56	0.36	0.59	0.93	0.71	0.73	0.83	0.78	0.74	0.04	0.040	0.459	0.946
MUC13	0.73	0.87	0.73	1.29	0.92	0.93	0.96	1.04	0.50	0.69	0.87	0.74	0.05	0.261	0.229	0.528
MUC20	0.74	1.37	1.89	1.43	0.75	1.39	1.31	1.18	0.58	1.11	1.13	0.90	0.11	0.732	0.954	0.196
TLR2	0.72	1.23	1.05	0.80	1.12	1.02	0.76	0.76	0.48	0.68	0.75	0.67	0.05	0.031	0.174	0.613
TLR4	0.89^{a}	1.71^{ab}	1.43^{ab}	0.69^{ab}	1.49^{ab}	1.35^{ab}	0.77^{ab}	0.99^{ab}	0.69^{ab}	0.69^{ab}	0.83^{ab}	0.63^{b}	0.07	0.002	0.012	0.500
IL-1β	0.30	1.18	1.62	0.64	0.67	0.50	0.81	2.00	0.81	2.03	3.32	0.64	0.28	0.345	0.327	0.690
IL-8	0.64	1.29	0.72	0.99	1.40	0.83	1.53	0.93	0.51	0.63	1.01	0.61	0.07	0.354	0.015	0.161
IL-10	0.47	1.76	1.46	0.79	1.39	1.98	1.82	1.75	1.31	1.89	2.31	0.78	0.20	0.845	0.483	0.553
IFN-γ	0.29	2.56	1.17	0.83	1.36	0.57	1.96	0.94	0.91	0.78	1.54	0.63	0.16	0.750	0.117	0.241
TGF-β	1.53	2.36	3.59	1.11	2.16	3.01	1.72	1.58	2.11	2.36	2.38	1.91	0.21	0.697	0.169	0.607

[1]Ninety-six 26 day weaned Landrace piglets were randomly allocated into three diets with low, medium and high dietary zinc (57, 164, and 2425 mg/kg diet from ZnO). Eight piglets in each dietary diet were killed at age of 33 ± 1, 40 ± 1, 47±1 and 54 ± 1 d. Data were performed by two-way ANOVA followed by Tukey post hoc test. The data were given as mean values in each group.

LZn: low dietary zinc; MZn: medium dietary zinc; HZn: high dietary zinc; MUC1: mucin 1; MUC2: mucin 2; MUC13: mucin 13; MUC20: mucin 20; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; IL-1β: interleukin 1β; IL-8: interleukin 8; IL-10: interleukin 10; IFN-γ: interferon-γ; TGF-β: transforming growth factor-β.

[ab]Means with different superscripts within a row indicate significant differences between groups ($P < 0.05$).

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Chapter 6: Discussion

Weaning forces piglets to adapt to stress factors (Kim et al., 2012). Zinc is an essential nutrient for various physiological processes and has been found to be a structural component of more than 300 enzymes (McCall et al., 2000). The recommendation for dietary zinc is 80 – 100 mg Zn/kg feed for weaned piglets (GfE, 2006). Swine can tolerate a high amount of zinc oxide, pharmacological levels from 2000 to 4000 mg Zn/kg diet from zinc oxide did not induce symptoms of zinc toxicity (Hahn and Baker, 1993; Kim et al., 2012). A reason might be that zinc oxide is less soluble and therefore less toxic than other inorganic zinc supplements (Hill and Miller, 1983; Hahn and Baker, 1993).

In addition, several studies showed that high concentrations of dietary zinc oxide decreased prevalence of diarrhea and improved growth performance in newly weaned piglets (Jensen-Waern et al., 1998; Huang et al., 1999; Sales, 2013). Therefore, high concentrations of dietary zinc are often considered as “pharmacological” supplementation, and are widely used in pig industry to prevent or overcome physiological disturbances in young pigs. However, the underlying mechanisms for the beneficial mode of action of such high zinc levels are yet not entirely clear. In this thesis, the effects of three levels of dietary zinc oxide from marginal to high concentrations in weaned piglets for a time period of four weeks were investigated. The study focused on morphological characteristics, mucin chemotypes, number of goblet cells, phenotyping of intraepithelial lymphocytes and gene expression related to innate and adaptive immunity in the host intestinal mucosa.

