Increased dietary zinc oxide changes the bacterial core and enterobacterial composition in the ileum of piglets

W. Vahjen, R. Pieper and J. Zentek

J ANIM SCI 2011, 89:2430-2439.
doi: 10.2527/jas.2010-3270 originally published online March 7, 2011

Increased dietary zinc oxide changes the bacterial core and enterobacterial composition in the ileum of piglets[1]

W. Vahjen,[2] R. Pieper, and J. Zentek

Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin, 14195 Berlin, Germany

ABSTRACT: This study was conducted to investigate the effects of increased dietary ZnO on the bacterial core and enterobacterial composition in the small intestine of piglets that were fed diets containing a total of 124 or 3,042 mg of Zn per kilogram of diet, respectively. Zinc was supplemented to the basal diet as ZnO. Bacterial 16S rRNA genes of ileal DNA extracts were PCR-amplified with 2 bar-coded primer sets and sequenced by 454 pyrosequencing. The bacterial core species were calculated from the relative abundances of reads present in 5 of 6 samples per group and at a minimum of 5 sequences per sample. The reference database SILVA was used to assign sequence reads at an alignment minimum of 200 bases and 100% identity. Lactic acid bacteria dominated the bacterial core, but showed diverse responses to dietary ZnO. Of the dominant *Lactobacillus* spp., *Lactobacillus reuteri* was reduced due to increased dietary ZnO (44.7 vs. 17.9%; $P = 0.042$), but *L. amylovorus* was not influenced. However, the changes of relative abundances of other lactic acid bacteria were more noteworthy; *Weissella cibaria* (10.7 vs. 23.0%; $P = 0.006$), *W. confusa* (10.0 vs. 22.4%; $P = 0.037$), *Leuconostoc citreum* (6.5 vs. 14.8%; $P = 0.009$), *Streptococcus equinus* (0.14 vs. 1.0%; $P = 0.044$), and *S. lutetiensis* (0.01 vs. 0.11%; $P = 0.016$) increased in relative abundance. Nonlactic acid bacteria that were influenced by increased dietary ZnO included the strict anaerobic species, *Sarcina ventriculi*, which showed a strong numerical decrease in relative abundance (14.6 vs. 5.1%). Species of the *Enterobacteriaceae* increased their relative abundance, as well as species diversity, in the high dietary ZnO experimental group. Bacterial diversity indices were increased due to increased dietary ZnO ($P < 0.05$), which was traced back to the increase of sequences from subdominant species. Increased dietary ZnO led to an increase of less prominent species and, thus, had a major impact on the bacterial composition and diversity in piglets. This effect may help to stabilize the intestinal microbiota in the sensitive postweaning period.

Key words: bacterial core, microbiota, piglet, pyrosequencing, zinc oxide

J. Anim. Sci. 2011. 89:2430–2439
doi:10.2527/jas.2010-3270

INTRODUCTION

In the last decades, many research efforts focused on ZnO for diarrhea prophylaxis in piglets (Holm, 1988; Holmgren, 1994; Poulsen, 1995; Carlson et al., 2008), and increased doses of ZnO have been suggested as a feed additive for weaning piglets (Hollis et al., 2005). However, the mode of action of ZnO is yet not fully understood. In addition to its effects on the host, increased dietary Zn affects the diversity of the intestinal microbial community. For instance, lactobacilli colony counts were reduced, but coliform colony counts increased using 2,500 mg·kg^{-1} of dietary Zn from ZnO in a study with piglets (Højberg et al., 2005). This is in agreement with a challenge study with postweaning piglets, in which an increased shedding of the inoculated pathogenic *Escherichia coli* strain was reported (Mores et al., 1998). Reduced anaerobic and lactic acid bacteria colony counts, but no effect on *E. coli*, were also described in piglets (Broom et al., 2006). Thus, the frequently observed reduction of postweaning diarrhea in piglets fed Zn-supplemented diets may not be related to a general reduction of *E. coli* (Fairbrother et al., 2005) and the impact of Zn on the diversity of the coliform community could be more important (Katouli et al., 1999). Because bacterial communities are in intimate contact with host tissues and induce different host reactions, knowledge of modulatory effects of ZnO on intestinal bacteria is a critical step to gain further insight in the mode of action of ZnO on pig health during the critical weaning period.

[1]This study was funded by the Collaborative Research Centre (SFB852) of the German Research Foundation (Bonn).
[2]Corresponding author: vahjen@zedat.fu-berlin.de
Received June 25, 2010.
Accepted February 28, 2011.

Recently, deep sequencing has changed the prospect of studying bacterial communities by parallel sequencing of relatively long bacterial nucleic acid sequences (Gill et al., 2006; Snyder et al., 2009). Combined with the technique of introducing nucleotide bar codes during PCR amplification, many samples can be processed at a relatively low cost per sequence (Armougom and Raoult, 2008; Hamady et al., 2008). Deep sequencing is therefore a promising tool to study the influence of nutritional factors on intestinal microbial communities and functionalities. The current study was conducted to evaluate the impact of ZnO on the small intestinal bacterial core composition in weaned piglets using deep sequencing technology.

MATERIALS AND METHODS

The study was approved by the German Tierschutzgesetz and the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, Berlin; LaGeSo Reg. Nr. 0096/2006).

Animals and Housing

Piglets were housed in groups of 2 in flat-deck cages, which were constructed of stainless steel to prevent uncontrolled Zn intake. The whole trial was conducted in 3 separate successive runs and 1 animal per pen was used in each run to determine the bacterial core composition in the ileum. A total of 12 piglets (German Landrace × Piétrain) weaned at 28 d with an average BW of 7.0 ± 0.7, 4.6 ± 0.5, and 6.8 ± 0.8 kg for trial runs 1 to 3, respectively, were used for this study. Feed and water were offered ad libitum. The light (15 h light/9 h darkness) and temperature (25°C) were kept constant during the experimental periods. Piglets were on their respective diets for 12 to 14 d before sampling. Body weight of the piglets at euthanasia was 10 to 12 kg.

Diets

Diets were based on a standard starter feed mixture. The composition of the basal diet is shown in Table 1. Analytical grade ZnO (Sigma Aldrich, Deisenhofen, Germany) was added to the diets. The analyzed dietary Zn concentration in the control group (low dietary Zn) was 124 mg/kg feed, whereas the treatment group (high dietary Zn) contained 3,042 mg/kg feed.

Sampling

The piglets were euthanized 3 h after the last meal. Piglets were anesthetized (Xylazin and Ketamine hydrochloride) followed by intracardial injection of embutramide. The gastrointestinal tracts were opened immediately, and the content of the ileum was collected, homogenized, deep frozen in liquid nitrogen, and stored at −80°C. After thawing, 1-g samples were taken for further analysis.

