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Studies on the Influence of the Probiotic *Enterococcus faecium* NCIMB 10415 and the Trace Element Zinc on Performance and Digestive Physiological Parameters in the Small Intestine of Piglets

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Lena Martin

Ernährungswissenschaftlerin
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Zweiter Gutachter:	Univ.-Prof. Dr. Dr. Ralf Einspanier
Dritter Gutachter:	Univ.-Prof. Dr. Jörg Rudolf Aschenbach

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Meiner Familie

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List of Abbreviations

ADFI	Average Daily Feed Intake
ADG	Average Daily Weight Gain
AGP	Antibiotic Growth Promoters
ANOVA	Analysis of Variance
APN	Aminopeptidase N
APN	Aminopeptidase N Gene
BBM	Brush Border Membrane
BW	Body Weight
CASP3	Caspase-3 Gene
cfu	Colony Forming Units
CIAD	Coefficient of Ileal Apparent Digestibility
CON	Control Group
CTTAD	Coefficient of Total Tract Apparent Digestibility
DM	Dry Matter
DMT1	Divalent Metal Transporter 1
EF	*Enterococcus faecium* NCIMB 10415
HRP	Horseradish Peroxidase
HZn	High Dietary Zinc Supplementation (group)
FCR	Feed Conversion Ratio
IAP	Intestinal Alkaline Phosphatase
IAP	Intestinal Alkaline Phosphatase Gene
IgG	Immunglobulin G
IL	Interleukins
IPEC-J2	Intestinal Porcine Epithelial Cell Line J2
GIT	Gastrointestinal Tract
KLF4	Krüppel-like Factor 4
LAC	Lactase
LPH	Lactase-Phlorizin-Hydrolase Gene
LZn	Low Dietary Zinc Supplementation (group)
mRNA	messenger Ribonucleic Acid
MT	Metallothionein-1
MTF-1	MRE-Transcription Factor 1
NZn	Normal Dietary Zinc Supplementation (group)
PCR	Polymerase Chain Reaction
PCNA	Proliferating-Cell Nuclear Antigene
ppm	Parts Per Million
PBS	Phosphate-Buffered Saline
PWD	Post-Weaning Diarrhoea
RPL19	60S Ribosomal Protein L19
RT-qPCR	Reverse-Transcription quantitative Polymerase Chain Reaction
SCFA	Short-Chain Fatty Acids

SGLT1	Sodium-/Glucose-Linked Co-Transporter 1
SI	Small Intestine
SUC	Sucrase
SUC	Sucrase-Isomaltase Gene
TiO_2	Titanium Dioxide
wk	Week
ZIP	Zrt-, Irt-like Protein Family (SLC39A)
ZIP4	Zinc Transporter SLC39A4
ZnO	Zinc Oxide
ZnT	Zinc Transport Protein Family (SLC30A)
ZnT1	Zinc Transporter SLC30A1
ZnT2	Zinc Transporter SLC30A2
ZnT5	Zinc Transporter SLC30A5

CHAPTER 1: General Introduction

The time around weaning is considered as the most stressful period in the life of a piglet (Kim et al., 2012). The piglets are exposed to a variety of stress factors, such as the separation from the mother, mixing with other litter mates, changes of the environment, and a more or less sudden dietary change from highly digestible and palatable sow's milk to less palatable and digestible cereal-based diets (Pluske et al., 1997). This frequently results in anorexia and a suboptimal nutrient and energy intake and imbalances in the gut microbiota leading to diet- or bacteria-induced post-weaning diarrhoea (PWD) (Pluske, 2013; Pluske et al., 1997, 2013).

In the 1980's, it was already stated that one of the detrimental consequences of sudden weaning are specific changes in the intestinal morphology. They are characterized by an increase in crypt depth, a decrease of villus height, a deregulated digestive function along with decreased digestive enzyme activity of the still underdeveloped digestive enzymatic system (Hampson, 1986; Hampson and Kidder, 1986). At the time of weaning (e.g. 21-28 d of age), the piglets have not yet reached their complete maturity referring to the function of their digestive tract (e. g. activity of pancreatic and jejunal enzymes) and the immune system (de Lange et al., 2010). Together with a still not fully established and stabilized intestinal microbial ecosystem, this sets the scene for overgrowth of opportunistic pathogens, leading, together with associated inflammatory processes and maldigestion/malabsorption, to the frequently observed post-weaning growth check or post-weaning syndrome (**Figure 1.1**).

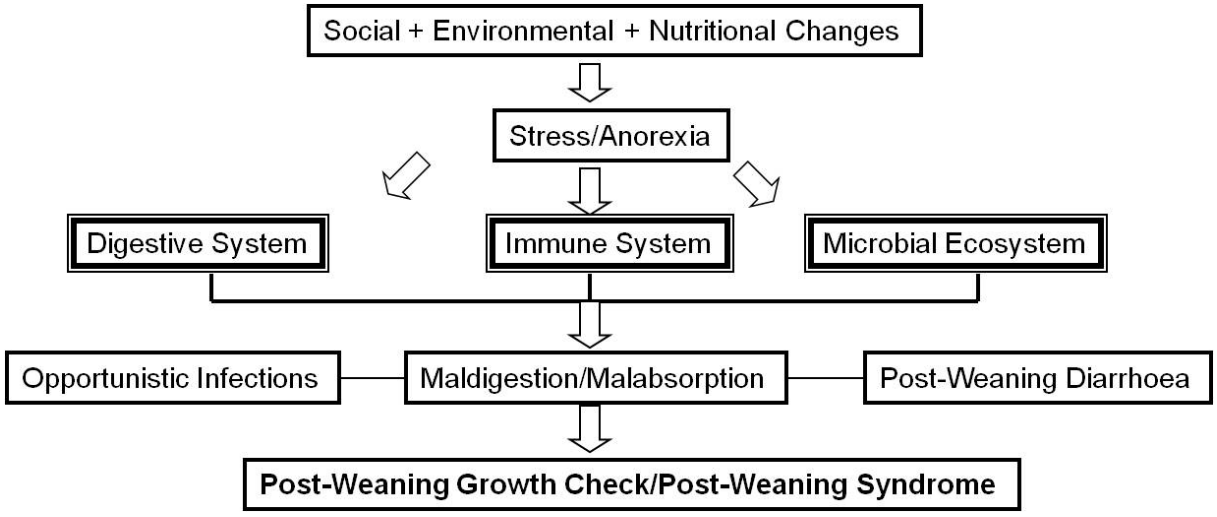

Figure 1.1: Influences and stress factors during the weaning process of young piglets leading to gut-associated disorders and post-weaning growth check

For more than 50 years, antibiotic growth promoters (AGP) have been an effective way to overcome these weaning problems and to maintain high production efficiency (Jagger, 2005). Besides their use for therapeutic reasons, it was common and legal practice to apply antibiotics for prophylaxis in high-density herds with positive effects like increasing feed intake and average daily weight gain (Jukes et al., 1950; Moore et al., 1946; Wegener, 2003). But these positive effects of extensive antimicrobial use in animal production were accompanied by increased reports on the transfer of antibiotic resistance genes among different bacteria with the risk of limiting the efficacy of certain antibiotics in human medicine (Wegener, 2003; Witte, 2000). Since the withdrawal of AGP as feed additives in the EU in January 2006, the attempts to identify alternatives have steadily been increasing, and new products and feeding concepts have been evaluated (Pettigrew, 2006; Pluske, 2013).

Recent research activities in this particular scientific field indicated, that a balanced intestinal microbiota – host interaction is of extreme importance to maintain gut health. Within these complex interactions, nutritional factors might be key factors to keep the balance in the gut. Some alternatives to AGP's are listed in **Table 1.1**, but this list is far apart from being complete.

Table 1.1: An overview of potential dietary tools and feed additives to influence overall health and productive performance in swine (listed by alphabetic order)

- Acids (benzoic-, formic-, lactic-, etc.)
- Alternative feeding concepts (diet composition in general, low protein diets, fermented liquid feeds, restricted feeding, hydrothermal treated cereals, etc.)
- Botanicals (phytobiotics, herbs, essential oils, microalgae (*Spirulina*))
- Enzymes (phytase, xylanase, β-glucanase)
- Fats (MCFA, unsaturated fatty acids)
- Prebiotics (fructans (Inulin), oligosaccharides (mannan-, fructo-oligosaccharides), polysaccharides
- Yeast cell walls, (1,3)-(1,6)- β-D-glucans
- Probiotics (direct-fed microbials, yeasts & yeast products)
- Proteins (conventional/ immunized egg products, milk protein products, spray-dried porcine plasma, bovine colostrum)
- Symbiotics (combination of pre- and probiotics)
- Trace elements (zinc, copper)

Adapted from Pettigrew, 2006; Pluske, 2013; Saeid et al., 2013; Windisch et al., 2008.

Some feed additives exert effects on microbial composition like inhibition of certain potential pathogenic microbes or promotion of selected potentially beneficial microbes; others affect or enhance the immune system or result in improvements of performance parameters like feed intake or daily weight gain. However, the mechanisms are often largely unknown. Within the scope of the present thesis, the focus was directed towards two possible strategies to support the development of gut physiology in young piglets:

1. the use of probiotics and,
2. the use of the trace element zinc as zinc oxide.

The first study of the PhD thesis concentrates on the probiotic strain *Enterococcus faecium* NCIMB 10415 (*E. faeciu*m) in suckling and weaned piglets. The strain was chosen since it is a frequently used feed additive for young piglets. It has been shown to decrease the occurrence of PWD and to affect immunological parameters after weaning (Scharek et al., 2005; Taras et al., 2006). Other potential beneficial effects of probiotics, amongst others, are the competitive exclusion of pathogens (Servin, 2004) or stimulation of other beneficial gut microbes in the small intestine, especially lactobacilli (de Lange et al., 2010; Vahjen et al., 2002). However, little is yet known about the influence of probiotic treatment on digestive physiological parameters such as expression and activities of digestive enzymes. Recent studies in simplified models such as gnotobiotic or mono-associated piglets indicate that specific microorganisms might influence these parameters in different ways (Kozakova et al., 2006; Willing and Van Kessel, 2009).

Another frequently used strategy and the main focus of the second part of the PhD thesis is the use of zinc oxide at high dietary levels to enhance growth performance and reduce the incidence of PWD syndrome in weanling piglets (Pettigrew, 2006). Although many studies have been conducted, the mechanisms have not yet been fully elucidated. Since zinc is an important co-factor for enzymes within the digestive process and due to its ubiquitous role in structural and regulatory processes in the organism, it can be hypothesized that zinc in very high dosages will affect manifold mechanisms including the expression and activity of digestive enzymes.

Therefore, the aim of the current thesis is to evaluate these two feed additives in three animal feeding trials including performance, health parameters, nutrient digestibility, and possible effects on physiological processes like gene expression and digestive enzyme activities in young piglets around the critical time of weaning.

CHAPTER 2: Literature Review

2.1 Development of Digestive Physiology in the GIT of Piglets

The gastrointestinal tract of humans and animals can be considered as a critical interface between the host and the external environment (Buddington and Sangild, 2011). The ontogenetic development of the mammalian intestine can be divided into five distinguished phases (Buddington and Malo, 1996): The phase before gestation in order to prepare the individual for birth, the phase of organogenesis and differentiation, and the third phase when growth and maturation take place. Directly after birth, the fourth phase occurs, when the intestine starts processing milk and the fifth phase equates the weaning phase in which the mammalian intestine acquires additional characteristics to cope with the shift from mother's milk to an adult's diet. The changes in the intestine will even continue for the rest of the lifetime as results of genetic determinations or adaptation processes due to diet, temperature or stress (Drozdowski et al., 2010).

Revealed by kinetic studies in rodents, the mammalian mucosa is the most rapidly replicating tissue in the body demonstrating a complete replacement of the small intestinal epithelium every two or three days (Ziegler et al., 2003). Addressing the anatomical background, the epithelial lining of the small intestine (SI) consists of finger-like projections known as villi and tubular glands called crypts which open into the intestinal lumen at the base of the villi, and contain epithelial stem cells required for the renewal of the epithelial cells (Heo et al. (2012). The brush border membrane (BBM) forms the digestive-absorptive surface of columnar epithelial cells covered by a luminal mucus layer. This builds a junctional barrier between the external environment and the host's body (Crane, 1968), and via tight junction formation and the secretion of mucus and antimicrobial peptides, it prevents from microbial penetration and translocation (Pieper, 2008). The brush border reveals itself as a structurally integrated unit, and is considered as an organelle of the epithelial cell. Its most prominent feature are the microvilli, formed like hollow tubes at a diameter of 100 nm at the luminal face of the epithelial cell increasing the surface of the gut to some 30- to 40-fold. The 'fuzzy appearance' of the microvilli led to the name 'brush border' as their structure reminded early anatomists of bristles of a paintbrush (Crane, 1968). The brush borders' main function in the small intestine reveals as varied digestive hydrolytic activity and furthermore absorptive functions via transport proteins. Several studies in the early 20^{th} century showed that all digestive enzymes, like the disaccharidases lactase, maltase, isomaltase and sucrase, as well as leucine-aminopeptidase and intestinal alkaline phosphatase are localized at the BBM (reviewed by Crane, 1968). These intrinsic enzymes are anchored in the apical plasma membrane of the microvilli of the BBM as integral membrane proteins closely located to transport proteins responsible for the absorption of digested nutrients. A further group of enzymes is termed 'adsorbed enzymes', such as amylase or lipase which originates from the pancreas (Crane, 1968).

The regulation of the BBM enzyme activity takes place on different levels like transcription, translation, post-translational glycosylation and via cell turnover (Willing and Van Kessel, 2009) and each BBM enzyme shows a special developmental pattern while the animal is aging (Simon et al., 1979). Furthermore, some results supported the premise of developmental changes of the hydrolytic capacity through age-related increases of BBM enzymes during postnatal growth of mammals, e. g. piglets (Adeola and King, 2006). Not only the enzyme kinetics change during maturational processes of the enterocytes (Adeola and King, 2006), but also functional changes occur in the gene expression patterns of the BBM enzymes (Fan et al., 2002).

2.2 Influence of Weaning on Physiological Processes at the Intestinal Wall

2.2.1 Morphological Changes

After substantial structural and functional changes in the early postnatal period, weaning exerts an even more dominating influence on the GIT of piglets (Walthall et al., 2005). The literature shows clear evidence that weaning causes immense changes in the structure of the porcine intestine and its functional properties (Hampson, 1986). Through an increase of the surface area for better digestion and absorption, long villi are desirable for an optimal function of the SI (Heo et al., 2012). After weaning, piglets show a significant decrease of the villus height (villus atrophy) and an increase of crypt depths (crypt hyperplasia). Furthermore, the shape of the villi changes from being finger-like to tongue-shaped and the epithelial cell mitosis increases (Dong and Pluske, 2007), cell proliferation and migration rate decline and cell losses plus apoptosis rate are elevated (Lalles and David, 2011). Pluske et al. (1996) revealed significant correlations between villus height/crypt depth and the dry matter intake in milk- and starter-fed piglets after weaning, but even continuous nutrient supply could not completely prevent these events (Kelly et al., 1991). Other researchers even concluded that the weaning-associated lack of feed ingestion and the following morphological changes after the critical first 48 h post-weaning could eventually lead to a more penetrable intestinal lining which results in hypersensitivity responses against (dietary) antigens (McCracken et al., 1995). Studies with germ-free or mono-associated pigs additionally revealed a significant influence of the bacterial colonization on villus length: Conventionally colonized piglets had shorter villi, deeper crypts and a higher enterocyte replacement rate than germ-free animals (Shirkey et al., 2006; Willing and Van Kessel, 2009). Thus, there are two main influencing factors: the change in nutrient supply to the enterocytes and the influence through changes in the microbial colonization. However, since the microbial ecosystem becomes unstable and its diversity declines (Janczyk et al., 2007; Pieper et al., 2008), the changes around weaning might be mainly related to the nutrient supply to both, enterocytes and bacteria.

2.2.2 Effects of Weaning on Cell Turnover and its Respective Gene Expression

While aging and maturation in postnatal animals occur, not only an increase in mucosal weight and length can be observed, but this also presupposes changes in gene expressional patterns (Adeola and King, 2006). Freshly weaned and fasted animals have an increased rate of apoptosis which takes place during the morphological remodelling process around weaning. This programmed cell death (apoptosis) is an important selective targeting cellular process in each multicellular organism in which the cell destroys itself, while neighbouring cells remain intact (Shimoda et al., 2003). Another form of cell death is necrosis, a cell death with distinct morphological and biochemical features within a living tissue and with the possibility to provoke inflammatory responses in the organism caused by toxins, trauma or injury (Proskuryakov et al., 2003). Proskuryakov et al. also state that disturbances in the homeostasis between necrosis and apoptosis, e.g., during embryogenesis, normal tissue renewal or immune reactions, are key factors in the development of certain diseases.

Caspases are highly-conserved, cysteine-aspartic acid specific proteases and they are activated by endonucleases to respond to different inducers of apoptosis. This activation process is a so-called 'key event' of the apoptosis (Shi, 2002). There are several methods developed to detect activated caspase. After cleavage of the pro-caspases, the detection of their products via electrophoresis or immunoblotting with caspase-specific antibodies is possible.

Another crucial protein in the complex mechanisms of the cell cycle is the proliferating cell nuclear antigen (PCNA), a ring-shaped replication factor which encircles the DNA like a fork in the nucleus of each cell and associates as a co-factor with the DNA polymerase δ during the DNA replication process (Moldovan et al., 2007). Therefore, it seems interesting to measure potential effects of dietary zinc concentrations, *E. faecium* and the weaning process on the *CASP-3* and *PCNA* gene expression level.

2.2.3 Effects of Weaning on Digestive Enzyme Activities and Gene Expression

Although prolonged suckling can help to delay all these weaning-related changes in the GIT related with the weaning process, it cannot completely prevent them. Moreover, this process can also be accelerated by advancing the transition from sow's milk to adult feed (Buddington and Sangild, 2011). Within this thesis, the focus will be on the activity and expression of BBM enzymes and the influence of weaning and nutritional factors on them.

