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Research article

EFFECTS OF DIETARY SUPPLEMENTATION WITH BENZOIC ACID AND CHELATED COPPER, ZINC AND MANGANESE SOURCES ON PRODUCTION PERFORMANCE IN PIGLETS

Dejan PERIĆ[1]*, Roberto BAREA[2], Sladjan NEŠIĆ[3], Lazar MAKIVIĆ[4], Jelena JANJIĆ[5], Dragan ŠEFER[1], Radmila MARKOVIĆ[1]

[1]University of Belgrade - Faculty of Veterinary Medicine, Department of Animal Nutrition and Botany, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia; [2]Novus Europe NV, Leuvensesteenweg 643, box 15, 1930 Zaventem, Belgium; [3]University of Belgrade - Faculty of Veterinary Medicine, Department of Pathology, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia; [4]Rapić d.o.o., Rovine bb, 78400 Gradiška, Bosnia and Herzegovina; [5]University of Belgrade - Faculty of Veterinary Medicine, Department of Economics and Statistics, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia.

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The aim of this experiment was to investigate the effects of benzoic acid and chelates in which copper (Cu), zinc (Zn) and manganese (Mn) were bound to methionine hydroxy analogue on growth performance, intestinal morphology, intestinal microbiota and digesta pH value of post-weaning piglets at 28 days of age. The experiment was conducted on 96 piglets randomly assigned to one of four treatments (6 replicate pens of 4 piglets each): 1) control (C) – microminerals were provided as sulfates of Cu, Zn and Mn at 130 (80 at second phase), 100, 120 mg/kg in the first phase, respectively; 2) chelates (CTM), microelements were provided as chelates of Cu, Zn and Mn at 130 (80 in second phase), 60, 60 mg/kg in the first phase, respectively; 3) benzoic acid (BA), with the addition of 2500 mg/kg during both periods; 4) chelates + benzoic acid (CTM + BA), microelements were provided as chelates of Cu, Zn and Mn at 130 (80 in second phase), 60, 60 mg/kg in the first phase, respectively, and 2500 mg/kg of benzoic acid during both periods. Results showed that chelates and benzoic acid supplementation not only improved the final body weight ($p<0.05$), average daily gain ($p<0.05$) and feed conversion ratio ($p<0.05$), but also increased the morphology performance and decreased the number of *E. coli* in the jejunum and ileum in the treated groups ($p<0.05$). This study provides the evidence that dietary supplementation has beneficial effects on the intestinal morphology and microflora of weaned pigs, which can partly explain why growth performance of the piglets was improved.

Keywords: benzoic acid, chelates, dietary supplementation, piglets, production results

*Corresponding author: e-mail: dperic@vet.bg.ac.rs

INTRODUCTION

The ban of using antibiotics in animal nutrition for growth promotion purposes has led to the need to find alternatives with the aim of promoting growth and improving the health status of weaned piglets that are exposed to stressogenic factors [1]. The intestine is a highly proliferative and secretory organ that is the main organ of digestion and absorption of nutrients [2]. Also, the intestines have a barrier function that protects the organism from pathogens and toxins [3]. The protective role of the intestine consists of non-specific mechanisms that include the regenerative capacity of the mucosa and epithelium, the intercellular junctions between the epithelial cells and the mucus gel, the specific immune response and the microbiota [4-7].

As a type of organic acidifier, benzoic acid ($C_7H_6O_2$) is a colorless crystalline solid and represents the simplest aromatic carboxylic acid [8]. In the small intestine, it is absorbed and transported via monocarboxylic acid transporters, and the rate of absorption for the jejunum is higher than that for the duodenum and ileum [9]. Benzoic acid is completely metabolized to hippuric acid and then excreted in the urine [10]. The use of benzoic acid in the feed can inhibit pathogenic microorganisms and is used for the purpose of feed preservation [11]. Benzoic acid has an antimicrobial effect that could be of interest in reducing the incidence of diarrhea in piglets after weaning [12]. Benzoic acid has been shown to improve growth rate, feed consumption and utilization [13-17]. The positive effects of using benzoic acid are associated with a decrease in the pH value of intestinal content in the ileum, cecum and colon of pigs [18], inhibition of harmful bacteria in the intestines [14,15], as well as improving the morphological integrity of the intestine and the activity of endogenous enzymes [15,18,19].

Mineral supplements that are used with the purpose of meeting the nutritional needs of pigs are crucial for growth, development of the immune system and reproduction [20]. The use of high concentrations of zinc is thought to have effects of promoting growth in weaned piglets and reducing the incidence of post-weaning diarrhea [21,22]. Also, feeding high concentrations of copper achieves similar effects, hence it has begun to be used as an alternative to antibiotics [23,24]. Interest in the use of organic minerals in pig nutrition has increased due to higher bioavailability and reduced excretion into the environment [25]. Organic minerals include chelates or complex forms with amino acids, organic acids, peptides, polysaccharides and proteins [26]. The mineral methionine hydroxy analog chelate (MMHAC) has been reported to have higher digestibility and utilization of Zn, Cu and Mn in piglets compared to inorganic forms of these minerals [27,28]. Also, the use of MMHAC reduces the body weight loss of lactating sows and increases the body weight of suckling piglets [27].

