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The effect of leonardite and lignite on the health of weaned piglets

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ABSTRACT

A three-week trial was conducted to evaluate the effects of leonardite and lignite, natural sources of humic substances, on selected indicators of health status of weaned piglets. A total of 45 weaned piglets were assigned to three dietary treatments: Control - basal diet without any medication; Leonardite or Lignite - diet supplemented with lignite or leonardite at a dose of 20 g/kg, respectively. Leonardite differed from lignite in the content of humic substances and minerals. Diarrhoea incidence and severity, growth performance, haematological and biochemical status, biomarkers of oxidative stress, serum fatty acid (FA) profile and faecal microbiota composition were monitored. Significantly lower faecal score, diarrhoea incidence, serum biomarkers of oxidative stress, higher body weight gain and no mortality were observed in leonardite and lignite group. The supplemented groups had or tended to have higher haematocrit, haemoglobin, erythrocyte counts, iron, cholesterol and lower urea in blood. Increased serum minerals (calcium, phosphorus, magnesium) were detected in the leonardite group. Different effects of leonardite and lignite on serum FA profile were detected. Significantly lower proportion of saturated FA, higher unsaturated, monounsaturated, polyunsaturated (PUFA) n-3 FA and PUFA n6/n3 ratio were detected in leonardite group compared to lignite group. Both treatments decreased microbial diversity and richness of faecal microbiota at the genus level. Specifically, lower relative abundance of *Firmicutes, Bacteroides, Anaerovibrio*, *Oscillospira*, SMB53, *Ruminococcus,* and a tendency to a higher abundance of *Prevotella* was found compared to control group. Natural humic materials may provide benefit to piglets' heath in the difficult post-weaning period.

1. Introduction

In conventional pig husbandry, piglets are weaned at the very early age of 21 to 28 weeks when they are physiologically not fully competent to deal with the multiple social, nutritional, environmental and immunological changes associated with weaning. Therefore, the post-weaning period is often characterized by a decreased nutrient intake and digestibility, weight loss and growth performance depression, increased oxidative stress and changes in intestinal barrier integrity and function which increase the susceptibility to diarrhoea.

Antibiotics and therapeutic doses of zinc oxide have been used to control post-weaning diarrhoea and to optimise growth performance in piglets. Due to increased concerns surrounding antimicrobial resistance and a residual risk in animal products, the European Union has banned the prophylactic use of antibiotics in feeds since January 2006 and limited their use for therapeutic purposes only. Nowadays, the European Commission has concluded that environmental risks outweigh the benefits of zinc oxide for the prevention of diarrhoea in pigs and has opted for phasing out the usage of high-level zinc oxide for veterinary purposes. Therefore, there is an imperative to find effective alternatives that could reduce the incidence and severity of health problems associated with the period immediately after weaning.

Among many alternatives, humic substances (HS) have been proposed for preventing diarrhoea in animals. Humic acids and their sodium salts are permitted for oral use in horses, ruminants, swine and poultry for the treatment of diarrhoea, dyspepsia and acute intoxications (EMEA 1999).

HS are major components of natural organic matter in soil, water and sediment. They are complex and heterogeneous mixtures of polydispersed materials formed by biochemical and chemical reactions during the decay and transformation of plant and microbial remains. In this process known as humification, a variety of organic compounds, such as phenolic compounds (e.g. lignins), carbohydrates and nitrogenous substances are resynthesized to form large complex polymeric aromatic structures. The extreme variability in composition and structure of HS relates to the precursor compounds and the environmental conditions under which they are formed. HS can be divided into three main fractions: humic acids, fulvic acids and humin according to their solubility. They are mainly composed of phenolic, carboxylic acid, enolic, quinone and ether functional groups but may also include a

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wide variety of other components such as polysaccharides, polypeptides, lignins, esters, phenols, ethers, lipids, fatty acids (FA), trace minerals etc. However, the phenolic and carboxylic groups are more prevalent in HS structure (de Melo et al. 2016).

Raw materials rich in HS are above all coal-type caustobiolites – lignite and leonardite. Lignite is a brown carbonaceous sedimentary rock with woody texture that consists of accumulated layers of partially decomposed vegetation. It was formed from naturally compressed peat at shallow depths and temperatures lower than 100 °C. It is often referred to as brown coal, and is considered the lowest rank of coal. Leonardite derives either from lignite that has undergone oxidation during surface exposure or it represents sediments enriched in HS that were leached from top soil or overlying lignite deposits. Leonardite is characterized by the highest content of HS in a natural source and, depending on the location, it may contain 80 to 95% HS.

The application potential of HS in the treatment of diarrhoea in weaned piglets has not been investigated properly. Nevertheless, their properties and verified effects in other species and categories of animals, in humans and in vitro indicated that they could be beneficial in piglets' heath too. HS have a multidirectional effect on the gastrointestinal tract, microbiota composition, enzymatic activity, digestibility of nutrients and their utilization, and thereby they can have a beneficial effect on the rate of growth (Bai et al. 2013; Ji et al. 2006; Kim et al. 2004; Wang et al. 2008). They possess antibacterial, antitoxic, anti-inflammatory and immunostimulatory effects (Chang et al. 2014; Islam et al. 2005; Joone and van Rensburg 2004; Kunavue and Lien 2012; Van Rensburg and Naude 2009; Van Rensburg et al. 2006; Van-Rensburg et al., 2001;Van Rensburg et al. 2000; Zraly et al. 2008). They suppress the development of oxidative stress and the formation of free radicals (de Melo et al. 2016; Khil'ko et al. 2011) that arise from metabolic processes, stress, inflammation or infections.

Most of the tested HS were commercially available products or purified fractions of humic and fulvic acids. However, the separation of HS into constituent fractions with specific chemical properties in order to reduce their heterogeneity remains challenging. Therefore, there is an ongoing effort to use natural humic materials containing the whole complex of humic fractions as well as some trace minerals. In addition, it appears that the efficacy of natural humic complexes could be greater than that of the isolated humic acids (Banaszkiewicz & Drobnik 1994).

Thus, the objective of this study was to evaluate the effect of leonardite and lignite, both of which are natural materials rich in HS, on selected indicators of health status in weaned piglets - diarrhoea incidence and severity, growth performance, oxidative stress, haematological and biochemical parameters, serum FA profile and faecal microbiota composition.