DNA Extraction

Two extraction procedures were used to maximize cell lysis of the diverse bacterial cells present in the ileum of piglets. First, total nucleic acids of the ileum (1 g) were extracted by using a guanidinisothiosulfate (4 *M*) containing lysis buffer at 90°C for 2 × 5 min., 2 × 1 min bead beating with acid-washed glass beads (diameter, 0.3 to 0.5 mm) and subsequent phenol/chloroform extraction and isopropanol precipitation. Crude extracts were purified to PCR-grade DNA with commercial silica gel spin columns (NucleoSpinKit Tissue, Machery-Nagel, Dueren, Germany). The amount of DNA was measured by fluorescence using SYBR Green I and calf thymus DNA as reference DNA.

Second, total nucleic acids were extracted using a commercial kit (Qiagen Stool kit, Qiagen, Hilden, Germany) from 200 mg of ileal chyme in triplicate according to the instructions of the manufacturer, except an increase in temperature during the lysis step to 90°C. Purified DNA was then pooled per sample, and the amount of DNA was measured as described before.

Table 1. Composition (as-fed basis) and results of proximate chemical analysis of the basal diet

Item	Diet
Ingredient, g/kg	
Thermally treated cereals[1]	250.0
Wheat	280.0
Soybean meal	220.0
Triticale	100.0
Whey powder	50.0
Soybean oil	38.0
Limestone	17.4
Monocalcium phosphate	17.4
Wheat bran	14.0
Trace mineral-vitamin premix[2]	12.0
L-Lys·HCl	1.1
Trp	0.1
Chemical analysis, g/kg	
DM	913
CP	200
Crude ash	68.4
Crude fat	58.9
Ca	12.4
Total P	8.8
Na	1.8

[1]Cereals: 50% wheat, 25% barley, and 25% maize (Optigrain; Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany).

[2]Provided per kilogram of diet: 7,200 IU of vitamin A; 1,440 IU of vitamin D_3; 96 mg of vitamin E; 1.2 mg of vitamin K_3; 3.0 mg of vitamin B_1; 3.0 mg of vitamin B_2; 4.8 mg of vitamin B_6; 24 mg of vitamin B_{12}; 30 mg of nicotinic acid; 0.30 mg of biotin; 66 mg of Ca pantothenate; 1.2 mg of folic acid; 960 mg of choline chloride; 60 mg of Fe as $FeCO_3$; 72 mg of Mn as MnO; 12 mg of Cu as $CuSO_4 \cdot 5\ H_2O$; 0.54 mg of I as $Ca(IO_3)_2$; 0.36 mg of Co as $CoSO_4 \cdot 7\ H_2O$; 0.42 mg of Se as $Na_2SeO_3 \cdot 5\ H_2O$; 1.56 g of Na as NaCl; 0.66 g of Mg as MgO.

Preparation of Sequencing PCR Amplicons

The DNA samples were diluted to 100 ng/μL, and 1 μL was used for 25-μL PCR reaction mix. Two primer sets at 0.3 μ*M* were used to amplify 2 regions of bacterial 16S rRNA genes. Primers were tagged with unique hexamer nucleotides to sort PCR products after sequencing (Table 2). A commercial master mix kit (HotStarTaq Plus Master Mix, Qiagen) with added SYBR green during cycle number optimization was used for PCR amplification under the following cycling conditions: 1 × 15 min at 95°C, 32 × (for 8f-534r) or 35 × (for 968f-1401r) 15 s at 95°C, 30 s at 55°C, 30 s at 72°C, and 1 × 1 min at 20°C. Optimal amplification conditions were defined for each primer combination by the cycle number, at which the real-time PCR amplification curve entered a plateau with no further increase of total fluorescence. Cycling was performed on a thermocycler (Mx3000P; Stratagene, Amsterdam, the Netherlands). The PCR products were removed immediately after the last cycle and stored at −20°C until further analysis.

Pyrosequencing Procedures

The PCR products were purified to remove unbound primer and salt using a commercial kit (Qiaquick nucleotide removal kit, Qiagen), and the amount of DNA was determined and equimolar dilutions of all samples were combined into 1 master sample per trial group. Pyrosequencing was performed by AGOWA (Berlin, Germany) on a genome sequencer (Roche Genome Sequencer FLX system using a Titanium series PicoTiterPlate, Roche Diagnostics GmbH, Mannheim, Germany).

Processing and Phylogenetic Assignment of Sequence Reads

Sequence reads were sorted according to sample tags and primer combination, resulting in 24 single data files. After removal of sample tags and primer sequences, data files were uploaded to a server (MG-RAST; Meyer et al., 2008) and processed by its SEED software tool (Overbeek et al., 2005).

The phylogenetic profile of each sample was computed with the following variables: maximum value of 1×10^{-5}, minimum percentage identity of 100%, and minimum alignment length of 200 bases. The SILVA reference database (Pruesse et al., 2007) was used for species identification. Sequence reads that were aligned as environmental isolates by the SILVA reference databases were checked again using the BLAST algorithm for their presence in known bacterial species and assigned to the respective species when the sequences could be aligned with a 100% similarity at a minimum of 200 bases (*Lactobacillus crispatus*, *L. vaginalis*, and *Arcobacter butzleri* in Table 3, and *Morganella morganii* and *Citrobacter murliniae* in Table 4).