The aim of this study was to indicate the effects of using the protected form of benzoic acid and MMHAC (copper, manganese, zinc) in piglet nutrition on production results, intestinal morphology and microbiota separately or in combination.

MATERIALS AND METHODS

Ethic statement

The experimental protocol was approved by the Veterinary Directorate of the Serbian Ministry of Agriculture, Forestry and Water Management and the Ethics Committee of the Faculty of Veterinary Medicine, University of Belgrade (Resolution number: 23/2020).

Animals, housing, and feeding

A total of 96 healthy weaned piglets originating from the same farm (Landrace x Yorkshire) with an average body weight (BW) of 6.83 ± 0.56 kg (28 days of age) were randomly allocated to four dietary treatments according to sex and weight in a completely randomized design. Male piglets were castrated on the third day of life. Each experimental group had six replicate pens with four piglets (two barrows and two gilts) per pen. All piglets were housed in an environmentally controlled nursery room, under 80 lux for at least 8 hours per day and they had access to daylight. Stainless steel pens 1.5 x 0.6 x 2.0 m with slatted plastic flooring perforated on the entire surface were used. Each pen was equipped with a self-feeder and a nipple drinker. The nursery barn was at 26 °C during the first week of the experiment and then gradually reduced to 22°C. Relative humidity was maintained at 60%–70%. All piglets had free access to feed and water *ad libitum* throughout the 42-d study period. The piglets were weaned at 28 d and the experiment period was divided into phase 1 from d 0 to d 22 and phase 2 from 22 d to 42 d. The dietary treatments were as follows: (1) a corn-soy-bean basal diet – control group - C; (2) basal diet + MMHAC (copper, manganese, zinc) - CTM; (3) basal diet + protected benzoic acid – BA; (4) basal diet + MMHAC (copper, manganese, zinc) + protected benzoic acid – CTM+BA (Table 1).

The nutritional feed program consisted of basal diets (Table 2), was formulated to meet or exceed the nutrient requirements for piglets, as recommended by the NRC [29]. No medications or other feed additives were included in any of the diets. Representative feed samples (1 kg) for each treatment were collected from the barn. All components of the diets were analyzed for dry matter, crude protein, crude fat, crude fiber, ash, calcium, and phosphorus and results are presented in Table 2 [30].

Table 1. Study treatments

Treatment code	Inclusion of inorganic source trace minerals	Inclusion of protected benzoic acid	Inclusion of chelated trace minerals[a]
Phase 1 (0 – 22 d)			
C - control	130 ppm Cu, 120 ppm Mn, 100 ppm Zn	–	–
CTM (MMHAC (Cu, Mn, Zn))		–	130 ppm Cu, 60 ppm Mn and 60 ppm Zn
BA (protected benzoic acid)		2500 ppm	–
CTM + BA (MMHAC (Cu, Mn, Zn) + protected benzoic acid)		2500 ppm	130 ppm Cu, 60 ppm Mn and 60 ppm Zn
Phase 2 (22 – 42 d)			
C – Control	80 ppm Cu, 120 ppm Mn, 100 ppm Zn	–	–
CTM (MMHAC (Cu, Mn, Zn)		–	80 ppm Cu, 60 ppm Mn and 60 ppm Zn
BA (protected benzoic acid)		2500 ppm	–
CTM + BA (MMHAC (Cu, Mn, Zn) + protected benzoic acid)		2500 ppm	80 ppm Cu, 60 ppm Mn and 60 ppm Zn

[a]Chelated trace minerals were added according to the "Reduce and Replace" concept (complete amount of inorganic Cu, Mn and Zn was replaced by the chelated form of the listed trace minerals) [69].

Growth performance

Each piglet was weighed on 0, 22 and 42 days, and feed consumption was also measured on a pen basis to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) in phase 1 (0 to 22 days) and in phase 2 (22 to 42 days), as well as for whole experimental period.

Intestinal morphology, pH and microbiota

At the end of the feeding trial, 1 piglet per pen, three males and three females per group (24 animals) were selected. Following a 12-hour fasting period, the animals were transported to the industrial slaughterhouse under continuous veterinary supervision. They were electrically stunned and promptly slaughtered.

Table 2. Ingredients and nutrient composition of basal diets

Item	Phase 1 (d 0 to d 22)	Phase 2 (d 22 to d 42)
Ingredients (% of DM)		
Corn	45.00	57.00
Barley	10.00	10.00
Soybean meal	12.00	15.00
Full fat soya	23.00	14.00
Vitamin-mineral premix[a,b]	10.00	4.00
Total	100.00	100.00
Nutrient composition		
Metabolic energy (MJ/kg)*	13.74	13.78
Moisture (%)	10.50	11.50
Crude protein (%)	20.35	18.25
Crude fat (%)	6.95	5.60
Crude fiber (%)	4.04	3.66
Ash	6.20	5.50
Calcium (%)	0.95	0.83
Total phosphorus (%)	0.68	0.63
Sodium (%)	0.19	0.18
Lysine (%)*	1.39	1.26
Methionine+cysteine (%)*	0.77	0.68
Threonine (%)*	0.81	0.75
Tryptophan (%)*	0.23	0.22