2. Material and methods

2.1. Animal management and dietary treatment

Testing of two natural humic materials - leonardite and lignite (Table 1) was performed on 45 weaned piglets (LW x (P x Du)) coming from different litters of commercial pig farm in Czech Republic (Bioprodukt, Knapovec). Piglets were transported to the experimental animal facility of the Veterinary Research Institute, Brno, Czech Republic on the day of weaning (28 days of life). They were identified by individual ear tags and housed in indoor pens. Animal handling followed the EU Directive 2010/63/EU 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.

Table 1
Content of humic substances and selected minerals in leonardite and lignite (in dry matter).

Parameter (wt%)	Leonardite	Lignite
Dry matter	88.62	94.45
Humic substances	76.22	36.65
Humic acids	62.50	26.50
Fulvic acids and other low molecular weight organic acids	13.72	10.15
Ca	15.36	11.01
Mg	0.76	2.32
P	0.11	0.79
Fe	14.93	12.80

Table 2
Composition of the diet for weaned piglets (as a feed basis).

Ingredient (%)	Basal diet
Wheat	40.0
Barley	30.0
Soybean meal, 47% CP	18.5
Dry whey and soy protein concentrate	5.0
Soybean oil	2.3
Synthetic amino acids and minerals[1]	2.7
Vitamin and mineral premix[2]	1.5
Chemical composition	
ME (MJ/kg)	12.27
Crude protein (g/kg)	189.00
Fat (g/kg)	4.10
Lysine (g/kg)	11.95
Methionine (g/kg)	4.00

[1] L-Lysine HCl, L-Threonine, DL-Methionine, $CaCO_3$, Na_2CO_3, NaCl, $CaHPO_4$.

[2] Provided per kg diet: 12000 IU of vitamin A, 2000 IU of vitamin D3, 100 IU of vitamin E, 152 mg of Cu (as $CuSO_4$), 22 mg of Zn (as ZnO), 88 mg of Zn (as $ZnSO_4$), 32 mg of Mn (as MnO), 110 mg of Fe (as $FeSO_4$), 1.0 mg of I (as $Ca(IO_3)_2$), 0.20 mg of Co (as $Co_2O_3 \times 7\ H_2O$), and 0.3 mg of Se (as $Na_2SeO_3 \times 5\ H_2O$).

Weaned piglets with an average initial body weight of 9.58 ± 0.92 kg were allocated into three groups balanced by live weight and sex. Each group was kept in one pen with 15 piglets (seven or eight males and seven or eight females) per pen. Dietary treatments were as follows: Control = non-granulated basal diet without antibiotics and medication; Leonardite = non-granulated basal diet mixed with leonardite at a dose of 20 g/kg; Lignite = non-granulated basal diet mixed with lignite at a dose of 20 g/kg. The basal diet (Table 2) was formulated according to animal requirements (National Research Council, 1998). Piglets were fed ad libitum, water was provided by automatic waterers. The dietary treatment was maintained for 21 days.

2.2. Diarrhoea and performance

Piglets were clinically monitored for sings of diarrhoea during the entire experimental period. When diarrhoea appeared, faecal samples from scouring piglets were taken and analysed in the National Reference Laboratory for *Escherichia coli* (VRI Brno, Czech Republic) for the presence of enterotoxigenic *E. coli* strains (ETEC) which are most frequently implicated in pathogenesis of post-weaning diarrhoea. The severity of clinical sings of diarrhoea was assessed every 3 days by individual scoring of faecal consistency: 0 solid, 1 pasty, 2 mushy, 3 watery. Mean faecal score (FS) was calculated as the group sum divided by the number of piglets in the group. Diarrhoea incidence (DI) was evaluated by the ratio of scouring piglets (with faecal consistency 2 and 3) in the group. Mortality rate was measured throughout the monitoring period.

Piglets were weighed at Day 1, 7, 14, 21 and individual body weight gains (BWG) were calculated. The average daily feed intake (FI) of the groups was recorded. The feed conversion ratio (FCR) was calculated from the FI and BWG.

2.3. Blood and serum samples

Blood samples (10 ml) from all animals except for two dead control piglets were drawn from the *vena cava cranialis* after 12-h fasting at the end of the trial (Day 21). Approximately 2 ml of blood samples were collected into heparinised tubes and immediately utilized for intended analyses. Following blood samples (8 ml) were collected into tubes, centrifuged at 3000 rpm for 15 min and obtained serum was stored at −20 °C until further analyses.

2.4. Biomarkers of oxidative stress

To assess oxidative status of piglets, serum levels of isoprostanes 8-isoPGF$_{2\alpha}$ and 8-isoPGE$_2$ were measured by liquid chromatography/tandem mass spectrometry (LCMS/MS) with an Agilent 1200 chromatographic system (Agilent Technologies, Germany), as was previously reported (Trckova et al. 2017).

2.5. Haematological and biochemical status

To assess the general health status of piglets after long-term treatments, haematological and serum biochemical parameters were measured. In blood, haematocrit, haemoglobin, erythrocyte and leukocyte counts were determined using the Coulter Counter M4 apparatus (Coulter Cientifica S.A., Mostoles, Spain). Differential leukocyte count in blood smears was determined using a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

In serum, total protein, albumin, urea, creatinine, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triacylglycerols, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), calcium, phosphorus, magnesium and iron were measured using a BS200 automated chemistry analyser (Mindray, Shenzhen, China). Serum globulin was calculated by subtracting the serum albumin from the total protein concentration (Thrall et al. 2013).

2.6. Serum FA profile

FA composition in serum was determined after extraction and methylation of samples by gas chromatography/mass spectrometry (GC/MS), as was previously reported (Trckova et al. 2017). Individual FA were identified by n-designation (carbon numbering starts from the methyl end of the molecule). A total of 50 FA were analysed. Only FA whose concentration was higher than 0.5 nmol/ml were summarised.

2.7. Faecal microbiota composition

Faecal samples were obtained from the rectum of animals at the end of the trial (Day 21) and immediately frozen at −20 °C until further analysis. Faecal DNA was isolated using DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Germany). Short fragments that could compromise library quality were removed using Agencourt AMPure XP (Beckman Coulter, Brea, USA) according to the manufacturer's instructions.

