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Effects of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on the health, oxidative stress status and fatty acid profile in weaned piglets

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ABSTRACT: The aim of this study was to investigate the effects of sodium humate (HNa), applied to feed or drinking water as a partial substitution for pharmacological doses of ZnO, on clinical, biochemical and haematological indicators of health status, oxidative stress and the serum fatty acid (FA) profile in weaned piglets. Weaned piglets (32) were allocated into four groups: Control = basal diet containing 110 mg ZnO/kg; ZnO 2.5 = pharmacological dose of ZnO (2.5 g ZnO/kg); ZnO 1.7 + HNa (f) = 1.7 g ZnO and 20.0 g HNa/kg; ZnO 1.7 + HNa (w) = 1.7 g ZnO/kg and drinking water with 0.2% HNa. All ZnO treatments resulted in good performance and clinical health of piglets in contrast to Control, which contained three diarrhoeic piglets. Increased triacylglycerols in the ZnO 2.5 and ZnO 1.7 + HNa (w) groups in comparison with Control suggested increased energy metabolism after treatments. Neither total cholesterol, nor HDL and LDL were affected by treatments. Piglets treated with ZnO and HNa had (ZnO 1.7 + HNa (w)) or tended to have (ZnO 1.7 + HNa (f)) lower urea in serum. Significantly (in ZnO 2.5 and ZnO 1.7 + HNa (f)) or non-significantly (ZnO 1.7 + HNa (w)) higher haematocrit and haemoglobin levels were detected in the blood of treated piglets. Long-term ZnO 2.5 treatment significantly increased serum 8-*iso*-$PGF_{2\alpha}$, the most reliable biomarker of oxidative stress. Partial substitution of ZnO by HNa positively affected the oxidative status of piglets as evidenced by significant (ZnO 1.7 + HNa (w)) or non-significant (ZnO 1.7 + HNa (f)) declines in serum 8-*iso*-$PGF_{2\alpha}$. ZnO 2.5 treatment significantly decreased saturated (SFA), monounsaturated (MUFA) and n-3 polyunsaturated FA (PUFA) and increased PUFA n-6 and n-6/n-3 ratios in serum compared to Control. The synthesis of some physiologically significant long-chain PUFA (LC-PUFA), namely n-6 dihomo-γ-linolenic acid and n-3 eicosapentaenoic, docosapentaenoic was negatively affected by ZnO 2.5 treatment. Both ZnO 1.7 + HNa (f)/(w) treatments significantly increased the proportion of SFA and PUFA n-3 and decreased PUFA n-6 and n-6/n-3 ratios in comparison with ZnO 2.5 and the effect was more considerable in ZnO 1.7 + HNa (w). Most individual PUFA n-3 and n-6 as well as the n-6/n-3 ratio in both treatments were similar to Control. The results indicate that the partial substitution of ZnO by HNa can benefit performance and health of weaned piglets to a similar extent as a high pharmacological dose of ZnO, whereas it can decrease the oxidative stress induced by prolonged over-supplementation of ZnO. Additionally, such a treatment can eliminate the unfavourable effect of high ZnO doses on the n-6/n-3 ratio and the proportion of some physiologically significant LC-PUFA in serum. Generally, it can be concluded that the effects of feed and water HNa supplementation are similar, but are more pronounced when HNa is applied to drinking water.

Keywords: humic substances; pig; lipid metabolism; eicosanoids; serum biochemistry; haematology; performance; GC/MS; LC/MS-MS

List of abbreviations

AA = arachidonic acid, **ALA** = α-linolenic acid, **ALP** = alkaline phosphatase, **ALT** = alanine transaminase, **AST** = aspartate aminotransferase, **BWG** = body weight gain, **DGLA** = dihomo-γ-linolenic acid, **DHA** = docosahexaenoic acid, **DPA** = docosapentaenoic acid, **EPA** = eicosapentaenoic acid, **ETEC** = enterotoxigenic *Escherichia coli*, **FA** = fatty acids, **FAME** = fatty acid methyl esters, **FCR** = feed conversion ratio, **FI** = feed intake, **GC/MS** = gas

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chromatography/mass spectrometry, **HA** = humic acid, **HCT** = haematocrit, **HDL** = high-density lipoprotein, **HGB** = haemoglobin, **HNa** = sodium humate, **HPLC** = high performance liquid chromatography, **HS** = humic substances, **LA** = linoleic acid, **LC-MS/MS** = liquid chromatography/tandem mass spectrometry, **LC-PUFA** = long-chain polyunsaturated fatty acid, **LDL** = low-density lipoprotein, **MUFA** = monounsaturated fatty acids, **OA** = oleic acid, **PA** = palmitic acid, **PUFA** = polyunsaturated fatty acids, **ROS** = reactive oxygen species, **SFA** = saturated fatty acids, **TGC** = triacylglycerols, **USFA** = unsaturated fatty acids

The beneficial effects of pharmacological doses of zinc oxide (ZnO) in piglets in the post-weaning period are well documented (Kim et al. 2012; Heo et al. 2013). In the last decades, the widespread use of high levels of ZnO has been criticised because of the subsequent high excretion of Zn into the environment. EFSA limits the maximum content of ZnO in complete feeding stuffs to 250 mg/kg for all animal species (EC Regulation 1831/2003), although the use of higher pharmacological doses is still permitted under veterinary prescription as the need arises. There are ongoing efforts aimed at finding effective alternatives, that could protect piglets from post-weaning diarrhoea and growth depression after weaning. In our previous studies (Trckova et al. 2015; Kaevska et al. 2016) we verified the possibility of the substitution of a pharmacological dose of ZnO (2.5 g/kg diet) by humic substances (HS). HS are natural bioactive agents primarily decomposed from organic matter by bacteria in the soil. They have been reported to improve performance and nutrient utilisation and show anti-bacterial, anti-viral and anti-inflammatory effects (Islam et al. 2005; Ji et al. 2006; Wang et al. 2008). EMEA (1999) has recommended humic acid (HA) and its sodium salt for the treatment of diarrhoea, dyspepsia and acute intoxications in animals. However, our previous results showed that in cases of severe post-weaning diarrhoeal infections caused by enterotoxigenic *Escherichia coli* strains (ETEC), supplementation of HS to piglets can be adequately effective only in combination with a specific dose of ZnO (1.7 g/kg; Trckova et al. 2015). Moreover, there is still a lack of data about other potential effects of this partial substitution of ZnO by HS on the young and vulnerable organism of weaning piglets.