Activities and gene expression patters of BBM enzymes have been used for several years as indicators to describe the maturation of the enterocytes, or more general, the small intestine of mammals (Willing and Van Kessel, 2009). Again, each of these enzymes shows a special developmental pattern during aging (Simon et al., 1979).

Expression patterns also change during the migration of enterocytes along the crypt-villus axis, e. g., the aminopeptidase N (APN), an enzyme responsible for digestion of oligopeptides

to dipeptides, shows a low expression at the immature crypt and a high expression at the mature villus tip enterocytes (Fan et al., 2001). This crypt-villus gradient was also reported for lactase-phlorizin hydrolase (LAC) with the highest activity at the villus tip, while disaccharidases reach maximal activity in the mid-villus region (Drozdowski et al., 2010). Common effects of weaning are a general reduction of digestive enzyme activities accompanied by changes in small intestinal architecture and absorptive capacity (Montagne et al., 2007). For example, LAC activity in suckling animals is high, but both, activity of LAC and mRNA abundance of its respective gene lactase-phlorizin-hydrolase (LPH) decline after weaning. This is genetically determined, except for humans in areas like North America or Western Europe (Drozdowski et al., 2010). In contrast to lactase, the activity of sucrase in mammals is low after birth, but this functional pattern reverses dramatically, while the animals are weaned (Cummins et al., 1988). Sucrase, maltase, and isomaltase are associated with each other as the bi-functional enterocyte BBM disaccharidase (sucrase-isomaltase). These enterocytes are programmed in the crypts before they migrate up the villus, so that all cells along the entire villus express the enzyme sucrase-isomaltase. This is also genetically determined and cannot be significantly affected by the diet (Drozdowski et al., 2010). However, most of these BBM enzymatic studies were performed in rabbits, mice and other rodents, but studies in pigs, which are interesting as model for the human gastrointestinal development, are still exceptional.

Fasting, as it happens in the first hours after weaning, as well as at the intestinal level via total parenteral nutrition, can also lead to alterations of the enzyme activity patterns, like of intestinal alkaline phosphatase (IAP). This enzyme is mainly expressed in villus-associated enterocytes in the SI and plays a major role in the intestinal homeostasis (Lalles, 2010). Besides the hydrolization of monophosphate esters, IAP contributes to the detoxification process of LPS and is deemed as an 'excellent marker for crypt-villus differentiation' and can additionally be linked to the innate immunity as key component of gut mucosal barrier function (Goldberg et al., 2008). Nevertheless, its activity is transiently declined post-weaning, thus leading to an increased susceptibility to enteric diseases in pigs post-weaning and to up-regulation of intestinal stress proteins downstream (Lackeyram et al., 2010).

Other digestive enzymes are secreted in the oral cavity (e.g. amylase), in the stomach (e.g. pepsinogen) or from the pancreas (e.g. chymotrypsinogen, trypsinogen, carboxypeptidases, pancreatic lipase, elastases, etc.) which secrets its enzyme-containing juice via pancreatic duct into the duodenum. Pancreatic enzyme activities are generally depressed during the first week after weaning, but no influence on gastric proteolytic effects could be detected (Lindemann et al., 1986).

2.2.4 Impact of the Intestinal Microbiota on Gut Physiology

The interaction between the GIT of the host, the diet and the residing microbiota starts at birth when the first colonizing bacteria encounter the gut and start digesting the very first feed (Buddington and Sangild, 2011). The host (animal or human) has to learn to accept the presence of numerous amounts of microbial species colonizing its former sterile GIT which is then as an adult colonized by approximately 10^{14} prokaryotic and eukaryotic microorganisms plus diverse fungi, protozoa, yeasts, viruses and bacteriophages (Buddington and Sangild, 2011; Hooper and Gordon, 2001). This whole number is more than 10-fold higher than the number of cells the host itself consists of – it is like an own organism within the host organism. Therefore, the intestinal autochthonous microbiota greatly affects the overall health status of the host organism and should be kept well-balanced to protect the host against invasion of pathogenic microbes and their toxins (Jensen, 1998; Katouli et al., 1999). Their composition is determined by autogenic factors which are mutual interactions between diverse microbes and the host himself and so-termed allogenic factors like the pH in the stomach, digestive enzymes, intestinal peristalsis, dietary nutrients, and the host's immunity (Vondruskova et al., 2010). However, an important fact is that not only nutrients, aging, or the weaning process exert effects on the development of the GIT, as mentioned in **Chapter 2.1**, but that also microbes themselves induce changes in an intensive 'cross-talk' during the maturation of the SI (Willing and Van Kessel, 2009). They might – depending on the colonizing species – exert effects on digestive and metabolic function (Kozakova et al., 2001), intestinal physiology including villus morphology and mucus secretion (Shirkey et al., 2006), immune development (Mazmanian et al., 2005) and enterocyte turnover in the host (Willing and Van Kessel, 2007). Nevertheless, most of these results were obtained with the help of more reductionistic approaches as used in studies with germ-free or mono-associated rodents and pigs. These gnotobiotic models revealed differences regarding digestive function and gene expressional patterns (Willing and Van Kessel, 2009). Unfortunately, only a few well-controlled studies have been performed in swine – much more in rodents and chicken – although the pig is documented as an adequate model for the human (neonatal) intestinal development (Shirkey et al., 2006).

2.3 Feed Additives and Nutritional Strategies during the Weaning Period

2.3.1 Probiotics: Literature Overview

One of the recently most cited definitions of the term 'probiotics' was set up by Fuller (1989): *'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.'*

This appears to be a rather vague definition, as it remains still unclear how a balanced intestinal microbiota is defined and by which mechanisms this may beneficially affect the host animal. With a more critical point of view, Tannock (1999) therefore stated about probiotics: *'They go in at one end of the digestive tract and come out the other, and hopefully something good happens along the way.'*

Although the term 'probiotics' was first used in 1974 for the description of microbial feed supplements, early experimental studies with '*Bacillus acidophilus*' in chicken were already documented in 1925 based on pioneer work by Metchnikoff at the beginning of the 20^{th} century (Tannock, 1999). In a study by Nurmi and Rantala (1973) with newly hatched chicken, the development of probiotic feeding in farm animals has even been enhanced. They discovered that applying a suspension of gut contents from healthy adult hens exerted protective effects against *Salmonella* invasion.

In the past few years, it has been shown that the application of probiotics seems to be a promising tool for improving the performance and stabilizing the health of farm animals in the livestock industry (Simon, 2010). Modern probiotics are mostly of bacterial origin, and as microbial feed additive have to pass several comprehensive approval tests (Regulation (EC) No 1831/2003) before they achieve permission to the market. They are normally applied to the animals via diet leading to a temporal colonization of the gut. Currently, there are diverse preparations of microorganisms of three different groups authorized as feed additives for pigs in the EU (summarized in **Table 2.2**): Lactic acid bacteria (mainly Enterococci, Lactobacilli and *Pediococcus acidilactici*), bacteria of the genus *Bacillus*, and yeasts of the genus *Saccharomyces*. Bacterial strains include *Enterococcus faecium*, *Streptococcus infantarius*, *Bacillus subtilis*, *B. licheniformis*, *B. cereus* var. *toyoi*, *Lactobacillus rhamnosus*, *L. casei*, *L. plantarum*, and the yeast *Saccharomyces cerevisiae* (European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003).

Preparations of these probiotics are commonly used at concentrations between 10^8 to 10^9 CFU/kg feed and are normally mixed into the animals' feed (Simon et al., 2003). Basic requirements of these probiotic strains are the ability to culture them on an industrial scale in suitable carriers or fermentable substrates, an acceptable shelf-life, sufficient stability against feed technological processes and the ability to remain viable in a sufficiently high number in the feed end product (Holzapfel et al., 1998). Last but not least, they should have the ability to colonize the gut.

Table 2.2: Licensed living microbial products (probiotics) authorized as feed additives for pigs in the European Union (without the claim of completeness)

Toyocerin (*B. cereus var. toyoi*)	Piglets, growing/finishing pigs, sows
Actisaf (*S. cerevisiae*)	Growing/finishing pigs
Biosaf (*S. cerevisiae*)	Piglets, sows
Biosprint Natural Yeast (*S. cerevisiae*)	Piglets
Levucell SB (*S. cerevisiae*)	Piglets, sows
Prosol S.p.A. (*S. cerevisiae*)	Sows
Bactocell P A, Fermaid P A (*Pediococcus acidilactici*)	Piglets, growing/finishing pigs
Cylactin, LBC (*E. faecium*)	Piglets, growing/finishing pigs, sows
Microferm (*E. faecium*)	Piglets
Oralin(*E. faecium*)	Piglets
Lactiferm (*E. faecium*)	Piglets
Fecinor Plus (*E. faecium*)	Piglets
Bonvital (*E. faecium*)	Piglets, growing/finishing pigs, sows
Biacton (*L. farciminis*)	Piglets
Provita LE (*L. rhamnosus; E. faecium*)	Piglets
Calsporin (*B. subtilis*)	Weaned piglets
Bio Plus 2B (*B. subtilis, B. licheniformis*)	Growing/finishing pigs, sows
Miya-Gold (*Clostridium butyricum*)	Weaned piglets, fattening pigs, pig species with a minor economic value

Source: European Union Register of Feed Additives pursuant to Regulation (EC) No. 1831/2003: http://ec.europa.eu/food/food/animalnutrition/feedadditives/comm_register_feed_additives_1831-03.pdf (Release date: 17.07.2013).

2.3.1.1 Application of Probiotics Aiming at Improved Gut and Overall Health in Pigs

Central motivation for most research studies on probiotic effects in animal health is the retrieval for alternative possibilities to treat or prevent diarrhoea, especially the *Escherichia coli* caused diarrhoea. Nevertheless, current recommendations to improve the efficacy of the applied probiotics suggest other technical measures and procedures, mainly affecting the feeding regime and the hygienic status in the stables (Hodgson and Barton, 2009). A positive, diarrhoea-prophylactic outcome was described by several authors for strains like *Bacillus subtilis* (Bhandari et al., 2008), *E. faecium* (Taras et al., 2006; Zeyner and Boldt, 2006), *Lactobacillus sobrius* (Konstantinov et al., 2008) as well as *Lactobacillus rhamnosus* (Zhang et al., 2010). Recent results of a probiotic study have shown that in-feed *Bacillus licheniformis* and *Bacillus subtilis* tended to reduce the morbidity, as well as the mortality of weaned pigs associated with *E. coli* diarrhoea (Alexopoulos et al., 2004). Previous reports showed that probiotic formulations containing lactobacilli have a positive effect on PWD and on other types of diarrhoea (Candy et al., 2000; Kyriakis et al., 1999; Marcin et al., 2000).

However, the results from probiotic application trials in pigs are not always consistent (Kritas and Morrison, 2003; Stavric et al., 1995), and other studies even reported that the strain *E. faecium* SF68 has no significant effect on the pig performance (Broom et al., 2006). Reasons for significant variability in results appear to be found in wide variations of the protocol designs, in the tested commensal microorganisms, probiotics and pathogens, in target of the animal species, and in the health status of farm or laboratory animals (Alexopoulos et al., 2004). The appropriate results were mainly based on the assessment of data from different studies – under more or less praxis-relevant conditions – or challenge trials measuring diarrhoea score and length of diarrhoea after the application of the above-mentioned probiotic bacteria. Results further depend on diverse other factors such as the initial microbiota in the host, the applied probiotic strain that was used in the study, etc.

2.3.1.2 Effects and Possible Modes of Action of Probiotics on the Composition of Intestinal Microbiota in the Pig

The aim of the application of probiotic feed additives is mainly the modulation of the intestinal gut microbiota. Potential mechanisms which are claimed for probiotics are summarized in **Table 2.3**. Current research in pigs has shown that both a reduction and the facilitation of representative gut bacteria of the autochthonous microbiota are possible, as well as the potential influence of some probiotics on pathogenic microbes (Servin, 2004; Zentek, 2012). Recent studies in the Institute of Animal Nutrition, Berlin, have shown an increase of selective lactobacilli in faecal samples through the application of *E. faecium* NCIMB 10415 (Vahjen et al., 2007). While most of the mechanisms have been well studied in cell or model organisms, general knowledge about probiotic modes of action in the GIT is still scarce (Zentek, 2012). Some mechanisms in swine have already been identified, but due to the variety of influencing factors, no clear picture is obtainable.

Possible explanations of the probiotic mode of action can be sought in the intensive crosstalk between specific bacteria of the gut microbiota, the applied probiotics and potential pathogenic strains at the gut lumen which is called 'quorum sensing'. This term includes several signal transduction pathways via substances with a low molecular weight on different levels between bacteria and regulates their colonizing density, for instance (Di Cagno et al., 2011). Peptide pheromones & other bioactive peptides, short chain fatty acids (SCFA), amino acids (e.g. β-alanine, GABA, glutamate), amines, biosurfactants, various enzymes and hormones for epigenetic regulations, stress proteins or antimicrobial compounds are only a small list of potential low-molecular substances participating in these 'quorum-sensing' mechanisms that have a regulatory impact on the intestinal microbiota (Shenderov, 2011). Most research has recently been done on parameters such as the concentration of short-chain fatty acids (SCFA) and lactic acid in the GIT. Feeding trials with *Bacillus cereus* showed significant reduced concentrations of ammonia, lactic acid and SCFA in jejunal digesta of piglets, while the picture in the caecum was less clear (Kirchgessner et al., 1993).

Table 2.3: What probiotics are claimed for - Overview on possible hypotheses of probiotic mechanisms and modes of action in pigs (in alphabetical order)

- Affecting gut permeability
- Improvements of feed efficiency via digestion of non-digestible dietary compounds not degradable by the pigs endogenous enzymes
- Increased resistance to infectious (particularly of the intestine) diseases
- Influencing nutrient uptake (amino acids, minerals) → promotion of growth and FCR
- Modulating the intestinal microbiota by:
  - competition with pathogens for epithelial receptors (competitive exclusion)
  - competition for nutrients
  - production of antimicrobial compounds (e. g. organic acids) with inhibitory effects on unwanted microbes
- Modulating the mucin expression in the host which serve as energy source for the bacteria
- Passive aggregation to pathogenic bacteria
- Promotion of gut health via prevention of PWD
- Stabilization of the gut microbial ecosystem
- Stimulation of the intestinal immune response (cytokine production, phagocytosis)

Adapted from Tannock, 1999; Doyle, 2001; Simon, 2003; Pettigrew, 2006.

Furthermore, Jadamus et al. (2002) demonstrated in a study with suckling piglets that *Bacillus cereus* var. *toyoi* led to a drop in microbial bile acid deconjugases in the small intestine, besides the reduction in jejunal lactic acid. *Bacillus cereus* var. *toyoi* was already discovered in 1966 and has now been used as active ingredient in the preparation TOYOCERIN for over 30 years in the field of animal nutrition for swine, poultry, cattle, aquaculture and rabbits. Additionally, it has to be mentioned that this strain *B. cereus* var. *toyoi* was initially classified as *B. cereus*, but showed significant differences to the species *B. cereus* in recent taxonomic studies and is therefore considered as an own species with the proposed name *Bacillus toyonensis* (Jimenez et al., 2013).

Studies with *Lactobacillus casei* strain Shirota have shown that shifts in the microbial fermentation patterns in the large intestine are possible, abeit with large inter-individual differences (Ohashi et al., 2004). Also yeasts might have an influence on fermentation patterns of the porcine GIT. Marinho et al. (2007), for example, detected a declined concentration of SCFA in the colon of piglets after application of *Saccharomyces cerevisiae*. Besides probiotic effects on microbiota in the digesta, effects on bacteria associated with the luminal wall are also

of great significance. Recent investigations showed a reduction in the adhesion of pathogenic germs after the application of diverse probiotics, but mechanisms are rarely documented. *In vitro* studies have shown co-aggregation processes of distinct lactobacilli strains with K88-positive *E. coli* which could result in a diminished adhesion potential of a enterotoxigenic *E. coli* strain on isolated porcine epithelial cells (Spencer and Chesson, 1994). The application of *Lactobacillus plantarum* combined with maltrodextrin and fructo-oligosaccharides in a feeding trial with weaned piglets resulted also in a lowered adhesion of pathogenic K88-positive *E. coli* in jejunum and in colon (Nemcova et al., 2007). Due to the well-known phenomenon of coprophagy in young pigs, several researchers have further demonstrated a maternal-neonatal transfer of probiotic bacteria in other species like mice and rats for strains like *Lactobacillus acidophilus* and *Bifidobacterium lactis* (Buddington et al., 2010). The hypothesis of vertical microbial transfer was also confirmed in swine for the strain *Bacillus cereus* var. *toyoi* (Taras et al., 2005) and has been described as the main source of microbes for the first colonisation of young animals (Jadamus et al., 2002; Macha et al., 2004).

This review shows that potential effects of probiotics on relevant signal molecules and mechanistic aspects affecting the luminal or mucosal sites of the porcine GIT and then on the health status of the host remain relatively hypothetically and need further clarification.

2.3.1.3 Special Application of *Enterococcus faecium* NCIMB 10415 as Probiotic Strain in Pigs

Interestingly, Buydens and Debeuckelaere (1996) treated adult humans suffering from acute diarrhoea with *E. faecium* NCIMB 10415 and observed a reduction of the diarrhoeal phase. Older studies with humans have shown that the oral administration of *E. faecium* SF68 can lead to a reduced incidence of and a faster recovery from diarrhoeal diseases (Wunderlich et al., 1989). In swine production, enterococci like the mentioned *E. faecium* NCIMB 10415 are commonly used as a probiotic supplement. In a piglet study, the dietary supplementation with *E. faecium* SF68 (Cylactin) led to an improved piglet performance in the first week post-weaning (Broom et al., 2006). Other researchers observed a reduction in diarrhoea incidence and an improved daily weight gain when *E. faecium* was administered orally to piglets from birth to weaning, but these results have to be considered critically because they had a very high incidence of diarrhoea (40 %) in the control group (Zeyner and Boldt, 2006). When *E. faecium* was fed to both sows and their piglets, a decreased incidence of diarrhoea and a lower occurrence of potentially pathogenic *E. coli* isolates were discovered (Taras et al., 2006). On the other side, no effect of dietary *E. faecium* on ADG or feed conversion efficiency could be detected, but an increase of serum IgG (Gardiner et al., 1999). For the application of the same *E. faecium* strain in combination with inulin, decreased faecal counts of bifidobacteria and lactobacilli in piglets were measured (Böhmer et al., 2005), while others could not detect any changes in gastrointestinal bacterial populations, but found an lower serum IgG concentration suggesting an immunomodulatory effect of this probiotic strain (Broom et al.,

2006). Additionally, there are also hints of immune-modulating effects on the intestinal epithelial immune response via *E. faecium* (Mafamane et al., 2011; Scharek et al., 2005). Support for this hypothesis has been found in still unpublished results of co-workers where a dysregulation in expressional patterns of pro- and anti-inflammatory cytokines, both in the intestinal and spleen tissue of *E. faecium*-fed piglets, has been discovered (Siepert et al., 2013).

