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# The Impact of Dietary Zinc Oxide on the Bacterial Diversity of the Small Intestinal Microbiota of Weaned Piglets

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## Abstract

Dietary zinc oxide is often used in pharmacological concentrations to promote health as well as performance of weaned piglets due to its bacteriostatic effects. This study was conducted to provide an in depth analysis of the bacterial composition in weaned piglets fed different amounts of dietary zinc oxide. Piglets were fed diets containing 57 (low), 164 (medium) or 2425 (high) mg/kg dietary zinc. Zinc above the basal dietary level was supplied from analytical grade zinc oxide (ZnO). DNA was extracted from stomach and ileum digesta samples of 32 and 53d old animals (n=4 per group) and used to generate bar-coded 16S ribosomal DNA amplicons for deep sequencing analysis. A total of 9 phyla, 40 orders, 75 families and 328 genera were detected in 8.76 x $10^5$ sequencing reads. *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the dominant phyla, but no significant differences between treatment groups were observed. *Lactobacillales* (16.3-59.9%), *Bacteroidales* (2.2-59.1%), *Clostridiales* (0.05-70.2%) and *Selenomonadales* (2.6-17.5%) were found as the dominating order. Noteworthy changes on the order level were found for numerically or significantly increased ratios of *Clostridiales*, but significantly decreased *Lactobacillales* in the high dietary zinc group. The bacterial diversity for the high dietary zinc diet was significantly higher for the total microbiota than the medium or low zinc diet. However, *Lactobacillales* diversity decreased, while *Clostridiales* and *Enterobacteriales* diversity increased significantly. Principal component analysis confirmed changes in the microbiota, most notably for the high dietary zinc treatment. This study has shown that pharmacological doses of high dietary zinc can drastically alter the bacterial composition and development of the microbiota in weaned piglets. The quantitative shift of bacterial groups due to high dietary zinc was most pronounced one week after weaning, while the more developed microbiota in older animals seemed to be able to adapt to high concentrations of dietary zinc.

**Keywords:** Pig; Nutrition; Microbiota; Zinc oxide

## Introduction

Zinc oxide has been shown to improve piglet performance and health [1] when it is used in high amounts (up to 3 g/kg feed). High ZnO concentrations act bactericidal and thus the reduction in frequently observed diarrhea in weaned piglets is believed to be due to the reduction of intestinal *E. coli*. However, studies on the bacterial composition in weaned piglets fed high dietary ZnO found reduced lactobacilli colony counts [2,3] or *Lactobacillus* spp. sequence reads, respectively [4], but also an increased coliform counts or *Enterobacteriales* sequence reads [3,4]. Additionally, an increased stability of coliform phenotypes was reported [5]. These studies imply that not all bacteria are inhibited by high doses of dietary ZnO and that a direct inhibition of coliforms seems unlikely. Furthermore, an *in vitro* study reported that minimal inhibitory concentrations for zinc oxide in a wide range of bacterial species vary for individual members of bacterial groups [6]. This also supports the hypothesis that dietary ZnO acts on the bacterial species level, inhibiting some, but not all bacteria alike.

The bactericidal action of zinc is expected to take place in the small intestine. As amphoteric molecule, ZnO is practically insoluble in water, but shows a high solubility at acidic pH. The low pH in the stomach of piglets transforms a considerable amount of insoluble ZnO into free Zn ions (54% free Zn ions at 164 mg·kg$^{-1}$ dietary ZnO) and thus high concentrations of free Zn ions occur in the stomach. On the other hand, the pH increase in the small and large intestine renders ZnO insoluble and only already solubilized $Zn^{2+}$ ions may act bactericidal. Therefore, the amount of free $Zn^{2+}$ is actually very much lower in the small and large intestine than in the stomach [7].

The stomach and small intestine of weaned piglets harbor large numbers of lactic acid bacteria and reducing its amount may change the development of the total intestinal microbiota. A reduction of small intestinal bacteria by pharmacological doses of dietary zinc may be beneficial for a short period after weaning, as documented by reduced diarrhea in piglets. However, detrimental effects may occur in the long run, if the intestinal microbiota cannot play its protective role against microbial invasion. The intense use of pharmacological doses of ZnO also raises environmental concerns, because pig manure is disposed into the environment [8] and manure is often used as additional and cheap fertilizer in crops for human consumption, for instance rice [9]. Recently, the use of high dietary zinc in piglets was also shown to increase the amount of multiresistant *E. coli* [10]. Finally, zinc accumulates in the pig liver [11], which is also processed for human consumption. Therefore, excessive use of dietary ZnO may lead to detrimental effects on soils, crops, animal products and antibiotic resistance development. As a consequence, some authors propose to shorten the application period to two weeks after weaning [5,12].

However, previous studies on the effect of dietary ZnO on the porcine bacterial composition have mostly focused on only one time point and thus the rapid development of the microbiota in piglets has been neglected. Therefore, this study was conducted to fill this gap in

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knowledge and performed an in-depth analysis of the development of the intestinal microbiota in piglets under the influence of different dietary ZnO concentrations.

## Material and Methods

### Animals and diets

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 based on a standard starter feed mixture (wheat/ barley/ soy bean meal) were fed after weaning until 53$^{th}$ d of life. Analytical grade ZnO (Sigma Aldrich, Taufkirchen, Germany) was added to the diets to adjust for 50, 150 and 2,500 mg/kg diet (analysed concentration: 57, 164 and 2425 mg/kg, respectively).