Weaning is the most difficult period in a piglet's life and is associated with social stress and dietary changes. These can lead to digestive disorders, which cause diarrhoea, and increased disease susceptibility resulting in economic losses in the post-weaning period (Metzler et al. 2005; Heo et al. 2013). Weaning stress, especially in early-weaned pigs, can also result in the suppression of antioxidant enzyme activity as well as in promotion of free radical production leading to the oxidative damage of lipids, proteins, and DNA (Zhu et al. 2012; Yin et al. 2014). This oxidative imbalance can result in immunosuppression with increased susceptibility to various diseases and in negative alterations in the growth performance of animals. Oxidative stress may also have a negative effect on meat quality and the shelf life of meat (Aksu et al. 2005; Bai et al. 2013).

In some reports, it was remarked that HS may have the potential to negate the effect of oxidative stress within the body (Ipek et al. 2008; Weber et al. 2014). On the other hand, HS, specifically HA, administered over a long period at high levels has been reported to induce damage in cells and tissues accompanied by an increase in oxidative stress and lipid peroxidation (Cheng et al. 1999; Varanka et al. 1999; Gau et al. 2001; Ho et al. 2003; Qi et al. 2008). HS are natural materials with variable composition, which may explain the varying efficacy described in the literature.

HS have been reported to influence saturated (SFA) and unsaturated fatty acid (USFA) concentrations and their ratios in the meat of finishing pigs (Wang et al. 2008). Nutrition, biosynthesis, lipogenesis and remodelling are the main sources of fatty acids (FA) and lipid composition in piglets. However, it can be supposed that the metabolism and biosynthesis of FA and lipids in young growing piglets could also be regulated in a different manner.

The aim of this study was to investigate the effects of sodium humate (HNa), applied to feed or drinking water as a partial substitution for pharmacological doses of ZnO, on clinical, biochemical and haematological indicators of health status, a biomarker of oxidative stress (serum 8-*iso*-$PGF_{2\alpha}$) and the serum FA profile in weaned piglets.

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MATERIAL AND METHODS

Animal management and dietary treatment. Testing of HNa and ZnO was performed on 32 weaned piglets [LW × (P × Du)] originating from a specific pathogen-free herd. Piglets were transported to the experimental animal facility of the Veterinary Research Institute, Brno, Czech Republic on the day of weaning (28th day of age). They were identified by individual ear tags and housed in indoor pens. 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).

Weaned piglets with an average initial body weight of 8.79 ± 1.31 kg were allocated into four groups. Each group was kept in one pen with eight piglets (with an equal sex ratio of four males and four females) per pen. Dietary treatments were as follows: Control = basal diet containing 110 mg ZnO/kg; ZnO 2.5 = basal diet supplemented with a pharmacological dose of ZnO (2.5 g ZnO/kg); ZnO 1.7 + HNa (f) = basal diet supplemented with 1.7 g ZnO and 20.0 g HNa/kg; ZnO 1.7 + HNa (w) = basal diet supplemented with 1.7 g ZnO/kg and drinking water supplemented with 0.2% HNa. The concentration of HNa in the drinking water was chosen so that the average daily intake of HNa per piglet by water was of approximately the same dose as the average daily intake of HNa by diet. The basal diet (Table 1) was formulated according to animal requirements (NRC 1998). HNa was made from oxyhumolite (Humatex, a.s., Bilina, Czech Republic) by neutralisation and extraction of the present humic acids with sodium hydroxide in aqueous medium. The chemical analysis revealed 93.9% dry matter, 34.3% ash, 52.4% HS, 15.2% HA and 37.2% fulvic acid in dry matter. Piglets were fed twice a day *ad libitum* and water was provided by automatic waterers. The dietary treatment was maintained for 21 days.

Clinical, biochemical and haematological indicators of health status. Piglets were observed daily for morbidity and mortality. Faecal consistency was recorded daily. To assess the general health status of piglets after long-term treatments, 10 ml samples of blood were drawn from the *vena cava cranialis* after 12-h fasting at the end of the trial for monitoring of the haematological and serum biochemical profile. From blood samples collected into heparinised tubes, haematocrit (HCT), haemoglobin (HGB), erythrocyte and leukocyte counts were determined using the Coulter Counter M4 apparatus (Coulter Cientifica S.A., Mostoles, Spain), whereas the differential leukocyte count in blood smears was determined using a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Serum was separated from blood samples by centrifugation at 4000 rpm for 20 min and stored at –20 °C until further analyses. In serum samples, glucose, total protein, albumin, triacylglycerol (TGC), cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), calcium, phosphorus,

Table 1. Composition of the diet for weaned piglets (as-fed basis)

Ingredient (g/kg)	Basal diet
Wheat	400.2
Barley	298.0
Soybean meal (47% CP)	186.0
Dry whey and soy protein concentrate	50.0
Soybean oil	23.0
Limestone, ground	12.0
Dicalcium phosphate	11.0
Salt	3.0
Sodium carbonate	1.0
L-Lysine HCl	0.2
L-Threonine	0.3
DL-Methionine	0.3
Vitamin and mineral premix[1]	15.0
Calculated 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
Ca (g/kg)	7.00
Na (g/kg)	1.90
P (g/kg)	4.55

[1]Provided per kg diet: 12 000 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 $Na_2SeO_3 \times 5\ H_2O$)

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magnesium, iron and zinc were measured using a BS200 automated chemistry analyser (Mindray, Shenzhen, China).

Piglets were weighed at the beginning (Day 1) and at the end (Day 21) of the trial. 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.

Biomarker of oxidative stress. Solid phase extraction was used for extraction of 8-*iso*-$PGF_{2\alpha}$ from blood serum. Select HLB SPE (1 ml, 30 mg) cartridges (Supelco, Prague, Czech Republic) were washed with 1 ml of ethyl acetate, 1 ml of methanol and 1 ml of water. Then, 0.5 ml of serum were loaded onto the SPE column. The columns were then washed with 2 ml of water. The cartridges were air dried for 3 min with vacuum and analytes were eluted with 1.5 ml of methanol. Samples were dried under a stream of nitrogen, dissolved in 60 µl of methanol and aliquots of 5 µl were injected into the high-performance liquid chromatography (HPLC) column.