Prior to PCR, DNA samples were diluted to 5 ng/μl and used as a template in PCR using primers, index and adaptor sequences as described earlier (Kubasova et al. 2017). The primers used allowed amplification of the V3/V4 region of eubacterial 16S rRNA genes. PCR amplification was performed using the KAPA Taq HotStart PCR Kit (Kapa Biosystems). In the next step the concentration of PCR products was determined spectrophotometrically, the DNA was diluted to 100 ng/μl and groups of 14 PCR products with different identification tag (MID) sequences were indexed with a Nextera XT Index Kit following the manufacturer's instructions (Illumina).

Sequencing was performed using MiSeq Reagent Kit v3 and MiSEQ 2000 according to the manufacturer's instructions (Illumina). The results were analysed using QIIME software (Caporaso et al. 2010). Quality trimming criteria were set to a value of 19 and no mismatch in the identification sequences. Reverse reads were shortened to a length of 250 bp and forward and reverse sequences were joined. Chimeric sequences were predicted by the slayer algorithm and excluded from subsequent analysis. The resulting sequences were then classified by RDP Seqmatch with an operational taxonomic units (OTU) discrimination level set to 97%. Only bacterial phyla and genera whose relative abundance was higher than 1% in at least one of the treatments were summarised. Bacterial diversity was calculated using Shannon's index and the Chao 1 index was used as species richness estimator.

2.8. Statistical analyses

The data were subjected to statistical analysis using the GraphPad Prism 5.04 (GraphPad Software, Inc., USA) and Statistica 12.6 (Dell, Inc., USA) software. The individual pig served as the experimental unit. The results were expressed as means ± SEM, mortality and DI as a number of occurrences. The normality of the data was tested with the D'Agostino and Pearson omnibus normality test and the homogeneity of variances using the Brown-Forsythe test. The effect of dietary treatments on performance parameters, biomarkers of oxidative stress, haematological and biochemical status, serum FA profile and faecal microbiota composition of piglets was analysed with one-way analyses of variance (ANOVA) in conjunction with the Tukey's multiple comparison test. The Kruskal-Wallis test in conjunction with the Dunn's multiple comparison test was used for nonparametric ANOVA. The dietary treatments (Control, Leonardite, Lignite) were included as fixed factor in the statistical model.For mortality and DI data, frequency of occurrence of the phenomena in experimental groups was counted and analysed using the Chi-squared test. Differences between means with $P < .05$ were accepted as being statistically significant. Differences between means with $.05 < P < .10$ were accepted as representing tendencies for differences.

3. Results

3.1. Diarrhoea and performance

The first signs of diarrhoea occurred in control as well as supplemented groups in the first week after weaning. On the 6th day, mushy to watery faeces were detected in 30–40% piglets of each group (Fig. 1). In faecal samples of scouring piglets, the presence of ETEC was confirmed. Although the onset of diarrhoea in control and supplemented groups was similar, the course in the following period differed significantly. From the second week after weaning, a significant decrease in the severity of diarrhoea, documented by significantly lower FS and DI, was observed in leonardite and lignite groups (Fig. 1). Mushy or watery scouring was seen in no more than three (20%) leonardite and four (27%) lignite piglets in particular days of the second and third week. Furthermore, nine (60%) leonardite and seven (46%) lignite piglets stayed clinically healthy without any signs of diarrhoea during the whole three-week period (Table 3). No mortality was observed in both supplemented groups. On the other hand, the course of diarrhoea in control group was very intense with a high incidence of scouring piglets and watery faeces, which were still observed during the third week after weaning (Fig. 1). Overall 13 (87%) control piglets were affected by diarrhoea during the experimental period (Table 3). Due to severe long-lasting diarrhoea with resultant dehydration, two control piglets died in the third week after weaning.

Post-weaning diarrhoea adversely affected growth performance of piglets (Table 3). In the first week after weaning, a loss of weight was seen in control group as well as supplemented groups. As expected, low FI was observed early after weaning. In the following period, leonardite and lignite piglets recovered and started to gain weight unlike the control group in which weight loss was still observed in seven (47%)

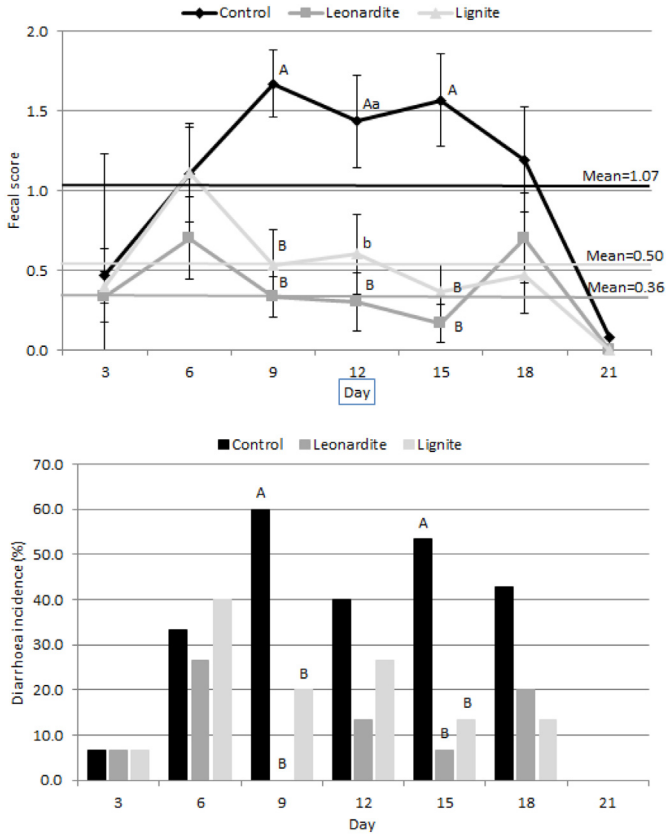

Fig. 1. Faecal score and diarrhoea incidence of weaned piglets. Treatments: Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite at a dose of 20 g/kg. Values are means ± SEM

[a,b] Values with different superscripts on the same day differ significantly ($P < .05$).