DM – dry matter; [a]Provided per kilogram of diet in phase 1 (d 0 to d 21): 20.000 IU vitamin A, 2.000 IU vitamin D_3, 80 mg vitamin E, 2.4 mg vitamin K_3, 2.4 mg vitamin B1, 6 mg vitamin B2, 6 mg vitamin B6, 0.4 mg vitamin B12, 0.3 mg biotin, 32 mg niacin, 14 mg Ca-pantothenate, 5 mg folic acid, 3 mg I, 0.4 mg Se, 0.6 mg Co, 550 mg choline chloride, 240 mg Fe, 130 mg Cu, 120 mg Mn, 100 mg Zn, 1000 mg phytase, 100 mg antioksidant BHT; [b]Provided per kilogram of diet in phase 2 (d 21 to d 42): 20.000 IU vitamin A, 1.800 IU vitamin D_3, 120 mg vitamin E, 2.8 mg vitamin K_3, 4 mg vitamin B1, 8.8 mg vitamin B2, 6.8 mg vitamin B6, 0.04 mg vitamin B12, 0.28 mg biotin, 28 mg niacin, 16 mg Ca-pantothenate, 0.8 mg folic acid, 0.8 mg I, 0.4 mg Se, 0.6 mg Co, 500 mg choline chloride, 200 mg Fe, 80 mg Cu, 120 mg Mn, 100 mg Zn, 1000 mg phytase, 40 mg antioxidant BHT; *Calculated values

Morphological and histological analyses

Samples of intestine tissue for morphometric examination from each group were taken immediately after slaughter. The duodenum, jejunum, ileum and caecum were parts of the intestine which were examined. The tissue samples were fixed for 72 hours in 10% pH neutral formalin. In order to prevent blinding and deformation of the intestinal wall, formalin was injected into the lumen of the ligated intestine. After fixation the samples were embedded in paraffin wax and then were cut into 5 μm transverse sections for staining. The tissue sections were stained by hematoxylin and eosin (HE) and periodic shiff acid (PAS) stain [31]. Intestinal mucosal morphology

including villus height, villus width and crypt depth was determined with an Olympus BX-51 microscope. For morphometric analyses Olympus cell Bsoftware was used.

The morphometric examinations were performed on histological tissue sections. The villi length was measured in a 400x field of view on 10 randomly selected correctly oriented villi. The length of the villi was measured in micrometers and was obtained by measuring the distance from the junction of the villus and crypt to the villus apex [32]. Crypt depth was measured as the vertical distance between the lowest and highest points of the crypt.

Microbiological and pH analyses

The pH values of the stomach, duodenum, jejunum, ileum, cecum, colon and rectum digesta were measured using a hand-held pH-meter Testo 205 (Testo AG, Lenzkirch, Germany). The microbiological examination of the duodenum, jejunum, ileum and cecum revealed the following results: total number of aerobic bacteria, total number of anaerobic bacteria, number of bacteria of the *Enterococcus* spp., *Escherichia coli*, *Clostridium perfringens* and *Lactobacillus* spp. number. ISO methods were used for counting bacteria, based on cultivation of targeted microorganisms with modification of the used matrix, incubation temperature, and media depending on the biological characteristics of the tested bacteria. The number of bacteria was determined according to ISO 7218:2007 [33] and SRPS ISO 4833-1:2014 [34] standards using the "pour on" seeding technique.

For the total number of aerobic and anaerobic bacteria, a plate count substrate (PCA, HiMedia) was used with aerobic or anaerobic incubation for 72 h at 30 °C. UTI agar (HiMedia) was used for the number of *Enterococcus* and *E. coli* bacteria, which were incubated aerobically at 37 °C for a period of 16 to 20 hours. ISO method 7937:2010 [35] was used for the numeration of *C. perfringens* by seeding sulfite cycloserine agar without yolk (Oxoid) and anaerobic incubation at 37 °C for 20 h ± 2 h (GasPack, BD BBL). The ISO 15214:1998 method [36] was used to isolate *Lactobacillus* bacteria with mesophilic growth by seeding MRS agar (Oxoid) in anaerobic conditions (GasPack, BD BBL) at 30 °C for 72 hours.

Statistical Analysis

Statistical analysis of the results was elaborated using software GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All parameters were described by means and standard deviation(SD). One-way ANOVA with Tukey's post hoc test was performed to assess the significance of differences among experimental groups. Statistical significance was considered at a level of $p<0.05$.

RESULTS

Growth performance of piglets

The results of the growth performance are presented in Table 3. Body weight of piglets did not differ on day 0 across the treatment groups.