Analysis of samples was performed using liquid chromatography/tandem mass spectrometry (LC-MS/MS). An Agilent 1200 chromatographic system (Agilent Technologies, Germany) was used. Separation of prostaglandins was carried out using a HPLC Ascentis Express C18 column (2.1 × 150 mm, 2.7 µm particle size, Supelco, Bellefonte, USA) maintained at 45 °C. Mobile phases consisted of 0.1% formic acid in water and acetonitrile. A 25-min linear gradient from 30 to 100% acetonitrile was used. The flow rate was 0.3 ml/min, and the injection volume was 5 µl. The HPLC system was coupled to an Agilent 6410 Triple Quad LC/MS triple quadrupole mass spectrometer (Agilent Technologies, USA). Electrospray ionisation was performed in negative ion mode with nitrogen as the nebulising and collision gas. 8-*iso*-$PGF_{2\alpha}$ was analysed using multiple reaction monitoring and mass transition; precursor and product ions were 353.2/193.1 *m*/*z*.

FA profile in diets and blood serum. The blood serum was protected from oxidation by the addition of butylated hydroxytoluene at a final concentration of 40 mg/ml and was stored at –80 °C. Samples were analysed within one month of collection. After protein precipitation, 0.5 ml of serum and about 1.0 g of diets and HNa were extracted according to a modified protocol of Bligh and Dyer (1959). Appropriate volumes of extracts were evaporated to dryness under a stream of nitrogen and a slightly modified method of Kang and Wang (2005) was used for the transesterification of FA from extracted lipids. Briefly, 20 µl of the internal standard solution (C23:0 FA, 80 µg/ml), 20 µl butylated hydroxytoluene (200 µg/ml), 0.5 ml of hexane and 0.5 ml of 10% BF_3 in methanol were added to the dry sample in a 4 ml silanised glass vial. Samples were vortexed, vials were capped under nitrogen and transferred to a heating block at 100 °C and heated for 60 min. Subsequently, the samples were cooled, uncapped, and neutralised by the addition of 1 ml of a 5% solution of K_2CO_3. The FA methyl esters (FAME) were then extracted twice with 1 ml of hexane. Combined extracts were evaporated to dryness under a stream of nitrogen and dissolved in 200 µl of 2,2,4-trimethylpentane. From the final samples, 1 µl was injected into the capillary column of a gas chromatography/mass spectrometry (GC/MS) system.

GC/MS analysis was based on determination of retention times and relative abundances of selected mass ions of FAME. GC separation was done in an Omegawax fused silica capillary column (30 m × 0.25 mm *i.d.*, 0.25 µm, Supelco, Bellefonte, USA). Helium at a column head pressure of 70 kPa was used as the carrier gas. An ion trap mass spectrometer Saturn 2100T (Varian, Walnut Creek, USA) was used for the detection and identification of analytes. The mass spectrometer was operated in electron ionisation and scan mode at an electron energy of 70 eV. Relative standard deviation for three parallel assays ranged from 1.8% (22:0) to 16.9% (20:4n3).

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.

Statistical analyses. All data were subjected to statistical analysis using the GraphPad Prism 5.04 (GraphPad, Inc., San Diego, USA) and Statistica 12.6 (Dell, Inc., Tulsa, USA) software. 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. When data passed the normality and homoscedasticity test, the statistical significance of the differences among the group means was determined by the analyses of variance (ANOVA) in conjunc-

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tion with the Tukey's multiple comparison test. Otherwise, the Kruskal-Wallis test in conjunction with the Dunn's multiple comparison test were used. Mortality and diarrhoea incidence data were analysed using the Chi-squared test. Differences between means with $P < 0.05$ were accepted as being statistically significant.

RESULTS AND DISCUSSION

Clinical, biochemical and haematological indicators of health status

All ZnO treatments, at pharmacological doses or at amounts supplemented with HNa in feed or drinking water, resulted in good health status of weaned piglets. None of the piglets from the treated groups expressed any clinical signs of illness over the course of the experimental period. In the untreated control group, clinical signs of diarrhoea (watery faeces) were recorded in three out of the eight piglets. Diarrhoea occurred on the second week after weaning (Day 8) and lasted for four to five days in individual piglets.

Partial substitution of ZnO by HNa resulted in good performance, which was comparable to pharmacological dosing with ZnO (Table 2). The BWG and FI of treated groups did not differ significantly; significantly higher ($P < 0.01$) BWG values were detected only in comparison with the untreated Control. Piglets from treated groups were also more efficient in converting feed to gain than Control. Differences in performance after HNa supplementation in feed and water were not significant, although there was a trend towards better BWG and FCR values in ZnO 1.7 + HNa (w).

The results are in accordance with previous findings that confirmed the positive effect of ZnO and HNa treatments on performance in weaned piglets (Trckova et al. 2015; Kaevska et al. 2016). ZnO is well known to enhance growth performance in piglets through the stabilisation of the intestinal microbiota and improvements in nutrient digestion and utilisation. HS may benefit animal performance even though the actual mechanism is not fully understood (Kim et al. 2004; Ji et al. 2006; Bai et al. 2013).

The high, pharmacological dose of ZnO as well as lower dose supplemented with HNa altered the serum chemistry of piglets, particularly the activity of liver enzymes (Table 3). Increases in liver enzyme activities after ZnO and HS dietary administration were reported previously (Poulsen 1995; Trckova et al. 2015). However, the increased levels of liver enzymes in treated piglets were found to be within the physiological ranges (Jackson and Cockcroft 2002) and they did not reflect any trend that would suggest any adverse effect of treatments.

