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Effect of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on faecal microbiota composition in weaned piglets

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ABSTRACT: The aim of this study was to use high throughput sequencing of piglet faeces to investigate if the favourable effects of sodium humate (HNa) and zinc oxide (ZnO) in post-weaning diarrhoea (PWD) treatment are related to changes in the faecal microbiota. Twenty four piglets weaned at 28 days of age were divided into three groups with eight animals per pen: a control group without any treatment (Control), a group treated with 2500 mg ZnO (ZnO), and a group treated with 20 g sodium humate and 1700 mg ZnO (HNa + ZnO) per kg of diet. Piglets of all three groups were challenged with two enterotoxigenic *Escherichia coli* (ETEC) strains (ETEC/O149/F4/LT and ETEC/O147/F18/LT) on Day 4 post-weaning. As a result very intense and severe diarrhoea with high mortality developed in the ETEC-infected control group, while the ZnO and HNa + ZnO dietary treatments both protected piglets from clinical signs of diarrhoea, mortality and depression of growth performance. A higher relative abundance of *Gammaproteobacteria* represented mainly by genus *Escherichia* on Day 10 post-weaning in faeces of the ETEC-infected control group in comparison with ZnO and HNa + ZnO was detected. On Day 21, the highest relative increase of beneficial lactobacilli was observed in the HNa + ZnO group. Correlation analysis showed a positive correlation of the ETEC-infected control with the genera *Turicibacter*, *Clostridium*, *Campylobacter*, *Dehalobacterium*, *Desulfuvibrio*, *Paludibacter* and a negative correlation with the genera *Prevotella*, *Blautia*, *Faecalibacterium*, *Lactobacillus*, and *Coprococcus*. The opposite correlations with these genera were observed in the supplemented groups, especially in the HNa + ZnO group. The results indicate that dietary supplementation with HNa and ZnO affects the microbial composition of faeces while maintaining good health condition and growth performance of ETEC-infected weaned pigs.

Keywords: dietary supplements; humic substances; zinc; bacterial community composition

Weaning is one of the most critical periods in the life of pigs. The change from a milk- to a solid-based diet is accompanied by stress and marked changes in gastrointestinal physiology, microbiology and immunology (Heo et al. 2013; Pluske 2013). In the first weeks post-weaning piglets are most susceptible to enteric disease with diarrhoea and depression of growth performance. Post-weaning diarrhoea (PWD) is considered to be a major problem in the swine industry and causes significant economic losses in pig herds (Fairbrother et al. 2005; Vondruskova et al. 2010; Heo et al. 2013). The most common strategy of prevention in previous decades was the usage of antibiotic growth promoters, until these were banned in the EU in 2006 due to increasing bacterial resistance and residual risk in animal products (Regulation (EC) No. 1831/2003). Different alternative strategies have

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been introduced to decrease the susceptibility of young piglets to post-weaning diarrheal infections (Vondruskova et al. 2010; Heo et al. 2013; Pluske 2013; Thacker 2013). High doses of ZnO (2500 to 3000 mg/kg of feed) have been widely used in PWD prophylaxis (Roselli et al. 2005; Heo et al. 2013; Sales 2013). The mechanism of action of zinc is still not fully understood. However, it was found that high dietary ZnO has a positive impact on the stability and diversity of the intestinal flora which contributes to a high colonisation resistance against pathogens and thereby to resistance to diarrhoeal infections and to better growth performance (Katouli et al. 1999; Vahjen et al. 2011; Pieper et al. 2012). Although ZnO has traditionally been included in diets for weaning piglets, its excretion in high amounts represents a hazard to the environment (Heo et al. 2013; Sales 2013). To reduce environmental pollution, this practice is prohibited in some European countries. There have been attempts at decreasing the doses of ZnO used as well as a search for alternatives which are effective in the prevention of PWD.