6.1 Effect of Dietary Zinc Oxide on Morphological Characteristics in the Intestine

The intestinal mucosal morphology can be characterized by villus height and crypt depth, which are considered as indicators of the maturity and functional capacity of enterocytes (Hampson, 1986). A large surface area with functional maturity of enterocytes is important for young growing pigs, in that case they may attain maximum digestive and absorptive

capacity (Cera et al., 1988). It has been well documented that weaning causes villus atrophy and crypt hyperplasia in the small intestine of pigs (Hampson, 1986; Hedemann et al., 2003). Shorter villus height and increased crypt depth suggest a severe deterioration of intestinal morphology during weaning (Hampson, 1986; Deprez et al., 1987). It was also demonstrated that the hindgut of pigs undergoes morphological changes around the time of weaning, including an increase in crypt size and colonocyte proliferative activity (Brunsgaard, 1997). The majority of previous studies showed that diet has a minor effect on intestinal morphology of young animals after weaning (Cera et al., 1988; Mavromichalis et al., 2000). However, piglets supplied with diosmectite-zinc oxide at 500 mg Zn/kg showed increased villus height of the jejunal mucosa compared to the control group (Hu et al., 2012). High levels of dietary zinc reduced the weaning-associated impairment of the small intestine and decreased alteration of mucosal morphology (Li et al., 2001).

In the present study, there was no effect of dietary zinc on jejunal morphology of piglets. However, the colonic crypt area was significantly affected by dietary zinc concentrations, and the greatest area was observed in the pigs fed with the medium level of dietary zinc. Normally, the change in crypt area was mainly related to the crypt depth (Brunsgaard, 1998) because an increased crypt depth indicates improved proliferative activity. However, no effect by diet was found on the colonic crypt depth. Increased colonic crypt area may suggest a potential for improved absorptive capacity of electrolytes and water. A previous study reported that extracellular zinc enhanced survival of HT29 colonocytes (Cohen et al., 2012). Whether an increased colonic crypt area was due to a regulatory role on intestinal microbes such as coliforms or enterococci (Hojberg et al., 2005) or a direct effect of high intestinal zinc concentration is not clear. Pigs fed the highest concentration of dietary zinc showed a lower colonic crypt area compared to the low and medium levels. The finding confirms a previous study in pigs fed 100 mg zinc/kg of diet possessing longer villi in the small intestine than the piglets fed with 2500 mg zinc/kg in the diet (Hedemann et al., 2006).

Another interesting change of intestinal mucosal morphology was time-dependent after the

weaning of the piglets. In the jejunum, villus height, crypt depth and the ratio of villus height to crypt depth were significantly higher in the third week post-weaning compared to the first two weeks. Similarly, the colonic crypt area was also greater in the third week post-weaning than in the first two weeks. In the small intestine, nutrients, electrolytes and water are absorbed by villus enterocytes, while electrolytes and water are secreted in crypt cells (Powell, 1987). Changes in villus height and crypt depth are relevant for the number of villus enterocytes and crypt cells, respectively (Hampson, 1986; Nabuurs et al., 1993). Shorter villi and deeper crypts may indicate poor absorption and increased secretion. After the acute post-weaning response, villus height was increased. Crypt cells migrate upward and become villus cells (Lipkin, 1973). The temporal changes in the gut by weaning include an early acute response for 1-5 days after weaning, followed by an adaptive phase from day 5 to day 15. In the proximal jejunum of piglets weaned at 21 days villus height decreased by 40% from day 0 to 2 after weaning. Afterwards, villus height increased, but it was 77% lower at day 15 compared to the values at day 0 (Montagne et al., 2007). In the same segment, the crypt depth shortened from day 2 onward, and returned to 64% height value at day 15 compared with pre-weaning level. In piglets weaned at 21 or 35 days of age, the jejunal villus height was reduced dramatically within 3 d post-weaning, and this change continued until 7 d after weaning (Cera et al., 1988). The ratio of villus height to crypt depth declined within 1 to 3 d post-weaning in pigs and returned to pre-weaning value by d 11 to 12 (Kenworthy, 1976). In the present study, the results showed jejunal villus height increased up to the third week post-weaning, and no obvious changes were observed between the 3rd wk and 4th wk after weaning.

6.2 Effect of Dietary Zinc Oxide on Mucin Distribution

Mucins are the major component of mucus, mainly secreted from goblet cells. They are high molecular weight glycoproteins with different polymeric, viscoelastic and protective properties (Dharmani et al., 2009). The oligosaccharide sugar moieties determine chemical properties and allow to detect different mucin types (Kiernan, 1990). In the present study, AB-PAS and HID-AB staining methods were used to differentiate neutral from acidic sialylated and sulphated mucins.