Apart from the activity of liver enzymes, TGC, urea, Ca and Zn were also significantly affected by treatments. Significantly increased TGC in ZnO 2.5 and ZnO 1.7 + HNa (w) in comparison with Control suggested increased energy metabolism in piglets after treatments. This is in accordance with Zhang et al. (2014), who reported a dose-dependent increase in TGC with supplementation of dietary Zn. On the other hand, HS were

Table 2. Performance and diarrhoea occurrence in piglets

Parameter	Treatments				Main test	Post-hoc tests					
	Control	ZnO 2.5	ZnO 1.7 + HNa (f)	ZnO 1.7 + HNa (w)		1:2	1:3	1:4	2:3	2:4	3:4
Initial body weight (kg)	8.78 ± 1.43	8.81 ± 1.47	8.80 ± 1.28	8.74 ± 1.32	ns						
Body weight gain (g/day)	191.5 ± 0.03	286.8 ± 0.03	249.5 ± 0.04	271.9 ± 0.04	**	**	**	**	ns	ns	ns
Feed intake (g/pig/day)	453.5 ± 287.5	500.9 ± 327.5	495.8 ± 324.6	492.4 ± 304.8	ns						
Feed conversion ratio	2.37	1.75	1.99	1.81							
Mortality (piglets)	0	0	0	0	ns						
Diarrhoea incidence (piglets)	3	0	0	0	ns						
Duration of diarrhoea (days)	1.75	0	0	0	ns						

Control = basal diet containing 110 mg ZnO/kg, ZnO 2.5 = basal diet supplemented with 2.5 g ZnO/kg, ZnO 1.7 + HNa (f) = basal diet supplemented with 1.7 g ZnO and 20.0 g HNa/kg, ZnO 1.7 + HNa (w) = basal diet supplemented with 1.7 g ZnO/kg and drinking water supplemented with 0.2% HNa

**$P < 0.01$

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Table 3. Serum biochemical and haematological parameters of piglets on Day 21 after weaning

Parameter	Treatments				Main test	Post-hoc tests					
	Control	ZnO 2.5	ZnO 1.7 + HNa (f)	ZnO 1.7 + HNa (w)		1:2	1:3	1:4	2:3	2:4	3:4
Albumin (g/l)	26.45 ± 2.77	29.42 ± 1.66	28.57 ± 1.28	27.45 ± 2.40	ns						
Total protein (g/l)	44.59 ± 3.24	45.11 ± 4.87	44.97 ± 4.09	44.89 ± 5.63	ns						
Triacylglycerols (mmol/l)	0.28 ± 0.07	0.45 ± 0.06	0.30 ± 0.08	0.42 ± 0.11	**	*	ns	*	*	ns	ns
Cholesterol (mmol/l)	2.05 ± 0.16	2.26 ± 0.19	2.14 ± 0.30	2.26 ± 0.39	ns						
HDL (mmol/l)	1.33 ± 0.19	1.24 ± 0.10	1.16 ± 0.19	1.20 ± 0.21	ns						
LDL (mmol/l)	0.70 ± 0.11	0.86 ± 0.11	0.76 ± 0.15	0.81 ± 0.17	ns						
Creatinine (µmol/l)	95.68 ± 15.34	92.98 ± 6.40	87.88 ± 10.68	92.09 ± 6.55	ns						
Urea (mmol/l)	4.25 ± 1.39	3.60 ± 0.89	2.86 ± 1.21	2.50 ± 0.93	*	ns	ns	*	ns	ns	ns
aLT (µkat/l)	0.78 ± 0.07	1.02 ± 0.09	0.98 ± 0.12	0.90 ± 0.15	**	**	**	ns	ns	ns	ns
aST (µkat/l)	0.57 ± 0.09	0.62 ± 0.12	0.84 ± 0.11	0.72 ± 0.08	**	ns	**	*	**	ns	ns
aLP (µkat/l)	3.59 ± 1.02	8.13 ± 1.56	5.43 ± 0.91	4.56 ± 1.07	**	**	*	ns	**	**	ns
Ca (mmol/l)	2.73 ± 0.17	3.08 ± 0.20	2.84 ± 0.11	2.79 ± 0.19	**	**	ns	ns	ns	*	ns
P (mmol/l)	2.78 ± 0.24	2.72 ± 0.21	2.86 ± 0.37	2.88 ± 0.28	ns						
Mg (mmol/l)	1.06 ± 0.08	1.16 ± 0.12	1.09 ± 0.07	1.15 ± 0.06	ns						
Fe (µmol/l)	16.18 ± 9.67	22.74 ± 12.22	20.46 ± 4.20	28.08 ± 14.06	ns						
Zn (mmol/l)	14.18 ± 2.20	42.18 ± 7.01	29.62 ± 5.48	29.88 ± 7.35	**	**	*	*	ns	ns	ns
Erythrocytes ($\times 10^{12}$/l)	6.38 ± 0.66	7.07 ± 0.45	6.96 ± 0.30	6.40 ± 1.28	ns						
Hematocrit (%)	26.53 ± 2.72	33.00 ± 1.23	31.94 ± 1.91	29.15 ± 4.78	**	**	*	ns	ns	ns	ns
Haemoglobin (g/l)	76.25 ± 7.65	92.40 ± 4.77	90.75 ± 5.85	83.63 ± 11.59	**	*	*	ns	ns	ns	ns
Leukocytes ($\times 10^{9}$/l)	15.70 ± 3.78	14.94 ± 2.52	14.43 ± 2.10	17.40 ± 6.54	ns						
Lymphocytes (%)	55.50 ± 8.83	65.00 ± 13.40	59.25 ± 6.58	56.50 ± 13.66	ns						
Neutrophils (%)	43.19 ± 8.77	32.60 ± 13.61	39.19 ± 7.40	41.50 ± 14.47	ns						
Monocytes (%)	0.25 ± 0.38	0.60 ± 0.42	0.25 ± 0.46	0.19 ± 0.37	ns						
Eosinophils (%)	0.88 ± 0.74	1.70 ± 0.84	1.31 ± 1.00	1.56 ± 1.27	ns						
Basophils (%)	0.19 ± 0.26	0.10 ± 0.22	0.25 ± 0.27	0.25 ± 0.38	ns						

Control = basal diet containing 110 mg ZnO/kg, ZnO 2.5 = basal diet supplemented with 2.5 g ZnO/kg, ZnO 1.7 + HNa (f) = basal diet supplemented with 1.7 g ZnO and 20.0 g HNa/kg, ZnO 1.7 + HNa (w) = basal diet supplemented with 1.7 g ZnO/kg and drinking water supplemented with 0.2% HNa

*$P < 0.05$, **$P < 0.01$

described to decrease serum TGC and cholesterol in animals (Samudovska and Demeterova 2010; Mista et al. 2012; Ozturk et al. 2012). In our study, partial substitution of ZnO by HNa in feed resulted in significantly lower serum TGC in comparison with ZnO 2.5, but this was not observed when HNa was supplemented in water. This variability may be because of differences in the form and amount of HNa received in feed and water. Neither total cholesterol, nor HDL and LDL were affected by HNa supplementation.