Table 3. Production results and parameters ($\overline{X}$±SD) of experimental groups of piglets

Fattening day	Parameters	Groups			
		C	CTM	BA	CTM+BA
0	Body weight (kg), n=24	6.83±0.58	6.84±0.62	6.83±0.53	6.83±0.53
22		12.80^{A}±1.47	13.22^{AB}±1.68	13.04^{AC}±1.64	13.68^{BC}±1.49
42		25.06^{A}±3.33	26.58^{BC}±2.05	25.78^{AC}±1.81	27.28^{B}±2.28
0 to 22	Body gain (kg), n=6	5.97±1.24	6.38±1.30	6.20±1.23	6.85±1.16
	Daily body gain (kg), n=6	0.28±0.06	0.30±0.06	0.30±0.06	0.33±0.06
	Feed consumption (kg), n=6	43.20±2.43	44.08±3.77	42.25±3.48	44.58±2.21
	Daily feed consumption (kg), n=6	0.51±0.03	0.52±0.05	0.50±0.04	0.53±0.03
	Feed conversion ratio, n=6	1.81^{A}±0.03	1.73^{B}±0.06	1.71^{B}±0.03	1.63^{C}±0.02
22 to 42	Body gain (kg), n=6	12.21^{A}±2.33	13.36^{B}±0.98	12.74^{AB}±1.09	13.60^{BC}±1.10
	Daily body gain (kg), n=6	0.58^{A}±0.11	0.64^{B}±0.05	0.61^{AB}±0.05	0.65^{BC}±0.05
	Feed consumption (kg), n=6	101.80±9.05	103.70±1.80	101.40±3.42	103.30±2.60
	Daily feed consumption (kg), n=6	1.21±0.11	1.24±0.02	1.21±0.04	1.23±0.03
	Feed conversion ratio, n=6	2.09^{A}±0.08	1.94^{B}±0.04	1.99^{AC}±0.05	1.90^{BC}±0.05
0 to 42	Body gain (kg), n=6	18.18^{A}±3.19	19.74^{AB}±1.67	18.95^{AC}±1.54	20.45^{BC}±1.97
	Daily body gain (kg), n=6	0.43^{A}±0.08	0.47^{AB}±0.04	0.45^{AC}±0.04	0.49^{BC}±0.05
	Feed consumption (kg), n=6	145.00±11.71	147.80±4.10	143.60±2.93	147.90±4.88
	Daily feed consumption (kg), n=6	0.87±0.07	0.88±0.03	0.85±0.02	0.88±0.03
	Feed conversion ratio, n=6	2.00^{A}±0.07	1.87^{BC}±0.04	1.90^{B}±0.05	1.81^{CD}±0.04

Legend: Different letter in superscript within row ^{A,B,C,D}-$p<0.05$;C – control group (basal diet); CTM – basal diet + MMHAC (copper, manganese, zinc); BA – basal diet + protected benzoic acid; CTM + BA – basal diet + MMHAC (copper, manganese, zinc) + protected benzoic acid

At the end of the experiment, body weight of the group with addition of CTM was higher ($p<0.05$) than the control group. Also, body weight of the group supplemented with CTM+BA was higher ($p<0.05$) than the control and BA group. Higher average daily weight gain ($p<0.05$) was found in the CTM+BA group than in the group fed with basic diet - control. There were no differences between experimental groups in

average daily feed intake. Feed conversion ratio was higher ($p<0.05$) in the control group than in the other groups. Also, feed conversion ratio was lower ($p<0.05$) for piglets fed diets supplemented with CTM+BA than in the BA group of piglets.

Intestinal morphology of piglets

The results of the intestinal morphology are presented in Table 4. All experimental diets increased villi length in the duodenum, jejunum, ileum and cecum ($p<0.05$).

Experimental diets also increased villi width in all segments of the intestine ($p<0.05$). Crypts depth of the control group was higher ($p<0.05$) than the experimental groups. Number of goblet cells per 100 enterocytes did not differ in duodenum, jejunum and ileum across treatment groups. Number of goblet cells in the cecum was higher ($p<0.05$) in the BA group than in the control group of piglets. Villus height and crypt depth ratio was higher ($p<0.05$) in group of piglets fed diets supplemented with CTM+BA compared to piglets fed the unsupplemented diet. This value was also higher ($p<0.05$) in CTM and BA group compared with piglets fed the basic diet i.e. control group. Experimental diets affect villus height, villus width, crypt depth, and the ratio of villus height to crypt depth in all analyzed intestine segments.

Bacterial counts and pH value in duodenal, jejunal, ileal and caecal digesta of piglets

The difference in microbial diversity of intestine content samples from the four groups is shown in Table 5. The total number of aerobic bacteria did not differ in the duodenum across the treatment groups, but there were differences ($p<0.05$, $p<0.05$) in the jejunum, ileum and cecum. The number of *E. coli* was not affected by dietary treatments in the duodenum and cecum, but in the jejunum and ileum the value was lower ($p<0.05$). The number of *Enterococcus* spp. was not affected by dietary treatments in the ileum and cecum, but a difference was detected in the BA group compared to the control group ($p<0.05$) in the duodenum and CTM+BA group compared to other treatments ($p<0.05$). Total number of anaerobic bacteria differed ($p<0.05$, $p<0.05$) in the duodenum, jejunum and cecum across the treatment groups, but there were no differences in the ileum. The number of *Clostridium perfringens* decreased ($p<0.05$) in the ileum of piglets fed diets supplemented with BA compared to other dietary treatments.