Table 2. Sample tag PCR primer sequences

Item	Sequence (5′ to 3′)
F-968–1	ACACACGAACGCGAAGAACCTTAC
F-968–2	ACATGCGAACGCGAAGAACCTTAC
F-968–3	ACGTACGAACGCGAAGAACCTTAC
F-968–4	AGCAGCGAACGCGAAGAACCTTAC
F-968–5	TGCGCGGAACGCGAAGAACCTTAC
F-968–6	TGTGTGGAACGCGAAGAACCTTAC
F-968–7	GCGTCTGAACGCGAAGAACCTTAC
F-968–8	GTCTCTGAACGCGAAGAACCTTAC
F-968–9	GCTGCTGAACGCGAAGAACCTTAC
F-968–10	GACGCTGAACGCGAAGAACCTTAC
F-968–11	GTGACTGAACGCGAAGAACCTTAC
F-968–12	GATGATGAACGCGAAGAACCTTAC
R-1401–1	ACACACGCGTGTGTACAAGACCC
R-1401–2	ACATGCGCGTGTGTACAAGACCC
R-1401–3	ACGTACGCGTGTGTACAAGACCC
R-1401–4	AGCAGCGCGTGTGTACAAGACCC
R-1401–5	TGCGCGGCGTGTGTACAAGACCC
R-1401–6	TGTGTGGCGTGTGTACAAGACCC
R-1401–7	GCGTCTGCGTGTGTACAAGACCC
R-1401–8	GTCTCTGCGTGTGTACAAGACCC
R-1401–9	GCTGCTGCGTGTGTACAAGACCC
R-1401–10	GACGCTGCGTGTGTACAAGACCC
R-1401–11	GTGACTGCGTGTGTACAAGACCC
R-1401–12	GATGATGCGTGTGTACAAGACCC
8 F-1	TGACACAGAGTTTGATCCTGGCTCAG
8 F-2	TGATGCAGAGTTTGATCCTGGCTCAG
8 F-3	TGCATCAGAGTTTGATCCTGGCTCAG
8 F-4	CTCTGTAGAGTTTGATCCTGGCTCAG
8 F-5	CGATGTAGAGTTTGATCCTGGCTCAG
8 F-6	GTAGCTAGAGTTTGATCCTGGCTCAG
8 F-7	GAGTATAGAGTTTGATCCTGGCTCAG
8 F-8	GTATATAGAGTTTGATCCTGGCTCAG
8 F-9	GTCGATAGAGTTTGATCCTGGCTCAG
8 F-10	GCAGATAGAGTTTGATCCTGGCTCAG
8 F-11	GTGCATAGAGTTTGATCCTGGCTCAG
8 F-12	CGTCGTAGAGTTTGATCCTGGCTCAG
534r-1	TGACACATTACCGCGGCTGCTGG
534r-2	TGATGCATTACCGCGGCTGCTGG
534r-3	TGCATCATTACCGCGGCTGCTGG
534r-4	CTCTGTATTACCGCGGCTGCTGG
534r-5	CGATGTATTACCGCGGCTGCTGG
534r-6	GTAGCTATTACCGCGGCTGCTGG
534r-7	GAGTATATTACCGCGGCTGCTGG
534r-8	GTATATATTACCGCGGCTGCTGG
534r-9	GTCGATATTACCGCGGCTGCTGG
534r-10	GCAGATATTACCGCGGCTGCTGG
534r-11	GTGCATATTACCGCGGCTGCTGG
534r-12	CGTCGTATTACCGCGGCTGCTGG

For statistical interpretation, the next step in the analysis was the deletion of all data with less than 5 identical sequence reads per sample to increase confidence of sequence reads and reduce bias by possible sequencing errors (Huse et al., 2007; Kunin et al., 2010). Also, sequence reads that only occurred in 1 sample were deleted to focus on the common bacterial species.

As the pyrosequencing results arose from 4 independent PCR amplifications for each sample (2 primer combinations with 2 extraction procedures), 4 sets of sequences were present. These data sets were merged by taking into account only relative abundances from the primer combination or extraction procedure that

Table 3. Influence of increased dietary zinc oxide on the bacterial core species (%) in the ileum of piglets (n = 6 per experimental group)

	Treatment[1]	
Item	Control	High ZnO
Lactobacillus reuteri	44.7 ± 26.9[x]	17.9 ± 13.2[y]
Weissella cibaria	10.7 ± 7.6[x]	23.0 ± 3.2[y]
Weissella confusa	10.0 ± 9.0[a]	22.4 ± 12.2[b]
Leuconostoc citreum	6.5 ± 5.1[a]	14.8 ± 3.8[b]
Sarcina ventriculi	14.6 ± 24.6	5.1 ± 9.0
Lactobacillus amylovorus	9.6 ± 9.1	11.6 ± 11.2
Microbacterium sp. SKJH23	1.8 ± 3.1	1.6 ± 1.3
Streptococcus equinus	0.14 ± 0.16[x]	1.0 ± 0.87[y]
Lactococcus lactis	0.24 ± 0.32	0.49 ± 0.60
Streptococcus gallolyticus	0.43 ± 0.72	0.21 ± 0.14
Lactobacillus salivarius	0.09 ± 0.11	0.37 ± 0.63
Uncultured *Microbacterium* sp.	0.23 ± 0.15	0.33 ± 0.26
Uncultured *Lactobacillus* sp.	0.22 ± 0.29	0.15 ± 0.04
Lactobacillus johnsonii	0.13 ± 0.13	0.04 ± 0.05
Streptococcus suis	0.05 ± 0.09	0.15 ± 0.12
Lactobacillus helveticus	0.12 ± 0.11[y]	0.02 ± 0.05[x]
Aerococcus sp. zf93I	0.03 ± 0.05[a]	0.12 ± 0.07[b]
Rhodococcus sp. djl62	0.01 ± 0.02[x]	0.12 ± 0.16[y]
Streptococcus lutetiensis	0.01 ± 0.03[x]	0.11 ± 0.07[y]
Neisseria canis	n.d.[2]	0.11 ± 0.15
Lactococcus plantarum	0.04 ± 0.07	0.11 ± 0.14
Lactobacillus crispatus	0.07 ± 0.09	0.04 ± 0.05
Lactobacillus vaginalis	0.04 ± 0.07	0.09 ± 0.08
Arcobacter butzleri	0.02 ± 0.06	0.08 ± 0.10
Actinomyces denticolens	0.06 ± 0.06	0.07 ± 0.06
Staphylococcus haemolyticus	n.d.	0.07 ± 0.06
Acidovorax sp. OS6	n.d.	0.05 ± 0.06
Bacillus cereus	n.d.	0.04 ± 0.05

[a,b]Within a row, means without a common superscript differ (ANOVA, $P < 0.05$).

[x,y]Within a row, means without a common superscript differ (Kruskal-Wallis test, $P < 0.05$).

[1]Control treatment with 124 mg·kg^{-1} of zinc from ZnO; high ZnO treatment with 3,042 mg·kg^{-1} of zinc from ZnO.

[2]n.d. = not detected.

yielded the greater relative abundance for a specific sequence assignment in a sample. 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. The bacterial core was constructed of species that were present in at least 5 of 6 samples in each trial group.

Phylogenetic Tree of the Bacterial Core and Ecological Indices

Full-length sequences of the members of the bacterial core were used to construct a phylogenetic tree. The tree was calculated using the ARB software (Ludwig et al., 2004) based on 1,000 bootstrap trees. The sum of sequence reads for each species in both experimental groups was then used to calculate the relative abundance of the species and shown as pie charts under the respective species name.

