RESEARCH ARTICLE

The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets

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Abstract
Weaned piglets were fed diets containing 57 (low) or 2425 (high) mg kg⁻¹ analytical grade ZnO for a period of 5 weeks. Intestinal contents were sampled in weekly intervals and analyzed for bacterial cell numbers and main bacterial metabolites. The most severe effects of high dietary zinc were observed 1 week after weaning in the stomach and small intestine. Pronounced reductions were observed for Enterobacteriaceae and the Escherichia group as well as for Lactobacillus spp. and for three of five studied Lactobacillus species. The impact of high dietary zinc diminished for enterobacteria with increasing age, but was permanent for Lactobacillus species. Bifidobacteria, enterococci, streptococci, Weissella spp. and Leuconostoc spp. as well as the Bacteroides-Prevotella-Porphyromonas group were not influenced by high dietary zinc throughout the trial. High dietary zinc reduced bacterial metabolite concentrations and increased molar acetate ratios at the expense of propionate in the proximal intestine, but differences diminished in older animals. Lower lactate concentrations were observed in the high dietary zinc group throughout the feeding trial. This study has shown that the application of dietary zinc at high concentrations leads to transient and lasting effects during the development of the intestinal microbiota, affecting composition as well as metabolic activity.

Introduction
The intestinal tract of young mammals is a dynamic entity within the animal and many different genetic, physiological and environmental factors govern its development into maturity (Mackie et al., 1999). Generally, the stomach functions as pre-digestive organ, as low pH, mixing of solids and liquids and peptidolytic enzymes prepare the ingested feed for digestion. In the small intestine, nutrients are hydrolyzed by digestive enzymes and transported across the epithelium. Finally, the large intestine acts as fermentation chamber, in which indigestible feed components are utilized by strict anaerobic bacteria to yield short chain fatty acids, which can be resorbed and used by the host. Birth and weaning are the most severe influences that lead to direct modification of the mammalian intestine, as nutrient supply and contact with the environment trigger a multitude of physiological and immunological responses (Lallès et al., 2007).

An integral part of this complex and dynamic ecosystem is the intestinal microbiota, which is inescapably associated with their host (Neish, 2009). Estimates on the total of bacterial species present in the intestine range from 500 (Eckburg et al., 2005; Steinhoff, 2005) to up to 1000 (Peris-Bondia et al., 2011) different bacterial species belonging to almost all branches of the bacterial tree of life (Leser et al., 2002). They outnumber the total of host cells by a factor of 10–100 (Shanahan, 2002) and their physiological diversity is considered as far higher than that of their host (O’Hara & Shanahan, 2006; Qin et al., 2010). Furthermore, the production of short chain fatty acids (SCFA), lactate and ammonia not only influences the microbiota itself by stimulating metabolic cross feeding (De Vuyst & Leroy, 2011; Muñoz-Tamayo et al., 2011) and regulation of the bacterial gut environment (Macfarlane and Macfarlane, 2003), but can also affect the host by delivering SCFA with possibly beneficial effects on health (Havenaar, 2011; Fung et al., 2012). On
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et al.

in vitro

FEMS Microbiol Ecol et al. 2011; Liedtke & Vahjen, 2012; Mohamed & Abo-Amer, 2012). A recent in vitro study on the minimal inhibitory concentration (MIC) of ZnO against a wide range of intestinal strains has shown that most bacterial phylogenetic groups contain members with low or high zinc resistance, but strict anaerobic strains generally showed more diverse MIC than lactic acid bacteria or enterobacteria (Liedtke & Vahjen, 2012).

Relative to the wide-spread use of high dietary zinc in animal nutrition, there are also surprisingly few in vivo studies available on the influence of zinc on intestinal bacteria. In a study by Hojberg et al. (2005) especially lactobacilli colony counts were reduced, but enterococci were less influenced. Furthermore, increased coliform colony counts were observed. This is in agreement with a challenge study by Mores et al. (1998) which also found increased shedding of an inoculated pathogenic Escherichia coli strain in post weaning piglets fed zinc oxide supplemented diets. Another study by Broom et al. (2006) also reported reduced anaerobic and lactic acid bacteria counts, but no effect on E. coli was found. Recently, sequencing studies on the effect of high dietary zinc on the microbiota in piglets have shown that the enterobacterial diversity increases due to high dietary zinc (Vahjen et al., 2011). Furthermore, changes on the genus level were observed in some bacterial groups without changing the total number of sequencing reads (Vahjen et al., 2010).