### Sampling

Piglets of each experimental group were sacrificed on 32 ± 1 and 53 ± 1 d 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 and terminal ileum, shock-frozen in liquid nitrogen and stored at -80 °C until further analysis.

### DNA-extraction

Total genomic DNA was extracted in triplicate from 3 × 200 mg sample using a commercial kit (Qiagen Stool kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions except for an increase in temperature during the lysis step to 90°C. Purified DNA was then pooled per sample and the DNA amount was measured by fluorescence using a real-time PCR cycler and SYBR® green with calf thymus DNA as calibrator.

### Preparation of sequencing PCR amplicons

DNA samples were diluted to 100ng/µl and 1µl was used for 25 µl PCR reaction mix. A primer set (0.3 µM) was used to amplify a region of bacterial 16S rRNA genes. Forward primers targeting *Escherichia coli* position 8–24 (GM3 5'-AGAGTTTGATCMTGGC-3') and reverse primer (926R 5'-CCGTCAATTCMTTTGAGTTT-3') were tagged with unique hexamer nucleotides in order to sort PCR products after sequencing (Table 1). A commercial master mix kit (HotStarTaq Plus Master Mix; Qiagen, Hilden, Germany; with added SYBR® green during cycle number optimization) was used for PCR amplification under the following cycling conditions: 1x 15 min at 95°C, 32×15 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C and 1×1 min 20°C. Optimal amplification conditions were defined by the cycle number at which the fluorescence curve entered a plateau with no further increase of total fluorescence. Cycling was performed on a Stratagene Mx3000P thermocycler (Stratagene, Amsterdam, The Netherlands). PCR products were removed immediately after the last cycle and stored at -20 °C until further analysis.

### Pyrosequencing procedures

The PCR products were purified using a commercial kit (Qiaquick nucleotide removal kit, Qiagen, Hilden, Germany), DNA amount was determined and equimolar dilutions of all samples were combined into one master sample per trial group. Pyrosequencing was performed by LGC Genomics (Berlin, Germany) on a Roche Genome Sequencer FLX system using a Titanium series PicoTiterPlate.

### Processing and phylogenetic assignment of sequence reads

After quality control, sequence reads were sorted according to sample tags and primer combination, resulting in 48 single data files. After removal of sample tags and primer sequences, data files were uploaded to the MG-RAST Server [13] and processed by its SEED software tool. The phylogenetic profile of each sample was computed with the following parameters: maximum e-value of 1e-8, minimum percent identity of 99% and minimum alignment length of 500 bases. The Green Gene reference data bank was used for identification.

For statistical interpretation, the next step in the analysis was the deletion of all data with four or less identical sequence reads per sample in order to increase confidence of sequence reads and reduce bias by possible sequencing errors [14,15]. Also, sequence reads that only

| | Stomach | | | Ileum | | |
|---|---|---|---|---|---|---|
| **32d** | **Low** | **Medium** | **High** | **Low** | **Medium** | **High** |
| **Total reads** | 1394 (± 153.1)$^{a}$ | 1805 (± 289.4)$^{b}$ | 2202.5 (± 490.3)$^{b}$ | 616.8 (± 350.2) | 677.3 (± 235.7) | 553.0 (± 242.3) |
| **Lactobacilliales** | 429.3 (± 70.5)$^{b}$ | 448.5 (± 167.8)$^{b}$ | 257.0 (± 134)$^{a}$ | 166.5 (± 40.1)$^{b}$ | 183.0 (± 77.0)$^{b}$ | 87.5 (± 41.8)$^{a}$ |
| **Bacteroidales** | 350.7 (± 72.9)$^{b}$ | 291.7 (± 139.5)$^{b}$ | 51.8 (± 13.3)$^{a}$ | 137.4 (± 93.4) | 76.5 (± 104.0) | 52.7 (± 56.4) |
| **Clostridiales** | 13.3 (± 11.0) | 10.5 (± 7.3) | 21.0 (± 12.1) | 13.2 (± 15.1)$^{a}$ | 9.5 (± 5.4)$^{a}$ | 280.5 (± 107.2)$^{b}$ |
| **Enterobacteriales** | 8.3 (± 7.57)$^{ab}$ | 4.2 (± 1.5)$^{a}$ | 23.5 (± 17.8)$^{b}$ | 23.1 (± 12.1)$^{ab}$ | 15.4 (± 12.9)$^{a}$ | 50.0 (± 24.8)$^{b}$ |
| **Selenomonadales** | 220.2 (± 169.8) | 269.2 (± 150.4) | 292.3 (± 144.5) | 89.7 (± 83.3) | 177.7 (± 109.3) | 63.2 (± 98.6) |
| **53d** | | | | | | |
| **Total reads** | 1919.9 (± 321.8) | 1391.9 (± 611.2) | 1413.3 (± 429) | 655.6 (± 297.9) | 833.3 (± 178.6) | 702.6 (± 304.2) |
| **Lactobacilliales** | 479.5 (± 283.9)$^{b}$ | 496.7 (± 292.5)$^{b}$ | 197.4 (± 62.5)$^{a}$ | 178.0 (± 166.9)$^{b}$ | 173.8 (± 187.9)$^{b}$ | 13.3 (± 6.9)$^{a}$ |
| **Bacteroidales** | 250.4 (± 98.2) | 275.7 (± 137.4) | 261.2 (± 99.1) | 37.0 (± 43.6) | 9.6 (± 4.8) | 14.5 (± 10.1) |
| **Clostridiales** | 21.4 (± 11.8)$^{a}$ | 20.8 (± 12.6)$^{a}$ | 68.8 (± 44.4)$^{b}$ | 84.2 (± 46.7)$^{a}$ | 41.7 (± 8.0)$^{a}$ | 290.3 (± 38.7)$^{b}$ |
| **Enterobacteriales** | 25.3 (± 23.3)$^{a}$ | 34.7 (± 25.0)$^{a}$ | 99.4 (± 41.2)$^{b}$ | 47.0 (± 28.0) | 67.2 (± 26.0) | 55.8 (± 20.3) |
| **Selenomonadales** | 89.9 (± 29.4)$^{a}$ | 116.6 (± 26.2)$^{a}$ | 226.6 (± 34.8)$^{b}$ | 50.8 (± 50.2) | 24.0 (± 12.4) | 95.5 (± 58.3) |