Several bacterial diversity indices were calculated (Kwak and Peterson, 2007). Richness is defined as the number of species in a sample (in this instance, species of the bacterial core), and the greater the richness value, the more species are present. The Shannon index was used to calculate the qualitative diversity of the bacterial core in a sample using the equation $-\Sigma[P_i \ln (P_i)]$, where P_i is the abundance of a species i relative to the total number of species and ln is the natural logarithm; the greater the Shannon index, the greater the diversity in the sample. Evenness estimates the similarity of species abundance, which was used to estimate evenness of the bacterial core species by the equation: H/ln(S), where H is Shannon index and S is richness. An evenness of 1 indicates identical proportions of each species in a sample. Finally, the Simpson index of diversity defines the probability that 2 randomly picked species in a habitat belong to different species $[(1 - \Sigma(P_i^2)]$ and is therefore a quantitative indicator for species diversity in a sample. A diversity of 1 would indicate infinite diversity.

Deposition of Nucleic Acid Sequences

The metagenomic data are available under public metagenomes (Meyer et al., 2008). The individual files

Table 4. Influence of increased dietary zinc oxide on the relative abundance (%)[1] of *Enterobacteriaceae* in the ileum of piglets (n = 6 per experimental group)

	Treatment[2]			
Item	Control	n[3]	High ZnO	n
Citrobacter freundii	0.091 ± 0.051	4	0.063 ± 0.076	6
Citrobacter gillenii	n.d.[4]	0	0.043 ± 0.017	3
Citrobacter murliniae	0.034 ± 0.007	3	0.094 ± 0.081	3
Citrobacter sp. F1–4	n.d.	0	0.033 ± 0.003	3
Citrobacter sp. F3–1	n.d.	0	0.031	1
Citrobacter werkmanii	n.d.	0	0.028	1
Enterobacter cloacae	0.026	1	n.d.	0
Enterobacter hormaechei	0.053	1	0.031	1
Enterobacter sp. 638	0.035	1	0.059 ± 0.004	3
Enterobacter sp. IMD1260	0.134	1	0.031	1
Enterobacter sp. pptphilum	0.026	1	0.031	1
Enterobacter sp. TUT1390	0.026	1	n.d.	0
Erwinia chrysanthemi	n.d.	0	0.033 ± 0.003	3
Erwinia rhapontici	0.024	1	n.d.	0
Escherichia coli	0.126 ± 0.094	2	0.676 ± 0.727	4
Klebsiella granulomatis	n.d.	0	2.893	1
Klebsiella oxytoca	0.111 ± 0.110	3	0.265 ± 0.209	3
Klebsiella pneumoniae	0.294 ± 0.304	6	0.916 ± 2.296	6
Klebsiella sp. HF13	n.d.	0	0.826	1
Klebsiella sp. LB-2	n.d.	0	0.059	1
Morganella morganii	0.053	1	0.031	1
Pantoea agglomerans	n.d.	0	0.100 ± 0.096	3
Pantoea sp. Co9941	n.d.	0	0.033 ± 0.003	2
Pantoea sp. TNT6	n.d.	0	0.029 ± 0.002	2
Proteus mirabilis	0.134	1	n.d.	0
Salmonella enterica	n.d.	0	0.031	1
Serratia grimesii	n.d.	0	0.571	1
Shigella flexneri	0.134	1	0.258 ± 0.220	2
Uncultured *Citrobacter* sp.	n.d.	0	0.031	1

[1]× 10^{-3}.

[2]Control treatment with 124 mg·kg^{-1} of Zn from ZnO; high ZnO treatment with 3,042 mg·kg^{-1} of Zn from ZnO.

[3]Number of animals in which the species was detected.

[4]n.d. = not detected.

are named 1–8f-1 to 1–8f-12, 1–968f-1 to 1–968f-12, and 2–8f-1 to 2–8f-12, and 2–968f-1 to 2–968f-12, respectively. In the file names, "1-" denotes sequences generated by DNA extraction procedure I, "2-" denotes sequences generated by DNA extraction procedure II, 8f and 968f are the respective upstream primer used in the PCR, and "-1" to "-12" stand for individual samples (1 to 6 = low dietary Zn, and 7 to 12 = high dietary Zn).

Statistical Analysis

Arithmetic means and SD were calculated for all. The piglet was used the experimental unit; piglets were selected from different pens and 3 different successive trial periods. A *t*-test was used with the SPSS software (15.0, IBM, Somers, NY) to determine statistically significant differences at the $P \leq 0.05$ level after testing for normality by the Shapiro-Wilk test and homogeneity of variances. Data that failed homogeneity tests were compared with the nonparametric Kruskal-Wallis test to determine their asymptotic significance at the $P \leq 0.05$ level. Differences in the total number of enterobacterial species detected in the 2 experimental groups were tested with the Pearson's χ^2 test.

RESULTS

The SILVA reference database assigned a total of 127 bacterial species with 5 or more sequence reads and in more than 1 sample from a total of 50,715 sequences (4,226 ± 1,367 sequences per sample). Table 3 shows the distribution of bacterial core species in the ileum of piglets fed 2 different amounts of ZnO, and Figure 1 shows a graphical display of the phylogenetic diversity of the bacterial core, as well as the relative abundance of each species in the experimental groups. The bacterial core represented species detected in 5 of 6 samples per experimental group and included a total of 28 species. The low dietary ZnO experimental group showed 24 of 28 species, compared with 28 species present in the high dietary ZnO experimental group.

Lactobacillus reuteri was the dominating species in both experimental groups. The strongest numerical impact of high dietary ZnO supplementation was ob-