Piglets treated with ZnO and HNa had (ZnO 1.7 + HNa (w)) or tended to have (ZnO 1.7 + HNa (f)) lower urea in serum. A trend towards a decrease in blood urea nitrogen after HS dietary supplementation was found previously in laying hens (Rath et al. 2006). Serum mineral levels (Ca, P, Mg and Fe) were not affected by ZnO + HNa treatments. HS appear to have metal-chelating effects and were reported to reduce levels of serum minerals in broilers (Rath et al. 2006; Celik et al. 2008; Samudovska and Demeterova 2010) and rabbits (Mista et al. 2012), although this effect was not observed with HNa in weaned piglets (Trckova et al. 2015). Significantly higher Zn levels in the serum of the ZnO 2.5 group reflected the higher Zn dietary intake in the group.

In the blood of treated piglets, significantly or non-significantly higher HCT and HGB were detected in comparison with Control (Table 2). This

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confirms the previous findings that dietary Zn appeared to be responsible for an increase of HCT and HGB in pigs (Rupic et al. 1998). The increase of HCT and HGB was also observed when HA was fed to broilers and rabbits (Ipek et al. 2008; Mista et al. 2012), although no data have as yet been reported from pigs. In our previous study, we observed non-significantly higher HCT and HGB when ZnO and HNa were fed to piglets challenged by *E. coli* (Trckova et al. 2015). Blood cell content was not affected by treatments.

Biomarker of oxidative stress

Isoprostanes are specific products of non-enzymatic free radical-catalysed peroxidation of arachidonic acid and bioactive F_2-isoprostanes (mainly 8-*iso*-$PGF_{2\alpha}$) detectable in tissues and body fluids were shown to be the most reliable *in vivo* biomarkers of lipid peroxidation and oxidative stress in animal and human studies (Kadiiska et al. 2005; Basu 2007; Basu 2008; Niki 2014). Quantification of F_2-isoprostanes in plasma or serum is a specific, reliable and non-invasive method used also for evaluation of various dietary antioxidants and other free radical scavengers (Basu 2007; Basu 2008).

In our study, significantly lower ($P < 0.05$) serum 8-*iso*-$PGF_{2\alpha}$ levels were found in ZnO 1.7 + HNa (w) (61 pg/ml) and Control (91 pg/ml) in comparison with ZnO 2.5 (454 pg/ml) after three weeks of dietary treatment. The levels in ZnO 1.7 + HNa (f) (132 pg/ml) were non-significantly lower compared to ZnO 2.5.

Zn is an essential trace element important for growth, immunity, and healing. It has also been shown to have antioxidant properties, although the mechanisms are not fully understood. Chronic Zn deprivation in animals generally results in increased free radical production and higher sensitivity to oxidative stress (Powell 2000). Wang et al. (2009) showed that high dietary Zn (3000 mg/kg as ZnO for 15 days) reduced levels of oxidative stress and prevented apoptosis in the jejunum of weaned piglets. Similarly, feeding 1000–2000 mg/kg Zn for 14 days was found to affect the expression of genes involved in reducing oxidative stress in newly weaning pigs (Martinez-Montemayor et al. 2008). Studies in poultry confirmed that dietary Zn improved antioxidant status and augmented the activity of enzymes (superoxide dismutase and glutathione peroxidase) involved in the antioxidant defence system (Sahin et al. 2005; Ma et al. 2011).

However, the results from our study do not suggest such an effect. The high pharmacological dose of ZnO fed to weaned piglets for 21 days seemed to negatively influence the oxidative status of piglets according to the levels of serum 8-*iso*-$PGF_{2\alpha}$. Some recent studies conducted in animals suggest that prolonged over-supplementation with dietary Zn can promote oxidative stress with adverse effects on growth performance. Our results are in accordance with Pieper et al. (2015) who found that long-term supplementation with high dietary Zn (2425 mg/kg for 28 days) triggers oxidative stress reactions in the pancreas of weaned piglets. Similarly, Zhao et al. (2014) found that while 20–60 mg/kg of ZnO nanoparticles in broiler diet stimulate antioxidative functions, a higher dose (100 mg/kg) can lead to hepatocyte damage and a gradual decrease in total antioxidant capacity in liver tissue. In support of the aforementioned results, Gazaryan et al. (2002) suggested that Zn^{2+} can interfere with mitochondrial antioxidant production and may stimulate the production of reactive oxygen species.

Our results showed that partial substitution of ZnO by HNa positively affected the oxidative status of piglets as judged by a decline of 8-*iso*-$PGF_{2\alpha}$ in serum in both ZnO + HNa-treated groups as compared with ZnO 2.5. This could be due to the lower dose of ZnO (1.7 mg/kg) which might rather act in an anti-oxidative manner in comparison to high pharmacological doses. Alternatively, the decrease in the levels of the oxidative stress biomarker could be the result of the added HNa. Differing effects of HS on oxidative stress and induction of lipid peroxidation in human and animals have been described. HS containing phenolic and polyphenolic compounds can act as an antioxidant, but also as an enzyme (including anti-oxidative enzymes, e.g. catalase) inhibitor in a time- and concentration-dependent manner (Varanka et al. 1999). Inhibition of these enzymes leads to higher levels of reactive oxygen species (ROS) and development of oxidative stress with subsequent negative effects in the body. However, HS are substances with very complex structures and it cannot be expected that two separate sources of HS will contain identical molecules and exert the same effects on the animal or human body (Vaskova et al. 2011).

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Weber et al. (2014) suggested that HS in the diet of young pigs (0.25% for 35 days) may play a role in negating the effects of oxidative stress within the body. The inclusion of HS in the diets of broilers and growing pigs was found to reduce lipid peroxidation in muscle tissues during storage which had a positive effect on meat quality (Aksu et al. 2005; Bai et al. 2013). The *in vitro* study of Vaskova et al. (2011) showed a decrease in the activity of superoxide dismutase; however, activities of other antioxidant enzymes were not influenced after HS treatment and HS-supported generation of ROS was not observed.