$^{a,b}$ = significantly different within rows and intestinal segment (Mann-Whitney-U test; P ≤ 0.05)

**Table 1:** Chao1 diversity index of the dominant bacterial order in the small intestine of piglets fed diets with different dietary ZnO concentrations.

occurred in one sample were deleted in order to focus on the common bacterial species. The remaining sequence reads were used to calculate the relative contribution of specifically assigned sequences to total sequence reads in a sample. These values were then used for further statistical analysis.

### Ecological diversity indices and bacterial core on the genus level

The CHAO1 index as well as Evenness was calculated using the Ribosomal Data base project pyrosequencing tools with original sequence data, i.e. without deletion of low abundance reads: sequence read alignments were sorted by bacterial order and merged, followed by a Complete Linkage Clustering with 3% maximum cluster distance [16]. Additionally, a core microbiota was computed on the genus level. Individual genera had to be present in 3 of 4 samples of each intestinal segment per treatment group and sampling day to qualify for the core microbiota.

### Principal component analysis

Distance metrics for samples from 32 and 53 days of life were calculated using the MG-RAST tool "PCoA" with normalized values (0-1) and Bray-Curtis distance.

### Statistics

All data management and statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL). Between group differences were calculated by the Mann-Whitney-U test. All tests were two-sided, and the level of significance was set to an α level of 0.05.

## Results

### 454-pyrosequencing statistics

The pyrosequencing procedure yielded a total of $1.34\times10^6$ sorted reads with correct hexamer tags (mean read length of 563 (± 53) bp). After correction for read length (minimum 500 bases) $8.76\times10^5$ sequences with an average of 28040 (± 19064) reads per sample were used for analysis.

### Bacterial phylogeny on the order level

A total of 9 phyla, 40 orders, 75 families and 328 genera were detected. *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the dominant phyla, but no significant differences between treatments were observed on the phylum level (data not shown). On the order level, *Lactobacillales* (16.3-59.9%), *Bacteroidales* (2.2-59.1%), *Clostridiales* (0.05-70.2%), *Selenomonadales* (2.6-17.5%) and *Enterobacteriales* (0.02-8.7%) dominated the bacterial communities in the stomach and ileum. All other order only occasionally exceeded 1% of total reads. Figure 1 shows the ratios of bacterial order on day 32 of life. The distributions of bacterial order in the stomach of the low and medium dietary zinc treatments were similar, but the high dietary zinc treatment led to a reduction of *Lactobacillales* at the expense of *Selenomonadales* and *Pasteurellales*, respectively. In the ileum, the medium dietary zinc treatment displayed a higher ratio of *Selenomonadales* than the low dietary zinc treatment, but the proportion of *Clostridiales* increased drastically in the high dietary zinc treatment. The high dietary zinc diet led to significantly higher *Clostridiales* and *Pseudomonadales* ratios in the stomach as well as increased *Bacilliales* reads in the ileum compared to the low and medium zinc diet (Table 2). Although not significant due to high individual variation, a numerical reduction of the *Lactobacillales* reads was observed only in the high dietary zinc diet on day 32.

On day 53 of life, the stomach presented a different picture, as *Lactobacillales* were dominant only in the medium dietary zinc treatment, while *Bacteroidales* was the foremost order in both the low

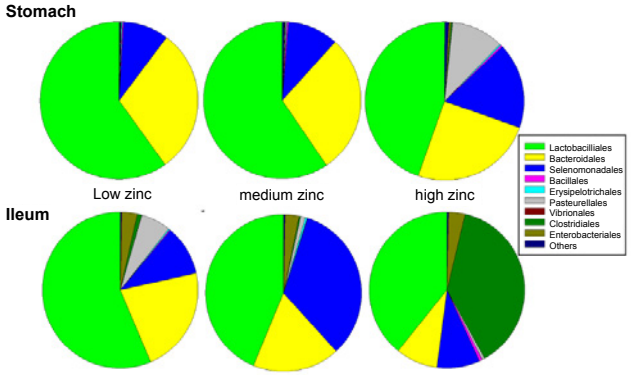


**Figure 1:** Distribution of bacterial order in the stomach and ileum of 32 day old piglets fed different concentrations of dietary zinc [% of total reads per sample].