In addition to the prophylactic effects due to *E. coli*-induced diseases, some probiotics have also been investigated for their preventive effects in piglets dealing with *Salmonella* infections. Studies with healthy piglets have shown that *E. faecium* has the ability to decrease the pathogenic bacterial load (Taras et al., 2006). On the other hand, increased faecal excretion and colonization of several organs with *Salmonella* serovar Typhimurium have been detected while using a porcine animal infection model after feeding *E. faecium* (Kreuzer et al., 2012a; Szabo et al., 2009). Current research further shows an influence of probiotics like *E. faecium* on virus-shedding in naturally infected pigs caused by immunological changes, depending on the virus type (Kreuzer et al., 2012b) or a reduction in the rate of porcine carry-over infections by obligate intracellular pathogens like *Chlamydiae* (Pollmann et al., 2005).

However, knowledge about the influence on other intestinal digestive and physiological parameters such as expression and activity of digestive enzymes via *E. faecium* administration in piglets is still scarce. Some researchers have corroborated with their results of gnotobiotic pig studies that early colonization with different bacterial species can dramatically modify the small intestinal development and the brush border membrane enzyme activities (Willing and Van Kessel, 2007, 2009).

Furthermore, very few data are available whether feeding of probiotics also influences the nutrient digestibility (Kornegay and Risley, 1996). Although an increase of ileal digestibility of most of the measured amino acids was observed in a former piglet trial with *E. faecium*, the results for the influence of *E. faecium* on ileal digestibility of amino acids could not be repeated in a further trial (Simon, 2010). While *B. cereus* showed an improved feed conversion ratio, the same effect was lacking for *E. faecium* (Simon, 2010). Therefore, it has been hypothesized that the nutrient utilization, the performance and the effects on the intestinal physiology are depending on the applied probiotic strain (Simon, 2010).

In *ex vivo*-trials in Ussing chambers, a positive effect of *E. faecium* application on glucose-induced I_{sc} on day 14 of life of suckling pigs has been detected, and a tendency to a forskolin-induced increase of I_{sc} on day 28 which has been interpreted as a positive outcome for the animal (Lodemann et al., 2006). After the supplementation of *B. cereus* var. *toyoi*, an enhanced Na^+/glucose co-transport and peptide transport (glycyl-L-glutamine, glycyl-L-sarcosine) has been measured (Breves et al., 2000), while others failed to reach significance for the same parameter after feeding *E. faecium* (Lodemann et al., 2006).

The study presented in the published manuscript in **Chapter 4** aimed at determining the effect of the supplementation of *E. faecium* NCIMB 10415 to sows and their piglets, on performance parameters, on digestibility, and on the development of physiological measures of

the SI, such as gene expression and activities of several BBM enzymes in the piglets during the pre- and post-weaning period.

2.3.2 Zinc: Literature Overview

'Zinc is indispensable for life from bacteria to man.' (Couinaud, 1984)

2.3.2.1 History of Zinc in Agriculture and Livestock Production

As Nielsen (2012) pointed out in his review about the history of zinc:
'The history of zinc in agriculture is an outstanding demonstration of the translation of research into practical application.'

It started in 1926, when zinc became established as an essential trace element for green plants and in 1934 also for mammals (Nielsen, 2012). Mendel and Hubbel stated 1927: *'It is not unlikely, however, that there is some variation in growth with varying amounts of zinc and that the metal is not merely an accidental factor in the nutrition of the mouse.'*

However, it took another 20 years, before first reports appeared on zinc deficiency symptoms in pigs in North America. Common signs were associated with the typically use of maize- and soybean meal based diets for pigs. These pigs exhibited symptoms including poor growth, retarded feed intake and abnormal skin, and were summarized under the disease parakeratosis (Kernkamp and Ferrin, 1953; Tucker and Salmon, 1955) which caused fatal economic losses in many commercial herds during the late 1940's and early 1950's (Luecke, 1984). It was also reported that parakeratosis of swine could be cured by additional zinc supply in the diet (Tucker and Salmon, 1955). In 1958, the same symptoms plus leg abnormalities, poor feathering and parakeratosis were described in chicken. In the 1960's, the symptoms of parakeratosis in grazing sheep and cattle were also alleviated by additional zinc supply (Nielsen, 2012). In the following years, dietary recommendations have been derived in feeding systems for most farm animal species. In the late 80's and early 90's, the positive effect of feeding very high levels of dietary zinc as zinc oxide have led to a series of studies which aimed at determining the mode of action. Details will be given below.

2.3.2.2 Zinc Metabolism: Zinc Distribution and Function under Physiological Supply

Zinc as an essential trace element is involved in multitudinous processes in the body (Suttle, 2010). Through its catalytic, structural and regulatory characteristics and its critical role in the stabilization of biomembranes (Chvapil, 1973), zinc is therefore, undeniable, an essential dietary nutrient for swine and man (Payne et al., 2006). As participant in physiological processes like as a constituent in over 300 metalloenzymes, in zinc-finger proteins, in DNA replication and cell proliferation processes, protein synthesis (Payne et al., 2006) and as well

as a regulator of the gene expression, zinc is involved in a multitude of different physiological processes in all mammals (Suttle, 2010). A cautious estimate has suggested that around three percent of the genes of the human genome code for zinc-finger proteins (Klug, 2010). Zinc-finger proteins are referred as 'prototypical structural zinc sites' (Hernick and Fierke, 2005).

Zinc as divalent cation plays an essential role as co-factor in the catalytic activity in diverse zinc hydrolases (zinc-dependent hydrolytic enzymes) and is foremost known as a strong Lewis acid. This chemical property allows to gather diverse structural rearrangements which occur necessarily during the process of chemical reactions (Hernick and Fierke, 2005).

Well known examples for zinc-metalloenzymes are, for instance, carbonic anhydrase, copper/zinc superoxide dismutase, alkaline phosphatase, various dehydrogenases (alcohol-, lactate-, malate-, glutamate-) and the pancreatic carboxipeptidase (Lloyd et al., 1978). Zinc is also co-factor in several enzyme systems like diverse peptidases (e.g. aminopeptidase-N), arginase, etc. A relationship is also ascertained between zinc and the activity of hormones such as insulin, glucagon, FSH, LH and corticotropin (Lloyd et al., 1978).

As with other essential elements, zinc uptake, distribution and excretion underlies a strong homeostatic regulation, and zinc ions are mainly transported actively across biological membranes (see **Chapter 2.3.2.3**). Common tissues in the animals where higher zinc concentrations can be found are muscles which provide the largest body pool, followed by bones and the erythrocytes in the blood (Suttle, 2010). The availability of free zinc ions is also an important factor for the zinc uptake from the gut lumen. Zinc oxide, for example, is relatively insoluble at neutral pH, but its solubility increases with a decrease in pH value. Under normal zinc supply, the proportion of free zinc in jejunal digesta of pigs is approx. 13 % from dietary zinc oxide (Dintzis et al., 1995). This is in good agreement with recent findings reporting similar values in jejunal digesta of piglets with normal (150 mg/kg) zinc supply (Pieper et al., 2013). Another point is the total amount of zinc ions in the jejunal digesta which potentially promotes the regulation of zinc-specific transport and intracellular binding mechanisms. Weigand and Kirchgessner (1978) showed that the homeostatic regulation of zinc concentrations in the body takes place in the intestine. Reliable markers for zinc status in animals are, for instance, classical ones such as zinc plasma content, zinc content in diverse organs (hair, liver, pancreas, bone, kidney) or activity of serum alkaline phosphatase, but concentrations underlie a strong deviation depending on the homeostatic regulation mechanisms and thus, several parameters should be combined to obtain a better picture about the zinc status (Suttle, 2010).

2.3.2.3 Zinc Absorption and Intracellular Binding and Distribution

In recent years, immense research has been done on zinc metabolism, and several zinc-depending transport mechanisms have been identified. Under normal dietary zinc supply, zinc homeostasis in the body is mainly controlled by intestinal absorption and secretion (Weigand and Kirchgessner, 1980; Windisch and Kirchgessner, 1995; Windisch and Kirchgessner, 1994).

Uptake primarily takes place via small intestine and even via skin (creams), and then absorption occurs via various zinc transport proteins in the small intestine. To date, altogether 24 zinc transporters are known, and two families of zinc transporters have been described. The first is the *ZnT*-like family (*SLC30*) which is mainly responsible for decreasing intracellular zinc levels by transporting zinc ions from the cytoplasm into the extracellular matrix or into cell organelles (Lichten and Cousins, 2009). The second family is the *ZIP* (or Zrt, Irt-like) proteins (*SLC39*) whose members mainly increase the intracellular zinc concentration (Lichten and Cousins, 2009). Some of these transport proteins, like ZnT1, are ubiquitously distributed in all epithelial cells of the jejunum, liver, pancreas and kidney in all mammals, while others show strong tissue specificity, and can only be found in specified organs of the body, such as ZIP3 only in blood cells or ZIP10 in the brain or kidney.

In the small intestine, for example, ZnT1, ZnT2, ZnT5, ZIP4 and ZIP5 are involved in zinc metabolism (Lichten and Cousins, 2009). The divalent metal-ion transporter 1 (DMT1) appears to play a more minor role in zinc homeostasis, and seemed more involved in iron and copper uptake (Kordas and Stoltzfus, 2004), but there is still an on-going discussion between cell biologists about its role in intestinal zinc uptake (Espinoza et al., 2012). Furthermore, Hill and Link (2009) hypothesized that the uptake of zinc by ZIP proteins is ATP-independent, and is driven by a concentration gradient. Within the cell, zinc is mainly bound to metallothionein (MT) which is the main zinc-binding protein in the cytosol of the cell in intestinal tissue. MT, in general, are a family of ubiquitous, low molecular weight proteins (0.5-14 kD) with a high cysteine content which consists of various isoforms, and are known to be involved in the regulation of zinc homeostasis (Sun et al., 2006). Furthermore, the metal-binding activity of this intracellular protein exerts protective effects against oxidative stress (i.e., reactive oxygen and nitrogen species) and heavy metal toxicity (Cousins et al., 2006). Studies in zebra fishes revealed that changes in the intracellular concentration of the labile Zn^{2+} are responsible for diverse zinc-signaling pathways which might result in genomic and non-genomic alterations (Hogstrand et al., 2008).

The responsible transcription factor is called MTF1 (metal-regulatory transcription factor 1) and, in response to zinc or cadmium, it binds to putative metal-response elements at the 5' regulatory region of the *mt* (metallothionein) genes being in charge for immense genomic responses (Andrews, 2000; Chen et al., 2007). This MTF1-connected regulation of *mt* gene expression might be sufficient enough to protect the cells from Zn^{2+} intoxication, but not sufficiently enough to prevent Cd^{2+} intoxication (Solis et al., 2002). This transcription factor was further termed as 'a master regulator of gene expression' during developmental processes (Hogstrand et al., 2008). Nevertheless, all of the above mentioned studies have been done in rodent or cell culture models. To date, too less information about these complex mechanisms in pigs are known. Therefore, the current thesis aimed at addressing these issues (**Chapter 4 & 5**).

2.3.2.4 Zinc Interactions with other Dietary Factors

Although many staple foods, like cereals, maize and vegetables, are relatively good zinc sources based on their total zinc content, the zinc uptake by the host can be negatively affected by a diversity of other dietary factors such as other divalent metal ions in huge amounts (calcium, magnesium, phosphorus, iron), chelating substances such as phytate or copper as a zinc antimetabolite (Lloyd et al., 1978) as well as the source and level of protein in the diet. For example, casein in milk can have negative effects on zinc absorption (Lönnerdal, 2000). Another important factor is phytate (myoinositol hexaphosphate) which can be found in all plant seeds and roots. It can build insoluble complexes with zinc ions and other divalent metal ions, thus leading to an inhibition of zinc absorption and even to zinc deficiency in cases when dietary zinc is very low, and the phytate content is very high (Schlegel and Windisch, 2006). A common approach to overcome this issue is the additional dietary supplementation with microbial phytases to raise zinc bioavailability (Adeola et al., 1995). No effects on zinc status have yet been reported by other feed ingredients such as lignin, pectin, wood cellulose, or gum arabic. On the other side, zinc complex building with high stability constants with several amino acids like histidine or methionine, facilitates the uptake of zinc (Cousins et al., 1986). Due to their weak chelating effects, citric, malic or lactic acid also bind zinc and improve its uptake at mucosal cells (Lönnerdal, 2000). The most studied antagonism is, for sure, the interaction of zinc with other trace elements like copper and iron (Baker and Ammerman, 1995). Another example might be the secondary zinc deficiency after long-term supply of demand-exceeding amounts of calcium (Jeroch et al., 2008).

2.3.2.5 Zinc Bioavailability of Different Zinc Sources

Zinc uptake into the enterocytes is primarily transporter-driven, and therefore requires soluble zinc ions. Zinc in the stable zinc oxide form is less soluble than zinc from other zinc sources, e.g., feed-grade zinc oxide has only 50 % of the bioavailability of zinc sulphate (Baker and Ammerman, 1995). Zinc accumulation in bones and growth rates of growing animals are counted as reliable indicators of relative zinc bioavailability. As indicated in chicken studies, the metallothionein level in the liver might be another useful indicator for zinc bioavailability (Sandoval et al., 1992).

After a reduction of zinc concentration in complete feedstuffs of 40 % by the Regulation (EC) No 1831/2003, one concern was, if the safety bands regarding possible interactions with other dietary ingredients were still adequate for animals with a high requirement, but a low feed intake like after weaning (Männer et al., 2006). In order to fulfill the dietary zinc requirements for newly weaned piglets, while these animals have a lower feed intake capacity, several studies in weaning pigs tried to replace dosages of dietary inorganic zinc oxide with lower levels of different organic zinc sources with a suspected higher bioavailability, such as zinc methionine, zinc proteinate, zinc amino acid chelates, zinc polysaccharide and zinc amino acid complexes (Hollis et al., 2005). Other authorized organic and inorganic zinc combinations

are zinc lactate, zinc acetate, zinc carbonate, glycine zinc chelates, zinc chloride monohydrate and zinc sulphate heptahydrate or monohydrate which differ very much between their zinc bioavailabilities (Regulation (EC) No 1831/2003).

Due to the fact that the feed-grade sources of zinc oxide vary extremely, a decade ago, relative bioavailability (RBV) became also an issue of interest. In chicks, differences in RBV of zinc between less than 40 % and more than 90 % were found by different authors (Edwards and Baker, 1999; Sandoval et al., 1997). In piglet performance studies by Mavromichalis et al. (2000), zinc oxide sources with a low RBV (39 %) manufactured by the Waelz process dominating, for instance, the US-market, were compared to high-RBV zinc oxide sources achieved by the hydrosulfide process. The results showed a significant increase of ADG in the first week of feeding zinc with a high RBV compared to zinc with a low RBV. Nevertheless, considering the whole study period, the RBV of zinc in these two different zinc oxide sources seemed not to have a substantially influence on the growth-promoting efficacy of zinc oxide in weaning pigs. A general higher feed intake in zinc oxide fed piglets is generally assumed to be the main reason for the differences in weight gain. A not yet fully evaluated reason might be an enhancement of the ghrelin production via zinc supplementation in the stomach of the young pigs. This might also enhance the appetite via a stimulus on appetite leading to increased feed intake and other complex feedback-mechanisms (Yin et al., 2008).

But bioavailability of zinc does not only depend on the interaction with other dietary nutrients and the source of zinc, but also in which compartment zinc content was measured. Experiments with chicken revealed that the bioavailability of zinc oxide is only 61 % compared to zinc sulphate when measured by tibial zinc content (Wedekind et al., 1992). Later, differences between loci were determined based on different response characteristics (e. g., metacarpal, coccygeal or plasma zinc content etc.) in pigs (Wedekind et al., 1994). Furthermore, beneficial effects of, for instance, zinc methionine complexes on immune function like macrophage recruitment and adherence in turkeys were also under discussion (Kidd et al., 1994), but it seems that potential benefits in pigs have not been too intensively and critically evaluated yet.

Zinc amino acid complexes have been described to exert positive effects on intestinal development and immune function in pigs in the 24 h after weaning (Caine et al., 2001). No differences could be detected in a pig trial comparing different organic zinc sources and zinc sulphate when looking at different immune parameters, organ zinc concentrations and performance parameters (Van Heugten et al., 2003).

Organically bound zinc (such as zinc-methionine 1:2 or zinc-yeast) seem to have the ability to replace higher doses of inorganic zinc salts because of an increased digestibility and significant enhanced retention. This fact indicates that the kind of chelates might be an important factor for the zinc retention process (Nitrayova et al., 2012).

An overview of some often cited studies' regarding the comparison of organic and inorganic zinc sources in piglet diets is given in **Table 2.4**. This short summary considers the possibility to replace inorganic zinc sources via organic zinc sources such as zinc-lysine, zinc-

polysaccharide or zinc-glycinates due to an environmentally friendly reduction of the excreted zinc amounts and the amounts which need to be fed to the animals. Nevertheless, results of these studies with organic zinc sources concerning performance parameters are relatively inconsistent.