[A,B] Values with different superscripts on the same day differ significantly ($P < .01$).

piglets in the second week after weaning. Significantly higher BWG and a tendency to higher FI was found in leonardite and lignite groups compared to control group. Negative or high FCR values reflected poor health status and grow performance of piglets.

3.2. Biomarkers of oxidative stress

Supplementation of leonardite and lignite to weaned piglets had a beneficial effect on their oxidative status. Significantly lower or a tendency to lower serum biomarkers levels were found in supplemented groups in comparison with control group (Table 4).

3.3. Haematological and biochemical status

Good health status of supplemented piglets was also documented by detected levels of haematological and biochemical parameters at the end of the three-week supplementation. The results are presented in Table 5. Compared to control piglets, significantly higher haematocrit and haemoglobin levels and a tendency to higher erythrocyte count were found in leonardite and lignite groups. No significant effect on total leukocyte count and differential blood count was found except significantly higher monocytes in lignite piglets.

In serum of control group, significantly higher globulin and hence total protein levels were found in comparison to leonardite and lignite groups. Supplemented groups had or tended to have lower urea and higher total cholesterol levels in serum. Although there was a tendency

Table 3
Performance, morbidity and mortality of piglets during 21 days after weaning.

Parameter	Period	Treatments[1]			SEM	*P*-value
		Control	Leonardite	Lignite		
Body weight (kg)	Day 1	9.57	9.70	9.47	0.16	0.84
	Day 21	9.44^{A}	11.57^{B}	10.54	0.28	0.01
Body weight gain (g/day)	1st week	−73.9	−40.6	−138.3	17.05	0.06
	2nd week	-17.2^{a}	127.2^{b}	107.8^{b}	21.81	0.01
	3rd week	68.3^{A}	225.0^{B}	208.9^{B}	20.34	< 0.01
Feed intake (g/pig/day)	1st week	138.9^{A}	185.2^{B}	166.7	4.25	0.01
	2nd week	194.4	251.3	207.5	14.43	0.10
	3rd week	346.9	595.2	484.7	44.10	0.06
Feed conversion ratio	1st week	−1.9	−4.6	−1.2	–	–
	2nd week	−11.3	2.0	1.9	–	–
	3rd week	5.1	2.6	2.3	–	–
Diarrhoea affected piglets[2]	overall	13^{a}	6^{b}	8	–	0.02
Mortality (pig)	overall	2	0	0	–	0.48

Values with different superscripts in the same row differ significantly ($P < .05$).

Values with different superscripts in the same row differ significantly ($P < .01$).

[1] Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite a dose of 20 g/kg.

[2] Piglets with clinical signs of diarrhoea (mushy or watery faecal consistency).

Table 4
Biomarkers of oxidative stress in piglet serum on day 21 after weaning.

Compound (pg/ml)	Treatments[1]			SEM	*P*-value
	Control	Leonardite	Lignite		
8-isoPGF$_{2\alpha}$	120.46	54.31	47.05	15.62	0.09
8-isoPGE$_2$	23.88^{a}	9.83^{b}	11.46	2.52	0.03

Means with different superscripts in the same row differ significantly ($P < .05$).

[1] Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite at a dose of 20 g/kg.

to higher proportions of HDL and LDL in the blood of control group, their ratio (HDL:LDL) was not affected by any treatment. Increased levels of serum minerals (calcium, phosphorus and magnesium) were detected in leonardite group. Both supplemented groups tended to have higher serum iron level.

3.4. Serum FA profile

FA composition in serum of piglets at the end of the three-week supplementation is shown in Table 6. In lignite group, significantly higher proportion of palmitic FA was detected and even though other saturated FA (SFA), e.g. stearic, arachidic, behemic and lignoceric acids were significantly decreased or not affected, total SFA in serum was significantly higher compared to control as well as leonardite group. None or non-significant effect of leonardite on specific SFA was found compared to control piglets.

Lignite supplementation significantly decreased the proportion of unsaturated FA (USFA) in serum of piglets. This effect was also not observed after leonardite supplementation.

Both supplemented groups had (leonardite) or tended to have (lignite) a lower proportion of polyunsaturated FA (PUFA) in serum, although different effects on the family of n-3 and n-6 PUFA and their ratio were detected. Supplementation of leonardite significantly decreased the proportion of n-6 (particularly linoleic, gamma-linolenic

Table 5
Haematological and biochemical parameters of piglets on day 21 after weaning.

Parameter	Treatments[1]			SEM	*P*-value
	Control	Leonardite	Lignite		
Haematocrit (%)	21.87^{Aa}	29.66^{B}	27.63^{b}	0.89	< 0.01
Haemoglobin (g/l)	73.38^{A}	83.86^{B}	78.00	1.24	< 0.01
Erythrocytes ($\times 10^{12}$/l)	5.75	7.23	6.90	0.23	0.08
Leukocytes ($\times 10^{9}$/l)	17.25	15.59	15.33	0.66	0.46
Lymphocytes (%)	62.65	60.46	69.20	1.84	0.12
Neutrophils (%)	34.69	38.12	29.00	1.78	0.11
Band neutrophils (%)	2.04	0.39	0.57	0.36	0.16
Monocytes (%)	0.19	0.21^{a}	0.54^{b}	0.09	0.02
Eosinophils (%)	0.31	0.61	0.43	0.08	0.31
Basophils (%)	0.12	0.14	0.17	0.03	0.84
Total protein (g/l)	44.13^{A}	36.18^{B}	38.50^{B}	0.92	< 0.01
Albumin (g/l)	27.78	25.98	26.07	0.40	0.15
Globulin (g/l)	16.35^{Aa}	10.20^{B}	12.43^{b}	0.68	< 0.01
Urea (mmol/l)	4.92^{A}	3.86	3.33^{B}	0.21	0.01
Creatinine (μmol/l)	84.5	86.1	90.8	2.50	0.56
Glucose (mmol/l)	2.00	2.02	1.88	0.11	0.86
ALT (μkat/l)	0.52	0.50	0.44	0.02	0.18
AST (μkat/l)	0.57	0.65	0.69	0.05	0.38
ALP (μkat/l)	2.46	2.70	2.58	0.17	0.95
Triacylglycerols (mmol/l)	0.57	0.55	0.66	0.06	0.42
Cholesterol (mmol/l)	2.00^{a}	2.40^{b}	2.39	0.07	0.03
HDL (mmol/l)	1.09	0.98	0.93	0.03	0.06
LDL (mmol/l)	1.03	0.89	0.90	0.03	0.10
Calcium (mmol/l)	2.57	2.64^{a}	2.52^{b}	0.02	0.04
Magnesium (mmol/l)	0.71^{a}	0.84^{bB}	0.66^{A}	0.02	< 0.01
Phosphorus (mmol/l)	2.23^{A}	2.67^{B}	2.58	0.07	0.02
Iron (μmol/l)	4.11	11.2	6.17	1.07	0.06