| | Stomach | | | Ileum | | |
|---|---|---|---|---|---|---|
| **32d** | **Low** | **Medium** | **High** | **Low** | **Medium** | **High** |
| **Total reads** | 0.698 (± 0.031)$^{a}$ | 0.717 (± 0.043)$^{ab}$ | 0.747 (± 0.030)$^{b}$ | 0.722 (± 0.078) | 0.735 (± 0.046) | 0.622 (± 0.056) |
| **Lactobacilliales** | 0.633 (± 0.061)$^{a}$ | 0.637 (± 0.056)$^{a}$ | 0.772 (± 0.034)$^{b}$ | 0.621 (± 0.088) | 0.639 (± 0.05) | 0.676 (± 0.063) |
| **Bacteroidales** | 0.706 (± 0.105) | 0.699 (± 0.056) | 0.771 (± 0.080) | 0.707 (± 0.095) | 0.801 (± 0.106) | 0.769 (± 0.119) |
| **Clostridiales** | 0.819 (± 0.091) | 0.864 (± 0.085) | 0.872 (± 0.033) | 0.853 (± 0.054)$^{a}$ | 0.772 (± 0.117)$^{a}$ | 0.624 (± 0.062)$^{b}$ |
| **Enterobacteriales** | 0.821 (± 0.127)$^{a}$ | 0.852 (± 0.053)$^{a}$ | 0.725 (± 0.101)$^{b}$ | 0.609 (± 0.057) | 0.733 (± 0.099) | 0.672 (± 0.086) |
| **Selenomonadales** | 0.801 (± 0.019) | 0.779 (± 0.021) | 0.786 (± 0.034) | 0.802 (± 0.044)$^{b}$ | 0.775 (± 0.031)$^{a}$ | 0.902 (± 0.03)$^{c}$ |
| **53d** | | | | | | |
| **Total reads** | 0.807 (± 0.026) | 0.797 (± 0.036) | 0.799 (± 0.052) | 0.733 (± 0.028) | 0.715 (± 0.072) | 0.712 (± 0.063) |
| **Lactobacilliales** | 0.787 (± 0.039) | 0.791 (± 0.019) | 0.798 (± 0.034) | 0.672 (± 0.051)$^{a}$ | 0.735 (± 0.058)$^{a}$ | 0.923 (± 0.04)$^{b}$ |
| **Bacteroidales** | 0.820 (± 0.021) | 0.779 (± 0.047) | 0.804 (± 0.009) | 0.816 (± 0.057) | 0.768 (± 0.045) | 0.845 (± 0.084) |
| **Clostridiales** | 0.906 (± 0.024) | 0.895 (± 0.039) | 0.853 (± 0.053) | 0.728 (± 0.056) | 0.714 (± 0.067) | 0.681 (± 0.041) |
| **Enterobacteriales** | 0.812 (± 0.062) | 0.793 (± 0.105) | 0.741 (± 0.06) | 0.717 (± 0.062) | 0.712 (± 0.051) | 0.807 (± 0.049) |
| **Selenomonadales** | 0.818 (± 0.038) | 0.828 (± 0.029) | 0.819 (± 0.014) | 0.846 (± 0.019) | 0.857 (± 0.018) | 0.862 (± 0.006) |

$^{a,b}$ = significantly different within rows and intestinal segment (Mann-Whitney-U test; $P \leq 0.05$)

**Table 2:** Evenness of the dominant bacterial order in the small intestine of piglets fed diets with different dietary ZnO concentrations.

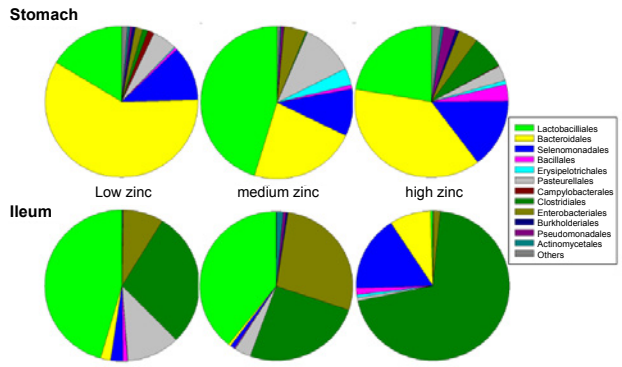


**Figure 2:** Distribution of bacterial order in the stomach and ileum of 53 day old piglets fed different concentrations of dietary zinc [% of total reads per sample].

and high dietary zinc treatments (Figure 2). The proportion of less dominant orders (i.e. the bacterial diversity) increased in all treatment groups. In the ileum, the low and medium dietary zinc treatments were more similar to each other, while the high dietary zinc treatment again showed drastically increased *Clostridiales* and *Selenomonadales* ratios, but the amount of *Lactobacillales* was strongly reduced.

## Bacterial phylogeny of the core microbiota at the genus level

A total of 16 genera were present in almost all samples from the stomach and ileum of 32d and 53d old animals (Tables 3a and 3b). The supplementation of high dietary zinc significantly modified a range of core genera, but fewer differences were visible between low and medium dietary zinc treatments. Thus, on 32d of age, the percentages of *Clostridium*, *Pantoea*, *Actinobacillus*, *Acidaminococcus*, *Dialister*, *Megasphaera*, *Selenomonas* and *Veillonella* were all significantly