Table 2.4: Overview of animal feeding trials evaluating the relative bioavailability of different zinc sources in pigs

Zinc sources	**Response criteria**	**Zinc (mg/kg diet)**	**No. of exp.**	**Results**	**Reference**
Zinc oxide Metallic zinc dust	Performance Serum zinc	25, 50	1	- Growth and serum Zn: independent of source - RBV of Zn dust: ~30 % greater than of ZnO	(Miller et al., 1981)
Zinc sulphate · $1H_2O$ Zinc methionine	Performance Bone zinc Serum zinc	9, 12, 15	2	- Zn sources are of similar biological value	(Hill et al., 1986)
Zinc sulphate · $7H_2O$ Zinc carbonate Zinc oxide	Absorption	127	1	- $ZnSO_4$ slightly lower bioavailable than ZnO	(Hap and Zeman, 1994)
Zinc sulphate · $1H_2O$ Zinc methionine Zinc lysine Zinc oxide	Performance Bone zinc Serum zinc	5, 10, 20, 40, 80	2	- bioavailability ranking: $ZnSO_4$ > Zn-Methionine > ZnO > Zn-Lys (organic Zn ≠ better available than inorganic)	(Wedekind et al., 1994)
Zinc sulphate · xH_2O Zinc methionine Zinc lysine Zinc oxide	Bone zinc Serum zinc	1000, 2000, 3000	3	- no differences between sources regarding performance - serum + liver Zn↑ (for $ZnSO_4$ > ZnO) - Bioavailability: ZnO < Zn-lysine = Zn-methionine < $ZnSO_4$	(Schell and Kornegay, 1996)
Zinc sulphate · xH_2O Zinc amino acid chelate	Zinc in serum and soft tissue	45	2	- clear effect of Zn content in the diet, but no effect of zinc source	(Swinkels et al., 1996)
Zinc sulphate · $1H_2O$ Zinc lysine	Absorption Serum zinc Bone zinc	100	4	- $ZnSO_4$ + Zn-lysine = equally effective in promoting growth performance, Zn absorption + tissue Zn storage	(Cheng et al., 1998)
Zinc-Polysaccharide Zinc oxide	Performance Plasma Zinc	Zn-PS: 150, 300, 450; ZnO: 2,000	1	- Equal effects on performance: for either 300/450 ppm (Zn-PS) or 2,000 ppm - 300 ppm Zn-PS: Zn shedding decreased (76 %) compared to ZnO	(Buff et al., 2005)

Zinc sources	Response criteria	Zinc (mg/kg diet)	No. of exp.	Results	Reference
Zinc oxide Zinc methionine	Performance	Zn-Met: 125, 250, 500; ZnO: 2,000; 2,500	2	- Zn-Met: not improved performance - 500 mg Zn (organic or inorganic) was not as efficient as 2,000 mg ZnO at improving performance	(Hollis et al., 2005)
Zinc sulfate Zinc chelate Zinc glycinate	Performance		1	- higher bioavailability of Zn glycinates plus plus reduced faecal excretion	(Männer et al., 2006)
Zinc oxide Zinc-methionine 1:2 Zinc-glycine Zn-yeast	Performance Serum zinc	10, 100 (ZnO); 10 (other Zn sources)	1	- Zn-bioavailability: Zn-yeast & Zn-Met better available than ZnO	(Nitrayova et al., 2012)
Zinc oxide Zinc-montmorillonite hybrid (ZnO-MMT)	Performance Intestinal permeability Enzyme activities	ZnO-MMT: 250, 500, 750; ZnO: 2,000	1	- performance: 500/ 750 mg ZnO-MMT = as efficiently as 2,000 mg ZnO	(Hu et al., 2012)

(after Jongbloed et al., 2002; plus own literature review: 2002-2013).

2.3.2.6 Zinc Deficiency

Nowadays, a clinically manifest zinc deficiency is a rare phenomenon in livestock industry (Hu et al., 2012). But if it comes to an inadequate dietary zinc uptake or zinc deprivation, the very first signs are loss of appetite followed by thickened, hardened and cracked skin and skin lesions over the extremities (Berger, 2003; Poulsen and Larsen, 1995). Furthermore, through its role in keratin synthesis, maturation and wound healing, zinc deprivation leads to abnormalities in fetal and skeletal growth, a general lower performance, dermatitis, impaired wound healing (Baker and Ammerman, 1995; Berger, 2003; Shelton et al., 2005) and a decreased T-cell function in mammals resulting in increased susceptibility to infection (Berger, 2003; Sugarman, 1983). These symptoms in swine have been summarized under the disease parakeratosis as it has been already mentioned in **Chapter 2.3.2.1** (Tucker and Salmon, 1955). Furthermore, shortened villi and crypts were observed (Whitenack et al., 1978), while activities of most mucosal enzymes remained unaffected (Lalles et al., 2007c). In addition, zinc deficiency generally leads to a decrease in plasma zinc and plasma alkaline phosphatases. Another aspect of zinc deficiency regards a potential reduction of growth hormone production, essential fatty acid metabolism and lipoprotein metabolism (Keen and Graham, 1989).

2.3.2.7 Zinc Intoxication

The extent of zinc tolerance depends partly on the species and on the nature of diet (like its calcium, copper, cadmium and iron content), but livestock in general exhibits a considerable tolerance to high amounts of ingested zinc. Nevertheless, several cases from zinc toxicity occurred in several species through the intake of material from galvanized surfaces or coins/nails in barns or errors in the diet formulation process (Pritchard et al., 1985). Feeding studies with weanling pigs with dietary zinc contents up to 3,000 ppm showed almost no effects, but from 4,000-8,000 ppm onwards, growth and appetite were depressed, and mortality rates increased (Suttle, 2010). Furthermore, joint and bone lesions were mentioned under experimental conditions in swine (Grimmett et al., 1937). An association between zinc intoxication and acinar cell necrosis, fibrosis and atrophy in the pancreas of piglets receiving TPN has been found which has not been observed in pigs before, but in ruminants, carnivores, laboratory animals and humans (Gabrielson et al., 1996). The crucial problem of supplying trace minerals via the intravenous route is that many possible homeostatic mechanisms in the intestine, e.g., absorption, binding to proteins, interactions with other trace elements or dietary fiber, and so on, are bypassed. Furthermore, the excretion route of zinc via serosal to mucosal flux and SI epithelial cell desquamation is diminished in TPN-patients and also in newly weaned piglets due to villus atrophy and declined enterocytes' renewal (Gabrielson et al., 1996). Reasons for this destructive appearance of zinc might be drawn back to its function as strong Lewis acid within cells (for more details in **Chapter 2.3.2.2**) which might act strongly corrosive via chemical reactions in the stomach with hydrochloric acid building zinc chloride. When it comes to a high zinc load and an outbalanced zinc homeostasis in the gut, the excess of zinc in the enterocytes might destroy the cell leading possibly – like in the pancreas – to atrophy and necrosis.

2.3.2.8 Zinc Requirements and Zinc Recommendations

Recommendations of GfE and NRC for dietary zinc supply consider not only the basal requirement of the pig, but also possible interactions with dietary phytate, calcium or other divalent metal ions in the diet, and additional 'safety allowances' (GfE, 2006; NRC, 2012). To date, the recommended dietary concentrations of zinc for weaned piglets are between 80 to 100 mg/kg (see **Table 2.5**), whereas the upper limit in the EU is set at 150 mg/kg to prevent an excessive input into the environment via the pigs' manure (European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003).

The zinc demand in swine differs due to the life weight (see **Table 2.5**), and is also related to sex and age. For example, castrated male pigs have a lower demand than sows or boars, and gilts have a higher requirement than barrows (GfE, 2006; NRC, 2012). For weanling pigs, an adequate conventional weaning diet containing 80 mg zinc/kg DM seems appropriate (Van Heugten et al., 2003), and for growing pigs even 50 mg zinc/kg DM can be adequate when the diet contains isolated protein sources, and covers the recommended calcium level (NRC, 2012).

Table 2.5: Recommended dietary allowances for zinc depending on body weight and sex

<table>
<tr><th>Category</th><th>ARC (1981)
mg/kg DM</th><th>INRA (1984)
mg/kg diet</th><th>NRC (1998, 2012)
mg/kg diet</th><th>GfE (2006)
mg/kg DM</th></tr>
<tr><td>3-10 kg BW</td><td rowspan="6">50</td><td rowspan="6">100</td><td>100</td><td>100</td></tr>
<tr><td>10-20 kg BW</td><td>80</td><td>80</td></tr>
<tr><td>20-50 kg BW</td><td>60</td><td rowspan="2">50-60</td></tr>
<tr><td>50-120 kg BW</td><td rowspan="3">50-60</td></tr>
<tr><td>Reproductive sows</td><td>50</td></tr>
<tr><td>Boars
(sexually active)</td><td>-</td></tr>
</table>

Adapted from: ARC, 1981; GfE, 2006; INRA, 1984; NRC, 1998, 2012.

But there are also differences between different agencies and commissions. Nevertheless, with all the safety margins included in these recommendations, the zinc demand for swine should be definitely covered under normal circumstances with the absence of diseases and other challenges and by feeding an appropriate and well balanced diet.

2.3.2.9 Supply of Very High Dietary Levels of Zinc in Piglet Nutrition

Impact of Zinc on Performance and Gut Health

During the last three decades, several supplementation studies with pharmacological levels of zinc oxide have shown to significantly improve health status and performance of newly weaned piglets (Hahn and Baker, 1993; Hill et al., 2001), and to reduce the incidence of diarrhoea in pigs (Fairbrother et al., 2005; Hahn and Baker, 1993) and in humans (Behrens, 1993). Several studies with these pharmacological dosages have demonstrated to have positive effects in piglets, e. g., preventing the development of post-weaning diarrhoea (Owusu-Asiedu et al., 2003). Additionally, there seems to be a growth-promoting effect at 2,000-2,500 mg zinc/kg diet (Carlson et al., 2008; Hollis et al., 2005), with the best results at 2,500 mg zinc/kg without affecting feed intake or feed efficiency (Poulsen, 1995). Furthermore, zinc oxide is influencing the intestinal microbiota (Hojberg et al., 2005), wound healing (Bhar et al., 2003), appetite and the immune system (Fischer Walker and Black, 2004).

Reasons are not yet completely clear, but possible modes of action have been accredited to the influence of zinc on the gut microbiota, digestive or epithelial barrier function (Hedemann et al., 2006; Hojberg et al., 2005; Vahjen et al., 2010, 2011; Zhang and Guo, 2009). Adjuvant effects of pharmacological dietary zinc levels on piglets' performance and intestinal growth

may also be exerted via increasing the *IGF-IR* and *IGF-I* expression in the small intestinal mucosa without affecting serum-IGF levels (Li et al., 2006). On the other side, Carlson et al. (2004) hypothesized that growth-enhancing effect of high zinc might be exerted via increased serum IGF-I. As a further possible pathway, the approach of Ou et al. (2007) showed that zinc oxide decreases stem cell factor expression (both on protein and on mRNA level) in the small intestine of piglets, the number of mast cells and histamine production resulting in the decreased appearance of diarrhoea.

Additionally, a further impact on zinc homeostasis has been reported via increasing zinc concentrations and the induction of metal binding proteins, such as metallothionein in various tissues, thus leading to an impact on animal health and performance (Carlson et al., 1999; Martinez et al., 2004; Schell and Kornegay, 1996; Williams et al., 2005). Unfortunately, up to now, almost only studies with extremely high dietary zinc intakes, mainly from zinc oxide, with 1,000-4,000 mg zinc/kg diet which are far beyond the maximum admitted concentration in the European Union, are published – studies on dose-dependent responses regarding 'normal' levels (approx. 150 mg zinc/kg diet or less) are still rare.

Effects of Zinc on the Intestinal Microbiota

Several mechanisms are discussed regarding the effects of high dietary zinc oxide including the influence on the gut microbiota (Vahjen et al., 2011). Pieper et al. (2012) showed that increasing zinc oxide levels in the diet can lead to an increased bacterial diversity in the ileum which might be an indicator for a more stable ecosystem, while others showed inhibiting effects through high dietary zinc levels on the bacterial metabolism (Choudhury and Srivastava, 2001), a decreased abundance of lactobacilli, an increased number and diversity of enterobacteria as well as an increase of coliforms and enterococci (Hojberg et al., 2005; Vahjen et al., 2010, 2011). Nevertheless, effects on total concentration of *E. coli* could not be shown (Broom et al., 2006). Earlier studies with high dietary zinc supply also reported a stabilized gut flora and diverse coliforms within the first two weeks after weaning which may compete with pathogenic strains for the colonizing receptor sites (Katouli et al., 1999). In the sensitive post-weaning period of piglets, an improved gut health via the stabilization of the intestinal microbiota with high dietary zinc levels might, so far, play an important role in the resistance against intestinal disorders (Vahjen et al., 2011; 2012).

Nevertheless, it should be mentioned here that the long-term use of zinc as alternative to antibiotics might result in increased antibiotic resistance via cross-resistance or co-selection etc., especially in the strain *E. coli*, as new studies indicate (Bednorz et al., 2013).

Effects of Zinc on Gene Expression

Zinc is an important regulator of the gene expression and a constituent of over 2,000 transcription factors (Suttle, 2010). An association between dietary zinc treatment and a marked decrease in the expression of immune response genes (cytokines) affecting

inflammation events in the gut has been shown in an ETEC challenge trial with weaning piglets (Sargeant et al., 2010). In general, zinc acts on different ways: as being a required catalytic unit in the RNA polymerase for RNA synthesis and via the increase of gene expression through the occupancy of special sites in transcription factors (Cousins, 1998). Some responsible constituents on gene expression are the zinc-finger motifs in zinc-finger proteins which are a class of DNA- or RNA-binding molecules acting as transcription factors. But there is a growing body of evidence that these domains also participate in interaction with proteins (Brayer and Segal, 2008). New research approaches are using the zinc-finger domains in order to selectively switch genes on and off which seems a promising tool for future approaches, for example, in biomedical therapies or in plant science for the adaptation of breeding improved crops (Klug, 2010).

Furthermore, zinc is known as a potent inhibitor of the effector caspase-3 (Perry et al., 1997). One potential mode of action might be the zinc-binding protein metallothionein (MT) which seems to be an important regulator of the apoptosis (Klaassen et al., 1999). MT can prevent apoptosis in cells induced by metals, like zinc or cadmium, and oxidative stress.

Studies with HepG2 cells (pre-treated with cadmium) showed a strong linear negative correlation of basal MT levels and etoposide (an anti-cancer drug)-induced apoptosis, while the pre-treatment of these cells with zinc led to increased MT synthesis (Shimoda et al., 2003). Another crucial protein in the complex mechanisms of the cell cycle is PCNA (Moldovan et al., 2007). Zinc might also play a role in this complex replication system through ubiquitin-binding of a zinc-finger domain of polymerase η (Masuda et al., 2010).

Therefore, an effect of dietary zinc supplementation in pharmacological dosages on gene expression patterns can be expected.

Effects of Zinc Supplementation on Gut Barrier Function

Generally, a single layer of adjacent epithelial cells lining the GIT builds a critical barrier limiting the uptake of antigens and noxious detergents from the lumen into the blood stream (Benjamin et al., 2000). This gut barrier is determined by a transcellular and a paracellular component. The paracellular permeability of enterocytes in the SI is determined by the integrity and composition of tight junctions (Barrett, 1997; Fasano, 2000). A marker for the barrier function is the unidirectional mannitol flux rate. Macromolecules are often used to measure the transcellular permeability, for instance, horseradish peroxidase (Benjamin et al., 2000).

The weaning process exerts a huge effect on these small intestinal barrier functions. The so-called 'first defense line' in the gut is thereby strongly affected. Four main factors play a major role: Weaning age, weaning stress, feed intake and diet composition (Wijtten et al., 2011). Both barrier functions are declined in the first two weeks after weaning, especially in jejunal regions, therefore it is important to maintain them: These weaning effects can be diminished through an adequate feed intake preventing the loss of villus height (Wijtten et al., 2011). Altogether, an increased mucosal permeability and a disturbed absorptive-secretory

balance characterise the transient alterations of piglets around weaning (Boudry et al., 2004). The effect of zinc on gut barrier function is not completely clear yet. On the one hand, it is known from human projects against malnutrition that prolonged zinc deficiency in the context with protein-calorie malnutrition might alter absorption capacity in the small intestinal mucosa in mammals. Furthermore, mucosal atrophy is induced leading to decreased absorption of water and electrolytes. Resulting in secretory diarrhoea, further zinc losses through watery digesta are the results which lead to even greater malabsorption of zinc and other nutrients (Ziegler et al., 2003). On the other hand, Carlson et al. (2008) showed in Ussing-chamber studies that the diarrhoea-reducing effects of zinc are not directly exerted via the basolateral side of the epithelium. This means zinc has to be absorbed and then circulates in the blood from where it could influence the secretion, while Hoque et al. (2005) detected a secretion-reducing effect of zinc only at the serosal side. Other possible mechanisms of zinc on reduced secretion might be the blocking of basolateral K^+ and/or Ca^{2+} channels, or altering the activity of membrane-bound enzymes like the adenylate cyclase which catalyzes the intracellular cAMP synthesis (Carlson et al., 2008).

Effects of Pharmacological Zinc Homeostasis and Zinc-Binding Proteins

To date, most research on zinc transporters and their role in the homeostasis of the body zinc pool has been done in rodents and in *in vitro* cell culture models by using adequate vs. marginal or suboptimal zinc concentrations. Balance studies with rodents have revealed that under minimal or exceeding dietary zinc supply, zinc homeostasis is mainly regulated through intestinal uptake and faecal excretion (Weigand and Kirchgessner, 1980; Windisch and Kirchgessner, 1995; Windisch and Kirchgessner, 1994). According to rat studies of Fujimura et al. (2012), a high zinc supply leads to a downregulation of *ZIP4* and *ZnT1* expression in the jejunum. These researchers could further show in deficiency studies with rodents that the zinc homeostasis is influenced by the level of dietary zinc which is then followed by a reduced or increased absorption from and an decreased or increased secretion into the gut lumen, with respect to the available amount of zinc (Weigand and Kirchgessner, 1980; Windisch and Kirchgessner, 1995; Windisch and Kirchgessner, 1994). Furthermore, it has been investigated that under marginal zinc supply, *ZIP4* expression is upregulated in enterocytes, likely to increase zinc uptake from the gut lumen, whereas the expression of *ZnT1* and *ZnT2* is downregulated (Cousins et al., 2006; Pfaffl and Windisch, 2003). The expression of metallothionein (*MT*) is likewise downregulated in intestinal tissue under marginal zinc supply (Cousins et al., 2006; Pfaffl and Windisch, 2003). However, to date, there are only very few data available in humans or their omnivorous counterpart, the pig (Hill and Link, 2009), because most research has been done in rodent models. Therefore, as shown in **Figure 2.2**, several questions remain, especially regarding the effects on zinc transporters after supplementation of high zinc dosages, and whether there are differences detectable in the gene expression patterns of these transporters between pig and rodent model. Contrary to

that, high/pharmacological dosages of zinc oxide are more and more used for the prevention or treatment of gastrointestinal disorders in humans, especially young children in developing countries (Dekate et al., 2013; Gupta et al., 2007) and in newly weaned piglets.

