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Bar-Coded Pyrosequencing of 16S rRNA Gene Amplicons Reveals Changes in Ileal Porcine Bacterial Communities Due to High Dietary Zinc Intake▽†

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Feeding high levels of zinc oxide to piglets significantly increased the relative abundance of ileal *Weissella* spp., *Leuconostoc* spp., and *Streptococcus* spp., reduced the occurrence of *Sarcina* spp. and *Neisseria* spp., and led to numerical increases of all Gram-negative facultative anaerobic genera. High dietary zinc oxide intake has a major impact on the porcine ileal bacterial composition.

Zinc oxide (ZnO) is used as a feed additive for diarrhea prophylaxis in piglets (23). However, the mode of action of ZnO is not fully understood. Besides its effects on the host (10, 30, 31), high dietary zinc levels may affect the diversity of intestinal microbial communities (2, 11, 20). The prevention of postweaning diarrhea in piglets due to high dietary ZnO intake may not be directly related to a reduction of pathogenic *E. coli* (8) but, rather, to the diversity of the coliform community (15). Studies on the impact of high ZnO levels on the porcine ileal bacterial community are scarce but nevertheless important, as bacterial diarrhea is initiated in the small intestine (9, 17). The small intestine is a very complex habitat with many different factors shaping the bacterial community. Studies on the ecophysiology (22) and maturation of the porcine ileal microbiota (13, 27) indicate a drastic impact directly after weaning and a gradual decline of modifications during the following 2 weeks. Thus, the time point for analysis chosen in this study (14 days postweaning) does reflect a more stable period of the ileal porcine microbiota. In this study, we used bar-coded pyrosequencing of 16S rRNA genes to gain further insight into the mode of action of pharmacological levels of ZnO in the gastrointestinal tract of young pigs.

Total DNA was extracted from the ileal digesta of 40- to 42-day-old piglets using a commercial kit (Qiagen stool kit; Qiagen, Hilden, Germany) and PCR amplified with unique bar-coded primer sets targeting the V1-to-V3 and the V6-to-V8 hypervariable regions (see the supplemental material for detailed methods). The rationale behind this approach was derived from the fact that no single "universal" primer pair can completely cover a complex bacterial habitat (4, 24, 32, 33). Furthermore, these studies also show that *in silico* information on the coverage of selected primer sets diverges from empirical results, and hence, two hypervariable regions were chosen in this study to maximize the detection of phylogenetically diverse bacterial groups.

Equimolar dilutions of all samples were combined into one master sample. Pyrosequencing was performed by Agowa (Berlin, Germany) on a Roche genome sequencer FLX system using a Titanium series PicoTiterPlate. The resulting data files were uploaded to the MG-RAST server (http://metagenomics.nmpdr.org/) (19) and processed with its SEED software tool using the RDP database (5) as the reference database. After automated sequence analysis, all sequences with less than five identical reads per sample were deleted in order to increase the confidence of sequence reads and reduce bias from possible sequencing errors (12, 16). Thus, 0.43% of all sequences were not considered (1,882 of 433,302 sequences). These sequences were assigned to a total of 238 genera, of which most only occurred in a few samples (see the supplemental material). Furthermore, all unclassified sequences were removed (8.7%; 41,467 of 474,769 sequences). Due to the use of the RDP reference database, the SEED software incorrectly assigned the majority of unclassified sequences as unclassified *Deferribacterales* (83%; 34,393 sequences), which were actually identified as 16S soybean or wheat chloroplasts by BLAST or as cyanobacterial chloroplasts by the RDP II seqmatch tool.

The pyrosequencing results for the two primer combinations were merged by taking only sequences from the primer combination that yielded the higher number of reads for a specific sequence assignment in a sample. The remaining reads were used to calculate the relative contribution of assigned sequences to total sequence reads in a sample.

The *Firmicutes* phylum dominated the small intestinal bacterial communities in both the control group and the group with high dietary ZnO intake, with 98.3% and 97.0% of total sequence reads, respectively. No significant influence of high dietary ZnO intake was found for the main phyla *Proteobacteria* (0.92% versus 1.84%), *Actinobacteria* (0.61% versus 0.75%), *Bacteroidetes* (0.15% versus 0.17%), and *Fusobacteria* (0.09% versus 0.12%).

On the order level, a total of 20 bacterial orders were detected (data not shown). *Lactobacillales* dominated bacterial communities in the control and high-dietary-ZnO-intake groups, with 83.37% and 93.24% of total reads. Lactic acid

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TABLE 1. Bacterial genera in the ileum of piglets fed diets supplemented with 200 or 3,000 ppm ZnO