However, no studies are available that combine bacterial composition and their major metabolites in a time dependent fashion. It is known that the microbiota in piglets is subjected to drastic changes during the first weeks after weaning (Konstantinov et al., 2006) and a succession of different dominating species has been shown to occur as the animal ages. The antibacterial agent zinc should have a massive impact on the natural development of the intestinal microbiota. Therefore, this study was conducted to advance the understanding of the effects of high concentrations of dietary zinc on the intestinal microbiota in piglets.

Materials and methods

Animals and housing

Landrace piglets were weaned at 26 ± 1 days of age with a mean body weight of 7.2 ± 1.2 kg and randomly allocated into the treatment groups balancing for gender, litter and body weight. Animals were housed in pens (n = 2 per pen) with straw bedding and ad libitum access to feed and water. The study was conducted according to the German Animal Welfare Act (TierSchG) and approved by the local state office of occupational health and technical safety ‘Landesamt für Gesundheit und Soziales, Berlin’ (LaGeSo Reg. Nr. 0347/09).

Diets

Diets based on a standard starter feed mixture (wheat/barley/soy bean meal) were fed after weaning until 54th day of life. The composition of the basal diet is shown in Supporting Information, Table S1. Analytical grade ZnO (Sigma Aldrich, Taufkirchen, Germany) was added to the diets. The analyzed dietary Zn concentration in the control group (low dietary ZnO) was 57 mg kg⁻¹ feed, whereas the treatment group (high dietary ZnO) contained 2,425 mg kg⁻¹ feed.

Performance

Body weight and feed intake were recorded on a weekly basis and average daily gain (ADG) and average daily feed intake (ADFI) were calculated. Fecal quality was monitored using a subjective scoring system ranging from 1 (entirely liquid) via 3 (normal) to 5 (hard pellets) and scoring was performed every day after the morning meal. No antibiotics were administered before and during the experiment.
Sampling

Piglets of each experimental group were sacrificed on 32 ± 1, 39 ± 1, 46 ± 1 and 53 ± 1 day of age such that treatment groups were balanced for litter and gender (n = 8). The piglets were sedated with 20 mg kg⁻¹ BW of ketamine hydrochloride (Ursotamin®, Serumwerk Bernburg AG, Germany) and 2 mg kg⁻¹ BW of azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) prior to euthanasia by intracardial injection of 10 mg kg⁻¹ BW of tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany). Intestinal contents were taken from the stomach, mid-jejunum, terminal ileum and colon ascendens. Samples were shock-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Determination of bacterial cell numbers

DNA extraction

DNA extraction was performed with a commercial kit (Qiagen Stool kit, Qiagen, Hilden, Germany) with 200 mg digesta in triplicate according to the instructions of the manufacture except for an increase in temperature during the to 90 °C lysis step. Purified DNA was then pooled per sample.

Realtime PCR – assays

Primer sequences and annealing temperatures are given in Table S2. All primers were purchased from MWG Biotech (Straubing, Germany). A Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) was used for PCR amplification and fluorescent data collection. The master mix consisted of 12.5 μL Brilliant II SYBR® Green QPCR Master Mix with Low ROX (Stratagene), 0.5 μL of each primer (10 μM) and 10.5 μL water. One microliter sample was added before PCR amplification. All amplification programs included an initial denaturation step at 95 °C for 10 min to activate the polymerase. All PCR programs featured an annealing time of 60 s and a 60 s extension at 72 °C.

Quantification of fluorescent signals

A detailed description of the quantification procedure is given by Vahjen et al. (2007). This quantification method employed extracts from a large number of reference strains inoculated in a sterile matrix and thus circumvents the bias of extraction efficiency and enables the expression of results as cell number per gram sample instead of target gene copy numbers. In short, a series of autoclaved (1 h, 121 °C, 2bar) sow faeces samples was spiked with overnight cultures of a wide range of bacterial species and known cell numbers (10⁸–10⁹ cells per gram wet weight). After extraction and purification with the same DNA extraction protocol (Qiagen Stool kit, see above), these extracts were used as PCR calibration samples. Results were therefore expressed as cell number per gram sample wet weight.