| | | Stomach | | | Ileum | | |
|---|---|---|---|---|---|---|---|
| **Order** | **Genus** | **Low** | **Medium** | **High** | **Low** | **Medium** | **High** |
| Clostridiales | *Clostridium* | 3.2 (± 0.9)$^{a}$ | 6.6 (± 1.2)$^{ab}$ | 8.4 (± 1.3)$^{b}$ | 1.7 (± 0.5)$^{a}$ | 6.5 (± 1.3)$^{a}$ | 33.4 (± 18.1)$^{b}$ |
| **Bacteroidales** | *Prevotella* | 0.21 (± 0.15)$^{a}$ | 7.5 (± 2.8)$^{b}$ | 0.88 (± 0.72)$^{a}$ | 1.6 (± 2.9) | 1.1 (± 1.4) | 0.45 (± 0.72) |
| **Enterobacteriales** | *Citrobacter* | n.d. | n.d. | n.d. | 0.61 (± 0.34) | 0.27 (± 0.22) | 0.41 (± 0.14) |
| | *Enterobacter* | n.d. | n.d. | 0.08 (± 0.05) | 1.1 (± 0.4) | 0.05 (± 0.03) | 0.16 (± 0.14) |
| | *Escherichia/Shigella* | 0.04 (± 0.01) | 0.06 (± 0.03) | 0.37 (± 0.29) | 4.2 (± 1.8)$^{a}$ | 3.52 (± 2.1)$^{a}$ | 7.4 (± 2.5)$^{b}$ |
| | *Pantoea* | 0.03 (± 0.01)$^{a}$ | 0.05 (± 0.04)$^{a}$ | 0.19 (± 0.15)$^{b}$ | n.d. | 0.07 (± 0.05)$^{a}$ | 0.23 (± 0.05)$^{b}$ |
| **Lactobacilliales** | *Lactobacillus* | 79.4 (± 16.4)$^{a}$ | 68.9 (± 19.2)$^{a}$ | 44.3 (± 16.8)$^{b}$ | 67.9 (± 21.8)$^{a}$ | 55.6 (± 12.5)$^{a}$ | 36.5 (± 9.9)$^{b}$ |
| | *Lactococcus* | 0.37 (± 0.33) | 0.90 (± 0.82) | 0.07 (± 0.02) | n.d. | 0.29 (± 0.14) | n.d. |
| | *Streptococcus* | 0.05 (± 0.03)$^{a}$ | 0.08[1] | 0.11 (± 0.09)$^{b}$ | n.d. | n.d. | 0.09 (± 0.02) |
| **Pasteurellales** | *Actinobacillus* | 0.11 (± 0.13)$^{a}$ | 0.22 (± 0.27)$^{a}$ | 12.5 (± 11.4)$^{b}$ | 2.3 (± 10.5) | 1.5 (± 1.4) | 1.4 (± 1.7) |
| **Selenomonadales** | *Acidaminococcus* | 0.80 (± 0.52)$^{a}$ | 0.99 (± 0.58)$^{a}$ | 2.2 (± 1.3)$^{b}$ | 1.3 (± 0.8) | 1.3 (± 1.0) | 0.13 (± 0.03) |
| | *Dialister* | 0.58 (± 0.11)$^{a}$ | 0.67 (± 0.31)$^{a}$ | 1.28 (± 0.21)$^{b}$ | 1.5 (± 1.9) | 0.75 (± 0.73) | 1.14 (± 1.3) |
| | *Megasphaera* | 6.8 (± 3.1)$^{a}$ | 7.5 (± 2.5)$^{a}$ | 15.0 (± 4.9)$^{b}$ | 7.6 (± 3.8) | 9.8 (± 4.2) | 2.7 (± 2.0) |
| | *Mitsuokella* | 4.3 (± 0.4) | 1.8 (± 2.4) | 4.1 (± 5.2) | 7.7 (± 6.2) | 8.2 (± 10.7) | 8.1 (± 11.1) |
| | *Selenomonas* | 4.5 (± 1.4)$^{a}$ | 2.6 (± 2.2)$^{a}$ | 7.9 (± 2.1)$^{b}$ | 4.5 (± 3.1)$^{a}$ | 8.4 (± 3.3)$^{ab}$ | 10.4 (± 5.5)$^{b}$ |
| | *Veillonella* | 0.06 (± 0.02)$^{a}$ | 0.11 (± 0.15)$^{ab}$ | 1.4 (± 0.4)$^{b}$ | 2.0 (± 1.2) | 0.40 (± 0.49) | 1.7 (± 2.1) |

$^{a,b}$ = significantly different within rows and intestinal segment (Mann-Whitney-U test; P ≤ 0.05)
[1] = single value

**Table 3a:** Core microbiota in the small intestine of 32d old piglets fed diets with different dietary ZnO concentrations [% of total reads].