Genus	Proportion (% ± SD) of ileal microbiota in group[a] receiving:	
	200 ppm ZnO	3,000 ppm ZnO
Lactobacillus	59.3 ± 30.6	40.7 ± 19.1
Weissella	11.6 ± 7.8 A	24.1 ± 8.3 B
Sarcina	11.4 ± 20.5 A	0.84 ± 1.2 B
Leuconostoc	4.7 ± 3.2 A	9.4 ± 3.1 B
Streptococcus	1.8 ± 1.6 A	5.7 ± 5.1 B
Lactococcus	1.6 ± 1.5	2.6 ± 3.1
Veillonella	0.57 ± 0.63	0.34 ± 0.30
Gemella	0.34 ± 0.67 A	0.45 ± 0.25 B
Acinetobacter	0.25 ± 0.21	0.44 ± 0.50
Clostridium	0.25 ± 0.40	0.22 ± 0.21
Enterococcus	0.19 ± 0.15	0.26 ± 0.24
Acidovorax	0.14 ± 0.04	0.16 ± 0.19
Arcobacter	0.14 ± 0.15	0.16 ± 0.17
Neisseria	0.14[b]	0.03 ± 0.01
Enterobacter	0.13 ± 0.09	0.29 ± 0.34
Lachnospira	0.12 ± 0.13	0.13 ± 0.03
Peptostreptococcus	0.11 ± 0.10	0.07 ± 0.09
Chryseobacterium	0.10 ± 0.07	0.15 ± 0.16
Actinomyces	0.09 ± 0.04	0.15 ± 0.16
Anaerobacter	0.07 ± 0.08	0.02 ± 0.01
Aerococcus	0.07 ± 0.04	0.07 ± 0.04
Dorea	0.07[b]	0.05 ± 0.05
Fusobacterium	0.06 ± 0.09	0.08 ± 0.11
Microbacterium	0.06 ± 0.01	0.07 ± 0.04
Carnobacterium	0.06 ± 0.02	0.08 ± 0.13
Granulicatella	0.06 ± 0.02	0.09 ± 0.10
Staphylococcus	0.06 ± 0.04	0.05 ± 0.02
Facklamia	0.05 ± 0.06	0.03 ± 0.01
Comamonas	0.05 ± 0.03	0.04 ± 0.02
Citrobacter	0.05 ± 0.02	0.07 ± 0.08
Erysipelothrix	0.05 ± 0.01	0.22 ± 0.40

[a] $n = 6$ piglets per trial group. A, B, results are significantly different by Kruskal-Wallis test.
[b] Single sample.

bacteria are well known to dominate the bacterial community in the ileum of piglets (11, 22). No significant difference between the control group and the group with high dietary ZnO intake was observed on the order level, although high dietary ZnO intake led to a strong numerical decrease for *Clostridiales* (14.4 ± 24.0% [mean ± standard deviation] versus 2.8 ± 1.7%), as well as to numerical increases for *Pseudomonadales* (0.3 ± 0.3% versus 0.6 ± 0.6%) and *Enterobacteriales* (0.2 ± 0.2% versus 0.5 ± 0.6%).

On the genus level, a total of 103 genera were detected. Table 1 summarizes the main 31 genera which exceeded 0.05% of total reads (see the supplemental material for a complete list). Lactobacilli clearly dominated the bacterial communities in both trial groups, but they also were numerically lower due to high dietary ZnO intake.

Significant changes due to high dietary ZnO intake were observed for other lactic acid bacteria, including *Weissella* spp., *Leuconostoc* spp., and *Streptococcus* spp. A significant and strong decrease was observed for *Sarcina* spp., which is a genus of acid-tolerant strictly anaerobic species found in the intestinal tract of piglets and other mammals (6, 28, 29). This genus thus appeared to be very sensitive to modifications induced by high dietary ZnO intake.

An interesting result was observed for Gram-negative *Proteobacteria*, (i.e., enterobacteria and relatives). Although not statistically significant, virtually all detected proteobacteria increased numerically due to high dietary ZnO intake (*Enterobacter* spp., *Microbacterium* spp., *Citrobacter* spp., *Neisseria* spp., and *Acinetobacter* spp.). Apparently, enterobacteria gained colonization potential by high dietary ZnO intake. This is in good agreement with the results of studies by Hojberg et al. (11), Amezcua et al. (1), and Castillo et al. (3). Therefore, the frequently observed diarrhea-reducing effect of zinc oxide may not be directly related to a reduction of pathogenic *E. coli* strains. Considering a possible antagonistic activity of lactobacilli against enterobacteria (25), it can be speculated that a numerical decrease of dominant lactobacilli may lead to increased colonization with Gram-negative enterobacteria. On the other hand, specific plasmid-borne genes for resistance against heavy metals have been reported for both Gram-positive and Gram-negative bacteria present in the intestine (21, 26), and an increased resistance against Zn ions may exist for Gram-negative enterobacteria. Zinc oxide is an amphoteric molecule and shows a high solubility at acid pH. The low pH in the stomach of piglets (pH 3.5 to 4.5) transforms a considerable amount of insoluble ZnO into zinc ions (54 to 84% free Zn^{2+} at 150 ppm and 24 ppm ZnO, respectively) (7), and thus, high concentrations of toxic zinc ions exist in the stomach. The stomach of piglets harbors large numbers of lactic acid bacteria, especially lactobacilli. Zn ions may thus lead to a modification of the lactic acid bacterial community in the stomach, and the changes observed in the ileum could have been created in the stomach. A reduction of dominant lactobacilli may thus point to an increased adaptation potential of Gram-negative facultative anaerobes and a generally increased bacterial diversity.

Additionally, the direct effects of dietary ZnO on intestinal tissues include altered expression of genes responsible for glutathione metabolism and apoptosis (30), enhanced gastric ghrelin secretion, which increases feed intake (31), and increased production of digestive enzymes (10). An analysis of the intestinal morphology was beyond the scope of this study, but although ZnO concentrations are markedly increased in intestinal tissue, the influence of ZnO on morphology is apparently not always observed (10, 14, 18). Consequently, any changes in epithelial cell turnover, feed intake, or digestive capacity may influence the composition of bacterial communities in the small intestine.

In conclusion, this study has shown that high dietary zinc oxide has a major impact on ileal bacterial communities in piglets. Future studies on the impact of zinc oxide in pigs should include a detailed analysis of host responses in order to identify the cause for the observed modifications of intestinal bacterial communities.

Metagenome sequence accession numbers. The sequence data are available under "Public Metagenomes" at http://metagenomics.nmpdr.org/. The individual files are named 2-8f-1 to 2-8f-12 and 2-968f-1 to 2-968f-12, respectively.

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