Humic substances (HS) are a class of compounds that are generated from the decomposition of organic matter in the soil. Their oral use is permitted in horses, ruminants, swine and poultry for the treatment of diarrhoea, dyspepsia and acute intoxications (EMEA 1999). HS in pig diet was also shown to improve growth performance, meat quality, increase the nutrient digestibility and reduce ammonia emission from manure (Ji et al. 2006; Wang et al. 2008; Pisarikova et al. 2010). Our previous studies showed that sodium humate (HNa) was insufficient for the treatment of diarrhoea in piglets challenged by pathogenic strains of *Escherichia coli* and that in the case of severe diarrhoeal infections supplementation of HNa to piglets can be effective only in combination with a specific dose of ZnO (1700 mg/kg; Trckova et al. 2015). However, to what extent the prophylactic effect of HNa and ZnO was due to the maintenance of intestinal health and stabilisation of microbiota after treatment was not determined.

Scientific articles about the effect of HS on intestinal health and composition of intestinal microflora are rather scarce (Shermer et al. 1998; Aksu and Bozkurt 2009). It has been reported that HS might favourably influence the performance of animals by altering the gastrointestinal ecosystem with subsequent stabilisation of intestinal flora, improvement of gut health and better utilisation of nutrients (Shermer et al. 1998; Islam et al. 2005).

The aim of this study was to investigate if the favourable effects of HNa and ZnO in PWD treatment are related to changes in intestinal microbiota. We used high throughput sequencing of piglet faeces to determine these changes.

MATERIAL AND METHODS

Animal management and treatment. The study was performed on twenty four weaned piglets (Large White × (Pietrain × Duroc)) originating from a specific pathogen-free herd. Piglets were transported into the experimental animal facility of the Veterinary Research Institute, Brno, Czech Republic at day of weaning (28th day of age). They were identified by individual ear tags and housed in indoor pens. The temperature in the rooms was 21 to 23 °C and humidity was 51 to 62%. Animal handling followed EU directive 86/609/EEC concerning animal care. The animal care protocol for this experiment followed the Czech guidelines for animal experimentation and was approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (Permission No. MZe 50-2011). Piglets were allocated to three treatment groups with eight piglets per pen: control group without any treatment (Control), group treated with 2500 mg ZnO (ZnO), and group treated with 20 g HNa and 1700 mg ZnO (HNa + ZnO) per kg of diet. The treatments were supplied to the basal diet formulated according to animal requirements (NRC 1998). Piglets were fed twice a day *ad libitum*, water was provided by automatic waterers. The dietary treatments were maintained for three weeks.

***Escherichia coli* challenge**. Piglets were orally challenged by two enterotoxigenic *Escherichia coli* strains, ETEC/O149/F4/LT (12549) and O147/F18/LT (12524), with a single dose of 1.5×10^{11} colony forming units (CFU) per piglet on Day 4 post weaning. The ETEC strains used for infection were grown in medium containing 12.5 g of acid casein hydrolysate, 12.5 g of enzymatic casein hydrolysate and 0.5 g of yeast extract (Oxoid) per litre and incubated at 37 °C for 16 h. For individual administration, the culture was concentrated by centrifugation and subsequently incorporated into semolina porridge paste.

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Diarrhoea evaluation. Piglets were clinically examined individually once per day and the presence of clinical signs of diarrhoea was documented. Diarrhoea incidence was evaluated by the ratio of scouring piglets in each group. The severity of diarrhoea was assessed visually and evaluated by individual scoring of the consistency of the faeces: 0 normal, 1 pasty, 2 mushy, 3 liquid, 4 liquid with blood. Mean daily diarrhoea score (DDS) was calculated as the group sum divided by the number of piglets in the group. The duration of diarrhoea was recorded individually and the mean duration for the group was calculated. Mortality rate throughout the monitoring period was recorded.

Performance. Piglets were weighed on Days 1, 7, 14 and 21 post-weaning. Individual body weight gains (BWG) were calculated. Average daily feed intake (FI) of the groups was recorded. Feed conversion ratio (FCR) was calculated from FI and BWG.