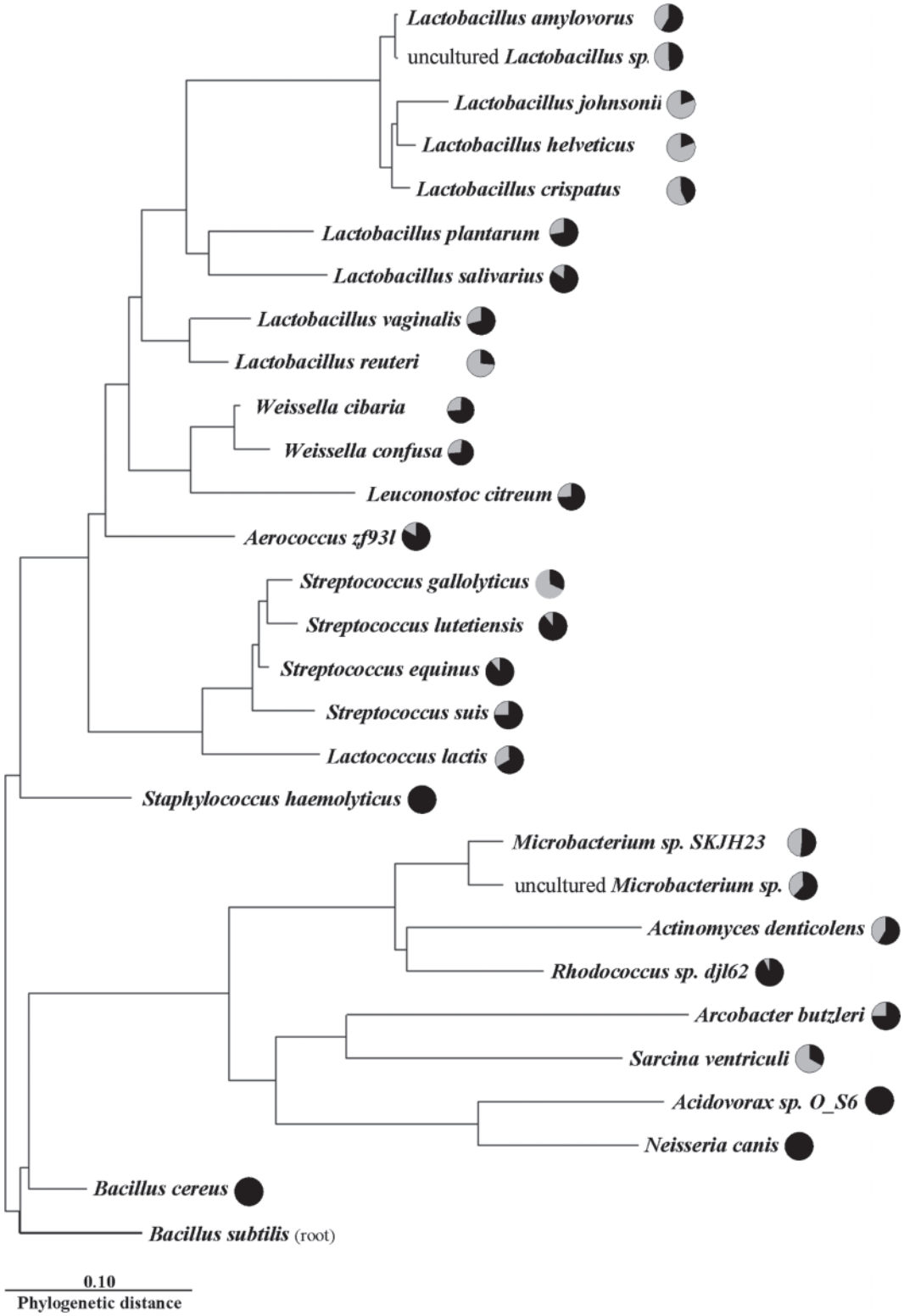

Figure 1. Phylogenetic tree of the bacterial core species in the ileum of piglets fed diets as influenced by increased dietary zinc oxide (gray = control, and black = high dietary ZnO experimental group). The tree was calculated based on 1,000 bootstrap trees using ARB software (Ludwig et al., 2004). *Bacillus subtilis* was used to root the tree. The scale bar represents the evolutionary distance.

served in a decrease of *Sarcina ventriculi,* as well as in increases of *Weissella cibaria* ($P = 0.006$) and *W. confusa* ($P = 0.037$). Other increases because of greater ZnO supplementation occurred for lactic acid bacteria such as *Leuconostoc citreum* ($P = 0.009$), *Streptococcus equinus* ($P = 0.044$), *S. lutetiensis* ($P = 0.016$), *Aerococcus* sp. zf93I ($P = 0.046$), and *L. helveticus* ($P = 0.049$). Species of the actinobacteria group also displayed increases (*Rhodococcus* sp. djl62, $P = 0.049$). Four species were only detected in the high dietary ZnO experimental group (*N. canis*, *Staphylococcus haemolyticus*, *Acidovorax* sp. OS6, and *Bacillus cereus*).

The relative abundance of *Enterobacteriaceae* is shown in Table 4. A total of 31 enterobacterial sequence reads was detected (17 in the low dietary ZnO experimental group, and 25 in the high dietary ZnO experimental group). According to the Pearson's χ^2 test, the total number of enterobacterial species detected in all piglets of each group was less in the control group than in the high dietary ZnO group (χ^2 value: 72.7; $P < 0.001$).

Klebsiella spp., *Citrobacter* spp., and *Escherichia coli* were the most prominent genera, but many species could only be detected in 1 of 12 piglets. *Pantoea* spp. were only detected in the high dietary ZnO group. No differences were observed due to increased individual variations between piglets, but the high dietary ZnO group generally displayed numerically greater abundances than the low dietary ZnO group.

Richness, Shannon, and Simpson indices as well as evenness are shown in Table 5. Richness ($P = 0.009$), Shannon ($P = 0.037$), and Simpson ($P = 0.025$) indices were all increased due to increased dietary ZnO intake, whereas the evenness was only numerically increased.

Table 5. Influence of increased dietary zinc oxide on the bacterial diversity in the ileum of piglets (n = 6 per trial group)

	Treatment[1]	
Item	Control	High ZnO
Richness	15.67 ± 3.14[x]	21.33 ± 3.50[y]
Shannon	1.35 ± 0.42[x]	1.77 ± 0.21[y]
Evenness	0.49 ± 0.13	0.58 ± 0.06
Simpson	0.62 ± 0.19[a]	0.79 ± 0.05[b]

[a,b]Within a row, means without a common superscript differ (ANOVA, $P < 0.05$).

[x,y]Within a row, means without a common superscript differ (Kruskal-Wallis test, $P < 0.05$).

[1]Control treatment with 124 mg·kg^{-1} of Zn from ZnO; High ZnO treatment with 3,042 mg·kg^{-1} of Zn from ZnO.

DISCUSSION

The current study investigated the influence of dietary ZnO on the bacterial core composition in the ileum of piglets. A pyrosequencing approach employing a bar code technique and restrictive analysis of sequence reads was chosen to apply statistical evaluation of multiple deep sequencing data, as well as to gain greater accuracy of assigning results at the species level. Furthermore, to enhance the recovery of the bacterial diversity in ileal samples, 2 different DNA extraction procedures and 2 primer combinations were used. It is known that extraction procedures (Morita et al., 2007; Nechvatal et al., 2008; Salonen et al., 2010), as well as primer combinations (Schmalenberger et al., 2001; Baker et al., 2003; Sipos et al., 2007), can have effects on the quality of PCR results.