Determination of bacterial metabolites

For sample preparation, 0.5 g of digesta was diluted with 1.0 mL of ice-cold 100 mM 3-(N-morpholino) propansulfonic acid buffer (pH 7.5), homogenized for 1 min, and incubated for 10 min on ice. Samples were then homogenized again and centrifuged at 17 000 g at 4 °C for 10 min. The supernatant was kept on ice, until 100 μL was taken for determination of the SCFA. The rest of the supernatant was mixed with 50 μL of Carrez-I and Carrez-II solutions and subsequently used for ammonia and lactate analysis. Samples were centrifuged and the supernatants were filtered by a 0.45-μm cellulose acetate syringe filter. Analysis of SCFA was carried out by gas chromatography (Agilent Technologies 6890N with auto sampler G2614A and auto injector G2613A; Santa Clara, CA). An Agilent 19095N-123 HP-INNOWAX polyethylene glycol column was used. Then, 100 μL of the sample supernatant was diluted with 900 μL of internal standard solution, containing 0.5 mmol L⁻¹ of caproic acid. The standard solution contained 50 mL of 10 mmol L⁻¹ stock solution (250 μL caproic acid, 2 g of oxalic acid dihydrate in 200 mL), 2.5 g of sodium azide and 10 g of oxalic acid dihydrate in 1000 mL.

Ammonia was quantified using a Berthelot reaction assay. Twenty microliters of the sample supernatant was mixed with 100 μL of phenol nitroprusside and 100 μL of alkaline hypochlorite in a 96-well microtiter plate. After incubation for 10 min at room temperature, a photometric measurement was carried out at 620 nm with a Tecan microtiter plate reader (Tecan Austria GmbH, Salzburg, Austria).

Analysis of D- and L-lactate was carried out with HPLC using an Agilent 1100 system with Phenomenex Chirex 3126 (D)-penicillamine 150 × 4, 6-mm column and Phenomenex C18 4.0-L × 2.0 ID mm precolumn (Agilent Technologies). Two hundred microliters of sample supernatant were filled up to 1 mL with copper-II-sulfate solution (0.5 mmol). The column temperature was 35 °C and the UV detector wavelength was 253 nm.
Determination of total, free and protein-associated zinc

Samples were initially diluted (1 : 2 vol:vol) in water, homogenized for 1 h at room temperature and centrifuged at 14 200 g for 10 min. Centrifugates were used to determine total insoluble zinc in the sample. Supernatants were withdrawn quantitatively and applied on polymeric reversed-phase sorbent columns (8B-S100-FBJ, Phenomenex, Aschaffenburg, Germany). The resulting eluents contained the total free inorganic zinc of the samples. Protein-associated zinc was determined from eluents after elution with acetonitrile/water (4 : 6 vol:vol) and acetonitrile/formic acid (7 : 3 vol:vol) and evaporation of the organic phase by vacuum centrifugation. Total zinc in the sample fractions was determined in an atomic absorption spectrometer (AAS vario 6, Analytik Jena, Germany) after total hydrolysis of sample fractions in hydrochloric acid (37%) for 90 min at 250 °C.

Graphical presentation of the impact of zinc on the development of the intestinal microbiota

Mean data of bacterial cell numbers and metabolite concentrations were used to construct heatmaps with the web tool ‘Heatmap’ (http://www.hiv.lanl.gov) which uses ‘heatmap.2’ of the gplots package of the statistical environment R. Significant differences between trial groups were then marked with asterisks. 3D plots showing the development of bacterial metabolite concentrations along the intestinal tract were generated with the software SIGMAPLOT 11.0 (Systat Software, Inc., Erkrath, Germany).

Statistics

Statistical analysis was carried out with spss 19.0 (SPSS Inc., IL). The Kolmogorov–Smirnov-Test was used to test normal data distribution. Normal distributed data was analyzed for significant differences using the Students t-test. Significant differences of non normal distributed data were determined by the Kruskal–Wallis Test followed by the Mann–Whitney-U test. Differences at \( P < 0.05 \) were considered significant.