Table 4. Morphological and histological parameters ($\overline{X}$±SD) of experimental groups of piglets

Intestinal part	Parameters (n=6)	Groups			
		C	CTM	BA	CTM+BA
Duodenum	Villi length, μm	301.20^{A}±51.69	374.50^{B}73.54	360.70^{B}±69.73	388.80^{B}±75.51
	Villi width, μm	119.80^{A}±15.52	151.10^{BC}±24.39	143.10^{B}±22.59	158.40^{BCD}±16.98
	Crypts depth, μm	133.30^{A}±17.37	115.40^{B}±13.67	112.50^{B}±12.10	92.88^{C}±11.16
	Number of goblet cells per 100 enterocytes	26.83±3.55	27.83±3.71	28.50±4.09	34.17±6.52
	Villus height and crypt depth ratio	2.27^{A}±0.28	3.24^{B}±0.33	3.22^{B}±0.61	4.21^{C}±0.65
Jejunum	Villi length, μm	295.80^{A}±56.21	363.00^{BC}±57.96	342.50^{B}±59.92	377.60^{BCD}±64.40
	Villi width, μm	72.60^{A}±10.72	132.50^{B}±15.83	134.60^{B}±13.10	141.80^{B}±12.80
	Crypts depth, μm	107.90^{A}±17.10	97.15^{B}±14.35	91.16^{B}±14.63	79.77^{C}±12.42
	Number of goblet cells per 100 enterocytes	19.83±4.26	20.83±4.07	21.83±3.60	23.50±4.97
	Villus height and crypt depth ratio	2.76^{A}±0.28	3.77^{ABC}±0.47	3.85^{BD}±0.93	4.75^{CD}±0.64
Ileum	Villi length, μm	303.50^{A}±55.03	372.90^{B}±66.79	342.90^{C}±49.45	375.60^{BD}±41.05
	Villi width, μm	109.80^{A}±15.43	147.50^{B}±17.03	161.20^{C}±19.84	158.30^{CD}±15.27
	Crypts depth, μm	131.70^{A}±15.17	124.60^{A}±16.03	125.20^{A}±18.84	83.04^{B}±14.04
	Number of goblet cells per 100 enterocytes	31.50±6.41	34.50±6.29	36.17±6.97	38.00±6.42
	Villus height and crypt depth ratio	2.32^{A}±0.33	3.00^{B}±0.18	2.76^{AB}±0.27	4.58^{CD}±0.60
Cecum	Villi length, μm	169.30^{A}±19.36	177.90^{A}±30.10	178.60^{A}±29.05	219.00^{B}±34.70
	Villi width, μm	64.68^{A}±11.09	89.96^{B}±13.39	99.92^{C}±11.40	98.36^{CD}±10.61
	Crypts depth, μm	140.70^{A}±19.86	118.80^{B}±15.04	124.40^{B}±19.74	75.46^{C}±12.61
	Number of goblet cells per 100 enterocytes	24.50^{A}±5.54	26.50^{AB}±4.59	32.83^{BC}±6.18	31.33^{AB}±3.01
	Villus height and crypt depth ratio	1.21^{A}±0.13	1.51^{A}±0.22	1.44^{A}±0.16	2.91^{B}±0.22

Legend: Differentletter in superscript withinrow ^{A,B,C,D}-$p<0.05$; C – control group (basal diet); CTM – basal diet + MMHAC (copper, manganese, zinc); BA – basal diet + protected benzoic acid; CTM + BA – basal diet + MMHAC (copper, manganese, zinc) + protected benzoic acid

In other parts of the intestine there were no differences across experimental groups. The population of *Lactobacillus* was increased in the ileum and cecum of piglets in BA group with addition of protected benzoic acid ($p<0.05$) and in the group fed diets with CTM+BA addition ($p<0.05$). Number of *Lactobacilli* did not differ in the duodenum and jejunum across dietary treatments. There were no differences in pH value in segments of gastrointestinal tract between dietary treatments (Table 6) except in the jejunum where the BA and CTM+BA groups measured a lower pH value compared to the control group ($p<0.05$).