In AB-PAS stained slides of both proximal and distal jejunum, the majority of goblet cells contained a mixture of neutral and acidic mucins. Goblet cells containing neutral mucins occurred occasionally in villi or at the base of crypts. In the proximal jejunum, goblet cells with acidic mucins, sialomucins and mixed sialo-sulfomucins were either not observed or rarely found in the villi. The goblet cells in the villi were mainly positively stained for sulfomucins. The distribution of these mucins was consistent with results of a previous study in pigs (Brown et al., 1988). Newly produced goblet cells seem to secret sialomucins preferentially, and sialomucins are mainly located in the base of crypt (Olubuyide et al., 1984). When mature goblet cells migrate up from crypt to villus, sialomucins switch to sulfomucins in the small intestine (Brown et al., 1988). The distribution of cells producing sialomucins and a mixture of sulfo-sialomucins in the villi of the distal jejunum was similar to the one in the proximal segment, but the cells with acidic mucins were more compared to the proximal jejunum.

In the colon, the distribution of neutral, acidic and mixed neutral-acidic mucins was similar to results of a previous study in pigs (Brown et al., 1988). It has been reported that sulfomucin abundance was greater than sialomucin production in colonic mucosa, both in humans (Croix et al., 2011) and pigs (Brown et al., 1988). This staining characteristic was also observed in the present study. The goblet cells with sulfomucins were mainly distributed in the upper crypt and surface epithelium, whereas sialomucins were located in the lower crypt area of the ascending colon. Reverse distributions have been shown in humans (Croix et al., 2011). This may be due to species differences, but also due to age effects and the studied segment of the large intestine.

6.3 Effect of Dietary Zinc Oxide on Mucin Chemotypes in Goblet Cells

The main function of goblet cells is the synthesis of water-soluble mucins (Forstner, 1978). Mucins are large glycoproteins and they protect the underlying mucosa from various insults, caused by antigens, bacteria, viruses and toxins (Allen et al., 1984). Mucins contribute to the lubrication of the epithelial surface for the passage of luminal contents

(Miller, 1987). They maintain a fairly constant pH value at mucosal surface (Flemstrom and Turnberg, 1984). Weaning has shown to be accompanied by a temporary increase in number of goblet cells and alteration in mucin composition, particularly characterized by increased sulphated mucins in the crypt (Brown et al., 1988).

Diets can induce mucin secretion (Lundin et al., 1993; Hedemann et al., 2005), and also affect the composition of intestinal mucins (Sharma and Schumacher, 1995). Dietary zinc can affect the mucosal micro-environment, increase cell proliferative activity and mucin production. For example, pigs fed a diet with 2500 mg/kg of zinc from zinc oxide showed a greater area of neutral, acidic and sulfomucins in the colon for 2 weeks post-weaning (Hedemann et al., 2006).

Goblet cells differentiate and mature as they migrate up the villus. The process is affected by many factors, such as the age of animal (Bruininx et al., 2002), diet (More et al., 1987) and composition of microbiota (Sharma and Schumacher, 1995). The production of different mucin types is related to the number of goblet cells and their mucin contents, as well as the rate of biosynthesis of different mucin types. In the present study, regardless of the diet factor, mucin chemotypes changed in a time-dependent manner. Interestingly, there was a consistent change of sulfomucins and sialomucins in the jejunum and colon of piglets depending on time. In the jejunum and colon, the sulfomucin production in goblet cells was significantly increased during the first two weeks followed by decrease in the 3rd and 4th week. The goblet cells with sialomucins increased during 4 weeks post-weaning. Increased sulfomucins after weaning have also been described in a previous study (Brown et al., 1988). This might be due to an immature phase of piglets resulting in prematurity of the mucin types produced by goblet cells (Shub et al., 1983). Moreover, sulfomucins decreased after 2 weeks, which coincides with transition from the acute phase post-weaning to the late phase response after weaning in piglets. The majority of physiological parameters returned to the levels of pre-weaning period. The changes of mucins in the small intestine of rats during the weaning period were similar to the ones observed in the present study (Turck et al., 1993).

It was reported that pigs at 180 days of age secreted more fucose and glucosamine, and only a small amount of sulfated moieties. The amount of sialic acid in mucins returned to a level of newborn piglets (Turck et al., 1993). In the present study, an increase of neutral mucins was observed in villi and crypts of the distal jejunum 4 weeks after weaning. The mixed neutral-acidic mucins increased in the villi of the proximal and distal jejunum also during 4 weeks post-weaning. In the crypts of the proximal jejunum, acidic mucins, mixed neutral-acidic mucins and total number of goblet cells increased in the first two weeks, afterwards they declined. Neutral mucins protect against invasion of pathogenic bacteria (Dean-Nystrom and Samuel, 1994). An increased mucin secretion in pigs, especially of sulfomucins may contribute to the declined susceptibility to intestinal infections in the time from weaning to the fattening period (Brunsgaard, 1997). The more mature mucins are considered as more effective defense against intestinal infections (Turck et al., 1993).