Results

Performance of animals

All animals remained clinically healthy during the entire period and diarrhoea (fecal scores < 2.5) occurred only very occasionally with no differences between treatments. The average daily weight gain (ADG) and average daily feed intake (ADFI) were higher \(( P < 0.05)\) in piglets fed high ZnO level during the first week as compared to the other group, but this effect almost reversed after 3 weeks with higher ADG in the low ZnO group (Martin et al., 2012).

Bacterial composition

Means and standard deviations for all data are shown in Tables S3–S6. Figure 1 displays a heatmap of all studied bacterial groups and species for all sampling days and intestinal segments. The Bacteroides–Prevotella–Porphyromonas group, bifidobacteria, enterococci, streptococci, Weissella spp. and Leuconostoc spp. showed no significant differences between trial groups at any sampling time or
intestinal location. Similarly, high dietary zinc invoked no or only marginal differences for three clostridial clusters, except for the clostridial cluster IV in the jejunum and ileum and for the clostridial cluster I in the colon on sampling day 42. *Enterobacteriaceae* as well as the *Escherichia-Hafnia-Shigella* group were significantly reduced due to high dietary zinc on day 35, but later sampling days showed no differences. Conversely, lactobacilli and especially three of five studied *Lactobacillus* species responded to high dietary ZnO with reduced cell numbers throughout the trial period, the exception being *L. johnsonii* and *L. reuteri*, which only showed significantly reduced cell numbers in the high dietary ZnO group on the 35th day of life. Generally, the most pronounced differences were observed in the small intestine (jejunum, ileum) and in the first sampling week.

**Bacterial metabolites**

Means and standard deviations for all data of short chain fatty acids and their molar ratios, lactate and ammonia as well as total metabolites are shown in Tables S7–S10. A heatmap for bacterial metabolites is shown in Fig. 2. As expected, lactate was the main metabolite in the stomach and small intestine, while acetate and propionate were the dominant SCFA in the large intestine. Although significant differences were infrequent due to high individual differences, reduced concentrations of SCFA, ammonia and lactate were visible at almost all sampling sites and sampling days. Figure 3 shows that high dietary zinc reduced concentrations of ammonia especially in the jejunum and colon. However, in the 2nd to 4th week after weaning, fewer differences were observed. Total lactate was drastically reduced in the small intestine throughout the trial (Fig. 4). Ratios of the major short chain fatty acid acetate were generally higher in animals fed the high dietary zinc concentration throughout the trial (Fig. 5). Concurrently, propionate generally showed higher numeric ratios in animals fed the low dietary zinc diet, but significant differences were infrequent (see Tables S7–S10). Overall, total metabolites were reduced considerably throughout the trial with often significant differences in the small intestine.

**Effects of protein associated and free inorganic zinc fractions on bacterial cell numbers and metabolites in different intestinal sites**

A spearman correlation analysis of protein-associated and free inorganic zinc fractions to bacterial cell numbers and free inorganic zinc combining all sampling days is shown in Table 1. While free inorganic zinc showed most interactions in the stomach and small intestine, no interactions were observed for protein associated zinc in the stomach. There were also generally less interactions between protein associated zinc and bacterial parameters than for free inorganic zinc.

As expected, most correlations were negative for free inorganic zinc, but some noteworthy exceptions were observed. Thus, combined for all sampling days, free inorganic zinc showed positive dependencies for the *E. coli* group in the proximal intestine. The same was true for the strict anaerobic gram-negative *Bacteroides-Prevotella-Porphyromonas* cluster as well as for *Enterococcus* spp. Also, the clostridial cluster XIVa correlated negatively with free inorganic zinc in the proximal intestine, but a
positive correlation was observed in the hind gut. All studied *Lactobacillus* spp. strains showed only negative dependencies for the zinc fractions, but bifidobacteria seemed not influenced at all.

Surprisingly, no significant correlations were visible for short chain fatty acids except positive dependencies in the jejunum. Ammonia was negatively correlated to free inorganic zinc, but except for negative dependencies in the stomach, t-lactate only showed negative correlations to protein-associated zinc in the small and large intestine.

**Discussion**

This study was conducted to describe the influence of high dietary zinc on the development of the intestinal microbiota and their metabolic activity in the intestine of weaned piglets.