Means with different superscripts in the same row differ significantly (P < .05).
Means with different superscripts in the same row differ significantly (P < .01).
[1] Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite at a dose of 20 g/kg.

and arachidonic) and increased n-3 (particularly docosahexaenoic) compared to control and lignite group. Consequently, a significantly lower serum n-6/n-3 ratio was detected in leonardite group.

The lignite group also was, or tended to be, lower in some specific serum n-6 PUFA (e.g. linoleic, gamma-linolenic, ardenic) but, unlike leonardite, it was significantly higher in serum arachidonic FA. Likewise, lignite group tended to be higher in n-3 docosahexaenoic PUFA, but it was lower in other n-3 (e.g. linolenic, eicosapentaenoic, docosapentaenoic). Therefore, serum n-6/n-3 ratio was significantly higher.

3.5. Faecal microbiota composition

The relative abundance of the most frequently identified bacterial phyla and genera and the characteristics of microbiota diversity in faeces of control and supplemented groups are shown in Table 7. Regardless of treatment, the faecal bacterial community was predominantly comprised of phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria.* The relative abundance of *Bacteroidetes* and *Proteobacteria* did not differ between treatments, but lower abundance of *Firmicutes* was detected in the supplemented groups.

At the genus level, *Prevotella* was the most abundant genus (comprising the majority in phylum *Bacteroidetes)* with a tendency to higher abundance in supplemented groups compared to control one. On the other hand, significantly lower abundance of *Bacteroides* and genera belonging to *Firmicutes* phylum*: Anaerovibrio*, *Oscillospira*, SMB53 (belonging to *Clostridiaceae* family) and *Ruminococcus* was found in the supplemented groups. No significant difference between leonardite and lignite groups was found either at phylum level or at genus level.

Table 6
Fatty acid composition in the serum of piglets on day 21 after weaning.

Fatty acid (%)	Structure	Treatments[1]			SEM	*P*-value
		Control	Leonardite	Lignite		
Lauric	C12:0	0.02	0.03	0.02	0.01	0.43
Myristic	C14:0	0.50	0.51	0.51	0.01	0.83
Pentadecanoic	C15:0	0.36	0.38	0.23	0.03	0.16
Palmitic	C16:0	11.16^{A}	12.47	17.49^{B}	0.80	< 0.01
Cis-7 hexadecenoic	C16:1 n-9	0.67^{A}	0.64^{A}	0.46^{B}	0.03	< 0.01
Palmitoleic	C16:1	2.03^{A}	2.36^{A}	1.35^{B}	0.12	< 0.01
Heptadecanoic	C17:0	2.24	1.68	1.71	0.22	0.54
Heptadecenoic	C17:1 n-7	0.36	0.35	0.32	0.03	0.90
Stearic	C18:0	16.32	16.53^{a}	14.11^{b}	0.36	0.01
Vaccenic	C18:1 n-11	0.17	0.10	0.12	0.03	0.65
Oleic	C18:1 n-9	19.10	20.06	18.06	0.40	0.14
Cis-vaccenic	C18:1 n-7	2.75	3.15^{a}	2.66^{b}	0.08	0.02
Linoleic	C18:2 n-6	17.93^{a}	15.72^{b}	16.19	0.36	0.02
Gamma-Linolenic	C18:3 n-6	0.53	0.58^{a}	0.35^{b}	0.04	0.02
Linolenic	C18:3 n-3	1.35	1.35	1.19	0.03	0.11
Nonadecylic	C19:0	0.10	0.07	0.88	0.35	0.83
Arachidic	C20:0	0.25^{A}	0.21^{a}	0.14^{Bb}	0.02	< 0.01
Eicosenoic	C20:1 n-9	0.20^{A}	0.21^{A}	0.14^{B}	0.01	< 0.01
Eicosadienoic	C20:2 n-6	0.28	0.27	0.27	0.01	0.83
Mead	C20:3 n-9	0.86	0.81	0.71	0.04	0.21
Dihomo-gamma-linolenic	C20:3 n-6	0.60	0.49	0.50	0.03	0.74
Arachidonic	C20:4 n-6	12.55	11.26^{a}	14.53^{b}	0.52	0.02
Eicosatrienoic	C20:3 n-3	0.10	0.04	0.06	0.01	0.33
Eicosapentaenoic	C20:5 n-3	1.82	1.79	1.46	0.08	0.09
Behemic	C22:0	0.19^{A}	0.16^{a}	0.08^{Bb}	0.02	0.01
Erucic	C22:1 n-9	0.08	0.06	0.04	0.01	0.16
Adrenic	C22:4 n-6	1.23^{a}	1.09	0.79^{b}	0.07	0.02
Docosapentaenoic	C22:5 n-3	2.52	2.02	1.76	0.14	0.09
Lignoceric	C24:0	0.17^{A}	0.13^{A}	0.05^{B}	0.01	< 0.01
Nervonic	C24:1 n-9	0.74	0.73	0.55	0.04	0.06
Docosahexaenoic	C22:6 n-3	2.78^{a}	4.72^{b}	3.19	0.04	0.03
Others fatty acids[2]		0.04	0.03	0.08	0.01	0.36
SFA[3]		31.31^{A}	32.17^{a}	35.22^{Bb}	0.56	< 0.01
USFA[4]		68.65^{A}	67.80^{A}	64.70^{B}	0.53	< 0.01
MUFA[5]		26.10	27.66^{a}	23.70^{b}	0.59	0.02
PUFA[6]		42.55^{a}	40.14^{b}	41.00	0.37	0.05
PUFA[6] n-6		33.12^{a}	29.41^{b}	32.63	0.62	0.02
PUFA[6] n-3		8.57	9.92^{a}	7.66^{b}	0.35	0.01
PUFA[6] n-6/n-3		3.86	2.96^{a}	4.26^{b}	0.22	0.01

Means with different superscripts in the same row differ significantly (*P* < .05).
Means with different superscripts in the same row differ significantly (*P* < .01).
[1] Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite at a dose of 20 g/kg.
[2] Others fatty acids = fatty acids whose concentration was lower than 0.02%.
[3] SFA = saturated fatty acids.
[4] USFA = unsaturated fatty acids.
[5] MUFA = monounsaturated fatty acids.
[6] PUFA = polyunsaturated fatty acids.