Irrespective of the time effect, piglets fed high level of dietary zinc had the greatest number of mixed neutral-acidic mucins and total mucins in goblet cell in the colon, consistent with a previous study (Hedemann et al., 2006). Speculatively, this might result in a better protection against diarrhea and pathogen invasion in the post-weaning period. A previous study has shown that colonic mucin synthesis and secretion is stimulated locally by short chain fatty acids (Sakata and Setoyama, 1995) as well as systemically by hormones (Finnie et al., 1996) and neurotransmitters (Phillips and Wilson, 1993). There are two possible mechanisms to interpret how zinc affects mucin secretion. Zinc is a potent regulator of gene expression because it has an effector site on a transcription factor (Blanchard and Cousins, 1996; Tako et al., 2005). Therefore, zinc may influence mucin secretion through the regulation of the mucin genes (Hedemann et al., 2006). The dynamic mucin secretion is determined by the interaction between mucins and microbes (Mack et al., 2003; Hedemann et al., 2006). Dietary zinc oxide increased the microbiota diversity and affected the metabolic activity of the intestinal microbiota (Katouli et al., 1999; Vahjen et al., 2011; Starke et al., 2013), which in turn may have an influence on the intestinal mucin secretion. The composition of mucins may change binding of intestinal microbiota during the post-weaning period.

Moreover, in the present study, piglets fed the high level of dietary zinc oxide produced more sialomucins and less sulfomucins in the jejunal crypts. Both sulfate and sialic acid groups have protective roles. Compared to sulfated mucins, sialylated mucins have more acetyl groups that could inhibit enteric bacterial sialidases (Corfield et al., 1992). The colonic mucins are highly sulfated and sialylated in newly born piglets (Turck et al., 1993). The appearance of acidic mucins seemed to be particularly important in the early development, acting in a protective role for the immature immune system (Cebra, 1999), and the transformation from predominately sulfated mucins to sialylated mucins might be a defense strategy in young animals (Forder et al., 2007). Their presence in the early development may be particularly important as an innate barrier, because the adaptive immune system is not fully functional in neonatal animals (Deplancke and Gaskins, 2001).

6.4 Effect of Dietary Zinc Oxide on Intraepithelial Lymphocytes in the Jejunum

In the present study, four major lymphocyte populations were monitored in the jejunal epithelium of the piglets. After weaning, the most frequent T cell type was $CD2^{+}CD5^{-}$. Based on prior studies with porcine blood immune cells, this population was most likely natural killer (NK) cells (Denyer et al., 2006; Gerner et al., 2009). Another frequent lymphocyte population was $CD4^{-}CD8\beta^{+}$ cells. This phenotype represents the classical major histocompatibility complex (MHC)-class I restricted cytolytic T cells in the pig (Denyer et al., 2006). The percentage of these cells increased with age, which has also been observed in blood samples from pigs. *In vitro* tests hint to an antigen dependent proliferation of the $CD4^{-}CD8\beta^{+}$ cells (Denyer et al., 2006). Furthermore, a minor population of $CD4^{+}CD8\beta^{+}$ cells was present in the jejunal epithelium of the piglets. Cells with this phenotype have been shown to express perforin, which was considered to be a marker for lymphocytes having active cytolytic function (Denyer et al., 2006). This intraepithelial lymphocyte (IEL) population was small in piglets and decreased with age. Neither the relative numbers of these two cytolytic T cell populations nor the portions of NK cells were clearly affected by dietary zinc concentrations. However, the relative numbers of the $CD8^{+}$ γδ T cells were significantly reduced in piglets fed with the highest dietary zinc concentration. Since γδ T cells recognize unprocessed non-peptide antigens in

a MHC-unrestricted manner (Tanaka et al., 1994), they are an important branch of the innate immune system. Although to date the expression of perforin has not been shown in porcine γδ T cells, the $CD8^+$ subtype is described to have cytotoxic capabilities (Pauly et al., 1996; de Bruin et al., 1997; Yang and Parkhouse, 1997). Interestingly, in contrast to all other lymphocyte populations in the porcine intestinal epithelium, these lymphocytes also act as antigen presenting cells (Takamatsu et al., 2002, 2006), and even a memory function of these γδ T cells is under discussion (Tsuji et al., 1996). The T cells represent a link between natural and adaptive immunity. On stimulation with the mitogen phorbol myristate acetate porcine γδ T cells produce a wide variety of cytokines and chemokines (Denyer et al., 2006). Nanoparticles from zinc oxide activated antigen-presenting cells and induced the expression of inflammatory signals to recruit neutrophils, macrophages and lymphocytes (Palomaki et al., 2010). However, since no increase in distinct immune cell populations was observed with the high dietary zinc supplementation, the observed relative decline in γδ T cells was unlikely due to an increase of the other monitored IEL populations. More likely, the decreased portion of γδ T cells could be a consequence of a lower pathogen load or a reduced necessity to eliminate infected epithelial cells. This could set a link to the frequently observed changes in intestinal microbial ecology with high dietary zinc oxide levels (Hojberg et al., 2005; Starke et al., 2013).