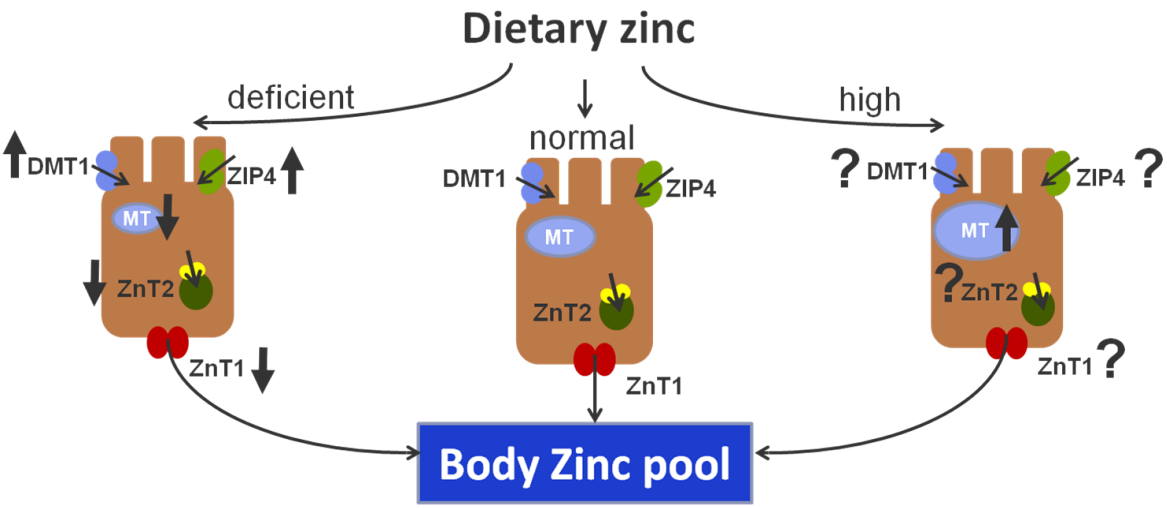

Figure 2.2: Zinc transporter in the jejunum of rodent models under marginal, normal and high zinc supplementation (and respectively with question marks for the pig)

After reports on increasing zinc concentrations in various tissues (Martinez et al., 2005; Schell and Kornegay, 1996), it is questionable whether a homeostatic regulation of zinc in the body can still be maintained at these high levels. Especially the ZIP4 transporter seems to be a crucial factor in zinc uptake mechanisms as evidenced in a review by Lichten and Cousins (2009). They showed that severe deficiency symptoms were discovered in various species including humans caused by mutations of the *ZIP4* gene (Liuzzi et al., 2009). This mutation causes a disease called *Acrodermatitis enteropathica* leading to a low intestinal zinc absorption and severe zinc deficiency symptoms, and was discovered in humans in the early 1960's (Moynahan, 1962). As revealed in a mouse model, a zinc-finger transcription factor called Krüppel-like factor 4 (*KLF4*) seems to be responsible for the adaptive regulation of *ZIP4* expression. This *KLF4* expression was enhanced during zinc-deficient conditions, and its response element then upregulates *ZIP4* expression in the small intestine (Liuzzi et al., 2009). Nevertheless, the mechanism in the pig is unclear to date. Latest research results on the genetic variations of the porcine ZIP4 transporter indicate that also the genotype might have an influence on the individual pig's ability to absorb zinc in the jejunum (Siebert et al., 2013). These results are suggesting an influence of the respective breed and the importance to consider these findings for the prospective breed selection regarding future zinc feeding trials.

However, assuming that different dietary zinc levels will influence the expression of zinc transport and zinc-binding proteins in the porcine intestine in order to maintain homeostasis, further research is necessary regarding the aspect of long-term zinc supply, because a

deregulated zinc homeostasis can have significant consequences with respect to health and organ function.

CHAPTER 3: Aims and Objectives of the Thesis

Nutritional factors exert manifold effects on different pathways in the organism and may have a great impact, for instance, on digestive physiological measures, on the complex host microbiota, the metabolism in diverse organs and on functional patterns of the immune system in the host. Therefore, they may all have an impact on the health of the host organism and on the respective performance. These interactions are summarized to some extent in **Figure 3.3**. Nevertheless, complex knowledge about the modes of action regarding diverse feed additives such as the probiotic *Enterococcus faecium* and trace elements like zinc (as zinc oxide) is still incomplete, and mechanisms are not fully understood.

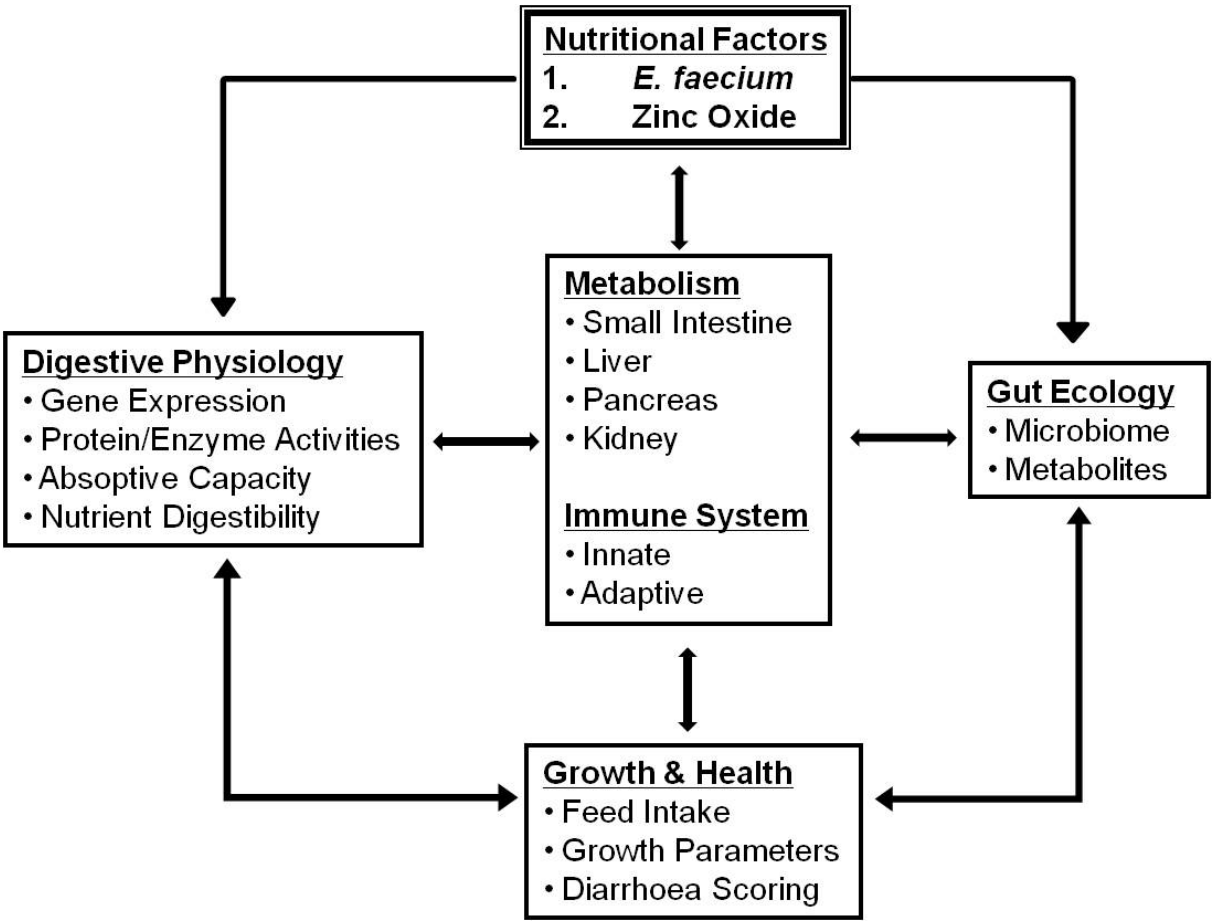

Figure 3.3: Interactions between the host, nutritional factors (the probiotic *Enterococcus faecium* NCIMB 10415 and zinc oxide) and the gut microbiota

Firstly, *E. faecium* NCIMB 10415 is a commonly used probiotic strain in pig production, but the results of its impact on piglet performance, gut health and possible modes of action are currently giving a not very consistent picture in the literature. Therefore, we conducted a feeding trial with sows and their respective piglets receiving *E. faecium* NCIMB 10415 hypothesizing

that the application of *E. faecium* NCIMB 10415 does have an influence on the performance and on the problem of PWD in piglets (under experimental, not farm conditions).
We further aim to address the following objectives:

1. Can *E. faecium* NCIMB 10415 lead to changes in small intestinal/jejunal physiology of these piglets and to an altered digestibility of the dietary nutrients?

2. Furthermore, it has to be considered if either the probiotic application or the weaning process itself has a greater impact on jejunal digestive physiology (gene expression patterns, enzyme activities) and morphometry in these piglets.

Secondly, besides the mentioned well-known positive effects of zinc oxide on diarrhoea and performance parameters (see **Chapter 2.3.2.9**), to date, it is not completely known whether a high dietary zinc supply for longer periods would have similar effects than for shorter periods. Also, whether an extended zinc supply after weaning could also negatively affect animal physiology and performance is yet not entirely clear. For obtaining a broader picture in the current thesis, two zinc studies were conducted within the SFB 852 collaboration to evaluate the effect of three different zinc concentrations: from a sub-optimal (57 mg/kg) over a medium (164 mg/kg) to a high or so-called 'pharmacological' (2,425 mg/kg) level of zinc in the diet, given as zinc oxide. We hypothesize that the supplementation of high dosages of zinc oxide will have a fundamental impact on performance, digestive physiological parameters and molecular zinc transport mechanisms in the jejunum of weaned piglets.

In detail, we aim to address the following objectives:

1. To which extent do different amounts of zinc have different effects on the piglets' performance, on the appearance or absence of post-weaning diarrhoea, or on measures of small intestinal physiology (such as enzyme activities and gene expressional patterns) after the weaning of these piglets?

2. High dietary zinc supply leads to an outbalanced zinc homeostasis in the body thus resulting in increased zinc accumulation in different tissues (kidney, spleen, pancreas, bone and liver) and in increased serum zinc levels in the pig.

3. Is there an optimal length of feeding additional zinc oxide after the weaning? And might there be a time frame when zinc supplementation becomes crucial for the pig?

4. Furthermore, another aim of the thesis is if we can achieve better insights into molecular transport mechanisms of zinc in the small intestine, respectively pig-specific regulation mechanisms for maintaining the homeostasis in the porcine gut.

5. Purely hypothetically: Can we find a more custom-fitted level of zinc supplementation in pig production in order to balance advantages of the zinc supplements and the disadvantage of environmental problems due to zinc shedding?

Although other factors regarding the influences of the nutrition factors on digestive physiology, immunology or microbiota of the host, are also of great importance, they do not play a role within the scope of the current thesis.

The results of the current thesis are carefully obtained with three different animal trials, and evaluated and summarized in three different manuscripts following in the next chapters (**Chapter 4-6**).

CHAPTER 4: Influence of Age and *Enterococcus faecium* NCIMB 10415 on the Development of Small Intestinal Digestive Physiology in Piglets.

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Authors: Lena Martin, Robert Pieper, Susan Kröger, Farshad Goodarzi Boroojeni, Wilfried Vahjen, Konrad Neumann, Andrew G. Van Kessel, Jürgen Zentek

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CHAPTER 5: Performance, Organ Zinc Concentration, Jejunal Brush Border Membrane Enzyme Activities and mRNA Expression in Piglets Fed Different Levels of Dietary Zinc.

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Authors: Lena Martin, Robert Pieper, Nadine Schunter, Wilfried Vahjen, Jürgen Zentek

http://dx.doi.org/10.1080/1745039X.2013.801138

CHAPTER 6: A High Amount of Dietary Zinc Changes the Expression of Zinc Transporters and Metallothionein in Jejunal Epithelial Cells *in vitro* and *in vivo*, but Does Not Prevent Zinc Accumulation in Jejunal Tissue of Piglets.

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CHAPTER 7: General Discussion and Conclusions

Weaning constitutes a stressful event for piglets and causes considerable economic problems for the livestock industry. Besides other problems like social and environmental stress factors, the young piglet has to cope with the immediate shift from highly digestible sow's milk to cereal based, plant derived feed with complex carbohydrates, proteins and other nutrients – and this situation requires rapid physiological adaptation processes of both the intestinal microbiota and the physiology of the host himself (as reviewed in **Chapter 1 & 2**). Most likely, the reduced feed consumption and health impairments, like a transient gut inflammation or compromised host immune response during the weaning process (Berg, 1995), lead to an increased risk of intestinal pathogen overgrowth and diarrhoea, reduced body weight gain and a higher mortality rate. It even culminates in diminished animal welfare and high economic losses in the pig production industry (Lalles et al., 2007a, b; Pie et al., 2004; Pluske et al., 1997). In order to overcome these weaning disturbances, in-feed antibiotic growth promoters have been the method of choice for several decades. But serious concerns of the public were raised about the association between the increasing use of antibiotics for growth promotion and the risk of emergence of antibiotic-resistant bacterial strains in human medicine (Broom et al., 2006). Finally in January 2006, the application of in-feed antibiotics as AGP has been banned in the European Union and since then, many studies have been focussing on alternatives to antibiotic growth promoters. One potential alternative might be the modification of intestinal microbial communities by using pre- and probiotics, as well as diverse trace elements, essential oils, short chain fatty acids and other additional feed ingredients as an approach to maintain 'gastrointestinal health', and promote growth of the animals (see **Chapter 1 & 2**).

Within this present thesis, three different studies were conducted to gain a deeper insight into the role of feed additives such as the probiotic *E. faecium* NCIMB 10415 and the trace metal zinc influencing digestive physiological parameters in suckling and weanling piglets. In the first project of this thesis (**Chapter 4**), we therefore aimed at determining the influence of *E. faecium* NCIMB 10415 on performance, intestinal health (diarrhoea scoring), total tract and ileal nutrient digestibility, as well as intestinal physiological measures in piglets before and after weaning.

Chapter 5 & Chapter 6 have been dedicated to the trace element zinc fed as zinc oxide to weaned piglets in order to achieve deeper insights in digestive physiological and performance parameters as well as the influence of zinc on the gene expression level – and how the porcine metabolism tries to maintain a homeostasis during high zinc supplementation.

Application of *Enterococcus faecium* NCIMB 10415 in young piglets

Regarding approaches for the evaluation of feeding concepts, classical parameters such as feed intake, feed digestion, and excretion, as well as nutrient digestibility (total tract and ileal) were taken into consideration for several decades. Besides this 'classical field' of animal nutrition, which is of great importance for the farmers, more and more other aspects and mechanistic approaches (including enzymatic analysis of BBM digestive enzymes and their respective gene expression, western blot analysis of specific proteins relevant for digestion, etc.) have been taken into account when evaluating a feed additive and the respective modes of action (**Chapter 2**).

Recent studies have documented that feeding of *E. faecium* NCIMB 10415 can improve the performance of piglets during the post-weaning period (Chen et al., 2006; Taras et al., 2006; Zeyner and Boldt, 2006). Significant positive effects on weight gain and feed consumption were reported during a four-month feeding-trial with *E. faecium* SF68 (Maeng et al., 1989). In earlier studies, on the one hand, *E. faecium* did not affect the piglets' growth performance (Broom et al., 2006; Pollmann et al., 1980). But on the other hand, performance-enhancing effects of dietary *E. faecium* SF68 were reported for one week after weaning (according to 'unpublished data' in Broom et al., 2006). A reduced rate of diarrhoea was also reported when *E. faecium* was fed to sows and their piglets (Taras et al., 2006). However, the picture in the literature seems pretty contradictory. Furthermore, it has been hypothesized that the nutrient utilization as well as the performance are affected via probiotic influences on the intestinal physiology (Simon, 2010), albeit spore forming probiotics might act differently. Especially the genus *Enterococcus*, in particular the species *E. faecium*, are regarded as normal components of the intestinal microbiota in pigs (Devriese et al., 1994). Only little and very inconsistent data has been yet available whether probiotic feeding, especially *E. faecium*, can influence the nutrient digestibility in pigs (Chen et al., 2006; Kornegay and Risley, 1996). So, we hypothesized that the commonly used probiotic *E. faecium* might positively influence the performance and the problem of PWD in piglets. This might be due to changes in small intestinal/jejunal physiology of these piglets or to the altered digestibility of the diet via this special probiotic application.