Faecal sample collection.The faecal samples were collected on the day of weaning (Day 1) before piglets were subjected to treatments and on Days 10 and 21. Faecal samples were obtained from the rectum of all animals and immediately frozen at –20 °C until further analysis.

DNA isolation, PCR and pyrosequencing. Faecal DNA was isolated from 200 mg of faecal samples, using a commercially available kit (Stool DNA Isolation kit, Qiagen, Germany) with previously described modifications (Kralik et al. 2011). The DNA was subsequently mixed from all the animals within a group and used for amplification. The gene coding for 16S rRNA was amplified using universal bacterial primers targeting the variable regions V3 and V4 (Nossa et al. 2010). The primers were flanked with standard MID sequences and tags needed for the pyrosequencing. PCR reactions were performed using the HotStarTaq Master Mix Kit following the manufacturer's instructions (Qiagen), with cycling conditions as follows: 95 °C for 15 min followed by 30 cycles of incubation at 94 °C for 40 s, 55 °C for 55 s and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. PCR products were visualised using electrophoresis on 1.5% agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen). The amplified products were sequenced on the 454 GS Junior platform from Roche, according to the recommended protocol (Roche).

Sequence analyses. Sequences were analysed using the QIIME software (Caporaso et al. 2010). Quality trimming criteria included no mismatch in MID sequences and a maximum of one mismatch in primer sequences. The obtained sequences with quality scores higher than 20 were shortened to the same length of 350 bp and classified with RDP Seqmatch with an operational taxonomic units (OTUs) discrimination level set to 97%.

Statistical analyses. Data on diarrhoea and performance evaluation were subjected to statistical analysis using the program GraphPadInstat 3.0. The differences among SDs were tested using the Bartlett test. The normality of the data was tested with the Kolmogorov-Smirnov test. When data passed the normality test, statistical significance of the differences among the group means was determined by the analyses of variance (ANOVA) in conjunction with the Tukey-Kramer test. When normality of the data was not validated ($P < 0.05$) Kruskal-Wallis nonparametric ANOVA was used. Differences between means with $P < 0.05$ were accepted as being statistically significant. Correlation coefficients of bacterial community composition with the diet of piglets, DDS, diarrhoea incidence, and BWG, were rearranged by biclustering using Ward's hierarchical method and visualised as a cluster heatmap using MATLAB 2014b (MathWorks).

RESULTS

Diarrhoea evaluation

Differences in clinical signs of diarrhoea among the ETEC-infected control and supplemented groups (ZnO and HNa + ZnO) were highly significant ($P < 0.001$). Severe cases of diarrhoea were observed in the ETEC-infected control group already a day after challenge (Day 5 post weaning). The course of diarrhoeal infection was very intense with a high incidence of scouring piglets and liquid faeces. Due to severe diarrhoea with resultant dehydration high mortality of ETEC-infected control piglets occurred in the first week after challenge. In survivors, clinical signs of diarrhoea lasted on average five days with a range of three to seven days in individuals. Clinical signs of diarrhoea were also observed in some individuals of the ZnO and HNa + ZnO groups, but the overall course of diarrhoeal infection was very mild (pasty or mushy faeces) and the piglets recovered within one or two days. No mortality was observed in these groups (Table 1).

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Table 1. Growth performance of piglets and diarrhoea evaluation

Parameter	Period post-weaning (week)	Treatments*		
		control	ZnO	HNa + ZnO
Body weight gain (g/day)	1	–111.10[a]	101.80[b]	85.70[b]
	2	189.30	285.70	273.20
	3	187.50	403.60	453.60
	1–3	88.70	263.70	270.80
Feed intake (g/pig/day)	1	215.89	305.00	291.86
	2	476.20	410.71	401.79
	3	773.84	765.40	794.64
	1–3	488.64	493.70	496.10
Feed conversion ratio	1–3	5.51	1.87	1.83
Mortality (%)	1–3	62.5	0	0
Diarrhoea incidence (%)	1–3	100[a]	50[b]	50[b]
Daily diarrhoea score	1–3	2.10[a]	0.64[b]	0.45[b]
Duration of diarrhoea (days)	1–3	5.00[a]	1.38[b]	1.13[b]