6.5 Effect of Dietary Zinc Oxide on Gene Expression Related to Immune Response

The maintenance of intestinal integrity and the digestive and absorptive functions during the weaning period depend on the function of the immune system (Bailey et al., 2001). In newly weaned animals, gene expression involved in immune response was upregulated in the gut-associated lymphoid tissues (Bar-Shira et al., 2003).

Mucins have multiple physiological roles, acting as protective barrier, microecological habitat and immune defense mechanism (Ganz, 2002; Lievin-Le Moal and Servin, 2006). MUC2 is one major secreted mucin expressed in the small and large intestine (Einerhand et al., 2002). MUC2 gene transcription is regulated by many factors, such as enteric microbiota, toxins, cytokines, hormones and growth factors (Andrianifahanana et al., 2006).

Mucins located on the surface of the intestinal epithelium inhibit the translocation of harmful bacterial (Lievin-Le Moal and Servin, 2006). Probiotic mediated inhibition of bacterial translocation is controlled by up-regulation of the secretory mucin MUC2 (Mattar et al., 2002). In the present study, the colonic MUC2 increased with time during 4 weeks after weaning. This observation confirmed a finding on early dietary intervention with a fermentable fibre in weaned rats (Paturi et al., 2012). The highly glycosylated MUC2 is mainly responsible for the formation of the mucus layer in the gastrointestinal tract (Johansson et al., 2011). A lack of MUC2 or any alteration of glycosylated structures will result in predisposition to diseases, such as colitis and colorectal cancer (Corfield et al., 2001; Kawashima, 2012). MUC1, 13 and 20 are membrane-bound mucins, which participate in cellular signaling and play an important role as sensors in response to pathogen invasion or epithelium inflammation (Carraway et al., 2003). However, only few studies evaluated the effect of nutritional factors on mucin gene expression in the gastrointestinal tract of pigs. MUC1 is a large transmembrane glycoprotein expressed on the apical surface of reproductive tract epithelia (Gendler and Spicer, 1995). Porcine MUC20 was reported to affect susceptibility to enterotoxigenic *Escherichia coli* F4ab/ac (Ji et al., 2011). MUC13 is one of the most abundant mucins in the gastrointestinal tract. High-protein diets up-regulated gene expression of MUC1, MUC 20 and pro-inflammatory biomarkers (Pieper et al., 2012).

Trefoil factor family (TFF) peptides are typically produced by mucin-secreting goblet cells (Thim, 1997), and it has been well documented that these peptides are important agents in preventing mucosal damage and assisting epithelial repair (Thim, 1997; Hoffmann et al., 2001). TFF3 is selectively expressed in high concentrations in the small and large intestine (Podolsky et al., 1993), and is generated in mucin-secreting goblet cells along with MUC2 (Podolsky, 1999). It has been reported that TFF3 was significantly affected by bovine colostrum supplementation in pigs (Huguet et al., 2007). However, the influence of zinc or other trace elements on TFF3 gene was not reported until the present study. In this study, the TFF3 expression on jejunum increased with age in the first three weeks post-weaning, afterwards it decreased. This may reflect the maturation of gut related immune mechanisms. *β*-defensins play an important role in mammalian innate immunity. In addition to their

antiviral and toxin-neutralizing characteristics, *β*-defensins possess antimicrobial and immunomodulatory effects (van der Marel. M, 2012). *In vitro* studies revealed that zinc sulfate induced the expression of *β*-defensins in a porcine epithelial cell line (Mao et al., 2013). In the present study, the expression of *β*-defensin 3 in jejunum tissue of piglets was found to be down-regulated after feeding high level of dietary zinc. This may be due to alleviation of weaning-associated intestinal inflammation by high dietary zinc treatment.