Therefore, a feeding trial with sows and their respective piglets was conducted within the SFB 852 in which sows received the probiotic *E. faecium* NCIMB 10415 from day 28 *ante partum* on, and piglets received the probiotic from day 12 until day 54 of life on. Contrary to previous data from above mentioned researchers, the results from our own study have shown that *E. faecium* NCIMB 10415 had neither an enhancing effect on growth performance, nor on feed intake, incidence of diarrhoea, proximate precaecal or total digestibility of nutrients in the small intestine of the piglets (**Paper 1, Chapter 4**). These findings are comparable with the results of a study by Böhmer et al. (2005) where piglets were fed an *E. faecium*/inulin mix. But unlike to the low diarrhoea incidence in our study, another study showed that *E. faecium* led to an increased ADG and a reduced occurrence of diarrhoea in the weanling pigs on farm-level (Zeyner and Boldt, 2006). In their study, moreover, the diarrhoea incidence was generally very

high (40 % of all piglets in the control group). Therefore, as mentioned in the literature review, it is possible to demonstrate positive and significant effects of probiotic supplementation in sick animals. In our study, the lacking effects of the probiotic application might have different reasons. One might be the good sanitary condition in the present *E. faecium* trial which could attribute to the fact that the piglets' faecal scores were relatively unaffected by the diet group in this study.

Further Ussing-chamber studies with the jejunum of the same piglets revealed that *E. faecium* supplementation led to increases in the absorptive and secretory capacity and indicated an improved intestinal barrier function (Klingspor et al., 2013). This might protect against invasion and colonization of pathogenic bacteria, as well as the exposure to their produced toxins, and thus can result in a reduction of diarrhoea. But as already mentioned, the incidence of softer feces/diarrhoea was generally very low which made it very difficult to draw conclusions whether an *E. faecium* application can have diarrhoea-preventing effects in piglets under these study conditions. It is also important to remark that several authors have already shown that both probiotic and antibiotic applications seem to have a more pronounced diarrhoea-decreasing and growth-promoting effect on farms where housing and hygiene are not optimal in comparison to relatively clean conditions in experimental stables (Janczyk et al., 2010; Mulder et al., 1997; Thomke and Elwinger, 1998).

However, due to the age difference of the animals, the comparability of the above mentioned positive effects of *E. faecium* on nutrient digestibility is restricted (Chen et al., 2006) – this study used growing-finishing pigs, while we conducted our study with weaning pigs. Nevertheless, others have proven that probiotic responses are age-related, and the best responses appear naturally directly after weaning, and then decline (Böhmer et al., 2005; Vanbelle et al., 1990).

Although ADG and ADFI were not markedly affected by the *E. faecium* treatment in the current study, a lower FCR in the *E. faecium* group could be detected. The FCR is of great economic value for the livestock industry in terms of production costs. However, the number of animals used in the present study was not high enough to draw final conclusions about the economic impact of the *E. faecium* supplementation in weaning piglets. Field trials with higher piglet numbers and under production conditions are necessary to determine a potential economic impact of *E. faecium* in piglets.

To evaluate possible modes of action of probiotics in pigs, their effects on digestive physiological parameters are of particular importance. The activities of disaccharidases in the brush border membranes, and especially their changes during postnatal development, have been consulted as good indicators of intestinal gut maturation in piglets (Willing and Van Kessel, 2009). We have established diverse assays to obtain brush border membranes from jejunal segments of the dissected pigs in order to measure the activities of disaccharidases (lactase, maltase, sucrase), aminopeptidase-N and intestinal alkaline phosphatase in microtiter plates (methods published in Martin et al., 2012; **Chapter 4**). Furthermore, several protocols

for quantitative real-time RT-PCR have been established in our laboratory to determine potential impacts of the investigated feed additives on the expression of genes related to gut development, digestive physiology and nutrient absorption (Martin et al., 2012; **Chapter 4**).

Previously, it has been shown in experiments with gnotobiotic pigs that intestinal colonization with different bacteria can influence the development of the small intestine and the brush border membrane enzyme activities (Willing and Van Kessel, 2007; 2009). Nevertheless, it is important to remark that these trials with gnotobiotic or germfree animals do not show the real situation in the gut, but they are only a very reductionistic approach to simulate influences of a single microbial strain, a specific nutrient or a nutritional process on the gut physiology without the interference with the host microbiota.

The results of our first feeding trial with the probiotic *E. faecium* connote that none of the investigated enzymes (both gene expression and activity) was significantly affected by the probiotic, neither during the pre-weaning (days 12 and 26 of life) nor during the post-weaning (days 33 and 54 of life) period. Similarly, intestinal morphometry and the gene expression of two cell turnover markers seemed also not significantly affected by the supplementation of *E. faecium*. While the effect of the probiotic on morphometric data like villus length and crypt depth was missing, a treatment-independent, but age-related atrophy of the gut mucosal villi and a respective crypt hyperplasia was observed one week after weaning. These are special weaning-associated characteristics which are connected with the post-weaning anorexia leading to a damage of the small intestinal mucosa, and corresponding to the gut adaptation process after the introduction of creep feeding (Lalles et al., 2007b; McCracken et al., 1999). Significant age-effects were observed for these parameters such that SUC and MAL activities increased continuously after the introduction of creep feed on day 12 until day 26, with a small decline of SUC activity in the week after weaning.

Lactase is a crucial enzyme in the neonate, such that it cleaves the disaccharide lactose (the major carbohydrate in mother's milk) into glucose and galactose. Therefore, a clear age-dependent decline in activity could be observed in our study, especially after introduction of creep feed on day 12 and around the weaning process when the cleavage of lactose becomes less important for the animal. As mentioned in **Chapter 2.2.3**, also expression patterns change during the migration of enterocytes along the villus-axis, such as the aminopeptidase-N (*APN*) which shows a low expression at the immature crypt and a high expression at the mature villus tip of the enterocytes (Fan et al., 2001). Before the weaning of the study piglets, *IAP*, *APN*, *LPH*, *SUC*, *SGLT1*, *CASP* and *PCNA* gene expression plus the enzyme activities of LAC, MAL and SUC were numerically, but not significantly reduced in the *E. faecium* group compared to the control group at all investigated time points. Gene expression profiles of BBM enzymes *LPH*, *APN* and *IAP* did not parallel their respective activity. It has also been observed that fasting periods shortly after weaning did not act on gene expression profiles, which means in contrast to the decline in enzyme activities, the respective mRNA coding for digestive enzymes was not influenced in a correlating manner (Montagne et al., 2007). However, an age-dependent

increase of *SUC* and *SGLT1* gene expression was observed indicating a differentiated pre- and post-weaning gene expression, irrespective of probiotic treatment (**Chapter 4, Table 4.10**). Klingspor et al. (2013) who investigated the *SGLT1* gene expression in a slightly different part of the jejunum of the same piglets confirmed with their gene expression results that *E. faecium* had no influence on *SGLT1* expression. In another experiment, a positive effect of *E. faecium* application on glucose-induced I_{sc} in suckling pigs was detected on the 14^{th} day of life (Lodemann et al., 2006), while another experiment with the same study set-up could not repeat this effect. However, they saw significant differences of glucose-induced I_{sc} in piglets on day 26 and day 54 of life compared to the control group (Klingspor et al., 2013). Nevertheless, also the increase in glucose uptake by *E. faecium* supplementation had no effect on the daily body weight gain, but acted rather contradictory: The ADG in the *E. faecium* group even tended to be smaller than in the control group, although no significant differences between the BW of the groups could be measured at the end.

Recent scientific literature has revealed a major role of intestinal alkaline phosphatase (IAP) in maintaining gut homeostasis and barrier function (Lalles, 2010), whereupon the most important function can possibly be seen in the involvement in bacterial LPS detoxification (Bates et al., 2007; Poelstra et al., 1997). As above mentioned, no correlation between IAP enzyme activity and gene expression could be detected, but both significantly declined after the weaning reflecting gut maturation processes. Although the trend towards the interaction (treatment x age) was not significant for *IAP* expression, this could presumably be considered as a direct or indirect effect of *E. faecium* on intestinal immunity through altered intestinal microbial communities. Fasting also seems to have a declining effect on IAP activity (Bamba et al., 1990), while studies in rats showed that dietary lactose level can increase the activity of IAP (Sogabe et al., 2004). However, as discussed in **Chapter 4**, interesting interactions with other factors such as morphological measures, expression of *SGLT1* and lactase activity were detected there. Furthermore, we observed a positive correlation of IAP activity and villus length as well as a negative correlation of IAP activity and crypt depth. These findings correlate well with the literature (Montagne et al., 2007; Pluske et al., 1997), where these results were associated with anorexia-induced mucosal atrophy and maturation processes in the small intestine of piglets post-weaning.

While feeding of *E. faecium* apparently had no spectacular effects on performance, nutrient digestibility and intestinal gut physiology of the piglets, several indicators could be observed in the experiments that this probiotic was responsible for diverse prominent immunological reactions in both the sows and their piglets. It has been previously shown that *E. faecium* treatment influences the immune response suggesting 'tolerogenic' effects of *E. faecium* feeding in piglets (Mafamane et al., 2011), while others documented alterations of the abundance of virulence factors from gram-negative *E. coli* (Scharek et al., 2005; Taras et al., 2006). These 'tolerogenic' findings were somehow comparable with a previous study where the effects of *E. faecium* feeding on B cells in the blood of sows and the influence of *E. faecium*

on enteric virus shedding were studied (Kreuzer et al.; 2012b). They observed that the relative proportions of B cell and T cell populations were altered in ileal Peyer's patches, ileal lymph nodes and in the blood. However, distinct mechanisms remain unclear.

An infection with *rotavirus A* can cause severe diarrhoea in both humans and in newborn and early weaned piglets (Martella et al., 2010). Interestingly, in a study with human adults suffering from acute diarrhoea, a shortening of the diarrhoeal phase via treatment with *E. faecium* could be detected (Buydens and Debeuckelaere, 1996). While *E. faecium* had no effect on the shedding of *hepatitis E* virus, encephalomyocarditis virus and norovirus compared to the unsupplemented control group, an additional result of this piglet trial conducted in our institute was that feeding *E. faecium* led to a reduced shedding of *rotavirus A* in sows and piglets and decreased their serum anti-rotavirus IgA and IgG levels. This might be an important point for minimizing the risk of the consumers (Kreuzer et al., 2012b).

Another interesting result of co-workers within this *E. faecium* trial was the antiviral influence of the probiotic on the coronavirus TGEV which leads to severe gastrointestinal infections in young pigs (Chai et al., 2013). *E. faecium* seems to have inhibitory effects on swine influenza viruses (H1N1, H3N2) which was discovered in diverse *in vitro* studies (Wang et al., 2013). They revealed two different mechanisms: Direct physical interaction of the virus with the probiotic and an increase of strength of the innate immune defense. These results sound promising, but further research, especially with piglet challenge trials, is necessary to validate single results partly achieved by cell culture experiments.

In two independent challenge trials with weaned piglets, however, it was shown that feeding *E. faecium* elevated the translocation and elongated the shedding of *S. Typhimurium* DT104 (Kreuzer et al., 2012a; Szabo et al., 2009). Another noteworthy finding within the SFB 852-/*E. faecium* trial was the unexpected impact of probiotic feeding on milk cell populations in the milk of *E. faecium*-treated sows. It was detected that probiotic-feeding led to a significant decrease of membrane-bound CD14 expressing epithelial cells in the milk of these sows (Scharek-Tedin – unpublished results). Together with the toll-like receptor 4 (TLR4), CD14 functions as co-receptor for the detection of bacterial lipopolysaccharides (LPS) (Chow et al., 1999). Others have reported that $CD14^+$ immune cells bind LPS which then activates the NF-κB pathway leading to an inflammatory response (Hawiger, 2001). The epithelial cells in the sow's milk, which are able to express CD14, are assumed to bind LPS competitively, and to prevent the activation of intestinal immune cells in the piglets. This competitive binding is believed to secure the neonatal porcine gut from LPS-induced inflammation which – in severe cases – might lead to necrotizing enterocolitis (Blais et al., 2006; Labéta et al., 2000). Data from co-workers of the group hypothesize that CD14 in the sow's milk might have an anti-inflammatory effect on the intestinal immune system in piglets. The above mentioned lower frequencies in $CD14^+$ cells in the milk of *E. faecium*-fed sows may lead to a dampened anti-inflammatory effect of mother's milk in the offspring. Data from other co-workers within the research collaboration suggest that *E. faecium* might induce an early up-regulation of a pro-

inflammatory response in pre-weaning piglets with a subsequent down-regulation of IL-8 and IL-10 in the post-weaning period (Siepert et al., 2013). It can be speculated that feeding *E. faecium* to piglets minimizes the ability of their immune system to combat pathogen infections after the weaning. However, it still needs to be elucidated to which extend this could affect physiological digestive function, and increase their susceptibility to enteric infections.

Furthermore, it has also been suggested that *E. faecium* might have an influence on cytokine expression (e. g interleukin 1-α especially in intestinal epithelium) in various tissues influencing secretory properties in the small intestine as another mode of action of probiotics in swine (Klingspor et al., 2013), but this needs further investigations as well. However, given that the Gram-positive lactic acid bacterium *E. faecium* is an autochthonous inhabitant of the porcine gastrointestinal tract, it is presumable that other bacterial species or even strains, which are also in common use as probiotics in animal production (with other properties and from other origin), will have different effects on the host.

In order to answer the question raised in this thesis whether the probiotic application or the weaning process itself has more impact on jejunal digestive physiology (gene expression patterns, enzyme activities) and morphometric parameters in piglets, we can clearly state that the weaning process is more relevant. The application of *E. faecium* had neither an effect on performance parameters, enzymatic gene expression or their respective activity nor on morphometry, but the small intestine of the piglets showed a clear weaning-associated pattern: Villus length declined with age, and recovered slightly after weaning, while crypts showed the typical weaning-associated hyperplasia over time which was also observed by several other authors (**Chapter 2.2.1**). Especially BBM enzyme activities showed clear and significant age- and weaning related patterns, independently from *E. faecium* application, with a strong decline of LAC and IAP activities, a major increase of disaccharidase activities, and an increase of *SGLT1* and *SUC* expression post-weaning which reflected the gut maturation process, and the adaptation to creep feed very well.

Application of Zinc Oxide in Young Piglets After Weaning

The knowledge about the mode of action of zinc, especially in young pigs, is still not completely understood. To date, it also seems not very clear whether a high dietary zinc supply for longer periods (3-4 weeks) would have similar effects as zinc given for a shorter period (1-2 weeks). Additionally, is not entirely clear yet whether long-term supply could affect animal physiology and performance in a negative way. The purpose of the second project within this present thesis was to determine the influence of the trace metal zinc (supplied as zinc oxide) as a commonly used feed additive on performance, intestinal health, and the influence on diverse digestive physiological parameters in the intestine of weanling piglets. So therefore, two different piglet studies with dietary zinc were conducted within the SFB 852 consortium, both ranging from a sub-optimal (57 mg/kg) over a medium (164 mg/kg) to a high or 'pharmacological' (2,425 mg/kg) concentration of zinc given as zinc oxide in the diet for four weeks after weaning.

The first study with 126 piglets was mainly addressing diverse performance parameters, the appearance or absence of diarrhoea (via diarrhoea score), digestive physiological measures such as gene expression and enzyme activity of diverse digestive BBM enzymes, and the absolute zinc concentration in serum and different tissues (kidney, spleen, pancreas, bone and liver). The second trial was performed with 30 extra animals from the main trial achieving the same diet and living conditions, but being actually dissected at exact one time period, while the other pigs from the main trial were kept and dissected on four consecutive periods over one year. Within this extra study, we tried to get deeper insights in the homeostasis of transport proteins on the gene expression level including the transport of zinc in the gut as well as the influence of different dietary zinc levels on these zinc transporter and the zinc binding protein metallothionein. Furthermore, we would like to address the question of the optimal length of feeding zinc oxide to achieve insights in a more custom-fitted level of zinc supplementation post-weaning in order to reduce the environmental aspect of zinc in pig manure in the long run.

The growth-promoting and diarrhoea-reducing effects of high dietary zinc concentations for one up to three weeks after weaning have been well known for more than three decades now (for more details: see **Chapter 2.3.2.9**), but the mechanisms and modes of action are still not fully elucidated.

In the current thesis, we hypothesized that different dietary zinc concentrations exert different effects on performance parameters, the appearance or absence of post-weaning diarrhoea, and on measures of small intestinal physiology (such as enzyme activities and gene expressional patterns) after the weaning of our piglets.

In both of the current zinc trials of this PhD thesis, all piglets remained in good health condition throughout the experimental period, and both a higher body weight gain, and an increase in ADFI have been documented for the high dietary zinc group (HZn) within the first week. This again resulted in a higher ADG of these piglets in the first two weeks. On the other hand, no differences could be seen regarding the health status of the three different zinc feeding groups in both studies, but in general, only a low incidence of diarrhoea was observed in the piglets. This could be attributed to the high sanitary status under these experimental conditions (Janczyk et al., 2010).

In previous piglet studies of several researchers, growth-promoting effects were reported frequently (Carlson et al., 1999; Case and Carlson, 2002; Hahn and Baker, 1993; Hollis et al., 2005; Wang et al., 2009; Zhang and Guo, 2009), but could not be observed by others (Martinez et al., 2005; Schell and Kornegay, 1996). These growth-promoting effects could be confirmed in the HZn group of our zinc trial during a short-term period (one week), not during a longer-term supplementation (four weeks) which was also measured by other researchers (Mavromichalis et al., 2000). It can be hypothesized that a short-term application of HZn exhibits a stimulus in the organs of the animals, increases their appetite (which can be measured as significantly increased feed intake), and the mentioned homeostatic regulation mechanisms in the gut (**Chapter 2.3.2.3**) work well to maintain the balance between uptake and excretion.

While it is scientifically proven that zinc deficiency leads to declined activity of the enzymes lactase and sucrase in the jejunum of rats (Southon et al., 1986), there is still an inconsistency in the conclusions about effects of high dietary zinc amounts on enzyme activity and other potential targets in piglets which need further investigation (Hedemann et al., 2006). In order to determine digestive physiological measures of the piglets from the zinc oxide feeding trial, brush border membranes of the scraped mucosal samples of the jejunum were isolated, the protein content was determined via Bradford protein assay, and enzyme activity of lactase, maltase, sucrase, IAP and APN was measured according to the protocols which were established during the *E. faecium* trial (**Chapter 4**). Nevertheless, the short-term growth-promoting effect of HZn could not be linked with improvements of small intestinal digestive physiology: Expected influences of high dietary zinc levels on digestive enzyme activities could not be observed, and the mRNA expression of diverse digestive enzymes have revealed no clear picture regarding influences of zinc as well (**Chapter 5**). But also mice studies showed that increasing dietary zinc amounts have only little impact on the activity of disaccharidases (Tran et al., 2011). Furthermore, it was shown in rat studies feeding a zinc-deficient diet that APN activity was significantly declined (Ying et al., 2011). However, feeding pharmacological zinc levels did not lead to the opposite effect (means: increases in APN activity) in our pigs. The measured age-dependent effects on disaccharidases are the same as discussed for the *E. faecium* trial and represent weaning-associated maturational processes and the adaptation to creep feed (see also **Chapter 4**; Martin et al., 2012).