Table 5. Intestinal microbiota ($\bar{X}$±SD) of experimental groups of piglets

Intestinal part	Cell number (n=6) log CFU/ml	Groups			
		C	CTM	BA	CTM+BA
Duodenum	TNB[1]	3.54±0.22	3.810.35	3.54±0.56	3.35±0.16
	E. coli	5.24±0.46	4.95±0.39	5.48±0.77	5.57±0.51
	ECC[2]	2.74^{A}±0.38	2.51^{AB}±0.14	2.10^{BC}±0.31	2.65^{A}±0.35
	TNB AN[3]	4.72^{AC}±0.52	5.44^{B}±0.26	4.54^{CD}±0.28	5.20^{AB}±0.35
	CP[4]	4.44±0.30	4.48±0.33	4.08±0.51	4.31±0.62
	LB[5]	4.58±0.30	4.56±0.55	5.13±0.46	5.13±0.59
Jejunum	TNB	5.32^{A}±0.30	4.36^{B}±0.33	5.21^{AC}±0.46	4.69^{AB}±0.58
	E. coli	4.78^{A}±0.21	4.06^{AB}±0.48	3.62^{BD}±0.43	3.01^{CD}±0.34
	ECC	3.85^{A}±0.29	3.80^{A}±0.41	3.75^{A}±0.16	3.12^{B}±0.22
	TNB AN	5.10^{AB}±0.33	5.46^{AC}±0.52	5.44^{A}±0.31	4.49^{BD}±0.31
	CP	4.39±0.36	4.62±0.31	4.26±0.29	4.21±0.21
	LB	5.80±0.38	6.05±0.33	6.03±0.81	6.21±0.59
Ileum	TNB	9.47^{A}±0.85	8.35^{B}±0.40	9.71^{AC}±0.68	12.67^{D}±0.65
	E. coli	6.33^{A}±0.42	5.68^{B}±0.25	5.96^{AB}±0.47	4.93^{CD}±0.24
	ECC	5.48±0.61	5.34±0.72	4.68±0.29	5.44±0.26
	TNB AN	7.40±0.68	7.44±1.29	7.29±0.12	7.27±0.75
	CP	7.96^{A}±0.47	7.37^{A}±0.76	6.27^{B}±0.58	7.55^{A}±0.29
	LB	7.86^{A}±0.53	8.05^{A}±0.67	9.31^{B}±0.35	8.47^{A}±0.35
Cecum	TNB	12.00^{A}±0.63	7.94^{B}±0.41	11.70^{A}±0.67	8.31^{B}±0.63
	E. coli	7.26±0.61	6.35±0.92	6.14±0.43	6.28±0.49
	ECC	5.59±0.39	5.89±0.46	5.60±0.37	5.82±0.39
	TNB AN	9.20^{A}±1.08	10.59^{B}±0.48	10.22^{AB}±0.67	9.12^{A}±0.60
	CP	7.98±0.63	7.84±0.42	7.88±0.44	7.91±0.45
	LB	7.92^{A}±0.47	8.70^{ABC}±0.74	9.08^{BD}±0.50	8.82^{CD}±0.39

Legend: Differentletter in superscript within row ^{A,B,C,D}-$p<0.05$; [1]Total number of bacteria; [2]*Enterococcus* spp.; [3]Total number of anaerobic bacteria; [4]*Clostridium perfringens;* [5] *Lactobacillus* spp.; C – control group (basal diet); CTM – basal diet + MMHAC (copper, manganese, zinc); BA – basal diet + protected benzoic acid; CTM + BA – basal diet + MMHAC (copper, manganese, zinc) + protected benzoic acid

Table 6. pH value ($\overline{X}$±SD) of experimental groups of piglets

Intestinal part (n=6)	Groups			
	C	CTM	BA	CTM+BA
Stomach	4.99±0.82	4.60±0.77	4.21±0.61	4.24±0.90
Duodenum	6.43±0.20	6.19±0.39	6.01±0.12	5.80±0.84
Jejunum	6.46^{A}±0.20	6.51^{A}±0.04	6.00^{B}±0.15	6.07^{B}±0.28
Ileum	6.62±0.11	6.49±0.31	6.43±0.27	6.37±0.21
Cecum	5.82±0.19	5.85±0.25	5.35±2.53	5.35±2.35
Colon	5.94^{AB}±0.09	5.86^{BC}±0.19	6.31^{C}±0.18	6.19^{AD}±0.18
Rectum	6.20±0.32	6.15±0.19	6.39±0.22	6.42±0.27

Legend: Different letter in superscript within row ^{A,B,C,D}-$p<0.05$; C – control group (basal diet); CTM – basal diet + MMHAC (copper, manganese, zinc); BA – basal diet + protected benzoic acid; CTM + BA – basal diet + MMHAC (copper, manganese, zinc) + protected benzoic acid

DISCUSSION

Weaning is the most stressful period of a pig's life. During this time the piglets must quickly adapt to a multitude of stressors such as separation from the mother, sudden changes in the diet and increased exposure to pathogens [37]. The intestinal microbiota faces a serious challenge due to the influence of mentioned stresses during the first few days after weaning. Acidity is one of the most important factors for the survival of microorganisms in the digestive tract [18]. At weaning, piglets have elevated pH values in the digestive tract, which can lead to greater opportunities for enteropathogenic bacteria to colonize the digestive tract and ultimately lead to infection with pathogenic bacteria [38].

Benzoic acid is an aromatic carboxylic acid with the simplest structure and can be detected in the stomach and small intestine of piglets since it is not metabolized as quickly as other organic acids [39]. Numerous researches were conducted on weaned piglets in order to prove the benefits of its use. Some of the beneficial effects that have been proven are an increase in acidity in the digestive tract [15], and improvement of the composition of the intestinal microbiota and enhanced utilization of feed [13,18], all of which are factors that affect the production results of piglets.