Compared to control piglets, supplemented groups had (lignite) or tended to have (leonardite) a lower microbial diversity and richness at the genus level measured by Shannon's index and Chao 1 estimator, respectively.

4. Discussion

Although veterinary pharmaceuticals containing HS have worked well in the treatment of digestive disorders and diarrhoea in pet animals (Kuhnert et al. 1991), scientific data on the use of HS in the prophylaxis of diarrhoea in piglets are still missing. The present results indicate that natural humic materials leonardite and lignite added to the diet cannot

Table 7
Mean percent relative abundance (%) of the most frequently identified[1] bacterial phyla and genera and characteristics of microbiota diversity in faeces of the piglets.

	Treatments[2]			SEM	*P*-value
	Control	Leonardite	Lignite		
Phylum					
Bacteroidetes	63.19	68.65	75.04	2.94	0.21
Firmicutes	30.01[a]	17.85	18.06[b]	2.51	0.04
Proteobacteria	5.77	13.02	6.53	1.73	0.26
Others	1.03[a]	0.49	0.36[b]	0.12	0.02
Shannon's index	0.84	0.82	0.70	0.05	0.52
Chao1 index	15.3	11.5	10.6	1.16	0.29
Genus					
Prevotella	50.11	63.56	69.05	3.77	0.06
Bacteroides	4.24[a]	0.01[b]	0.02[b]	0.81	0.03
Lactobacillus	3.93	1.35	3.92	0.63	0.18
[Prevotella]	3.04	2.75	2.90	0.27	0.92
Anaerovibrio	2.69[a]	0.98	0.70[b]	0.38	0.03
Oscillospira	2.48[a]	0.97	0.90[b]	0.28	0.03
Escherichia	2.20	1.88	1.07	0.75	0.65
Succinivibrio	2.14	10.24	4.86	1.59	0.16
Megasphaera	1.87	0.81	2.30	0.42	0.06
SMB53	1.46[a]	0.01	0.00[b]	0.27	0.02
Roseburia	1.32	3.54	1.50	0.51	0.56
Ruminococcus	1.31[a]	0.33	0.28[b]	0.18	0.03
Phascolarctobacterium	1.11	0.68	0.75	0.10	0.23
Faecalibacterium	0.95	1.70	1.08	0.28	0.74
Mitsuokella	0.40	1.35	0.64	0.20	0.18
Others	20.76[a]	9.82[b]	10.02[b]	2.04	0.01
Shannon's index	1.89[a]	1.41	1.24[b]	0.12	0.04
Chao1 index	106.2[a]	86.5	62.8[b]	7.93	0.03

Means with different superscripts in the same row differ significantly ($P < .05$).

[1] Only bacterial phyla and genera whose relative abundance was higher than 1% in at least one of the treatments were summarised.

[2] Control = non-granulated basal diet without antibiotics and medication; Leonardite = basal diet supplemented with leonardite at a dose of 20 g/kg; Lignite = basal diet supplemented with lignite at a dose of 20 g/kg.

completely prevent diarrhoea in piglets after weaning, but they can significantly reduce its severity and related mortality, enhance feed intake and promote piglet growth in the post-weaning period. The content of HS and their individual fractions may to a certain extent determine the effectiveness of different humic materials. Leonardite with a considerably higher amount of HS showed a slightly more pronounced effect than lignite, although the differences were not statistically significant.

The response to HS treatment may also depend on the severity of diarrhoeal disease. In our previous study (Trckova et al. 2015), sodium humate, the product made from leonardite by extraction of the present humic acids, was investigated in piglets challenged by a high infectious dose of ETEC, which induced severe watery diarrhoea and high mortality. The results indicated that in the case of heavy diarrheal infection, sodium humate with a high content of HS did not have a sufficient therapeutic effect and had to be combined with a specific dose of zinc oxide. Anyway, in the previous study the possibility of partial replacement of a high therapeutic dose of zinc oxide with sodium humate in treatment of serious diarrhoeal ETEC infection was confirmed.

HS have been reported to improve pig performance (Bai et al. 2013; Ji et al. 2006; Kim et al. 2004; Wang et al. 2008), even though the actual mechanism has not been fully understood. It is supposed that the high biological activity of HS is related to their pronounced affinity for biological membranes and participation in ion transport (Pena-Mendez et al., 2005; Wershaw 1989). The improved BWG after HS administration is usually associated with the protection of the intestinal mucosa, improving its morphology, stabilisation of the intestinal microbiota, increased activity of several enzymes and the subsequent improvement in nutrient digestion and utilization, especially protein digestion and trace element utilization (Chang et al. 2014; Islam et al. 2005; Kunavue and Lien 2012; Wang et al. 2008; Yasar et al. 2002). The present results concerning improved piglet performance after HS treatment are in agreement with results of previous studies, although they cannot be exactly comparable, because they were obtained from growing and finishing pigs or clinically healthy piglets. We suppose that better growth rates of leonardite and lignite supplemented piglets observed in the present study resulted in particular from maintenance of gut health.

Weaning is one of the most stressful periods accompanied by the overproduction of reactive-oxygen species (ROS) inducing oxidative stress in piglets. Oxidative stress is connected with alterations in physiology of animals resulting in immunosuppression and poor nutrient digestibility and growth performance (Yin et al. 2014). Isoprostanes, mainly 8-isoPGF$_{2\alpha}$ and 8-isoPGE$_2$, formed by non-enzymatic free radical mediated peroxidation of arachidonic acid, have been suggested as most reliable, sensitive and noninvasive biomarkers of oxidative stress in animals and humans (Basu 2007; Niki 2014).