The most drastic impact of high dietary zinc was the lasting reduction of three of five studied *Lactobacillus* species. Lactic acid bacteria and especially lactobacilli are dominant in the stomach and small intestine of piglets (Sghir et al., 1998; Hill et al., 2005; Pieper et al., 2008; Vahjen et al., 2010). Among the lactobacilli, *Lactobacillus amylovorus* and *L. reuteri* are frequently found to dominate the *Lactobacillus* spp. populations in the small intestine (Konstantinov et al., 2004; Hojberg et al., 2005; Metzler-Zebeli et al., 2010). *Lactobacillus amylovorus* was also the most prevalent species among the *Lactobacillus* spp. that were analysed in this study and one of the species most severely influenced by high dietary zinc.
However, as cell numbers of total lactobacilli were much higher than the combined cell numbers of analysed species, other *Lactobacillus* spp. not analysed in this study have contributed to total lactobacilli. Nevertheless, given the enormous diversity of lactobacilli in the small intestine of piglets (Leser et al., 2002; Vahjen et al., 2011), it is probable that many other *Lactobacillus* spp. contributed to the total lactobacilli amount at lower cell numbers.

The decrease of *L. amylovorus* coincided with lower lactic acid concentrations due to high dietary zinc throughout the feeding trial. This effect seemed to be confined to lactobacilli only, as other lactic acid producing bacteria were not significantly reduced in cell numbers (*bifidobacteria, enterococci, streptococci, Weissella* spp. and *Leuconostoc* spp.).

Interestingly, *L. reuteri* and especially *L. johnsonii* were less affected by high dietary zinc. Thus, the antibacterial action of zinc *in vivo* is probably not intrinsic to lactobacilli *per se*, but may rather be species specific. This was also shown by Liedtke & Vahjen (2012), who observed differing *in vitro* zinc resistance among nine *Lactobacillus* species of intestinal origin.

A pyrosequencing study on the bacterial core in the ileum of 40–42 day old piglets with similar high zinc concentrations also reported that *Lactobacillus* species were reduced, but *Weissella* spp. and *Leuconostoc* spp. significantly increased due to high dietary zinc (Vahjen et al., 2011). Those results could only in part be confirmed in this study, as ileal *Weissella* spp. and *Leuconostoc* spp. cell numbers increased only numerically on day 42 and...
instead of significant decreases of *L. reuteri*, significant decreases of *L. amylovorus* were observed. Nevertheless, it seems that the niches opened by reduction of one or more bacterial species can be used in part by other species, in this case *Weissella* and *Leuconostoc* species.

The second most drastic influence of high dietary zinc was noted for enterobacteria. However, contrary to lactobacilli, the reduction in total enterobacterial cell numbers was confined to the first week after weaning. As individual enterobacterial species were not analyzed in detail, it remains unknown whether zinc sensitive species were replaced by resistant species or if enterobacterial species adapted to the high zinc conditions. However, a pyrosequencing study with 40–42 day old piglets has shown that the diversity of enterobacteria increased due to high dietary zinc (Vahjen et al., 2011). Furthermore, other studies on high dietary zinc in piglets also show either no effect (Broom et al., 2006) or an increase in coliforms (Hojberg et al., 2005) as well as an increased phenotype stability of coliform isolates after weaning (Katouli et al., 1999). Therefore, it is likely that enterobacteria possess mechanisms to successfully counteract high dietary zinc.
concentrations. This effect may be achieved actively by more efficient heavy metal efflux systems to expel intracellular zinc (Nies & Silver, 1995; Nies, 2003) or due to gene transfer of specific heavy metal plasmids, which have been detected in both gram-positive and gram-negative intestinal bacteria (Silver & Walderhaug, 1992; O’Brien et al., 2008). Less information is available on specific heavy metal plasmids of lactobacilli (Fortina et al., 1993). An enhanced colonization may also occur by passive means such as reduced bacterial competition with other bacterial groups. In this study, the reduction of the resident dominant lactobacilli as well as their main metabolite lactate may have led to a more undisturbed intestinal colonization of enterobacteria. Finally, an increased colonization potential and possibly increased diversity of enterobacteria due to high intestinal zinc concentrations may also have an impact on pathogenic E. coli strains. Under field conditions, E. coli induced diarrhoea is often reduced in piglets fed high dietary zinc diets. Therefore, pathogenic E. coli strains may encounter a higher intra-group competition due to increased diversity of enterobacteria or their resistance to zinc and may thus be at a disadvantage to colonize the porcine small intestine.