*control = ETEC-infected control group without any treatment; ZnO = treated with 2500 mg ZnO; HNa + ZnO = treated with 20 g HNa and 1700 mg ZnO per kg of diet. Means within a column with different letters differ significantly

Performance

Signs of growth depression in the ETEC-infected control group were observed during the first week post-weaning. The animals of the ZnO and HNa + ZnO groups gained weight in this period and significantly higher BWG values ($P < 0.05$) were calculated in comparison with the ETEC-infected control group. Differences in total BWG for the whole experimental period were not quite significant among treatments. FI was not significantly affected by treatments. A higher FCR in the ETEC-infected control group in comparison with the ZnO and HNa + ZnO groups was calculated (Table 1).

Pyrosequencing

There were altogether 135 898 reads obtained after pyrosequencing of the amplicons. These reads were distributed among 10 612 OTUs. The faecal microflora in weaned piglets consisted mainly of the following phyla: *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Notable shifts in abundance included a decrease in *Bacteroides* and *Clostridium* groups and an increase in *Prevotella*, *Lactobacillus* and *Streptococcus* groups during the studied period (Table 2). An increase in *Gammaproteobacteria* represented by the genus *Escherichia* was detected in all groups on Day 10; however, the highest rela-

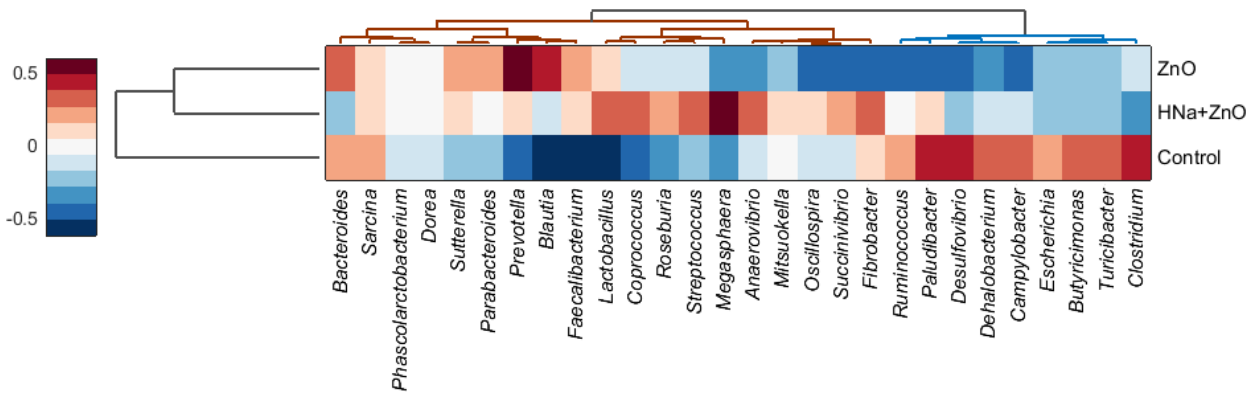

Figure 1.Correlation of abundance of faecal bacterial genera with feeding supplements in ETEC-infected piglets during three weeks post-weaning

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Table 2. Relative abundance (in %) of the most frequently identified bacterial genera in the piglet faeces