Differing effects of HS on oxidative stress and induction of lipid peroxidation have been described previously. Khil'ko et al. (2011) demonstrated that HS extracted from brown coal were an effective inhibitor of radical-chain oxidation in vitro. Bai et al. (2013) detected a reduction of lipid peroxidation in meat from pigs supplemented with HS. Results of Weber et al. (2014) suggested the potential for HS to mitigate some oxidative stress in weaned piglets. On the contrary, Ipek et al. (2008) found that high levels of HS increased oxidative stress in Japanese quails, whilst lower levels did not. According to some studies, long-term exposure to HS in humans induced the generation of ROS and promoted oxidative stress and lipid peroxidation in both a dose- and time-dependent manner (Ho et al. 2003; Qi et al. 2008; Peng et al., 1999). The inconsistent results may be due to a large variability in structure and redox properties of HS. It is thought that quinones present in macromolecules of HS can cause the formation of ROS which promote oxidative stress and lipid peroxidation. On the other hand, oxygen-containing functional groups, mainly carboxylic and phenolic groups, are responsible for antioxidant properties (de Melo et al. 2016; Khil'ko et al. 2011).

In the present study, supplementation of leonardite and lignite to weaned piglets had a beneficial effect on their oxidative status. The results are consistent with our previous study in which sodium humate supplemented to the diet as a partial replacement of zinc oxide in prophylaxis of post-weaning diarrhoea decreased the level of 8-isoPGF$_{2\alpha}$ in serum of weaned piglets (Trckova et al. 2017).

Literature data point out that HS can influence blood parameters in animals. Dietary HS appeared to be responsible for an increase in haematocrit, haemoglobin and erythrocyte in poultry, rabbits and rats (Banaszkiewicz & Drobnik 1994; Cetin et al. 2006; Ipek et al. 2008; Mista et al. 2012). Kim et al. (2004) observed significantly increased erythrocyte counts in pigs supplemented with HS. Non-significantly higher haematocrit, haemoglobin and erythrocyte were found in weaned piglets treated with sodium humate (Trckova et al. 2015). The increase in haematocrit and haemoglobin levels and erythrocyte counts induced by leonardite and lignite supplementation in the present study may also be connected with improved growth performance of supplemented piglets. A positive correlation between these parameters and BWG of piglets in the three-week post-weaning period was found previously (Bhattarai and Nielsen 2015). Higher haematocrit and haemoglobin levels in supplemented groups as well match up with the detected tendency to higher serum iron levels. Haematocrit and haemoglobin both reflect the amount of functioning iron in the body and have been used diagnostically for monitoring the iron status of pigs.

Increased serum globulin in the absence of increased serum albumin was found in control group. It usually results from an increased synthesis of alpha or/and beta globulin, for example during acute or chronic inflammatory processes in the body or antigenic stimulation.

Increased blood protein levels are also usually seen in dehydration states due to inadequate water intake or excessive water loss, e.g. during diarrhoea.

A trend towards a decrease in blood urea nitrogen after HS dietary supplementation was found previously in piglets (Trckova et al. 2017) and laying hens (Rath et al. 2006). It is being explained by improved protein digestion and utilization caused by HS (Islam et al. 2005; Tohid et al. 2010). Higher serum urea concentration in control group without alterations in creatinine levels (low molecular weight nitrogenous substance) could also be a sign of intense protein catabolism which increases during reduced dietary intake and fasting. It is also possible that insufficient dietary intake could reduce total serum cholesterol level in control piglets.

HS are known to act as a strong chelator responsible for chelating minerals and placing them in a chemical state that is readily absorbed by living cells. Chelating ability of HS arising from the presence of many reactive functional groups (mainly carboxyl and hydroxyl) located in the structure may affect the bioavailability of minerals in the body (Ipek et al. 2008; Islam et al. 2005). In addition, HS contain some minerals, among which iron is most abundant, but their bioavailability to piglets is not known. Only Kim et al. (2004) reported the relative bioavailability of iron in HS to pigs as 71% of iron sulphate.

In the present study, increased levels of serum minerals (calcium, phosphorus and magnesium) were detected in leonardite group. This effect was not found in lignite except a tendency to higher serum iron after both treatments. The observed different effects of leonardite and lignite supplementation on mineral utilization can be attributed to the different contents of HS and their fractions in supplemented materials. Leonardite, as a highly oxidized form of humic materials, contained considerably higher amounts of HS than lignite and therefore its supportive effect on mineral utilization could be more profound. It is not clear if serum mineral levels could also be affected by additional intake and utilization of minerals contained in leonardite, but there was no relationship between higher amounts of magnesium and phosphorus in lignite and their serum levels in lignite supplemented piglets. Moreover, significantly lower serum magnesium was even detected in the lignite group compared to the control and leonardite group.

The heterogeneity in composition and structure of HS obtained from different sources and deposits may be a reason of inconsistent results in previous studies. Stepchenko et al. (1991) observed increased levels of some essential minerals (calcium, iron, aluminium) in serum of broiler chickens after HS feeding. Ipek et al. (2008) and Mista et al. (2012) found a significant increase in plasma iron in quails and rabbits and Celik et al. (2008) a non-significant increase of serum iron in broiler chickens when HS was administered. On the contrary, HS were reported to reduce serum minerals (calcium, phosphorus, magnesium and iron) in broilers (Celik et al. 2008; Demeterova et al. 2009; Rath et al. 2006; Samudovska and Demeterova 2010) and rabbits (Mista et al. 2012).

FA are the major energy source, important components of the cell membrane, metabolic substrates in many biochemical pathways, and play a critical role as immune modulators. Nutrition, biosynthesis, lipogenesis and remodelling are the main sources of lipids and FA in the body. HS have been reported to influence FA composition in meat of finishing pigs (Wang et al. 2008) and eggs of layers (Macit et al. 2009). The mechanism whereby HS affect the FA composition is largely unknown. In general, HS contain negligible amounts of lipids, FA, and saccharides that could be involved in metabolism and synthesis of FA. However, their proportion is minimal and it does not alter FA content and composition in supplemented diet (Trckova et al. 2017). Based on the previous findings, it may be assumed that HS may influence lipid digestion and utilization, the activity of enzymes involved in lipid metabolism (Chang et al. 2014), and thus affect metabolic processes related to FA profile in the body. In the present study, both leonardite and lignite supplementation influenced the serum FA profile in piglets, but their effects were different.