On the other hand, Ipek et al. (2008) demonstrated that high levels of HS dietary supplementation (480–600 mg/kg) for five weeks increased oxidative stress in Japanese quails, although low levels (360 mg/kg) did not. Also, an *in vitro* study on endothelial cells showed that HA enhanced the accumulation of intracellular iron resulting in ROS generation and lipid peroxidation when administered for a long time at high levels (Gau et al. 2001). According to previous studies in humans, long-term exposure to HA and fulvic acids (through drinking water) induces the generation of ROS and promotes oxidative stress and lipid peroxidation in both a dose- and time-dependent manner. It can lead to oxidative DNA and protein damage, increased permeability of cell membranes and possibly cell death, and also to a reduction in the activity of antioxidant enzymes. This mechanism may contribute to the pathogenesis of Blackfoot disease (peripheral vascular disease), Kashin-Beck disease (chronic osteoarthritic disorder), lung emphysema and fibrosis and other pathologies (Cheng et al. 1999; Peng et al. 1999; Gau et al. 2001; Ho et al. 2003; Qi et al. 2008).

Despite these reports on the effects of long-term supplementation of high levels of HS, in our study, supplemenation with HNa in a dose of 20 g/kg in feed or 0.2% in drinking water for 21 days positively influenced the oxidative status of piglets in comparison with ZnO 2.5 treatment as judged by 8-*iso*-$PGF_{2\alpha}$ levels in serum. It should also be taken into account that wide variations in basal isoprostane levels among individuals, within a single day and also between days, exist (Basu 2008). However, as was described, these variations occur in individual subjects rather than on a group level, the level at which this study was performed.

FA profile in diets and blood serum of piglets

FA composition in the diets and HNa is presented in Table 4. HNa contained a low amount of total FA and its supplementation to the basal diet in a dose of 20 g/kg did not alter the FA content and composition in diet. Differences between the relative content of individual FA in the diet and serum of control and treated groups are shown in Figure 1. The results reflect the transformation of

Table 4. Fatty acid (FA) composition in the diets and sodium humate (HNa)

Fatty acids (%)	Structure	Basal diet	Basal diet supplemented with HNa	HNa
Lauric	C12:0	0.29	0.29	1.72
Myristic	C14:0	1.37	1.39	5.00
Pentadecanoic	C15:0	0.22	0.23	2.68
Palmitic	C16:0	21.88	21.89	32.51
Cis-7 hexadecenoic	C16:1 n-9	0.30	0.30	2.93
Palmoleic	C16:1	2.11	2.11	1.23
Heptadecanoic	C17:0	0.31	0.31	1.87
Heptadecenoic	C17:1 n-7	0.20	0.20	0.35
Stearic	C18:0	4.46	4.47	11.45
Vaccenic	C18:1 n-11	0.00	0.00	0.76
Oleic	C18:1 n-9	16.50	16.50	13.30
Cis-vaccenic	C18:1 n-7	3.79	3.77	0.01
Linoleic	C18:2 n-6	38.19	38.18	18.39
γ-Linolenic	C18:3 n-6	0.06	0.06	0.07
Linolenic	C18:3 n-3	5.15	5.15	0.64
Nonadecylic	C19:0	0.01	0.01	0.23
Arachidic	C20:0	0.21	0.20	1.30
Eicosenoic	C20:1 n-9	0.47	0.47	0.42
Eicosadienoic	C20:2 n-6	0.69	0.69	0.00
Mead	C20:3 n-9	0.01	0.01	0.06
Dihomo-γ-linolenic	C20:3 n-6	0.01	0.01	0.00
Arachidonic	C20:4 n-6	0.39	0.39	0.00
Eicosatrienoic	C20:3 n-3	0.03	0.02	0.00
Eicosapentaenoic	C20:5 n-3	0.73	0.72	0.00
Behemic	C22:0	0.18	0.18	1.46
Erucic	C22:1 n-9	0.12	0.12	0.00
Adrenic	C22:4 n-6	0.22	0.22	0.00
Docosapentaenoic	C22:5 n-3	0.30	0.30	0.00
Lignoceric	C24:0	0.24	0.24	2.89
Nervonic	C24:1 n-9	0.06	0.06	0.00
Docosahexaenoic	C22:6 n-3	0.86	0.86	0.00
Other FA		0.64	0.65	0.73
Total FA (nmol/g)		25571.4	25574.7	165.3

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FA after digestion of lipids. In the diet, linoleic (LA) was the most abundant FA, followed by palmitic (PA), oleic (OA) and α-linolenic acids (ALA). In blood serum, the content of essential FA (LA and ALA) decreased by about half, while levels of the other n-3 and n-6 long chain polyunsaturated FA (LC-PUFA), mainly eicosapentaenoic (EPA), docosapentaenoic (DPA), docosahexaenoic (DHA), dihomo-γ-linolenic (DGLA), arachidonic (AA) and ardenic acids, increased in comparison to the diet. It is evident that young growing piglets can efficiently metabolise digested essential FA into physiologically significant n-3 and n-6 LC-PUFA, mainly AA, EPA, DPA and DHA.

ZnO 2.5 and both ZnO 1.7 + HNa (f)/(w) treatments significantly modified FA content and composition in the serum of piglets in comparison with Control (Table 5). Significantly higher total serum FA was found in ZnO 2.5 compare to Control. This finding is in accordance with the significantly higher serum TGC detected in the group. Although studies about the effect of high dietary ZnO on serum FA in weaned piglets are missing, Zhang et al. (2014) reported that Zn participates in cellular lipid metabolism as a structural and functional component for some lipid metabolic enzymes and suggested that it plays an important role in FA metabolism.

A pharmacologic dose of ZnO significantly decreased SFA, monounsaturated (MUFA) and polyunsaturated (PUFA) n-3 and increased PUFA n-6 in serum compared to Control. In particular, the proportion of myristic, palmoleic, stearic, nonadecylic, arachidic, vaccenic, OA, *cis*-vaccenic, EPA, DPA, eicosadienoic, DGLA and adrenic acids was decreased, while the content of PA, AA and LA was increased. These results indicate that high ZnO treatment negatively affected the synthesis of some physiologically significant LC-PUFA, namely n-3 EPA, DPA and n-6 DGLA derived from ALA and LA. The changes in PUFA n-6 and n-3 caused by high ZnO treatment resulted in an unfavourable, higher n-6/n-3 ratio compared to the control animals.