	Treatments*								
	Day 1			Day 10			Day 21		
	Control	ZnO	HNa + ZnO	Control	ZnO	HNa + ZnO	Control	ZnO	HNa + ZnO
Anaerovibrio	0.23	0.48	0.43	1.75	0.24	3.47	7.02	0.53	1.41
Bacteroides	2.48	8.05	1.09	2.13	2.48	0.58	0.02	2.48	0.10
Blautia	0.22	0.22	0.34	0.70	3.79	0.34	0.46	0.96	0.50
Campylobacter	0.38	0.35	0.40	5.18	0.16	0.03	0.13	0.00	0.08
Clostridium	1.37	1.49	0.67	0.00	0.16	0.12	0.07	0.09	0.03
Coprococcus	0.17	0.22	0.18	0.29	1.72	0.95	0.43	0.19	0.57
Desulfovibrio	1.71	0.37	0.12	0.22	0.08	0.15	0.24	0.00	0.08
Dorea	0.28	0.29	0.34	0.19	0.92	0.34	0.25	0.13	0.24
Escherichia	0.11	1.84	0.62	13.12	4.67	3.68	0.02	0.00	0.00
Faecalibacterium	0.11	1.01	0.42	1.30	3.19	1.69	0.68	6.07	0.78
Lactobacillus	1.22	2.09	6.18	1.46	3.51	2.55	5.15	4.12	12.23
Megasphaera	0.00	0.00	0.10	2.41	0.00	2.24	2.04	0.37	4.67
Oscillospira	6.53	2.81	6.53	2.89	4.67	3.32	6.03	2.04	7.33
Paludibacter	1.37	0.15	0.51	0.22	0.00	0.28	0.34	0.02	0.38
Parabacteroides	1.59	1.12	0.82	1.02	4.39	0.80	0.40	1.43	1.20
Phascolarctobacterium	2.78	1.10	2.06	2.16	0.52	2.70	4.88	5.62	1.90
Prevotella	17.30	30.72	24.61	24.05	32.10	24.90	29.68	55.14	27.91
Streptococcus	0.18	0.00	0.20	0.00	0.00	0.03	1.77	0.35	1.63
Succinivibrio	0.20	0.11	0.22	7.75	0.12	2.39	0.63	0.06	0.34
Roseburia	0.34	1.76	0.57	4.42	5.87	5.19	3.47	0.77	2.55
Ruminococcus	6.00	3.33	4.44	3.40	2.67	4.05	4.81	0.54	3.68
Other genera	0.09	0.11	0.07	0.10	0.08	0.03	0.09	0.04	0.01

*control = ETEC-infected control group without any treatment; ZnO = treated with 2500 mg ZnO; HNa + ZnO = treated with 20 g HNa and 1700 mg ZnO per kg of diet

tive abundance of *Escherichia* was observed in the ETEC-infected control group on this sampling day (Table 2).

On Day 21 post-weaning, the relative proportion of *Bacilli* represented namely by genus *Lactobacillus* increased in all groups, especially in the HNa + ZnO group, after a drop on Day 10. The relative dominance of *Escherichia* decreased dramatically in all groups on this sampling day.

Correlation analysis of faecal microbiota composition to the feeding supplements performed at the genus level (Figure 1) showed two main microbial clusters. In the ETEC-infected control group, we observed a positive correlation with the genera *Turicibacter*, *Clostridium*, *Campylobacter*, *Dehalobacterium*, *Desulfuvibrio*, *Paludibacter*, and a negative correlation with the genera *Prevotella*, *Blautia*, *Faecalibacterium*, *Lactobacillus*, and *Coprococcus*. In general, the opposite correlations with these genera were observed in the supplemented groups, especially in the HNa + ZnO group (Figure 1).

The correlation between bacterial genera detected in faeces and the severity of diarrhoea and/or BWG is shown in Figure 2. Four bacterial clusters could be distinguished. Genera within cluster I (namely *Escherichia*, *Campylobacter*, *Bacteroides*, *Sarcina*, *Butyricimonas* and *Desulfovibrio*) negatively correlated with BWG and exhibited a more or less positive correlation with diarrhoea incidence and/or DDS. In contrast, bacterial cluster IV represented namely by genera *Prevotella*, *Lactobacillus* and *Streptococcus* showed the opposite correlations with BWG. Genera of cluster II generally negatively