IAP needs one Mg^{2+} and two Zn^{2+} as co-factors in its active center for enzymatic reactions (Coleman, 1992). *In vitro* studies with calf intestinal alkaline phosphatase and various zinc concentrations in the media showed that higher zinc concentrations led to a change in enzyme confirmation which resulted in higher IAP enzyme activities (Yan et al., 2003). Furthermore, an increase in IAP activities in plasma was detected (Poulsen, 1995) which has not been measured in our study. Instead, we observed significantly higher IAP activities in BBM of the HZn group at all four measured time points which is considered as a positive sign for the animals' health: It has been revealed that IAP plays a major role in maintaining gut barrier function via detoxification processes of LPS and other bacterial membrane compounds in the porcine gut (Geddes and Philpott, 2008). In pig studies with DSS-induced inflammatory bowel disease, it has just recently been discovered that IAP might also be an important factor in maintaining gut mucosal integrity (Lackeyram et al., 2012). But in order to elucidate the role of IAP and zinc in, for example, defense mechanisms against *E. coli*, further studies are necessary.

Another aspect concerning zinc and gut health in the piglets is the influence of zinc on porcine viruses in the intestine. Infection studies with swine testicles have shown a specific inhibition of two zinc salts (zinc chloride and zinc sulfate) on the replication process in the porcine coronavirus TGEV (transmissible gastroenteritis virus) in a concentration-dependent manner and they reduced markedly the viral titers in the investigated swine testicles, while

effects of zinc on virus-binding have not been observed (Wei et al., 2012). These first results have been considered quite promising, given that TGEV which causes gastroenteritis with high mortality rates in suckling pigs, is still a threat for the pig industry, but further research is necessary to fully elucidate direct modes of action of zinc in virus-related issues.

Looking again on the growth parameters in our zinc studies, the growth-promoting effects on the pigs of the HZn group even reversed relatively unexpected in the third week post-weaning. This can be underlined by similar findings of other researchers (Broom et al., 2006; Buff et al., 2005; Janczyk et al., 2013; Martinez et al., 2005; Walk et al., 2013). Reasons for the absence of growth-promoting effects after supplementation of HZn for periods longer than two weeks have still been relatively unclear, and most of the zinc studies in the present research literature focused only on short-term zinc application. Therefore, the question has been raised whether there are long-term effects of zinc oxide supplementation on the absolute zinc concentration in serum and different tissues (kidney, spleen, pancreas, bone and liver) in the pigs, and if they can give an explanation. Therefore, in one zinc oxide feeding trial (see **Chapter 5**), we measured organ zinc concentration in diverse organs at all four time points during these four weeks of zinc oxide supplementation, and could observe a significant accumulation of zinc in bone tissue and diverse organs such as the liver, kidney, spleen and pancreas over time course which is in good agreement with other previous findings in pigs (Schell and Kornegay, 1996; Williams et al., 2005) and rats (Fujimura et al., 2012). So far, a longer supplementation of pharmacological zinc oxide dosages can most likely be attributed to the fact that the zinc concentration in various organs in the animals' bodies have increased – counting as a relating stress response which might have led to a solid reduction of their performance. Recent data within the collaboration even support the hypothesis that these HZn diets cause an imbalance in zinc homeostasis after periods longer than two weeks, indicating that increased zinc accumulation in the liver and pancreas of piglets is associated with increased expression of stress proteins in hepatic and pancreatic tissue of pigs after long-term supplementation with high dietary levels of zinc oxide (Bondzio et al., 2012; 2013). Also the zinc concentrations in jejunal tissue and in jejunal digesta were two-fold greater with HZn levels as compared to the low zinc (LZn) or normal zinc (NZn) group (**Chapter 6, Table 6.18**). Similarly to the results of organ zinc concentration, serum zinc was also increased in our study with pharmacological zinc supplementation which is in good agreement with other researchers (Carlson et al., 1999; Martinez et al., 2005; Rincker et al., 2005; Williams et al., 2005), and suggests an outbalanced homeostatic regulation. The measured increases in organ zinc concentration or even the overstrained accumulation of zinc in metabolic highly relevant organs like liver, kidney and pancreas probably represents a risk for the animal. Overstressing compensatory mechanisms might have led to metabolic reactions in these organs which the piglets had to cope with, and which also might explain the retarded weight gain in these HZn fed animals compared to animals fed with NZn and LZn. Possible interactions with other essential trace elements such as copper and iron are currently under evaluation.

So far, this hypothesized outbalanced zinc homeostasis in the body indicates that the normal metabolic regulation mechanisms seems overstrained. As summarized in **Chapter 2.3.2.3**, zinc homeostasis is mainly managed in the gut via absorption and excretion, as several experiments with rodents revealed (Kirchgessner et al., 1994a; Kirchgessner and Roth, 1975; Kirchgessner et al., 1994b; Weigand and Kirchgessner, 1977a, 1978, 1977b, 1980; Windisch and Kirchgessner, 1999; Windisch and Kirchgessner, 1995; Windisch and Kirchgessner, 1994; Windisch et al., 2002). Absorption is realized via a variety of zinc transporters of two different zinc transporter families, and zinc transport is organized via binding to albumin in the blood or, beforehand, to a zinc binding protein namely metallothionein (MT) which plays an important role against heavy metal toxicity. The zinc transporter ZIP4 of the *ZIP* protein family increases the concentration of intracellular zinc, while zinc transporter of the *ZnT*-like protein family, such as *ZnT1*, decrease the intracellular zinc level through the export of zinc ions from the cytoplasm of the enterocytes into the extracellular matrix (Lichten and Cousins, 2009). The role of the divalent metal-ion transporter 1 (DMT1) in zinc homeostasis is less clear yet, but it might play an only minor role in the zinc metabolism (Kordas and Stoltzfus, 2004).

Several studies in rodent models under zinc-deficient conditions showed a strong upregulation of *ZIP4* and a strong downregulation of *ZnT1* (Cousins et al., 2006; Pfaffl and Windisch, 2003), while others surprisingly found a decreased expression of both genes during HZn supplementation in rats (Fujimura et al., 2012). Nevertheless, most of these results were revealed via rodent or *in vitro* cell culture models under zinc-deficient or normal conditions, and so far, only scarce knowledge about these molecular mechanisms with HZn supplementation in the pig as a model is available.

In order to reveal specific regulation patterns of intestinal mechanisms which might be important to maintain the homeostasis in the gut of piglets, five very important specific zinc transport proteins (ZnT1, ZnT2, ZnT5, ZIP4, DMT1) and MT as zinc-binding protein were chosen and their mRNA expressional patterns in jejunal tissue were examined via real-time RT-qPCR. The results are summarized in **Chapter 6** (Martin et al., 2013a). Additionally, we measured the gene expression of the two most important zinc transport-regulating proteins *ZnT1* and *ZIP4* in the main zinc feeding trial described in **Chapter 5** (Martin et al., 2013b).

In both of the current studies, we could reveal a highly upregulated *ZnT1* expression during HZn supplementation, while the expression of *ZIP4* was strongly downregulated (compared to the other groups with LZn and NZn supply), most likely to counteract the excessive zinc ingestion and the resulting zinc overload in the porcine organism. Previous studies in rodents showed similar results (McMahon and Cousins, 1998). If there are zinc-dependent transcription factors including *Krüppel-like factor* (*KLF*) 4 involved in these processes around the zinc transporter, will be cleared in further research studies. *ZnT5* and *DMT1* expression remained relatively unaffected by the dietary zinc concentration (see: **Chapter 6, Table 6.19**). These latter data only support the hypothesis that *DMT1* plays a minor role in zinc homeostasis during HZn supplementation, but this transporter might be more

involved in zinc recovery from the digesta when the dietary zinc supply is on a very low level. Another interesting point was that the expression of *ZnT2* transporter, which is mainly involved in zinc transport into intracellular vesicles (Lichten and Cousins, 2009), was only downregulated in the LZn group. This is suggesting that this zinc transporter plays only a minor role during elevated intracellular zinc amounts.

These *in vivo* data can also be confirmed by *in vitro* studies using an IPEC-J2 cell line model which showed a dose-dependent response of *ZIP4*, *ZnT1* and *MT* expression. It has already been shown in another *in vitro* study where they also investigated the effects of zinc (as zinc sulfate) on IPEC-J2 cell line that this cell line seems to be an appropriate model to study dietary effects on porcine intestinal cells (Lodemann et al., 2013). Confirmatory to our *in vivo* results, a dose-dependent increase in the expression of *ZnT1* (Chapter 6, Figure 6.5A) and a dose-dependent decrease in the expression of *ZIP4* (**Chapter 6, Figure 6.5B**) could be demonstrated in our studies after 24 h of zinc exposure. The mRNA expression (**Chapter 6, Figure 6.6A**) and protein abundance (**Chapter 6, Figure 6.6B**) of the zinc-binding protein metallothionein was significantly up-regulated with 200 μmol zinc in the incubation medium. There are also hints that enhanced *MT* expression after feeding high levels of dietary zinc to piglets might be another reason for enhanced growth performance (Carlson et al., 1999). Whether zinc also might have an influence on the anti-oxidative capacity in these pig of the two zinc feeding trials will be revealed in upcoming experiments, for example, by using TEAC and FRAP assays in diverse tissues and plasma.

In both *in vivo* and *in vitro* experiments, no influences of the different dietary zinc levels on the *ZnT5* gene expression could be demonstrated. All these results are already intensively discussed in **Chapter 5 & 6**. It was further hypothesized that intestinal zinc uptake possibly occurs through passive diffusion at higher intestinal zinc concentrations, thus leading to an out-balanced zinc homeostasis and then to the above mentioned accumulation of zinc in diverse organs and bone tissue during longer supplementation with very high dietary zinc supply (Menard and Cousins, 1983).

Another negative aspect of zinc supplementation in high dosages regarding *E. coli* populations has to be mentioned as well: Researchers have observed a higher diversity of *E. coli* clones in piglets supplemented with HZn compared to the LZn group, and also the proportion of multi-resistant *E. coli* was significantly inclined in the HZn group compared to the LZn group (18.6 % vs. 0 %). This suggests increased antimicrobial resistance via influences of zinc on resistance-plasmid uptake (Bednorz et al., 2013).

This have been reasons for the European Commission why the maximum concentration for zinc in feed-stuff has been limited to 150 mg/kg (88 % DM) for piglets. The applied pharmacological supplementation with 2,500 mg/kg DM used in our trial is far beyond the maximum allowance level. Model calculations have indicated that approx. 80-95 % of the ingested minerals from inorganic sources (like zinc oxide or copper sulfate) are ultimately excreted by the animal which can cause severe environmental problems (Buff et al., 2005;

Close, 2003; Veum et al., 2004), for example, accumulation of this heavy metal in soils through traditional manure application, and thus leading to reduced plant growth in areas with intensive pig production (McKenna et al., 1993). This fact might even potentiate through the global increase and intensification of pig production (Jondreville et al., 2003; Mullan et al., 2005).

So, if feed levels for piglets would be increased, as it is common practice in some other countries (NRC, 2005), an urgent question arises: What would be the optimal length of supplementing additional high levels of zinc oxide after weaning? Summarizing all performance data and measured results from our zinc oxide trials, it seems to tender that supplementing pharmacological zinc oxide does not make sense for longer than one week after weaning. Positive effects on performance could be observed only during the first week of application of zinc oxide and diminished after longer supplementation, and even seemed to be reversed in the third week. Regarding the length of feeding zinc to piglets, a further question emerged whether we would find a more custom-fitted level of zinc for pig production. But a prediction is only possible, if we can achieve better insights in intestinal molecular transport mechanisms of zinc. As mRNA expression results of *ZnT1* and *ZIP4* in the main zinc trial have revealed (**Chapter 5, Table 5.15**), there has not been a significant difference regarding the age of the animal respectively the length of HZn feeding to the animals. That means, the pharmacological zinc supply immediately has led to an up-, respectively downregulation of *ZnT1* and *ZIP4* in the piglets and have remained on that level during the whole time course without any changes in expression level.

In order to assess the impact of zinc on health, and further include the excretion problem of zinc into the environment into this assessment, it is necessary to calculate zinc excretion, while feeding different dietary amounts of zinc. This has been calculated assuming a general fattening phase of approx. 120 days to reach the end body mass of ~120 kg for slaughtering, with a BW of around 8 or 9 kg at weaning and a BW of ~30 kg at the beginning of the fattening phase. Furthermore, weaning would take place at an age of 28 days of life, and we have assumed a mean FCR of around 1.7, an ADFI of ~450 g in the weaning phase and a FCR of ~2.9, an ADG of ~700 g until the slaughtering, and a zinc excretion rate of 90 % later in the fattening phase. Scenario A was keeping exactly our study conditions where we feed our pigs with 2,500 mg zinc (per kg DM) for four weeks after weaning and further on with 80 mg zinc (per kg DM) until slaughtering (in theory). In scenario B, we assumed to feed a pig with 150 mg zing (per kg DM) for the whole time after weaning until slaughtering as it is allowed for pig production in the EU. And in scenario C, the assumption was to feed a pig with 2,500 mg zinc per kg DM for one week to overcome the weaning problems and for the rest of the growing/fattening phase until slaughtering, it was assumed to feed 80 mg zinc/ kg DM. In **Table 7.20**, the summarized calculations for a model pig can be found.

Table 6.20: Calculations of zinc excretion assuming three different scenarios in pig feeding after weaning

	Body weight (in kg)	**Zinc (in mg zinc/kg feed in the DM)**	**Feed (kg) demand in this period**	**Scenario A (ingested zinc in g)**	**Scenario B (ingested zinc in g)**	**Scenario C (ingested zinc in g)**
Weaning pig	8-9	2,500	2			5
Weaning pig	8-20	2,500	20	56		
Weaning pig	8-20	150	20		3.4	
Weaning/ growing pig	9-30	80	36			3
Growing pig	20-30	80	17	1.5		
Growing pig	20-30	150	17		2.9	
Fattening pig	30-120	80	260	23.4		23.4
Fattening pig	30-120	150	260		44.2	
			Sum of ingested zinc (in g)	**80.9**	**50.5**	**31.4**
			Sum of excreted zinc (in g)	**72,8**	**45,5**	**28,3**

These calculations of three different feeding scenarios show clearly that feeding 2,500 mg zinc/kg feed (in the DM) to a pig for four weeks would have the greatest impact on the environment regarding the amount of excreted zinc (~73 g zinc over the whole lifespan of each pig), while scenario C would have the lowest environmental impact (~28 g zinc), with scenario B having an intermediate position (45.5 g zinc). There is an estimated difference of ~17 g excreted zinc per pig between scenario B and C. An extrapolation to the 60 million slaughtered pigs in Germany would make a difference of ~1,000 tons (2,730 t vs. 1,698 t). Although it is not legal to feed 2,500 mg zinc/kg feed, the scenario C would have the lowest environmental impact compared to the other scenarios. Taking into consideration that zinc from a grain and legume-based diet has only a low bioavailability due to the high phytate content, the zinc bioavailability in this case can be increased via microbial in-feed enzyme supplementation as it is common practice the livestock industry (Bikker et al., 2012).

Conclusion and Perspectives

Post-weaning gut disturbances along with the development of PWD constitute a major challenge for a freshly weaned piglet and for the swine producers. Three animal trials with the feed additives *E. faecium* NCIMB 10415 and zinc oxide were conducted in order to achieve further insights into their modes of action. The current study suggests that *E. faecium* supplementation of sow and piglet diets did not markedly affect the measured physiological parameters associated with digestive function. The two present zinc studies showed that the growth-promoting effect of high dietary zinc levels have a positive effect in the initial period after weaning. Furthermore, after evaluating the zinc transporter experiments, it was evidenced that a very fast response of the intestinal epithelium occurred. However, counter-regulatory adaptation of the intestinal zinc transporters did not prevent the pigs from tissue zinc accumulation suggesting an overstrained homeostatic regulation. Whether this was the cause for impaired performance observed four weeks after weaning, needs further clarification.

CHAPTER 8: Summary/Zusammenfassung

Summary of the PhD Thesis:

Studies on the Influence of the Probiotic *Enterococcus faecium* NCIMB 10415 and the Trace Element Zinc on Performance and Digestive Physiological Parameters in the Small Intestine of Piglets.

Weaning in the early stage of the piglets' life can lead to post-weaning diarrhoea. For several decades, it was tried to overcome weaning problems by the application of antibiotics, but this has led to the risk of antibiotic resistance. In the last years, the search for natural alternatives as feed additives was immensely enhanced, as it is summarized in **Chapter 1**.

Chapter 2 reviews the current literature about the physiological development in the GIT of piglets with a special emphasis on the critical phase of weaning. Diverse studies in rodent and pig models have revealed that specific dietary factors (such as trace metals or probiotics) can have a major impact on gene expression and on other digestive physiological functions. Therefore, an overview is given on two feeding strategies (probiotics and the trace element zinc) used for this PhD thesis. Besides the 'classical field' of animal nutrition, other aspects and approaches including enzymatic analysis of digestive enzymes and the respective gene expression, or western blot analysis of specific proteins relevant for digestion and so on, have been taken into account when evaluating feed additives and the respective mode of action.

Chapter 3 explains the main aims and hypotheses of this PhD thesis. The main work of the current PhD thesis consists of three published manuscripts summarized in **Chapter 4, 5 and 6**. The main interest was to increase the knowledge of possible underlying mechanisms of the probiotic *Enterococcus faecium* NCIMB 10415 and the trace mineral zinc. Therefore, three studies with suckling or weaning piglets were conducted within this thesis.