| | | Stomach | | | Ileum | | |
|---|---|---|---|---|---|---|---|
| **Order** | **Genus** | **Low** | **Medium** | **High** | **Low** | **Medium** | **High** |
| Clostridiales | *Clostridium* | 3.5 (± 1.3)$^{a}$ | 7.3 (± 2.4)$^{ab}$ | 14.3 (± 3.0)$^{b}$ | 11.9 (± 3.7)$^{a}$ | 24.2 (± 11.9)$^{ab}$ | 56.1 (± 15.4)$^{b}$ |
| Bacteroidales | *Prevotella* | 6.0 (± 2.1) | 4.8 (± 4.3) | 4.3 (± 4.0) | 0.16 (± 0.03)$^{a}$ | 0.09 (± 0.12)$^{a}$ | 1.3 (± 0.33)$^{b}$ |
| Enterobacteriales | *Citrobacter* | 0.50 (± 0.32)$^{a}$ | 0.6 (± 0.9)$^{a}$ | 1.86 (± 1.3)$^{ab}$ | 1.1 (± 1.7)$^{ab}$ | 0.53 (± 0.43)$^{a}$ | 2.3 (± 0.34)$^{b}$ |
| | *Enterobacter* | 2.6 (± 0.4)$^{a}$ | 2.8 (± 0.9)$^{ab}$ | 4.41 (± 1.3)$^{b}$ | 1.5 (± 1.4)$^{a}$ | 2.33 (± 0.26)$^{a}$ | 4.3 (± 0.2)$^{b}$ |
| | *Escherichia/Shigella* | 0.12 (± 0.07)$^{a}$ | 2.1 (± 1.4)$^{ab}$ | 2.8 (± 0.8)$^{b}$ | 1.4 (± 1.6)$^{a}$ | 6.3 (± 2.2)$^{ab}$ | 8.1 (± 1.9)$^{b}$ |
| | *Pantoea* | 1.3 (± 1.1) | 0.79 (± 0.84) | 1.2 (± 0.6) | 2.8 (± 1.5)$^{a}$ | 2.8 (± 0.86)$^{a}$ | 4.3 (± 0.21)$^{b}$ |
| Lactobacilliales | *Lactobacillus* | 41.5 (± 14.0)$^{ab}$ | 50.2 (± 11.6)$^{b}$ | 30.9 (± 8.5)$^{a}$ | 64.9 (± 24.5)$^{b}$ | 49.3 (± 15.7)$^{ab}$ | 2.3 (± 0.28)$^{a}$ |
| | *Lactococcus* | 1.0 (± 0.86) | 2.3 (± 0.48) | 3.2 (± 2.9) | 0.10 (± 0.04) | 0.14 (± 0.14) | 0.03 (± 0.01) |
| | *Streptococcus* | 0.36 (± 0.31)$^{a}$ | 0.25 (± 0.19)$^{a}$ | 2.1 (± 0.3)$^{b}$ | 0.10 (± 0.09)$^{a}$ | 0.42 (± 0.37)$^{ab}$ | 1.3 (± 0.1)$^{b}$ |
| Pasteurellales | *Actinobacillus* | 10.3 (± 3.7)$^{b}$ | 8.4 (± 2.2)$^{ab}$ | 4.1 (± 1.3)$^{a}$ | 10.2 (± 3.9)$^{b}$ | 2.7 (± 2.8)$^{a}$ | 0.67 (± 0.03)$^{a}$ |
| Selenomonadales | *Acidaminococcus* | 0.41 (± 0.43)$^{a}$ | 1.6 (± 0.44)$^{b}$ | 1.9 (± 0.5)$^{b}$ | 0.12 (± 0.07)$^{a}$ | n.d. | 0.28 (± 0.07)$^{b}$ |
| | *Dialister* | 4.3 (± 1.8)$^{b}$ | 0.21 (± 0.09)$^{a}$ | 1.0 (± 0.3)$^{a}$ | 0.11 (± 0.05)$^{a}$ | 0.03 (± 0.02)$^{a}$ | 2.7 (± 0.9)$^{b}$ |
| | *Megasphaera* | 14.1 (± 11.1) | 13.7 (± 8.6) | 11.8 (± 5.4) | 1.1 (± 0.6)$^{a}$ | 0.24 (± 0.07)$^{a}$ | 11.2 (± 2.8)$^{b}$ |
| | *Mitsuokella* | 7.1 (± 5.6) | 4.9 (± 2.9) | 9.1 (± 6.1) | 0.35 (± 0.29)$^{a}$ | 0.10 (± 0.15)$^{a}$ | 2.3 (± 0.5)$^{b}$ |
| | *Selenomonas* | 2.7 (± 3.4) | 1.0 (± 0.8) | 2.4 (± 0.51) | 0.51 (± 0.10)$^{b}$ | 0.19 (± 0.13)$^{a}$ | 3.5 (± 0.6)$^{b}$ |
| | *Veillonella* | 2.1 (± 1.5) | 2.6 (± 3.9) | 0.39 (± 0.46) | 0.11 (± 0.04) | 0.43 (± 0.33) | 0.61 (± 0.68) |

$^{a,b}$ = significantly different within rows and intestinal segment (Mann-Whitney-U test; P ≤ 0.05)
[1] = single value

**Table 3b:** Core microbiota in the small intestine of 53d old piglets fed diets with different dietary ZnO concentrations [% of total reads].

increased, while *Lactobacillus*, but not *Streptococcus* was decreased in the stomach of the high dietary zinc group. This trend was less clear in the ileum, but high dietary zinc still led to significantly increased *Clostridium*, *Pantoea* and *Selenomonas* ratios as well as to significantly reduced *Lactobacillus*. Furthermore, the numeric increase for *Escherichia/Shigella* in the stomach was significant in the ileum of animals fed the high zinc diet.

On day 53, the stomach core microbiota was still influenced by high dietary zinc, but to a different degree. *Clostridium*, *Acidaminococcus* and *Dialister* were again increased and *Lactobacillus* (but not *Streptococcus*) decreased, but three of four genera of the *Enterobacteriales* (*Citrobacter*, *Enterobacter* and *Escherichia/Shigella*) showed increased ratios. Contrary to the increased *Actinobacillus* ratios on 32d, high dietary zinc led to a decrease for this genus. A similar trend was observed in the ileum, but genera of the *Selenomondales* order (*Megasphaera*, *Mitsuokella* and *Selenomonas*) were also enhanced in the high dietary zinc group.