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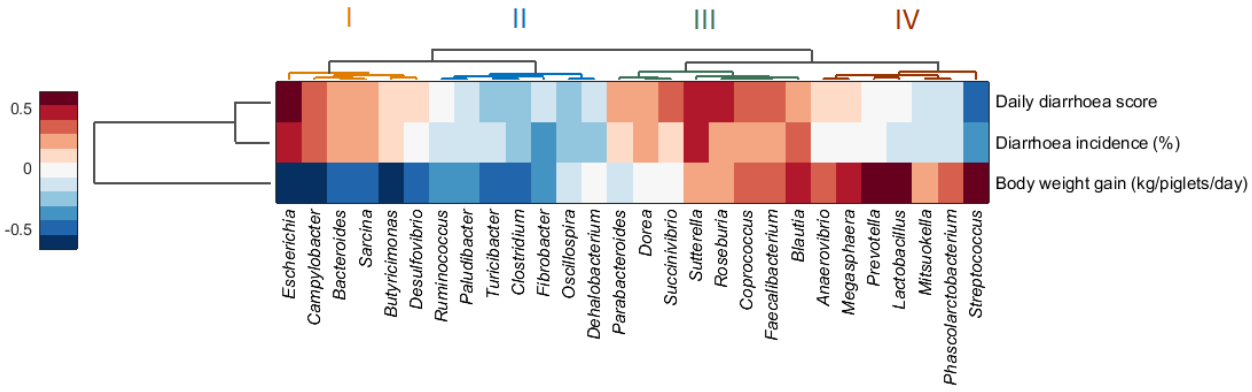

Figure 2. Correlation of abundance of faecal bacterial genera with diarrhoea incidence, daily diarrhoea score and body weight gain in ETEC-infected piglets during three weeks post-weaning

correlated with all three signs of health status and performance. Genera within cluster III exhibited, on the other hand, a predominantly positive correlation with DDS and with diarrhoeal incidence.

DISCUSSION

All piglets in this experiment were challenged with two pathogenic strains, ETEC O149:F4/LT and O147:F18/LT, which are commonly present in the environment and frequently cause diarrhoeal infection in weaned piglets (Frydendahl 2002; Fairbrother et al. 2005). The conjunction of two serotypes with different fimbrial adhesins can induce severe disease in piglets (Madec et al. 2000). Therefore, we used the composite inoculum in the experimental challenge. While very intense and severe diarrhoea with high mortality developed in the ETEC-infected control groups, the ZnO and HNa + ZnO dietary treatments both protected piglets from clinical sings of diarrhoea, mortality and depression of growth performance. The high FCR in the ETEC-infected control group reflected the poor health status and growth depression of piglets in comparison with the lower FCR in groups supplemented with ZnO and HNa + ZnO. These results are in accordance with those in our previous experiments in which we confirmed the possibility of reducing the high pharmacological dose of ZnO in PWD treatment through partial replacement with HNa (Trckova et al. 2015). To the best of our knowledge, there are no data on the use of HS in the prophylaxis of ETEC-induced diarrhoea in piglets. ZnO and HS have been reported to improve pig performance probably through stabilisation of the intestinal microflora and the subsequent improvement in nutrient absorption, especially protein digestion and trace element utilisation (Jensen-Waern et al. 1998; Islam et al. 2005; Wang et al. 2008; Kim et al. 2012).

To determine the possible effects of ZnO and HNa on the microbial community we have, unlike other studies which focused on ilea digest or mucosa, analysed only the faecal bacterial composition (Vahjen et al. 2011; Pieper et al. 2012). This approach enabled us to follow the composition in the same animals over time, thus minimizing individual differences, while at the same time sparing the animals' lives.

The genus *Prevotella* was the most abundant in faecal samples with an observable time-dependent increase in weaned piglets similarly as in the study of Pajarillo et al. (2014). Bacteria of the genus *Prevotella* predominated also in samples of fresh piglet manure (69.45%, Lu et al. 2014).