Furthermore, several protocols for quantitative real-time RT-PCR plus diverse enzyme assays have been established in order to measure activities of disaccharidases (lactase, maltase, sucrase), aminopeptidase-N and intestinal alkaline phosphatase and the respective gene expression in jejunal brush border membranes of the piglets (methods have been published in Martin et al., 2012; **Chapter 4**).

In the first feeding trial, the impact of *E. faecium* NCIMB 10415 on performance parameters, digestibility and small intestinal digestive measures such as gene expression and digestive enzyme activities has been evaluated in piglets before and after weaning (**Chapter 4**). The results of the study suggest *E. faecium* supplementation of sow and piglet diets did not markedly affect physiological parameters associated with digestive function, likely due to good sanitary conditions in this trial. The age-dependent effects on brush border disaccharidase activities and epithelial morphology reflected the weaning transition, as well as maturation and

adaptation to feed. Taking all results from the collaboration into consideration (see: **Chapter 7**), the study failed to prove a positive impact of the tested *Enterococcus* strain.

Whether changes in the activity of intestinal alkaline phosphatase can be associated with other parameters, such as the intestinal microbial ecology or influences on the immune system due to probiotic application, will be determined in future trials. Also of great interest are specific effects of certain probiotic strains when animal health is impaired or when other stress factors exert negative effects on digestive physiological parameters. Therefore, further investigation of probiotic effects in pigs should include challenge conditions via conscious provocation tests with viruses or enteropathogenic bacteria, or studies should be conducted under sanitary conditions which are closer to the situations in farms.

The essential trace element zinc as a constituent of over 300 metalloenzymes has been used for decades in high concentrations as a feed additive to prevent or treat diarrhoea in animals and humans. It plays a vital role in the regulation of gene expression, and is seen as a key factor in maintaining different physiological processes. While zinc deficiency impairs growth performance and clinical signs as parakeratosis, high amounts of dietary zinc oxide have been proven in the literature to reduce the incidence of post-weaning diarrhoea, and improve the performance of pigs after weaning (**Chapter 2.3**). Intestinal zinc uptake is facilitated through members of the zinc transporter families SLC30 (ZnT) and SLC39 (ZIP). Whereas ZIP proteins help to increase intracellular zinc concentration, zinc transport proteins from the ZnT family reduce intracellular concentrations through transport into the extracellular matrix (ZnT1) or into vesicles (ZnT2). The divalent metal-ion transporter 1 (DMT1) appears to play a minor role in zinc homeostasis. Although it has been shown that low levels of dietary zinc can modify the expression of these transporters in the gut and other organs of rodents, the reaction to pharmacological zinc levels is still not fully clarified. Older studies on true zinc digestibility in rats have suggested a homeostatic regulation of zinc absorption. To date, studies about influences of dietary zinc on homeostatic mechanisms in the jejunum of piglets are still lacking.

In the studies in **Chapter 5 & 6**, it was aimed to feed piglets with marginal, normal and high dietary zinc amounts for four weeks after weaning as compared to current nutritional recommendations (GfE, 2006). The main interest of the two studies was to determine whether these different dietary zinc concentrations would have an influence on the performance of the weaned animals, on jejunal mRNA expression or activities of digestive enzymes. Furthermore, investigations on gene expression of the above mentioned specific key zinc transporters (*ZnT1*, *ZnT2*, *ZnT5*, *ZIP4*, *DMT1*, *MT*) played a main role because of their essentiality in maintaining a homeostatic balance in the GIT of the piglets. Furthermore, it has been aimed looking at short-term (1-2 weeks) versus long-term (3-4 weeks) effects of feeding high dietary zinc levels.

In both of the current zinc studies, piglets in the HZn group showed a higher ADFI and ADG in the first week post-weaning, suggesting a stimulus on appetite in the HZn group. Furthermore, it was possible to show that high levels of dietary zinc can influence the expression of zinc transporters and zinc-binding proteins in the jejunum of weaned piglets. A

decreased jejunal expression of zinc transporter *ZIP4* and the increase of the jejunal expression of *ZnT1* and *MT* suggest increased intracellular zinc concentrations, an increased zinc export from intestinal tissues into extracellular compartments and a decreased zinc uptake from the gut lumen at high dietary zinc supply. If there are zinc-dependent transcription factors such as the *Krüppel-like factor 4* (*KLF4*) involved in these processes, needs to be further elucidated for clearance. As the *in vitro* experiments with the intestinal porcine cell line IPEC-J2 have revealed, the adaptive process appears to be established within 24h; however, it does not prevent the pigs from tissue zinc accumulation in their bone, kidney, pancreas, intestine and liver tissue of the piglets suggesting an overstrained homeostatic regulation. Whether this is the cause for impaired performance during longer supply, as observed four weeks after weaning, needs further clarification.

In conclusion, high dietary zinc level improved the performance of piglets in the short-term which means the first two weeks after the weaning. Although we could show that jejunal mRNA levels of key zinc transporters changed with a high dietary zinc supply, this did not prevent the animal from zinc accumulation in various tissues suggesting an outbalanced homeostatic regulation. Besides the evaluation of critical points in the use of high zinc concentrations in pigs' diets, **Chapter 7** tried to contribute to the on-going discussion about the balance between environmental issues and animal health with a model calculation of the use of different zinc dosages in modern pig production.

Zusammenfassung der Dissertation zum Thema:

Studien zum Einfluss des Probiotikums *Enterococcus faecium* NCIMB 10415 und des Spurenelements Zink auf Leistung und diverse verdauungsphysiologische Parameter beim Ferkel.

In der modernen Ferkelproduktion birgt das Absetzen von der Muttersau für die jungen Ferkel ein hohes Stresspotenzial, bedingt durch die Fütterungsumstellung und Rangordnungskämpfe in neuer Umgebung und das Risiko, dass Tiere an Durchfall mit einer hohen Morbidität und Mortalität erkranken. Jahrzehntelang wurden diese absetzbedingten Probleme mittels Verabreichung von Antibiotika umgangen bzw. minimiert, jedoch wird dieses aufgrund des Risikos von Antibiotikaresistenzen kritisch diskutiert. Daher wurden Forschungen zu natürlichen Futterzusätzen als Alternativen zu Antibiotika in den vergangenen Jahren stark intensiviert, was im Rahmen einer Literaturübersicht in **Kapitel 1** zusammengefasst wird. **Kapitel 2** gibt einen Überblick über den aktuellen Stand der Literatur zur physiologischen Entwicklung des porzinen Verdauungstrakts mit dem Schwerpunkt auf der intestinalen Entwicklung während der kritischen Absatzphase der Ferkel. Diverse Modellstudien am Nagetier bzw. Schwein haben aufgezeigt, dass spezifische Faktoren im Futter, wie zum Beispiel Spurenelemente oder probiotische Kulturen, einen Einfluss auf verdauungsrelevante Stoffwechselvorgänge einschließlich der Genexpression haben können. Deshalb wird ein Überblick über zwei relevante Fütterungsstrategien (Probiotika und das Spurenelement Zink) gegeben, welche die Hauptbestandteile dieser Dissertation sind. Neben den „klassischen" Fragen der Tierernährung, die mit Parametern zu Leistung und Futteraufwand besondere Relevanz für den Landwirt haben, werden neue Aspekte zur Bewertung von Futterzusätzen und deren Wirkungsweisen, wie z. B. die Analyse verschiedenster Verdauungsenzyme und deren jeweils zugehörige Genexpression oder die Western-Blot-Analyse spezifischer, verdauungsrelevanter Proteine mit einbezogen.

Kapitel 3 stellt die Hauptziele und Hypothesen der Dissertation vor. Die drei veröffentlichten Beiträge liegen in **Kapitel 4, 5 und 6** vor. Hauptinteresse war dabei, den Wissensstand über mögliche zugrunde liegende Mechanismen und Wirkungsweisen des Probiotikums *Enterococcus faecium* NCIMB 10415 und des Spurenelements Zink auszubauen. Dazu wurden drei Studien mit Saug- und Absatzferkeln durchgeführt. Diverse Protokolle für die quantitative Real-Time Reverse-Transkriptase-PCR und für die Bestimmung verschiedener Enzymaktivitäten im jejunalen Bürstensaum der Ferkel (Laktase, Maltase und Saccharase sowie intestinale alkalische Phosphatase und Aminopeptidase-N) sind dafür etabliert worden (publiziert in Martin et al., 2012; **Kapitel 4**).

Im ersten Fütterungsversuch wurde der Einfluss von *E. faecium* NCIMB 10415 auf Leistungsparameter, Nährstoffverdaulichkeit, sowie auf die Genexpression und Aktivität verschiedener Verdauungsenzyme vor und nach dem Absetzen der Ferkel untersucht (**Kapitel**

4). Das Ergebnis der Studie zeigt, dass die Supplementierung des Sauen- und Ferkelfutters mit *E. faecium* NCIMB 10415 keinen Einfluss auf die untersuchten verdauungsphysiologischen Parameter hatte, was möglicherweise auf die guten sanitären Bedingungen während des Versuchs zurückgeführt werden kann. Die beobachteten altersbezogenen Effekte auf die Aktivitäten der Bürstensaum-Disaccharidasen und die Dünndarmmorphologie reflektieren altersabhängige Veränderungs- und Anpassungsvorgänge, die mit Darmreifung und der Anpassung an die Festfutteraufnahme einhergehen. Werden die Ergebnisse anderer Arbeitsgruppen aus dem SFB 852 mit in die Evaluierung einbezogen, kann kein positiver Einfluss auf die Tiergesundheit konstatiert werden. Ob jedoch die beobachteten Veränderungen der IAP-Aktivität mit anderen Parametern wie einer Veränderung der mikrobiellen Zusammensetzung im Darm oder einer Beeinflussung des Immunsystems aufgrund des applizierten Probiotikums assoziiert werden können, muss in zukünftigen Studien weiter untersucht werden.

Das Spurenelement Zink, das Bestandteil von über 300 Metalloenzymen ist, wird als Futterzusatz in der Tierernährung, u. a. beim Ferkel, und beim Menschen zur Durchfall-Prophylaxe eingesetzt. Zink spielt eine grundlegende Rolle in der Regulierung der Genexpression und gilt als Schlüsselfaktor zur Aufrechterhaltung lebensnotwendiger physiologischer Stoffwechselprozesse. Während ein Zinkdefizit im Allgemeinen zu Wachstumsverzögerungen und beim Schwein zu Parakeratose führt, wird sehr hohen Zinkoxid-Supplementierungen im Absetzfutter ein positiver Effekt in Bezug auf Leistung und auf die Reduktion von absetzbedingten Durchfallerkrankungen zugeschrieben (**Kapitel 2.3**).

Die intestinale Zinkaufnahme wird im Allgemeinen durch Mitglieder zweier Zinktransporter-Familien ermöglicht, namentlich SLC30 (ZnT) und SLC39 (ZIP). Während es Proteine der ZIP-Familie ermöglichen, die intrazelluläre Zinkkonzentration zu erhöhen, sorgen Proteine der ZnT-Familie durch Zinktransport in die extrazelluläre Matrix (ZnT1) oder in Vesikel (ZnT2) für eine Reduzierung der intrazellulären Zinkkonzentration. Die Rolle des divalenten Metallionen-Transporters DMT1 ist noch nicht abschließend geklärt. Obwohl bereits im Nagetiermodell gezeigt werden konnte, dass niedrige Zinkkonzentrationen im Futter eine modifizierende Wirkung auf die Genexpression dieser Zinktransporter im Jejunum und anderen Geweben haben können, sind die Auswirkungen pharmakologischer Zinkdosierungen beim Schwein noch nicht ausreichend erforscht. Ältere Untersuchungen an Ratten legen eine homöostatische Regulierung der Zinkabsorption nahe. Jedoch fehlten bisher noch Belege für die Effekte pharmakologischer Zinkdosierungen auf mögliche homöostatische Regelmechanismen im Intestinaltrakt des Schweins.

Die Versuche in **Kapitel 5 & 6** zielten darauf ab, Ferkel über vier Wochen nach dem Absetzen mit marginalen, normalen und sehr hohen Zinkkonzentrationen (verglichen mit aktuellen Fütterungsempfehlungen der GfE, 2006) zu füttern. Eine Hauptintention bestand darin, Einflüsse auf Leistungsparameter sowie die Expression und Aktivität diverser Verdauungsenzyme im Jejunum zu untersuchen. Weiterhin spielten die Untersuchungen der Genexpressionen der Transportproteine (ZnT1, ZnT2, ZnT5, ZIP4, DMT1, Metallothionein)

aufgrund ihrer Bedeutung in der Aufrechterhaltung der Zink-Homöostase im Darm der Ferkel eine große Rolle. Ein nicht unwesentliches Ziel war zudem der Zeitaspekt der Zinkfütterung, insbesondere, ob in der kurz- (1-2 Wochen) oder längerfristigen (3-4 Wochen) Gabe hoher Zinkmengen verdauungsphysiologische Unterschiede beim Ferkel festgestellt werden können.

In beiden Studien zeigten die Tiere in der Gruppe mit der hohen Zinksupplementierung in der ersten Woche nach dem Absetzen eine höhere tägliche Futteraufnahme sowie eine höhere tägliche Gewichtszunahme, was auf eine appetitstimulierende Wirkung hindeuten könnte. Weiterhin war es möglich zu zeigen, dass eine hohe Zinkzufuhr mit dem Futter die Expression verschiedener Zinktransporter (*ZnT1*, *ZIP4*) und zinkbindender Proteine (Metallothionein) im Jejunum der Absatzferkel beeinflusst. Eine verringerte jejunale Expression des *ZIP4* und eine gesteigerte Expression des Zinktransporters *ZnT1* und des Zink bindenden Proteins Metallothionein während der hohen Zinksupplementierung lassen auf erhöhte intrazelluläre Zinkkonzentrationen, einen erhöhten Zinkexport aus dem Darmgewebe in extrazelluläre Kompartimente sowie auf eine verringerte Zinkaufnahme aus dem Darmlumen schließen. Ob an diesen Prozessen weitere zinkabhängige Transkriptionsfaktoren, wie z. B. der *Krüppel-like factor 4* (*KLF4*), beteiligt sind, muss in weiterführenden Experimenten untersucht werden.

In vitro-Experimente mit der porzinen Zelllinie IPEC-J2 konnten zeigen, dass sich dieser Adaptierungsprozess auf Genebene innerhalb der ersten 24 Stunden manifestiert. Trotz effizienter Regulation der Transporterexpression waren die Tiere nicht vor einer Zinkakkumulierung in Knochen-, Nieren-, Pankreas-, Darm- und Lebergewebe geschützt. Die Daten deuten auf eine Überforderung der Zinkhomöostase hin. Ob dies als Grund für eine verminderte Leistung der Tiere während längerfristiger hoher Zinksupplementierung verantwortlich gemacht werden kann, muss in weiteren Studien geklärt werden.

Zusammenfassend zeigt sich, dass hohe Zinkkonzentrationen in der Fütterung helfen können, die Leistung von Absatzferkeln kurzfristig, d. h., in den ersten beiden Wochen nach dem Absetzen, zu verbessern. Die Ergebnisse der längerfristigen Messungen zeigen eine unausgeglichene Zinkhomöostase.

Neben der Evaluierung kritischer Aspekte des hohen Zinkeinsatzes im Futter von Absetzferkeln wurde in **Kapitel 7** versucht, mittels Modellrechnungen einen Beitrag zur anhaltenden Diskussion über umweltrelevante Aspekte der Zinkfütterung und der Tiergesundheit durch kurzfristig hohe Zinkgaben in der Tierproduktion zu leisten.

Personal Contribution of the PhD Student Lena Martin to the Published Manuscripts

Manuscript 1 (Chapter 4):
R. P., W. V. and J. Z. designed experiments; L. M., R. P., S. K., and F. G. B. conducted the research. L. M. specifically was involved in the sampling, tissue storage, isolated the brush border membranes, established and performed the enzyme activity assays, the mRNA extraction and the RT-qPCR assays. L. M., R. P., W. V. and K. N. analyzed the data. L. M. and R. P. wrote the manuscript. R.P., W. V., A. V. K and J. Z. had primary responsibility for the final content. All authors read and approved the final manuscript.

Manuscript 2 (Chapter 5):
R. P. and J. Z. designed the experiments. L. M., R. P., and N. S. conducted research. L. M. specifically was involved in the sampling, tissue storage, and conducted the brush border membrane isolation, the enzyme activity assays, the mRNA extraction and the RT-qPCR assays for gene expression analysis. N. S. was responsible for the determination of the organ zinc concentration. L. M., R. P., and W. V. analyzed the data. L. M. wrote the manuscript. R. P., W. V. and J. Z. and had primary responsibility for the final content. All authors read and approved the final manuscript.

Manuscript 3 (Chapter 6):
R. P. and J. Z. designed the experiments; L. M. and R. P. developed the initial research hypothesis; L. M. and R. P. performed sampling. L. M., U. L., A. B., E.-M. G., and R. P. conducted the research. L. M. specifically established target primers, and all mRNA expression analyses via RT-qPCR from jejunal tissue and from supplied cell culture RNA extracts (supplied by U. L. and J. R. A.). L. M., A. B., J. R. A. and R. P. analyzed the data. R. P. and L. M. wrote the manuscript. J. R. A., W. V., J. Z., and R. P. had primary responsibility for the final content. All authors read and approved the final manuscript.

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Publication List

Publications (peer reviewed)

2013

L. Martin, U. Lodemann, A. Bondzio, E.-M. Gefeller, W. Vahjen, J.R. Aschenbach, J. Zentek, R. Pieper. A high level of dietary zinc changes the expression of zinc transporters and metallothionein *in vitro* and in the jejunum, but does not prevent zinc accumulation in jejunal tissue of piglets. *Journal of Nutrition 143* (8): 1205-1210. IF 3.9

L. Martin, R. Pieper, N. Schunter, W. Vahjen, J. Zentek. Performance, organ zinc concentration and jejunal brush border membrane enzyme activity and mRNA expression in piglets receiving suboptimal or very high dietary zinc levels. *Archives of Animal Nutrition* 67 (3): 248-261. IF 0.987

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

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Lena Martin