## Ecological indices

Table 1 shows the effect of dietary zinc oxide on the bacterial diversity as measured by the CHAO1 index for total reads of dominant bacterial order. The total bacterial diversity was significantly increased in the stomach of 32d old animals fed the high zinc diet, possibly due to a significant increase in *Enterobacteriales* as well as a numeric increase in *Clostridiales* diversity. On the other hand, *Lactobacillales* and *Bacteroidales* diversity decreased in the stomach of animals fed the high zinc diet. The same trend was observed in the ileum, as *Lactobacillales* diversity was drastically reduced, but *Clostridiales* and *Enterobacteriales* diversity increased.

On day 53 of life, no significant differences were observed for total bacterial diversity in the stomach or ileum. High dietary zinc still significantly lowered the *Lactobacillales* diversity together with a significantly increased *Clostridiales* diversity in both intestinal segments, while *Enterobacteriales* diversity was only increased in the stomach with no differences in the ileum. *Bacteroidales* diversity was unchanged in both intestinal segments, but *Selenomonadales* diversity was significantly higher in the stomach of animals fed the high zinc diet.

The estimation of the bacterial Evenness is shown in Table 2. Significant effects of high dietary zinc on the distribution of pyrosequencing reads were found on day 32. The total bacterial Evenness in the stomach increased in animals fed the high zinc diet, possibly due to a significantly increased *Lactobacillales* Evenness as well as numerically for *Bacteroidales* and *Clostridiales*. However, the distribution of *Enterobacteriales* reads was significantly reduced for high dietary zinc. In the ileum, a numerically higher Evenness was found for *Lactobacillales*, but contrary to the stomach, Evenness for *Clostridiales* was significantly reduced, while *Selenomonadales* showed an increased Evenness. No significant differences between low and high dietary zinc were found on day 53 of life, except for a significantly increased *Lactobacillales* Evenness and a strong numeric increase for *Enterobacteriales* in the ileum.

## Principal component analysis

Principal component analysis grouping showed that samples from 32 day old animals clustered according to intestinal segment rather than dietary treatment (Figure 3a). However, sequence reads from the high dietary zinc treatment were separate from medium and low dietary zinc treatments for both stomach and ileum samples. A similar, but less clear grouping was noted for samples of 53 days of life (Figure 3b). Again, reads from stomach samples of the high dietary zinc treatment formed a separate cluster from the rest of the samples.

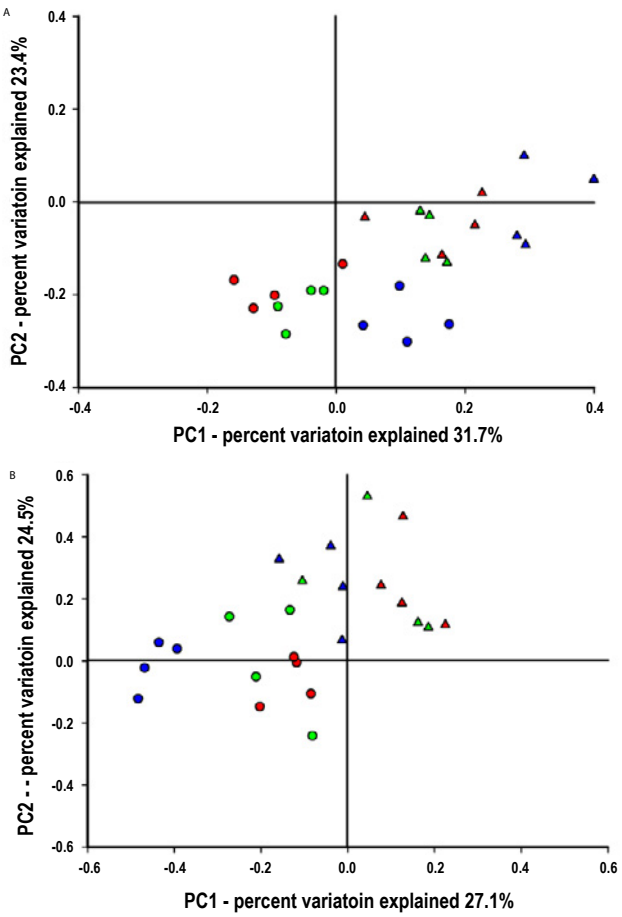


**Figure 3: a)** Principal component analysis of the bacterial composition in the stomach and ileum of 32 day old piglets fed different concentrations of dietary zinc.

**b)** Principal component analysis of the bacterial composition in the stomach and ileum of 53 day old piglets fed different concentrations of dietary zinc.

## Discussion

This study compared the impact of increasing concentrations of dietary zinc oxide on the development of stomach and small intestinal bacterial composition in freshly weaned piglets and piglets four weeks after weaning.

The most pronounced and lasting impact of pharmacological doses of zinc oxide was found for the *Lactobacillales*. More specifically, lactobacilli were affected on day 32 and 53 of life, while other member of the *Lactobacillales,* for instance streptococci increased. The stomach and small intestine of weaned piglets is typically dominated by lactic acid bacteria and lactobacilli are the most abundant genus found in many studies [4,17-19]. However, the proximal intestine of young piglets also harbors strict anaerobic bacteria that are dominant in the hind gut [4,20].

The increase of less dominant bacteria in the absence or reduction of dominant bacteria is a typical mode of action in the development of complex bacterial communities. This model is called "niche concept", although many different definitions of this concept exist [21,22]. It is

conceivable that the reduction of dominant members of a bacterial community will lead to the colonization with other members, i.e. new niches will be filled, if the opportunity arises. This may also be the cause for the drastic increase of the *Clostridiales*, but also for increase of the *Selenomonadales* order in this study. An *in vitro* study on the resistance of a large range of intestinal bacteria against zinc oxide suggests that zinc resistance cannot be assigned to whole bacterial groups, but to individual species [6]. In that *in vitro* study, *Lactobacillus* spp. reference strains showed a rather high resistance compared to other tested species, but interestingly *L. amylovorus*, a species known to dominate the small intestine of piglets, displayed a lower ZnO resistance than other lactic acid bacteria. If the lower zinc resistance of *L. amylovorus* holds true *in vivo*, this would indicate that the reduction of one dominating species could induce a major change in the bacterial composition.