Previous reports using culture-based techniques described streptococci (44.2%) as the predominant bacteria in swine faeces, while *Eubacterium* and *Clostridium* bacteria were present to lesser extents. Streptococci (46.9%) predominated also among organisms isolated from the colonic contents obtained from weaned healthy pigs, followed mainly by *Bacteroides*, *Eubacterium*, and *Fusobacterium* (Robinson et al. 1984). These bacterial genera were found to be relatively infrequently present in faecal or manure samples using culture-independent molecular methods (Lu et al. 2014; Pajarillo et al. 2014), including in our study (Table 2). These differences are probably due to a high percentage of non-cultivable bacteria in the faecal bacterial community. In a recent study on the gut microbiome

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in nursing and weaned pigs (Frese et al. 2015), the authors found that weaned pigs had stable intestinal microbiota, similar to the microbiota we have described in the piglets before the treatment.

As expected, we observed a higher relative abundance of *Gammaproteobacteria* represented mainly by genus *Escherichia* on Day 10 post weaning (Day 6 post challenge) in the faeces of the ETEC-infected control group in comparison to the ZnO and HNa + ZnO groups. This was associated with the very intense course of diarrheal infection in this untreated, ETEC-infected control group in comparison with the very mild course of diarrhoea in both treated groups. In agreement with our results, Slade et al. (2011) showed that supplementation with ZnO (3100 mg/kg of diet) significantly reduced faecal ETEC counts. HS were also shown to reduce *E. coli* counts in the ileo-caecal digest in broilers (Aksu and Bozkurt 2009).

The reduction of PWD prevalence after pharmacological levels of ZnO may not only be related to direct reduction of pathogenic *E. coli* in the intestine but can also be associated with the increase of diversity and maintenance of the stability of intestinal flora (Katouli et al. 1999; Vahjen et al. 2011; Pieper et al. 2012; Starke et al. 2013). Unlike previous studies, which found an increase in the enterobacterial group including *Escherichia coli* in the ileum in piglets supplemented with high dietary ZnO (Vahjen et al. 2011; Pieper et al. 2012), in our ETEC infection model, we did not observe this trend in faecal samples after three weeks of supplementation (Day 21, Table 2) with neither 2500 mg/kg nor 1700 mg/kg ZnO. Similarly, Jensen-Wearn et al. (1998) reported decreased numbers of *E. coli* excreted over the course of four weeks post-weaning in the faeces of piglets supplemented with 2500 mg/kg ZnO.

Over the course of three weeks of dietary supplementation, there was an observable increase in the relative proportion of lactobacilli in all groups. *Lactobacillus* spp. are the most abundant bacterial genera in the ileum and are considered as a beneficial bacterium for the balance of intestinal microbiota that may repress pathogenic microorganisms (Vahjen et al. 2011). In faeces, lactobacilli constitute only a minor group of the bacterial community (Pajarillo et al. 2014). We observed a lower relative abundance of lactobacilli after three weeks in the ZnO group in comparison with the ETEC-infected control and HNa + ZnO groups. The high dose of ZnO in diet was found to reduce lactobacilli counts in pig intestine in previous studies (Vahjen et al. 2011). Interestingly, the highest relative abundance of lactobacilli was observed in the HNa + ZnO group at this sampling time. It is noteworthy in this regard that HS may influence metabolism in microbes and can be a source of nutrients for some microorganisms with subsequent stimulation of their growth (Tikhonov et al. 2010). Shermer et al. (1998) observed that dietary HS (0–50 g/kg feed) may promote higher *Lactobacillus* population levels in the caecal bacterial flora of broilers. Similar results were described by Aksu and Bozkurt (2009) in the ileo-caecal digest of broilers supplemented with 1.5 g/kg humic acid.

CONCLUSION

In conclusion, dietary supplementation with HNa and ZnO appeared to reduce pathogenic *E. coli* and affected the microbial composition of faeces while maintaining good health condition and growth performance in spite of ETEC infection in the post-weaning period. Proliferation of beneficial bacteria such as lactobacilli and strepococci contributes to improved gut health in piglets in the critical post-weaning period. Additionally, we have here determined some of the other bacterial taxa whose abundance changes in response to dietary supplements.

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