Pattern recognition receptors such as toll-like receptors (TLRs) are important for innate immunity. TLRs can activate a common signaling pathway leading to the activation of mitogen-activated protein kinase and nuclear translocation of transcription factor NF-κB, which activates immune cell response and leads to production of inflammatory cytokines and co-stimulatory molecules (Moncada et al., 2003b). TLR2 and 4 are expressed in various lymphoid tissues of the porcine intestinal tract and play an important role in innate immunity of young pigs (Tohno et al., 2005; Tohno et al., 2006). TLR4 is responsible for recognizing endotoxins of LPS from gram-negative bacteria. Gene expression of TLR4 was up-regulated in calves after weaning, and the expression level increased 2-fold on day 7, suggesting an immune response under weaning stress (O'Loughlin et al., 2011). Activation of TLR4 increases the expression of pro-inflammatory cytokines (Shahrara et al., 2006). Only limited data showed the effect of dietary factors, such as yeast extract (Badia et al., 2012), fish oil (Liu et al., 2012) and arginine (Chen et al., 2012) on TLRs. In the present study, the highest level of dietary zinc down-regulated the expression level of TLR4，indicating alleviation of intestinal inflammatory responses after weaning. In addition, the expression of TLR2 and TLR4 was down-regulated in piglets during 4 wk after weaning, which is in accordance with previous findings (Bering et al., 2012; Uddin et al., 2013).

Weaning is associated with a transient up-regulation of pro-inflammatory cytokine gene expression, which may be related to intestinal functional disorders (Moeser et al., 2007a; Moeser et al., 2007b; Smith et al., 2010). It was reported that pro-inflammatory cytokine mRNA of IL-1β, IL-6, and TNF-α was up-regulated during the first 2 d post-weaning in pigs (Pie et al., 2004). After 2 d post-weaning, the majority of mRNA levels of these

cytokines returned to pre-weaning levels. However, TNF-α mRNA remained high in the distal intestine from d 2 to 8 (Pie et al., 2004). An excessive inflammatory response can lead to tissue damage. Therefore, reduction of early intestinal inflammation should have potential benefits in alleviating gut disturbance after post-weaning in piglets. Zinc can have a beneficial role on inflammatory precesses. For example, mRNA levels of TNF-α, IL-6 and IFN-γ decreased with high concentrations of dietary zinc in pigs (Hu et al., 2012; Hu et al., 2013a). In the present study, the relative gene expression of pro-inflammatory (IL-1β, IL-8, IFN-γ) and anti-inflammatory cytokines (IL-10 and TGF-β) was determined in order to try to assess the effect of zinc dietary treatments for 4 weeks after weaning. No significant difference was observed on IL-1β, IFN-γ IL-10 and TGF-β expression, but IL-8 was affected by zinc supplementation. IL-8 is a signal protein, which is essential for neutrophil recruitment and plays an important role in establishing protective immunity (Kelly and Conway, 2005). Normally, it is barely detectable in healthy tissues, but rapidly induced in response to pro-inflammatory cytokines. The variation of expression is one of the remarkable properties of IL-8 (Hoffmann et al., 2002). IL-8 expression increased over 2 fold on the first day post-weaning and reached a peak value at 24 hours, then it returned to baseline level (O'Loughlin et al., 2011). An *in vitro* study indicated that zinc supplementation inhibited NF-κB activation, leading to reduced expression of several NF-κB target genes including IL-8 (Uzzo et al., 2006). Zinc oxide also counteracted the expression of inflammatory IL-8 caused by enterotoxigenic *Escherichia coli* (Roselli et al., 2003). Accordingly, in this study, the mRNA level of IL-8 was down-regulated by the highest concentration of dietary zinc. TGF-β and IL-10 are considered as anti-inflammatory cytokines, protecting the intestinal barrier function. The mRNA levels of TGF-β and IL-10 were increased with high dietary zinc oxide supported on zeolite on day 7 post-weaning (Hu et al., 2012). It was shown that weaning induced transient increase of inflammatory cytokines for 2 days, then most of the tested cytokines rapidly decreased to the pre-weaning levels after day 9 post-weaning (Pie et al., 2004).

Chapter 7: Summary/Zusammenfassung

Summary

Title of the PhD thesis: Influence of Dietary Zinc Oxide on Morphological and Immunological Characteristics in the Jejunum and Colon of Weaned Piglets

High concentrations of dietary zinc have been reported to improve growth performance and health in piglets. It was the goal of the present study to characterize underlying mechanisms of different levels of dietary zinc oxide on the gut morphology and immune responses during four weeks after weaning.

In this thesis, **Chapter 1** introduces zinc oxide as feed additive which is known to have effects against diarrhea problems in pigs, and stresses the significance to investigate the underlying mechanisms of dietary zinc on intestinal mucosal immunity in weaned piglets.