To the best of the authors' knowledge, there is currently a lack of information for discussing the present results. However, interestingly, a significant increase in the proportion of SFA and PUFA n-3 and a decrease in PUFA n-6 and PUFA n-6/n-3 ratio in serum of weaned piglets was also found when sodium humate was supplemented to diet as a partial substitution of high-dose zinc oxide (Trckova et al. 2017). However, it was not clear whether the effect of either the low-dose of ZnO or the supplementation of humate, or a combined effect of both was responsible for the obtained results. Based on the present results, it seems that leonardite affected serum FA composition in a similar way as sodium humate obtained from leonardite by extraction of the present humic acids.

Significant changes in some SFA after HS supplementation were also found in the meat of finishing pigs (Wang et al. 2007). Significant increase in the proportion of myristic, palmitic and stearic SFA and a decrease in arachidic FA were found in lean and/or fat samples. USFA and the USFA/SFA ratio were increased in lean samples and decreased in fat samples. Macit et al. (2009) reported an increase in myristic acid and a decrease in stearic SFA in egg yolks of layers fed a HS-supplemented diet. Some MUFA (myristoleic, palmitoleic and heptadecenoic) were also increased, whereas PUFA n-3 and PUFA n-6 remained unchanged by HS dietary treatments.

The period after weaning is accompanied by large changes in the composition and activity of intestinal microbiota whose instability together with weaning stress are important factors in the development of post-weaning diarrhoea and other intestinal problems. It has been suggested that dietary HS may stabilize microbial population and influence it through affecting substrate preferences of various microbial groups (Islam et al. 2005; Shermer et al. 1998).

Although the investigation of faecal samples has obvious limitations when describing the microbial population of the anterior gastrointestinal tract, faeces are widely used because of their easy accessibility. However, the evaluation should take into account that the microbiota present in faeces does not necessarily reflect the specific features of that in the upper gastrointestinal tract, and different species may dominate in different parts of the gut.

Regardless of treatment, the faecal bacterial community was predominantly comprised of phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria,* which is in accordance with previous findings in weaned piglets (Kim et al. 2011; Pajarillo et al. 2014).

A marked shift from *Bacteroides* to *Prevotella* in piglets during weaning was observed previously by Pajarillo et al. (2014). The authors hypothesized that it was connected with a dietary change after weaning and the ability of *Prevotella* to degrade polysaccharides in plant-based feeds in comparison to *Bacteroides* which are able to utilize predominantly saccharides from sow's milk during the pre-weaning period. In the present study, higher intake of plant-based feed supplemented with leonardite or lignite compared to the control one may contribute to prompt development of gut microbiota in response to the dietary change after weaning.

The decrease in abundance of *Prevotella* and increase in abundance of *Oscillospira* was observed by Kohl et al. (2014) in caecal communities of various vertebrates (tilapia, quail and mice) during prolonged fasting. It was speculated that glycoside hydrolases of *Oscillospira* allow them to forage on host-produced glycans in times of nutrient deprivation. Thus, decreased feed intake and nutrient loss in piglets affected by diarrhoea may participate in higher abundance of *Oscillospira* in the control group. However, contrary to our results, Kohl et al. (2014) also observed a decline in the abundance of *Ruminococcus* during fasting because of a lack of dietary substrates for these fibre-degrading bacteria.

As Kohl et al. (2014) demonstrated, fasting may also cause a higher phylogenetic diversity of microbial communities in the colon. In the present study, higher microbial diversity and richness at the genus level measured by Shannon's index and Chao 1 estimator was found in control piglets with low FI.

In general, higher bacterial diversity and richness is linked to the

enhancement of metabolic functions, stabilisation of the bacterial gut community and reduction of pathogen proliferation, which is beneficial to health and performance of host animals (Roca et al. 2014). However, the inclusion of antibiotics or feed additives with antimicrobial properties (organic acids, plant extracts) in the diet caused a decrease in microbial diversity and this effect coincided with improved growth performance in weaned piglets (Namkung et al. 2004; Roca et al. 2014).

HS have been shown to inhibit the growth of some bacterial groups in vitro and conversely, stimulate others (Tikhonov et al. 2010; van Rensburg et al. 2000). However, there are only limited data about the effect of dietary HS on gut microbial population in animals, specifically in monogastric animals. The study conducted with healthy volunteers orally supplemented with humic acids for 45 days showed an increase in colonic microbiota sum concentration in each person due to the growth of pre-existing groups, while the individual microbial profile and bacterial diversity remained unchanged. The concentration of pioneer groups of *Bifidobacteriaceae, Enterobacteriaceae* and *Clostridium difficile* increased, but the observed differences were not significant (Swidsinski et al. 2017). In chicken, dietary HS were shown to reduce *E. coli* counts and increase beneficial lactobacilli in the intestinal digesta (Aksu and Bozkurt 2009; Shermer et al. 1998). Kaevska et al. (2016) observed similar changes in faecal microbiota in ETEC challenged weaned piglets treated with sodium humate and ZnO. In the present study, no significant effect of leonardite or lignite on the abundance of *Escherichia* and *Lactobacillus* in piglets was observed.

5. Conclusion

Natural humic materials – leonardite and lignite cannot completely prevent diarrhoea in piglets after weaning but they may significantly reduce its severity and related mortality, decrease oxidative stress, enhance feed intake and promote growth in the post-weaning period. This may also involve enhanced haematological parameters associated with the amount of functioning iron in the body, serum minerals, or even the serum n-6/n-3 ratio, and prompt the development of new microbiota in response to the dietary change after weaning. However, an important issue to consider about the supplementation of natural humic materials is the content of HS, their fractions as well as minerals. Leonardite as a highly oxidized form of humic materials with a considerably higher amount of HS may promote more substantial effect than lignite.

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