The addition of protected benzoic acid in mixtures has an important role in the regulation of intestinal bacteria by stimulating the growth of beneficial bacteria (*Lactobacillus*) and reducing the growth of potential pathogenic bacteria (*E. coli, C.perfringens*), which is in accordance with the results of previous research [13,18]. Organic acids maintain a lower gastrointestinal pH, providing a favorable environment for the growth of *Lactobacillus* [40,41]. Consequently, the beneficial bacteria suppress the colonization of *E. coli* by blocking adhesion sites and producing acidic metabolites [42], which is one of the mechanisms by which benzoic acid regulates the intestinal microbiota. In this trial, the treatment group of piglets with supplementation of protected benzoic acid

were fed with a dose of 2500 mg/kg benzoic acid, unlike the experiments published by Kluge et al. [13] and Gao et al. [43] who used a dose of 5000 mg/kg benzoic acid in *in vivo* and *in vitro* experiments. From these results, we conclude that high concentration of benzoic acid can reduce the growth of *Lactobacillus* in piglets. In this trial, the treatment group of piglets with addition of protected benzoic acid was fed with a dose of 2500 mg/kg benzoic acid, had a lower pH in the jejunum, while other researchers with a dose of 5000 mg/kg recorded a decrease in pH in the ileum and cecum [18].

A preserved intestinal mucosa is the first condition for a good health condition of animals, because it directly affects the process of digestion and absorption of nutrients, and its role is also crucial in the resistance to invasion of pathogenic bacteria [44]. Early weaning of piglets, which is a practice in intensive pig farms today, has been associated with impaired gut mucosal integrity of piglets, resulting in poorer production performance [45]. Early weaned piglets have higher pH values than piglets that are weaned later. Abnormal acidity in the digestive tract can affect the activity of intestinal digestive enzymes and cause the proliferation of pathogenic bacteria, leading to diarrhea. With the loss of epithelial cells, atrophy of intestinal villi occurs, while with reduced cell differentiation, deepening of the crypts occurs. Numerous studies have shown that the intestinal barrier is damaged in the early weaning process of piglets, which affects the intestinal resistance function and the incidence of diarrhea [45,46]. In this study, the benzoic acid diet increased the integrity of the mucosa of the small intestine, especially the jejunum, by improving villus height, intestinal villus width, and the ratio of villus height to crypt depth and decreasing crypt depth, which is consistent with previous results [15,18]. According to some studies, the expression levels of growth-stimulating factors were increased in piglets fed benzoic acid, and insulin-like growth factor-1 (IGF-1) was shown to be an important mediator of intestinal cell proliferation and differentiation [47]. Also, epidermal growth factor (EGF) can stimulate proliferation and differentiation of the small intestinal epithelium, promote intestinal maturation and prevent colonization of enteropathogens on the intestinal epithelium [48].

The increased interest in the use of chelated mineral complexes as mineral sources for weaned piglets has been due to their greater potential bioavailability compared to minerals from inorganic sources. Absorption of minerals is the main factor that limits the fulfillment of their biological functions in the body. The trace mineral can be absorbed, but it does not have to be used afterwards. Mineral ions of inorganic origin easily combine with other feed components to form insoluble complexes, which reduce mineral absorption. The organic trace mineral or chelated trace mineral is in a chemically inert form through ligand binding and as such is protected from negative interactions with feed components. Organic trace minerals use amino acid or peptide absorption mechanisms and circulate very efficiently to the target tissue [49]. Piglets fed with 50% Zn, Cu, Fe and Mn from chelated metal proteins showed better production results than those fed similar concentrations of trace minerals exclusively from inorganic sulfate forms [50]. The replacement of part of the inorganic microelements

with protein forms improved feed utilization weaned piglets. In some studies, pigs fed protein forms of Zn and Cu had higher concentrations of Zn and Cu in the liver than pigs fed sulfate forms of these metals [51]. This indicates greater utilization of Zn and Cu from proteinates compared to sulfate sources.

This study showed that the addition of chelated forms of copper, manganese and zinc that are bound to the methionine molecule, which replaces inorganic minerals, improves the production results of piglets after weaning. In accordance with our results also, Castillo et al. [52] reported that dietary supplementation with 80 mg/kg organic Zn improved gain to feed ratio during the first two weeks after weaning. Mullan et al. [53] also showed improvements in the feeding of post-decision pigs supplemented with organic Zn. However, Carlson et al. [54] did not find positive effects on production results in piglets that were fed with the addition of an organic form of trace elements, but in that study only organic zinc was used, not copper and manganese. Also, Creech et al. [55] found that piglets fed with chelated Zn achieved better production results than piglets that were fed similar concentrations of trace minerals exclusively from inorganic sulfate forms during the rearing phase, with the fact that in our research, lower concentrations of organic forms of microelements were used.

In this research better production results testify to the increased digestibility of nutrients in groups of piglets with the addition of organic forms of microelements, and the reason for this may be the stimulation of the secretion of digestive enzymes from the stomach, pancreas and intestinal mucosa by chelates [56]. Given that there is an increase in intestinal absorption, chelates can increase the permeability of the intestinal mucosa, which increases the bioavailability of some amino acids [57]. Also, chelates have antimicrobial properties, which can lead to an improvement in the immune function of the intestine [58,59]. On the other hand, the trace minerals used in this research have an important role in the synthesis of enzymes, which are necessary for the digestion of nutrients [60]. Carlson et al. [61] reported that the supplementation of the recommended dose of zinc to piglets after weaning had a positive effect with a trend towards longer intestinal villi, consequently improving piglet production performance. The same was observed in in our research, the addition of chelated forms of microelements increased the length and width of the intestinal villi, as well as the ratio of the length of the villi to the depth of the crypts. Taken together, these findings indicate that chelated forms of micronutrients contribute to enterocyte metabolism through their more efficient absorption [62].