Afterwards, **Chapter 2** reviews protective mechanisms in the intestinal mucosa and specially focuses on the innate defense factors including mucins, trefoil factors, antimicrobial peptides, and toll-like receptors. The characteristics of weaning-associated changes in gut morphology and intestinal immune response in piglets are described. Also the role of trace element zinc in the gastrointestinal tract is illustrated.

The aim and hypotheses of this thesis are emphasized in **Chapter 3**.

The effects of dietary zinc oxide on intestinal morphological and immunological characteristics in the jejunum (**Chapter 4**) and the colon (**Chapter 5**) of weaned piglets were evaluated, respectively.

Chapter 4 focuses on the investigation of changes of the proximal and distal jejunum to different dietary zinc levels. The results showed that villus height and crypt depth differed

age-dependently, whereas the dietary concentration of zinc oxide had no effect. Similarly, the mucin chemotypes were modified by age. Dietary zinc oxide had no effect in the proximal jejunum. In the distal jejunum, age and zinc impacted the mucin chemotypes in goblet cells. The abundance of sulfomucins decreased and sialomucins increased with age. High level of dietary zinc oxide reduced the sulfomucins and increased sialomucins in the jejunal crypts. The phenotypes of lymphocytes in the epithelium of jejunum showed a relatively constant percentage of T cells as well as NK cells. The highest concentration of dietary zinc led to a reduced abundance of $CD8^+$ γδ T cells. The relative proportion of different cytotoxic T cells was age-dependent. The percentage of $CD4^-CD8\beta^+$ increased, whereas the relative amount of $CD4^+CD8\beta^+$ decreased with age. The expression of MUC2 and MUC20 was not influenced by age or dietary zinc concentration. High zinc intakes resulted in a reduced gene expression of *β*-defensin 3 and did not affect the expression of TFF3. Thus, it was concluded that zinc in the form of zinc oxide appears to have specific effects on the gut associated immune system of piglets that may explain improved health and barrier function when piglets were fed with high levels of zinc.

In **Chapter 5**, the changes of the proximal colon to dietary zinc were studied. The colonic crypt area increased in an age-depending manner, and the greatest area was found after feeding medium concentration of dietary zinc. After feeding the high concentration of dietary zinc, the number of goblet cells containing mixed neutral-acidic mucins and total mucins increased. Sialomucin containing goblet cells increased age-dependently. The expression of MUC2 increased with age and reached the highest level at 47 days of age. The expression levels of TLR2 and 4 decreased with age. The mRNA expression of TLR4 and the pro-inflammatory cytokine IL-8 was down-regulated with high dietary zinc treatment, while piglets fed medium dietary zinc had the highest expression.

The effects of dietary zinc oxide on morphological and gut immunological characteristics in jejunum and colon of weaned piglets are discussed **in Chapter 6**.

Taken together, the present study has revealed some interesting impacts of dietary zinc levels on morphology, mucins and epithelial immune parameters, which may help to

understand the protective mode of high dietary levels of zinc against post-weaning diarrhea in piglets. Dietary zinc levels had a clear impact on colonic morphology, mucin profiles and immunological traits in piglets after weaning. Those changes might support local defense mechanisms and affect colonic physiology and contribute to the reported reduction of post-weaning diarrhea.

Zusammenfassung

Dissertation zum Thema: Einfluss von Zinkoxid auf morphologishe und immunologische Charakteristika im Jejunum und Kolon von abgesetzten Ferkeln.

Studien weisen darauf hin, dass sehr hohe Zinkkonzentrationen Einfluss auf Wachstum und Gesundheit von Ferkeln nehmen. Das Ziel der vorliegenden Arbeit war es, Effekte von Zinkoxid auf Morphologie und immunologische Parameter des Darms und die zugrundeliegenden Mechanismen nach dem Absetzen von Ferkeln zu untersuchen.

In **Kapitel 1** dieser Arbeit wird Zinkoxid als Futterzusatzstoff diskutiert einschließlich der Wirkungen gegen Durchfallerkrankungen beim Schwein und der möglichen Wirkmechanismen von Zink auf den Verdauungstrakt bei abgesetzten Ferkeln.

Im **Kapitel 2** werden protektive Mechanismen der intestinalen Mukosa besprochen, wobei im Besonderen auf die angeborenen Abwehrfaktoren einschließlich Muzinen, Trefoil Faktoren, antimikrobiellen Peptiden und Toll-like Rezeptoren eingegangen wird. Die Charakteristika von Veränderungen der Darmmorphologie und Immunantwort, die mit dem Absetzen von Ferkeln verbunden sind, werden beschrieben. Ebenso wird die Rolle des Spurenelements Zink im Gastrointestinaltrakt dargestellt.