Zinc is an essential nutrient with diverse regulatory, metabolic, and structural functions. Direct effects of pharmacological Zn doses on the piglet include altered expression of genes responsible for glutathione metabolism and apoptosis (Wang et al., 2009), enhanced gastric ghrelin secretion, which increases feed intake (Yin et al., 2009) or increased production of digestive enzymes (Hedemann et al., 2006). Indirect effects on the animal include modifications of the gastrointestinal bacterial composition and their metabolic activity (Mores et al., 1998; Højberg et al., 2005; Broom et al., 2006).

As expected, lactic acid bacteria (**LAB**) dominated the bacterial core in the ileum. The dominance of LAB in the porcine intestine has been shown in many studies (Sghir et al., 1998; Hill et al., 2005; Dowd et al., 2008; Pieper et al., 2008). In this study, their dominance was mainly due to *Lactobacillus* and *Weissella* species, more specifically *L. reuteri*, *W. confusa*, and *W. cibaria*. Other LAB such as enterococci and streptococci were less prominent than *Leuconostoc* spp. The increased relative abundance of *Weissella* spp. and *Leuconostoc* spp. has not been documented in piglets. These genera form a separate group ("leuconostoc-like" LAB) and largely differ in their metabolic potential from lactobacilli (Salminen et al., 2004). The key physiological features of the leuconostoc-like LAB are the exclusive production of L-lactate (some Weissella strains produce L- and L-lactate) and generation of CO_2 from glucose; however, fructose is preferred over glucose during growth. Furthermore, all leuconostoc-like LAB produce dextran from sucrose.

The dominating *L. reuteri* was influenced by greater ZnO supplementation, whereas the second most dominant *L. amylovorus* was not affected. Other lactobacilli, like *L. salivarius*, *L. johnsonii*, or *L. helveticus*, seemed to trade places in rank. The species specific response to ZnO supplementation may indicate different tolerance mechanisms against greater Zn inclusion; however, it could also be a result of interspecies competition. Considering the drastic increase in abundance of *Weissella* spp. and *Leuconostoc* spp., bacteriocin production may also have played a role. Like many other LAB, both genera are known to produce bacteriocins active against other LAB (Papathanasopoulos et al., 1997; Srionnual et al., 2007).

Sequences of the *Clostridiales* order in the bacterial core mainly consisted of *Sarcina ventriculi*, an acid-tolerant strict anaerobic species, found in the intestinal tract of piglets and other mammals (Crowther, 1971; Vatn et al., 2000; Thanantong et al., 2006). This species seemed to be very sensitive against ZnO or indirect modifications induced by increased dietary ZnO. The fact that *S. ventriculi* has also been found in the stomach of horses (Husted et al., 2010), lambs, dogs (Vatn et al., 2000), and free-living Colobus monkeys (Owaki et al., 1974), together with its ability to grow at low pH, indicates that this species may be autochthonous to the stomach of mammals.

An interesting result was observed for *Enterobacteriaceae*. Although not statistically significant, most detected species increased numerically in relative abun-

dance because of increased dietary ZnO. It should be mentioned that sequence data were generated from a PCR using the same concentration of target DNA, and thus, some species with low relative abundance may have fallen below the detection limit because of the high abundance of a few dominant members. Nevertheless, enterobacteria seemingly gained colonization potential by increased dietary ZnO; substantially more enterobacterial species were detected in animals from the high dietary ZnO trial group. This is in agreement with a previous study by Højberg et al. (2005). Furthermore, a survey of Canadian pig farms revealed an increased presence of an enteropathogenic *E. coli* strain in farms using dietary greater dietary ZnO (Amezcua et al., 2008). Also, an increase in enterobacterial diversity was observed in a study with piglets (Katouli et al., 1999). These results indicate that dietary ZnO causes an enhanced colonization with enterobacteria in the small intestine of piglets. Consequently, the frequently observed diarrhea-reducing effect of ZnO may not be related to direct reducing effects on pathogenic *E. coli*, but rather to an increase of the enterobacterial group, which would increase competition among enterobacteria.

On the other hand, considering the assumed antagonistic nature of LAB against enterobacteria, it can be speculated that a dramatic decrease of the dominant *L. reuteri* in animals fed the high ZnO-supplemented diet may be another reason for an increased colonization with gram-negative enterobacteria. Strains of *L. reuteri* are known to produce the broad-spectrum bacteriocin Reuterin, which is active against gram-negative enterobacteria (Cleusix et al., 2007).

The antimicrobial activity of ZnO has been long used in dental medicine (Smith, 1991), and several mechanisms for bacterial susceptibility and resistance against Zn have been suggested (Silver, 1996; Choudhury and Srivastava, 2001). For instance, gram-positive bacteria seem to rely on a P-type efflux ATPase (Nies and Silver, 1995) to expel Zn from their cells, whereas resistance to Zn^{2+} in gram-negative bacteria is mediated by proton-cation antiporter proteins. Specific plasmid-coded genes for resistance against heavy metals have been reported for both gram-positive and gram-negative bacteria present in the intestine (Nies, 1992; Silver and Walderhaug, 1992; Chen et al., 2008). There are no studies available on the inhibition of a range of gram-positive bacteria, except for some studies on *Bacillus cereus* (Sawai et al., 1996) and *Staphylococcus aureus* (Aarestrup and Hasman, 2004), but there are many studies on gram-negative enteric bacteria (Choudhury and Srivastava, 2001). Combined with results from studies from feeding trials in piglets (Mores et al., 1998; Højberg et al., 2005; Broom et al., 2006), it seems that gram-positive bacteria are more susceptible to Zn than gram-negative bacteria. Furthermore, fecal *E. coli* isolates showed increased minimal inhibitory concentration values for Zn in a study with cattle fed 30 or 300 $mg \cdot kg^{-1}$ of dietary Zn, respectively (Jacob et al., 2010). Considering the increase of enterobacteria diversity in this study, a greater resistance to heavy metal ions may be expected for this bacterial group.

Zinc oxide is an amphoteric molecule and practically insoluble in water, but shows greater 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 Zn ions (54% free Zn ions at 164 $mg \cdot kg^{-1}$ of dietary ZnO; Dintzis et al., 1995), and thus increased concentrations of free Zn ions may occur in the stomach. As the stomach of piglets harbors large numbers of LAB, modifications of ileal LAB could originate in the stomach because of the antibacterial effect of increased dietary ZnO.

The bacterial core of piglets in the low or high dietary ZnO experimental group differed both quantitatively and qualitatively. The bacterial core of the high dietary ZnO experimental group displayed more species, indicating a greater bacterial diversity. This was also reflected in the greater ecological diversity indices and may be a direct result of the reduction of dominant species such as *L. reuteri* and *S. ventriculi*, allowing more different species to gain colonization capacity. Finally, although changes in the ileal composition occurred, the dominance of LAB remained. Quantitatively, none of the other species exceeded 1% of total sequence reads except for the strict anaerobic *S. ventriculi*. Most notably, *Weissella* spp., *Streptococcus* spp., and *Leuconostoc* spp. seemed to quantitatively replace *L. reuteri* as a major player in the ileum.