A different effect on serum FA composition was detected after a lower dose ZnO treatment supplemented with HNa. Both ZnO 1.7 + HNa (f)/(w) treatments significantly increased the proportion of SFA and PUFA n-3 and decreased PUFA n-6 in comparison with ZnO 2.5, although the influence of water and feed HNa treatment on individual FA was different. Generally, it can be concluded that the effect of both treatments on individual FA was similar, but the efficiency was different. Partial substitution of the pharmacological dose of ZnO by HNa decreased the PUFA n-6/n-3 ratio in comparison with ZnO 2.5 and the effect was more pronounced (statistically significant), when HNa was applied to drinking water. In contrast to the high ZnO 2.5 treatment, the lower dose of ZnO supplemented by HNa did not have an unfavourable effect on the metabolism of physiologically

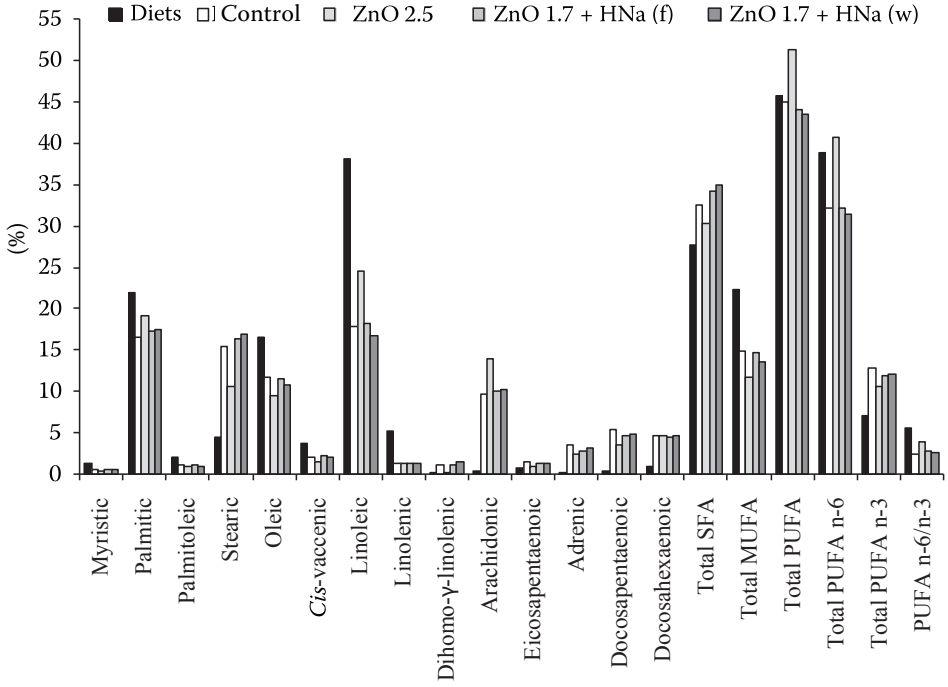

Figure 1. Differences between fatty acid composition in the diets and blood serum of piglets

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Table 5. Fatty acid (FA) composition in the serum of piglets