Ziel und Hypothese dieser Doktorarbeit werden in **Kapitel 3** zusammengefasst.

Im **Kapitel 4** wird die Publikation „Effect of Dietary Zinc Oxide on Jejunal Morphological and Immunological Characteristics in Weaned Piglets“ vorgestellt. Es werden die Untersuchungen zum Effekt von Zink auf das proximale und distale Jejunum geschildert. Die Ergebnisse zeigen, dass sich zwar Höhe der Zotten und die Kryptentiefe altersabhängig veränderten, Zink in den verschiedenen im Futter eingesetzten Konzentrationen jedoch keinen Effekt hatte. Die untersuchten Chemotypen von Muzinen zeigten Veränderungen in den verschiedenen Altersstufen, Zinkoxid hatte im proximalen Jejunum keinen Effekt auf die Muzinqualität. Im distalen Jejunum war sowohl ein alters- als auch zinkbedingter

Unterschied der Muzinchemotypen in den Becherzellen feststellbar. Das Vorkommen von Sulfomuzinen verringerte sich und das von Sialomuzinen erhöhte sich altersabhängig. Hohe diätetische Zinkoxidkonzentrationen führten zu einer Verringerung der Sulfomuzine und einer Erhöhung der Sialomuzine in den jejunalen Krypten. Die Phänotypen der Lymphozyten im Epithelium des Jejunums zeigten einen relativ konstanten Anteil von T- als auch NK-Zellen. Die höchste Zinkkonzentration führte zu einem reduzierten Vorkommen von $CD8^+$ γδ T-Zellen. Der relative Anteil an den verschiedenen zytotoxischen T-Zellen war altersabhängig. Der Prozentsatz der $CD4^-CD8\beta^+$ Zellen nahm zu, während der relative Anteil der $CD4^+CD8\beta^+$ Zellen altersabhängig zurückging. Die Expression von Muc2 und Muc20 wurde weder durch das Alter noch durch die Zinkkonzentrationen des Futters beeinflusst. Die höchste Zinkgabe resultierte in einer reduzierten Genexpression von *β*-Defensin 3, veränderte jedoch nicht die Expression von TFF3. Zink in Form des Zinkoxids hat offenbarspezifische Effekte auf das darmassoziierte Immunsystem von Ferkeln, welche die verbesserte Gesundheit und Barrierefunktion nach hohen Zinkgaben erklären könnten.

Das **Kapitel 5** enthält die Publikation „Effect of Dietary Zinc Oxide on Morphological Characteristics, Mucin Composition and Gene Expression in the Colon of Weaned Piglets“. Hier werden die Effekte von Zink auf das proximale Kolon dargestellt. Das Kryptenareal des Kolons nahm altersabhängig zu, wobei die höchsten Werte nach Gabe der mittleren Zinkkonzentrationen zu finden waren. Nach Gabe von Futter mit hohen Zinkkonzentrationen nahm die Zahl der Becherzellen mit gemischten neutral-sauren Muzinen sowie die Gesamtzahl der Becherzellen zu. Becherzellen mit Sialomuzinen stiegen altersabhängig an. Die Expression von MUC2 nahm mit zunehmendem Alter zu und erreichte das höchste Niveau mit 47 Tagen. Die Expression von TLR2 und 4 nahm demgegenüber zeitabhängig ab. Die mRNA-Expression von TLR4 und des proinflammatorischen Interleukin 8 wurde durch hohe Zinkkonzentrationen herunterreguliert, während mittlere Zinkkonzentrationen zur höchsten Expression führten. Zusammenfassend kann gesagt werden, dass Zink einen deutlichen Einfluss auf die Morphologie des Kolons, das Muzinprofil und immunologische Merkmale bei Ferkeln nach dem Absetzen zeigt.

Im **Kapitel 6** wird der Effekt von Zinkoxid auf morphologische und immunologische Charakteristika des Darms, insbesondere Jejunum und Kolon, von abgesetzten Schweinen diskutiert. Insgesamt zeigt die vorliegende Studie einige interessante Wirkungen von Zink auf Morphologie, Muzinbildung und epitheliale Immunparameter, welche dazu beitragen können, die protektiven Mechanismen von hohen Zinkkonzentrationen gegen den mit dem Absetzen verbundenen Durchfall bei Ferkeln zu erklären.

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Publication List

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Selbstständigkeitserklärung

Hiermit bestätige ich, Ping Liu, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 16. April 2014 Ping Liu