Studies also showed that the addition of chelated Zn achieved increased antimicrobial activity [63]. Hojberg et al. [64] reported that the number of lactic acid bacteria was reduced and the number of coliforms was increased in piglets fed high doses of zinc oxide. Broom et al. [65] showed that the number of lactic acid bacteria was reduced, but that zinc oxide had no effect on the number of *E. coli* in the intestines. In this study, there was a decrease in the number of *E. coli* in the jejunum and ileum in groups of piglets fed with the addition of chelated forms of microelements, while the number

of *Lactobacillus* in the cecum was increased. Similarly, Castillo et al. [52] reported that the number of *Lactobacillus* in the intestinal contents of the jejunum did not differ between pigs fed a control and a chelated Zn mixture.

The addition of large amounts of Zn to the diet of piglets, with the aim of reducing the incidence of diarrhea, has brought many problems. A high content of Zn in the diet leads to the accumulation of Zn in the soil, which contributes to the appearance of soil phytotoxicity in areas of intensive agriculture. Zinc, copper and manganese is bound to soil particles and can cause pollution of lakes, streams and coastal waters through runoff and soil erosion [66]. Zinc from manure complexes can potentially contaminate groundwater through leaching [67]. Taking into account the long-term impact of Zn excretion on the environment, it is important to use a lower concentration of Zn from organic sources to achieve adequate production results of the selected piglets [68]. Nutritional strategies that reduce the excretion of trace elements will be beneficial for the environment and the sustainable development of pig production, due to the prohibition of the use of antibiotics for the purpose of growth stimulation.

CONCLUSION

Dietary supplementation with protected benzoic acid and MMHAC (copper, manganese, zinc) in weaned piglets improved growth performance and the use of these additives in the feed has its nutritional justification. Improved intestinal morphometric measures, as well as a positive impact on the composition of intestinal microbiota resulted in better production results.

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Authors' contributions

DP participated in conducting the experiment and drafting the manuscript. RB participated in the design of the experiment. SN performed the pathohistological analyses of the intestine. LM participated in conducting the experiment. JJ performed the statistical analysis of the experiment results. DŠ made feed formulations and participated as coordinator in all activities. RM designed the study, participated in drafting the manuscript and was coordinator of all activities. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKTI UPOTREBE BENZOEVE KISELINE I HELATNIH FORMI BAKRA, CINKA I MANGANA NA PROIZVODNE REZULTATE PRASADI

Dejan PERIĆ, Roberto BAREA, Sladjan NEŠIĆ, Lazar MAKIVIĆ, Jelena JANJIĆ, Dragan ŠEFER, Radmila MARKOVIĆ

Cilj ovog eksperimenta bio je da se ispitaju efekti upotrebe benzoeve kiseline i helatnih formi mikroelemenata u kojima su bakar (Cu), cink (Zn) i mangan (Mn) vezani za metionin hidroksi analog na proizvodne rezultate, morfologiju creva, crevnu mikrobiotu i pH vrednost u digestivnom traktu odlučene prasadi starosti 28 dana. Eksperiment je sproveden sa 96 prasadi nasumično raspoređenih u četiri grupe (6 replikacija sa po 4 praseta): 1) kontrolna grupa (C) – mikroelementi sulfatnog porekla i to Cu, Zn i Mn u količini od 130 (80 u drugoj fazi), 100, 120 mg/kg u prvoj fazi, tim redosledom; 2) helati (CTM) – mikroelementi u helatnom obliku i to Cu, Zn i Mn u količini od 130 (80 u drugoj fazi), 60, 60 mg/kg u prvoj fazi, tim redosledom; 3) benzoeva kiselina (BA), sa dodatkom 2500 mg/kg u obe faze; 4) helati + benzoeva kiselina (CTM + BA) - mikroelementi u helatnom obliku i to Cu, Zn i Mn u količini od 130 (80 u drugoj fazi), 60, 60 mg/kg u prvoj fazi, tim redosledom; i 2500 mg/kg benzoeve kiseline u obe faze. Rezultati su pokazali da dodatak helatnih formi mikroelemenatai benzoeve kiseline nije samo poboljšao konačnu telesnu masu ($p<0,05$), prosečan dnevni prirast ($p<0,05$) i konverziju ($p<0,05$), već je poboljšao i performanse morfologije creva i smanjio broj *Escherichia coli* u jejunumu i ileumu u eksperimentalnim grupama ($p<0,05$). Ovo istraživanje pokazuje da helati i benzoeva kiselina imaju blagotvorno dejstvo na crevnu morfologiju i mikrofloru odlučene prasadi, što delimično može da objasni zašto su proizvodni rezultati prasadi poboljšani.