In conclusion, the dietary supplementation of ZnO in pharmacological concentrations modified the ileal bacterial composition at the species level, most notably for dominating LAB. The differential response of closely related lactobacilli indicates different susceptibilities to a greater concentration of ZnO among genetically relatively closely related phylotypes. An increase of enterobacteria because of increased dietary ZnO may point to an increased colonization potential of this bacterial group. This could lead to an increased competition among enterobacteria and, consequently, reduce the occurrence of pathogenic *E. coli* strains, which are the main factor for postweaning diarrhea.

LITERATURE CITED

Aarestrup, F. M., and H. Hasman. 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet. Microbiol. 100:83–89.

Amezcua, R., M. Friendship, and C. E. Dewey. 2008. An investigation of the presence of *Escherichia coli* O149:K91:F4 on pig farms in southern Ontario and the use of antimicrobials and risk factors associated with the presence of this serogroup. Can. Vet. J. 49:39–45.

Armougom, F., and D. Raoult. 2008. Use of pyrosequencing and DNA barcodes to monitor variations in *Firmicutes* and *Bacteroidetes* communities in the gut microbiota of obese humans. BMC Genomics 9:576–588.

Baker, G. C., J. J. Smith, and D. A. Cowan. 2003. Review and reanalysis of domain-specific 16S primers. J. Microbiol. Methods 55:541–555.

Broom, L. J., H. M. Miller, K. G. Kerr, and J. S. Knapp. 2006. Effects of zinc oxide and *Enterococcus faecium* SF68 dietary supplementation on the performance, intestinal microbiota and immune status of weaned piglets. Res. Vet. Sci. 80:45–54.

Carlson, D., J. Sehested, Z. Feng, and H. D. Poulson. 2008. Serosal zinc attenuate serotonin and vasoactive intestinal peptide induced secretion in piglet small intestinal epithelium in vitro. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 149:51–58.

Chen, Y. Y., C. W. Feng, C. F. Chiu, and R. A. Burne. 2008. cadDX operon of *Streptococcus salivarius* 57.I. Appl. Environ. Microbiol. 74:1642–1645.

Choudhury, R., and S. Srivastava. 2001. Zinc resistance mechanisms in bacteria. Curr. Sci. 81:768–775.

Cleusix, V., C. Lacroix, S. Vollenweider, M. Duboux, and G. Le Blay. 2007. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. BMC Microbiol. 7:101–110.

Crowther, J. S. 1971. *Sarcina ventriculi* in human faeces. J. Med. Microbiol. 4:343–350.

Dintzis, F. R., J. A. Laszlo, T. C. Nelsen, F. L. Baker, and C. C. Calvert. 1995. Free and total ion concentrations in pig digesta. J. Anim. Sci. 73:1138–1146.

Dowd, S. E., Y. Sun, R. D. Wolcott, A. Domingo, and J. A. Carroll. 2008. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: Bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. Foodborne Pathog. Dis. 5:459–472.

Fairbrother, J. M., E. Nadeau, and C. L. Gyles. 2005. *Escherichia coli* in postweaning diarrhea in pigs; an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6:17–39.

Gill, S. R., M. Pop, R. T. Deboy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. L. Gordon, D. A. Relman, C. M. Fraser-Liggett, and K. E. Nelson. 2006. Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359.

Hamady, M., J. J. Walker, J. K. Harris, N. J. Gold, and R. Knight. 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat. Methods 5:235–237.

Hedemann, M. S., B. B. Jensen, and H. D. Poulsen. 2006. Influence of dietary zinc and copper on digestive enzyme activity and intestinal morphology in weaned pigs. J. Anim. Sci. 84:3310–3320.

Hill, J. E., S. M. Hemmingsen, B. G. Goldade, T. J. Dumonceaux, J. Klassen, R. T. Zijlstra, S. H. Goh, and A. G. Van Kessel. 2005. Comparison of ileum microflora of pigs fed corn-, wheat-, or barley-based diets by chaperonin-60 sequencing and quantitative PCR. Appl. Environ. Microbiol. 71:867–875.

Højberg, O., N. Canibe, H. D. Poulsen, M. S. Hedemann, and B. B. Jensen. 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Appl. Environ. Microbiol. 71:2267–2277.

Hollis, G. R., S. D. Carter, T. R. Cline, T. D. Crenshaw, G. L. Cromwell, G. M. Hill, S. W. Kim, A. J. Lewis, D. C. Mahan, P. S. Miller, H. H. Stein, and T. L. Veum. 2005. Effects of replacing pharmacological levels of dietary zinc oxide with lower dietary levels of various organic zinc sources for weanling pigs. J. Anim. Sci. 83:2123–2129.

Holm, A. 1988. *Escherichia coli*-associated post-weaning diarrhoea in piglets. Zinc oxide added to feed as an antibacterial agent? Dansk Veterinaertidsskrift 71:1118–1126.

Holmgren, N. 1994. Prophylactic effects of zinc oxide or olaquindox against weaning diarrhoea in pigs. Svensk Veterinartidning 46:217–222.

Huse, S. M., J. A. Huber, H. G. Morrison, M. L. Sogin, and D. M. Welch. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol. 8:R143.2–143.9.

Husted, L., T. K. Jensen, S. N. Olsen, and L. Molbak. 2010. Examination of equine glandular stomach lesions for bacteria, including *Helicobacter* spp. by fluorescence in situ hybridisation. BMC Microbiol. 10:84–91.

Jacob, M. E., J. T. Fox, T. G. Nagaraja, J. S. Drouillard, R. G. Amachawadi, and S. K. Narayanan. 2010. Effects of feeding elevated concentrations of copper and zinc on the antimicrobial susceptibilities of fecal bacteria in feedlot cattle. Foodborne Pathog. Dis. 7:643–648.

Katouli, M., L. Melin, M. Jensen-Waern, P. Wallgren, and R. Mollby. 1999. The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. J. Appl. Microbiol. 87:564–573.

Kunin, V., A. Engelbrektson, H. Ochman, and P. Hugenholtz. 2010. Wrinkles in the rare biosphere: Pyrosequencing errors lead to artificial inflation of diversity estimates. Environ. Microbiol. 12:118–123.