The cell numbers of all studied clostridial cluster and the large Gram-negative Bacteroides-Prevotella-Porphyromonas cluster seemed to be unaffected by high dietary zinc concentrations on the whole. Again, as individual species of these bacterial groups were not analyzed, it remains unknown if a similar effect as observed for lactobacilli occurred, i.e. certain sensitive species may have been affected, while other more resistant species gained in cell number, resulting in no visible changes overall. Both bacterial groups are strict anaerobic, fastidious and therefore attain high cell numbers predominantly in the large intestine, although they can be detected at lower cell numbers in the small intestine. The combined correlation analysis for all sampling days showed that the clostridial cluster XIVa exhibited a negative dependency to free zinc ions in the small intestine like most other bacterial groups, but a positive dependence in the hind gut. It may therefore be possible that the already dominant clostridial cluster XIVa gained an additional colonization advantage in the hind gut during the development of the

Table 1. Spearman correlation coefficients of protein-associated and free inorganic zinc fractions to bacterial cell numbers and their metabolites in different intestinal locations (combined for all sampling days)

<table>
<thead>
<tr>
<th>Item</th>
<th>Stomach</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli/Hafnia/Shigella</td>
<td>0.534</td>
<td>0.373</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>-0.399</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridial cluster I</td>
<td>-0.513</td>
<td>-0.329</td>
<td>-0.28</td>
<td>0.451</td>
</tr>
<tr>
<td>Clostridial cluster XIVa</td>
<td>-0.502</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides-Prevotella-Porphyromonas</td>
<td>-0.484</td>
<td>-0.48</td>
<td>-0.318</td>
<td>-0.254</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>-0.325</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>-0.364</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>-0.333</td>
<td>0.622</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>-0.315</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Weissella spp.</td>
<td>-0.315</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leuconostoc spp.</td>
<td>-0.392</td>
<td>-0.534</td>
<td>-0.484</td>
<td>-0.368</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>-0.373</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. amylovorus</td>
<td>-0.354</td>
<td>-0.586</td>
<td>-0.467</td>
<td>-</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>0.531</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. mucosae</td>
<td>-0.389</td>
<td>-0.444</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>-0.378</td>
<td>-0.467</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total volatile fatty acids</td>
<td>-0.327</td>
<td>0.522</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Acetate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Propionate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% n-Butyrate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% i-Butyrate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% n-Valerate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% i-Valerate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% n-Valerate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>i-Lactate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.534</td>
<td>0.373</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*No significant correlation (P > 0.05).
microbiota. The cause for this effect may again be based on a higher zinc resistance of certain species, which would increase their cell number. On the other hand, a displacement of other bacterial groups such as lactobacilli would lead to reduced competition in the small intestine. This could be especially true for the Bacteroides-Prevotella–Porphyromonas cluster, as positive correlations to free zinc were also found in the small intestine, where lactobacilli usually dominate.

The total concentration of bacterial metabolites was almost always lower in all intestinal segments and all sampling days. This confirms the bacteriostatic effect of pharmacological doses of dietary zinc. Interestingly, propionate concentrations were also reduced in the small intestine. Propionate is not only produced from carbohydrate fermentation, but also by lactate fermenting bacteria. Among the lactate fermenting bacteria found in the stomach and small intestine, Veillonella spp. (McGillivray & Cranwell, 1992; Kraatz & Taras, 2008) are known to heavily ferment lactate. However, clostridia that are present in the stomach and small intestine of young piglets as well as other strict anaerobic bacteria such as Megaplasma elsdii or Selenomonas ruminantium also use lactate as an energy source (Ingham et al., 1998; Nagaraja & Titgemeyer, 2007). The metabolism of lactate fermenting species may therefore also be limited due to reduced substrate input in the small intestine.