Fatty acids (%)	Structure	Treatments				Main test	Post-hoc tests					
		Control	ZnO 2.5	ZnO 1.7 + HNa (f)	ZnO 1.7 + HNa (w)		1:2	1:3	1:4	2:3	2:4	3:4
Lauric	C12:0	0.041 ± 0.016	0.044 ± 0.014	0.083 ± 0.005	0.088 ± 0.016	**	ns	**	**	**	**	ns
Myristic	C14:0	0.527 ± 0.038	0.438 ± 0.028	0.617 ± 0.048	0.554 ± 0.061	**	*	**	ns	**	**	ns
Pentadecanoic	C15:0	0.47 ± 0.133	0.429 ± 0.077	0.458 ± 0.123	0.512 ± 0.121	ns						
Palmitic	C16:0	16.64 ± 0.709	19.18 ± 0.309	17.2 ± 0.547	17.41 ± 0.744	**	**	ns	ns	**	**	ns
Cis-7 hexadecenoic	C16:1 n-9	0.614 ± 0.062	0.556 ± 0.006	0.715 ± 0.016	0.579 ± 0.066	**	ns	ns	ns	**	ns	*
Palmoleic	C16:1	1.132 ± 0.153	0.874 ± 0.116	1.091 ± 0.133	0.864 ± 0.096	**	**	ns	**	*	ns	*
Heptadecanoic	C17:0	2.0 ± 0.583	1.453 ± 0.299	1.845 ± 0.461	2.642 ± 1.169	*	ns	ns	ns	ns	*	ns
Heptadecenoic	C17:1 n-7	0.405 ± 0.083	0.338 ± 0.067	0.369 ± 0.095	0.455 ± 0.202	ns						
Stearic	C18:0	15.39 ± 1.018	10.63 ± 0.424	16.28 ± 1.719	16.96 ± 1.512	**	**	ns	ns	**	**	ns
Vaccenic	C18:1 n-11	0.132 ± 0.04	0.06 ± 0.015	0.086 ± 0.045	0.105 ± 0.054	*	*	ns	ns	ns	ns	ns
Oleic	C18:1 n-9	11.52 ± 0.344	9.445 ± 0.591	11.74 ± 0.096	10.9 ± 0.808	**	*	ns	ns	**	ns	ns
Cis-vaccenic	C18:1 n-7	2.048 ± 0.177	1.459 ± 0.05	2.06 ± 0.046	1.972 ± 0.204	**	*	ns	ns	**	ns	ns
Linoleic	C18:2 n-6	17.86 ± 1.393	24.49 ± 0.938	18.2 ± 1.674	16.8 ± 0.919	**	**	ns	ns	**	**	ns
γ-Linolenic	C18:3 n-6	0.349 ± 0.034	0.271 ± 0.069	0.359 ± 0.052	0.249 ± 0.048	**	ns	ns	**	*	ns	**
Linolenic	C18:3 n-3	1.354 ± 0.109	1.294 ± 0.07	1.241 ± 0.234	1.271 ± 0.146	ns						
Nonadecylic	C19:0	0.073 ± 0.014	0.043 ± 0.006	0.07 ± 0.015	0.08 ± 0.019	**	**	ns	ns	*	**	ns
Arachidic	C20:0	0.3 ± 0.043	0.142 ± 0.015	0.259 ± 0.044	0.208 ± 0.06	**	**	ns	**	**	ns	ns
Eicosenoic	C20:1 n-9	0.19 ± 0.015	0.157 ± 0.019	0.182 ± 0.027	0.22 ± 0.099	ns						
Eicosadienoic	C20:2 n-6	0.73 ± 0.046	0.452 ± 0.055	0.710 ± 0.074	0.664 ± 0.153	**	**	ns	ns	*	ns	ns
Mead	C20:3 n-9	0.312 ± 0.088	0.282 ± 0.029	0.346 ± 0.081	0.396 ± 0.126	ns						
Dihomo-γ-linolenic	C20:3 n-6	1.053 ± 0.109	0.076 ± 0.008	1.15 ± 0.298	1.4 ± 0.312	**	ns	ns	ns	ns	**	ns
Arachidonic	C20:4 n-6	9.706 ± 0.86	13.860 ± 1.148	10.02 ± 0.714	10.17 ± 0.696	**	**	ns	ns	**	**	ns
Eicosatrienoic	C20:3 n-3	0.112 ± 0.043	0.056 ± 0.026	0.051 ± 0.032	0.069 ± 0.038	*	ns	*	ns	ns	ns	ns
Eicosapentaenoic	C20:5 n-3	1.544 ± 0.168	0.977 ± 0.113	1.31 ± 0.196	1.26 ± 0.075	**	**	*	**	**	*	ns
Behemic	C22:0	0.194 ± 0.022	0.105 ± 0.012	0.174 ± 0.036	0.15 ± 0.04	**	**	ns	ns	**	ns	ns
Erucic	C22:1 n-9	0.081 ± 0.027	0.092 ± 0.056	0.048 ± 0.035	0.084 ± 0.053	ns						
Adrenic	C22:4 n-6	3.582 ± 0.576	2.388 ± 0.168	2.838 ± 0.26	3.267 ± 0.637	**	**	ns	ns	ns	*	ns
Docosapentaenoic	C22:5 n-3	5.429 ± 0.425	3.55 ± 0.304	5.145 ± 1.338	5.088 ± 0.954	**	**	ns	ns	ns	ns	ns
Lignoceric	C24:0	0.339 ± 0.04	0.354 ± 0.026	0.278 ± 0.04	0.21 ± 0.097	**	ns	ns	**	ns	**	ns
Nervonic	C24:1 n-9	0.719 ± 0.042	0.654 ± 0.05	0.682 ± 0.03	0.474 ± 0.159	**	ns	ns	**	ns	ns	*
Docosahexaenoic	C22:6 n-3	4.535 ± 0.481	4.698 ± 0.468	4.528 ± 0.395	4.929 ± 0.285	ns						
Other FA		0.059 ± 0.019	0.032 ± 0.019	0.057 ± 0.021	0.066 ± 0.036	ns						
SFA		35.99 ± 1.061	32.83 ± 0.657	37.32 ± 1.538	38.89 ± 0.649	**	**	ns	**	**	**	*
MUFA		17.11 ± 0.876	14.75 ± 2.547	16.72 ± 1.058	15.66 ± 1.06	*	*	ns	ns	ns	ns	ns
PUFA		46.82 ± 0.889	52.39 ± 2.03	45.9 ± 0.971	45.390 ± 1.168	**	**	ns	ns	**	**	ns
PUFA n-3		13.2 ± 0.594	10.57 ± 0.562	12.28 ± 1.12	12.440 ± 0.878	**	**	ns	ns	*	**	ns
PUFA n-6		33.31 ± 0.879	41.53 ± 1.502	33.28 ± 1.756	32.550 ± 0.767	**	**	ns	ns	**	**	ns
PUFA n-6/n-3		2.528 ± 0.149	3.931 ± 0.103	2.739 ± 0.397	2.627 ± 0.184	**	**	ns	ns	ns	*	ns
Total FA (nmol/ml)		1118.5 ± 123.6	1435 ± 133.1	1127.4 ± 188.7	1154.8 ± 246.6	*	*	ns	ns	ns	ns	ns

Control = basal diet containing 110 mg ZnO/kg, FA = fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids, ZnO 2.5 = basal diet supplemented with 2.5 g ZnO/kg, ZnO 1.7 + HNa (f) = basal diet supplemented with 1.7 g ZnO and 20.0 g HNa/kg, ZnO 1.7 + HNa (w) = basal diet supplemented with 1.7 g ZnO/kg and drinking water supplemented with 0.2% HNa

*$P < 0.05$, **$P < 0.01$

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significant LC-PUFA n-3 and n-6. Levels of most individual PUFA n-3 and n-6 as well as the n-6/n-3 ratio in both treatments were similar to Control without treatment. On the other hand, the composition of some individual SFA (lauric, myristic, heptadecanoic, arachidic, lignoceric) was significantly higher. The effect on SFA was more pronounced when HNa was applied to drinking water. It is not clear whether the lower dose of ZnO, the presence of HNa, or a combined effect of both was responsible for the obtained results.

To the authors' knowledge, there are currently no data available on the effect of HS on serum FA composition in weaned piglets. The results can be partly compared to the findings of Wang et al. (2008), who reported a significant increase in SFA, mainly myristic, palmitic and stearic acids, and USFA in the meat of finishing pigs after 5% HS dietary supplementation. Those authors also detected a significant increase in the USFA : SFA ratio in meat after 10% HS dietary supplementation. Macit et al. (2009) reported an increase in SFA myristic acid and MUFA myristoleic, palmitoleic and heptadecenoic acids in egg yolks of layers fed a HS-supplemented diet. PUFA n-3 and PUFA n-6 levels in egg yolks remained unchanged by HS dietary treatments.

In conclusion, the results reported here indicate that the partial substitution of ZnO by HNa can benefit the performance and health of weaned piglets to a similar extent as a high pharmacological dose of ZnO, and at the same time can partially protect against the oxidative stress induced by prolonged over-supplementation of ZnO in weaned piglets. Additionally, such a treatment can eliminate the unfavourable effects of high ZnO doses on the n-6/n-3 ratio and the proportion of some physiologically significant LC-PUFA in serum. Generally, it can be concluded that the effects of both feed and water HNa supplementation are similar, but that these effects are more pronounced when HNa is applied to drinking water.

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