Kwak, T. J., and J. T. Peterson. 2007. Community indices, parameters, and comparisons. Pages 677–763 in Analysis and Interpretation of Freshwater Fisheries Data. C. S. Guy and M. L. Brown, ed. American Fisheries Society, Bethesda, MD.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: A software environment for sequence data. Nucleic Acids Res. 32:1363–1371.

Meyer, F., D. Paarmann, M. D'Souza, R. Olson, E. M. Glass, M. Kubal, T. Paczian, A. Rodriguez, R. Stevens, A. Wilke, J. Wilkening, and R. A. Edwards. 2008. The Metagenomics RAST server—A public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386.

Mores, N., J. Christani, I. A. Piffer, W. Barioni, and G. M. M. Lima. 1998. Effects of zinc oxide on postweaning diarrhea control in pigs experimentally infected with *E. coli*. Arq. Brasil Med. Vet. Zootec. 50:513–523.

Morita, H., T. Kuwahara, K. Ohshima, H. Sasamoto, K. Itoh, M. Hattori, T. Hayashi, and H. Takami. 2007. An improved DNA isolation method for metagenomic analysis of the microbial flora of the human intestine. Microbes Environ. 22:214–222.

Nechvatal, J. M., J. L. Ram, M. D. Basson, P. Namprachan, S. R. Niec, K. Z. Badsha, L. H. Matherly, A. P. Majumdar, and I. Kato. 2008. Fecal collection ambient preservation and DNA extraction for PCR amplification of bacterial and human markers from human feces. J. Microbiol. Methods 72:124–132.

Nies, D. H. 1992. Resistance to cadmium, cobalt, zinc, and nickel in microbes. Plasmid 27:17–28.

Nies, D. H., and S. Silver. 1995. Ion efflux systems involved in bacterial metal resistances. J. Ind. Microbiol. 14:186–199.

Overbeek, R., T. Begley, R. M. Butler, J. V. Choudhuri, H. Y. Chuang, M. de Cohoon, V. Crécy-Lagard, N. Diaz, T. Disz, R. Edwards, M. Fonstein, E. D. Frank, S. Gerdes, E. M. Glass, A. Goesmann, A. Hanson, D. Iwata-Reuyl, R. Jensen, N. Jamshidi, L. Krause, M. Kubal, N. Larsen, B. Linke, A. C. McHardy, F. Meyer, H. Neuweger, G. Olsen, R. Olson, A. Osterman, V. Portnoy, G. D. Pusch, D. A. Rodionov, C. Rückert, J. Steiner, R. Stevens, I. Thiele, O. Vassieva, Y. Ye, O. Zagnitko, and V. Vonstein. 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33:5691–5702.

Owaki, K., R. E. Hungate, L. Lotter, R. R. Hofmann, and G. Maloiy. 1974. Stomach fermentation in East African Colobus monkeys in their natural state. Appl. Microbiol. 27:713–723.

Papathanasopoulos, M. A., F. Krier, A. M. Revol-Junelles, G. Lefebvre, J. P. Le Caer, A. von Holy, and J. W. Hastings. 1997. Multiple bacteriocin production by *Leuconostoc mesenteroides* TA33a and other *Leuconostoc*/*Weissella* strains. Curr. Microbiol. 35:331–335.

Pieper, R., P. Janczyk, A. Zeyner, H. Smidt, V. Guiard, and W. B. Souffrant. 2008. Ecophysiology of the developing total bacterial

and lactobacillus communities in the terminal small intestine of weaning piglets. Microb. Ecol. 56:474–483.
Poulsen, H. D. 1995. Zinc oxide for weanling piglets. Acta Agric. Scand. Anim. Sci. 45:159–167.
Pruesse, E., C. Quast, K. Knittel, B. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35:7188–7196.
Salminen, S., A. von Wright, and A. C. Ouwehand. 2004. Lactic Acid Bacteria: Microbiological and Functional Aspects. 3rd ed. Page 11 in Classification and Physiology. S. Salminen, A. von Wright, A. C. Ouwehand, and S. Lahtinen, ed. Marcel Dekker Inc., New York, NY.
Salonen, A., J. Nikkila, J. Jalanka-Tuovinen, O. Immonen, M. Rajilic-Stojanovic, R. A. Kekkonen, A. Palva, and W. M. de Vos. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 81:127–134.
Sawai, J., H. Igarashi, A. Hashimoto, T. Kokugan, and M. Shimizu. 1996. Effect of particle size and heating temperature of ceramic powders on antibacterial activity of their slurries. J. Chem. Eng. Jpn. 29:251–256.
Schmalenberger, A., F. Schwieger, and C. C. Tebbe. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. 67:3557–3563.
Sghir, A., D. Antonopoulos, and R. I. Mackie. 1998. Design and evaluation of a *Lactobacillus* group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs. Syst. Appl. Microbiol. 21:291–296.
Silver, S. 1996. Bacterial heavy metal resistance. New surprises. Annu. Rev. Microbiol. 50:753–789.
Silver, S., and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56:195–228.
Sipos, R., A. J. Székely, M. Palatinszky, S. Révész, K. Márialigeti, and M. Nikolausz. 2007. Effect of primer mismatch annealing temperature and PCR cycle number on 16S rRNA gene-targeting bacterial community analysis. FEMS Microbiol. Ecol. 60:341–350.
Smith, D. C. 1991. Dental cements. Curr. Opin. Dent. 1:228–234.
Snyder, L. A., N. Loman, M. J. Pallen, and C. W. Penn. 2009. Next-generation sequencing—The promise and perils of charting the great microbial unknown. Microb. Ecol. 57:1–3.
Srionnual, S., F. Yanagida, L. H. Lin, K. N. Hsiao, and Y. S. Chen. 2007. Weissellicin 110, a newly discovered bacteriocin from *Weissella cibaria* 110, isolated from plaa-som, a fermented fish product from Thailand. Appl. Environ. Microbiol. 73:2247–2250.
Thanantong, N., S. Edwards, and O. A. Sparagano. 2006. Characterization of lactic acid bacteria and other gut bacteria in pigs by a macroarraying method. Ann. N. Y. Acad. Sci. 1081:276–279.
Vatn, S., G. Gunnes, K. Nybø, and H. M. Juul. 2000. Possible involvement of *Sarcina ventriculi* in canine and equine acute gastric dilatation. Acta Vet. Scand. 41:333–337.
Wang, X., D. Ou, J. Yin, G. Wu, and J. Wang. 2009. Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets. Amino Acids 37:209–218.
Yin, J., X. Li, D. Li, T. Yue, Q. Fang, J. Ni, X. Zhou, and G. Wu. 2009. Dietary supplementation with zinc oxide stimulates ghrelin secretion from the stomach of young pigs. J. Nutr. Biochem. 20:783–790.