Also, an antagonism between lactobacilli and enterobacteria has been proposed [23,24] and lactobacilli are generally regarded as beneficial for the host [25,26]. Therefore, the observed increase in enterobacterial reads as well as enterobacterial diversity may indicate that the massive reduction of lactobacilli is directly responsible for this effect. These results seemingly contradict the observed reduction of *E. coli* induced diarrhea in post weaning piglets fed pharmacological doses of dietary zinc. The obvious assumption would be that only a reduction of enterobacteria can reduce the amount of pathogenic *E. coli*. However, this study and an earlier study on the ileal bacterial composition with 56d old piglets [27] showed that the diversity of the *Enterobacteriales* increased drastically in animals fed pharmacological doses of zinc oxide. This effect was also in part observed in the analysis of the dominating microbiota. Therefore, another explanation for the reduction of *E. coli* induced diarrhea may be plausible. Applying the niche concept, one can contend that new niches are filled by an increased variety of autochthonous enterobacteria, thereby increasing the competition for pathogenic *E. coli*.

Although the direct beneficial effect of dietary zinc oxide after weaning has been documented and may be claimed to be due to an increase of enterobacteria diversity, detrimental effects of enterobacterial diversity may occur upon prolonged application. Animals from the same study also show an increase of multi-resistant *E. coli* [10]. Additional studies are under way to differentiate the development of several antibiotic resistance genes during that trial. Preliminary results indicate that the abundance of antibiotic resistance genes increases with time (W. Vahjen, unpublished data). Thus, a prolonged application of dietary zinc oxide may increase the probability of antibiotic resistant enterobacteria and must therefore be viewed as a negative outcome.

Other changes in the stomach and small intestine include a drastic increase in *Clostridiales*, specifically the genus *Clostridium*, as well as for the order *Selenomonadales*, which showed five of six species in the core microbiota that were positively influenced by dietary zinc oxide.

Members of the *Clostridiales* and *Selenomonadales* are autochthonous to the intestine of piglets. Applying the niche concept, their increase may also be related to the lactobacilli reduction, similar to the increase in *Enterobacteriales*. Although member of the genus *Clostridium* are normal inhabitants of the porcine intestine, they also include a range of pathogenic species such as *C. difficile* or *C. perfringens*. Nevertheless, regarding the accumulation of pathogenic *Clostridium* species in the small intestine, the same argument as for *Enterobacteriales* can be made, i.e. an increased diversity of physiologically similar bacteria will reduce the probability of overgrowth of one pathogenic species. However, the dominant microbiota of animals fed the high dietary zinc diet also showed an increase in the genus *Actinobacillus* on day 32, but a decrease on day 53 compared to medium or low dietary zinc. Contrary to the genus *Clostridium*, most members of the *Actinobacillus* are regarded as pathogenic for animals [28,29] and an increase of *Actinobacillus*, especially in freshly weaned piglets must be viewed as unfavorable due to the increased probability of dissemination of this pathogenic genus.

Generally, only the high dietary zinc concentration (2425 mg Zn/ kg feed) showed drastic variations of the bacterial composition, while differences between low (57 mg Zn/ kg feed) and medium (164 mg Zn/ kg feed) zinc concentrations were negligible. This would imply that there is a threshold of inhibitory action for zinc oxide. Indeed, in a study on the dose dependent impact of ZnO in piglets, pronounced effects were observed at 1000 mg and 2500 mgZn/ kg feed, respectively [30]. However, marginal zinc supplementation (50 mg/kg feed) also led to shifts in bacterial composition and activity. Thus, there seems to be a threshold of activity for dietary ZnO in piglets.

Regarding the time frame of the antibacterial activity of zinc, it could be shown that diversity and read amount of several bacterial groups were already modified by high dietary zinc one week after weaning. Given the fact that weaning leads to reduced feed intake in piglets and therefore less zinc is taken up by the animal, dietary ZnO seems to impose an immediate and strong stress on intestinal bacteria. Comparison between the core microbiota of 32d and 53d old animals showed that the reduction of lactobacilli as well as the diversity of the *Lactobacillales* was permanent in animals fed high dietary zinc diets. Similarly, *Clostridium* spp. as well as *Escherichia* spp. showed increased sequence reads. However, other members of the core microbiota or the diversity of other bacterial order showed no consistent trends. Applying the niche concept, this indicates that lactobacilli, clostridia and enterobacteria shared a closer level of interaction than other bacterial groups throughout the development of the small intestinal microbiota.

## Conclusions

The most pronounced impact of high dietary zinc was found in 32d old piglets, while changes in older piglets were more moderate. The increased diversity of the *Enterobacteriales* may act beneficial during the first week after weaning to combat *E. coli* induced diarrhea, but a continuous reduction of lactobacilli in the small intestine may lead to unfavorable effects later in life. Due to concerns regarding environmental pollution and possible development of antibiotic resistant enterobacteria, it is proposed that the application of pharmacological doses of zinc oxide should be restricted to the first week after weaning.

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