Total metabolites in the large intestine were also always lower in the high dietary zinc group; mainly because of a decrease of propionate concentrations (significantly so on day 49 and 56) and in part due to decreased n-butyrate concentrations, while acetate concentrations remained unchanged. As mentioned, lactate serves as substrate for propionate, but also for n-butyrate production in the intestinal tract. While the human microbiota seems to produce mainly n-butyrate from lactate (Bourrïaud et al., 2005), the rumen microbiota produces more propionate from lactate (Nagaraja & Titgemeyer, 2007).

Knowledge about the composition of lactate fermenting bacteria in the large intestine of pigs is scarce, but similar species as in humans and ruminants have been described. The use of lactate as substrate in the hind gut is of course dependent on lactate production and thus, it is reasonable to conclude that a reduced lactate concentration would reduce a metabolic cross-feeding. Lower lactate concentrations could directly lead to reduced propionate concentrations and may therefore also have an impact on hind gut microbiota, which could be modified due to reduced small intestinal input of metabolites and a different bacterial species composition. A reduced lactate production would also have implications on bacterial species that rely on lactate as sole energy source (Nagaraja & Titgemeyer, 2007).

From the comparison of bacterial cell numbers and metabolites it can be concluded that high dietary zinc acted bacteriostatic on the entire microbiota, because total detected cell numbers were only marginally reduced during the first week after weaning, but total metabolite concentrations were still reduced after 4 weeks. As total bacterial cell numbers were not much influenced by zinc, this also indicates that the intestinal microbiota as a whole community has a high capacity to adapt by replacement of certain species by other species that are able to thrive under the given conditions.

Overall, the first week after weaning showed the highest differences between low and high dietary zinc intake. Weaning forces the animal into an extreme stress situation, because it has to adjust to a new environment, to the change from liquid mother milk to uptake of solid feed as well as to social stress. The consequences are readily visible by refusal or only marginal intake of feed during the first 3–4 days after weaning and therefore the intestinal physiology and immune response of the animal are deeply disturbed. As a result, the intestinal microbiota itself is imbalanced, causing diarrhoea due to opportunistic pathogens such as E. coli. As the microbiota is far from equilibrium, any further factor that takes effect on bacteria during this time must induce additional modifications in the already perturbed habitat. In this case, zinc acted as a powerful modifier, which affected certain bacterial groups (Lactobacilli/enterobacteria). It remains unknown, if the lasting reductions of certain Lactobacillus spp. species are due to their inability to adapt to high zinc concentrations or if their initially slower development led to reduced colonization due to later competition from already established bacterial species.

Conclusions

The cell numbers of a range of bacterial groups indicate that pharmacological doses of dietary zinc oxide act mainly in the stomach and small intestine primarily through a reduction of certain Lactobacillus species. A reduced competition may give rise to increased colonization by other bacterial groups or species. The most drastic effects occurred during the first 2 weeks after weaning. Therefore, and in accordance with Broom et al. (2006) we propose that it may be sufficient to supplement diets with ZnO during this early post weaning phase. This would also reduce the environmental hazard of ZnO containing pig manures (Vellenga et al., 1992; Jondreville et al., 2003; Shi et al., 2011).

References

Blacher F, Mariotti F, Huneau IJ & Tomé D (2007) Effects of amino acid-derived luminal metabolites on the colonic


Martin L, Pieper R, Vahjen W & Zentek J (2012) Influence of high levels of dietary zinc oxide on performance and small intestinal gene expression in weaned piglets. XIIth International Symposium on Digestive Physiology in Pigs, May 29-June 1, Keystone, US. abstract #1104


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Composition of diets (as-is basis).

**Table S2.** Primer sequences, product length and annealing temperatures.

**Table S3.** Bacterial cell numbers in the intestinal tract of piglets on 35 days of age.

**Table S4.** Bacterial cell numbers in the intestinal tract of piglets on 42 days of age.

**Table S5.** Bacterial cell numbers in the intestinal tract of piglets on 49 days of age.

**Table S6.** Bacterial cell numbers in the intestinal tract of piglets on 56 days of age.

**Table S7.** Bacterial metabolites in the intestinal tract of piglets on 35 days of age.

**Table S8.** Bacterial metabolites in the intestinal tract of piglets on 42 days of age.

**Table S9.** Bacterial metabolites in the intestinal tract of piglets on 49 days of age.

**Table S10.** Bacterial metabolites in the intestinal tract of piglets on 56 days of age.