PERIODICUM BIOLOGORUM
VOL. 119, No 1, 63–73, 2017
DOI: 10.18054/pb.v119i1.4407

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

original research article

Influence of dietary mannan oligosaccharide and clinoptilolite on hematological, biochemical and gut histological parameters in weaned pigs

HRVOJE VALPOTIĆ[1]
RENATA BARIĆ-RAFAJ[2]
VLADIMIR MRLJAK[3]
ŽELJKO GRABAREVIĆ[4]
MARKO SAMARDŽIJA[5]*
MARCELA ŠPERANDA[6]
IVONA ŽURA ŽAJA[7]
DRAŽEN ĐURIČIĆ[8]
ANA BACH[9]
IVICA HARAPIN[3]
IVAN FOLNOŽIĆ[5]
SILVIJO VINCE[5]
IVICA VALPOTIĆ[10]

[1] Department of Animal Nutrition and Dietetics
Veterinary Faculty, University of Zagreb
Heinzelova 55, 10000 Zagreb, Croatia

[2] Department of Chemistry and Biochemistry
Veterinary Faculty, University of Zagreb
Heinzelova 55, 10000 Zagreb, Croatia

[3] Clinic for Internal Diseases, Veterinary Faculty
University of Zagreb, Heinzelova 55
10000 Zagreb, Croatia

[4] Department of Veterinary Pathology
Veterinary Faculty, University of Zagreb
Heinzelova 55, 10000 Zagreb, Croatia

[5] Clinic for Obstetrics and Reproduction
Veterinary Faculty, University of Zagreb
Heinzelova 55, 10000 Zagreb, Croatia

[6] Department of Animal Science
Faculty of Agriculture, University of Osijek
Petra Svačića 1d, 31000 Osijek, Croatia

[7] Department of Physiology and Radiobiology,
Veterinary Faculty, University of Zagreb,
Heinzelova 55, 10000 Zagreb, Croatia

[8] Veterinary Practice, Malinov trg 7
48350 Đurđevac, Croatia

[9] Passage West Veterinary Centre
County Cork, Cork, Ireland

[10] valpotic@vef.hr

***Correspondence:**
Marko Samardžija
E-mail: smarko@vef.hr

Key words: mannan, zeolite, dietary supplements, blood/intestinal parameters, pigs.

Received July 6, 2016.
Revised September 10, 2016.
Accepted September 14, 2016.

Abstract

Background and Purpose: *Yeast derivative mannan oligosaccharide (MOS) and zeolite clinoptilolite (CPL) well known nutritional supplements acting as either prebiotic or immunobiotic, respectively, in animal husbandry. The aim of this study was to investigate whether or not these agents referred to as potential alternatives to antibiotics may positively influence systemic immune parameters of weaned pigs without negatively affecting their hematological, serum biochemical and gut histological homeostasis.*

Materials and Methods: *Sixty pigs from a commercial farm were divided into three groups comprising 20 pigs each and treated at 4 weeks of age as follows: controls received standard weaner diet, whereas diet for principals was supplemented with 0.2% of MOS or 0.5% of CPL. The experiment was conducted throughout 35 days and at either Day 7 or Day 35 intervals starting at Day 0 before the treatments hematological, serum biochemical and intestinal histopathological parameters were monitored, respectively.*

Results: *Pigs fed CPL supplement had lower proportion of lymphocytes ($P<0.05$), but higher proportions of neutrophils and eosinophils ($P<0.05$) at Day 21. Neither MOS nor CPL affected values of hematological parameters. Serum levels of hepatic enzymes in pigs fed CPL supplemen, with exception of lower value of creatinine kinase (CK) at Day 35 ($P<0.01$) were not changed. These pigs had lower level of total proteins ($P<0.05$) at Day 28, but higher levels of urea ($P<0.05$) and creatinine ($P<0.01$) at Day 35. Generally, principal pigs had milder damages of villi, similar infiltration of lymphoid/myeloid cells within jejunal/ileal lamina propria and follicular hyperplasia in mesenterc lymph node, but more extensive proliferation within Peyer's patches.*

Conclusions: *MOS did not affect any of monitored parameters, and CPL only sporadically induced lymphopaenia and granulocytosis, decrease of CK and total proteins and increase of urea and creatinine levels, indicating that the agents were not associated with any harmful side effects on monitored blood and gut parameters and impairment in general health status of pigs.*

INTRODUCTION

The EU ban on in-feed antibiotic growth promoters (AGP) in swine production has stimulated research for alternative strategies on weaning diets as a way of reducing postweaning gut infections of bacterial etiology and growth retardation as well as of modulating immune functions in order to increase the resistance of pigs to enteric diseases.

Since the 1980's intriguing reports have appeared suggesting that vast variety of substances of natural or synthetic origin act as immune response modifiers (IRMs) and can restore, stimulate or suppress the innate and adaptive immunity in domestic food animals and, hence improve their health, growth and performance (*1*). Based on their biological/genetic or chemical origin and immunomodulating properties these substances have been classified as prebiotics, probiotics, IRMs in narrower sense, recently termed immunobiotics (including microbiotics, fungibiotics, phytobiotics and zoobiotics), chemical compounds (natural or synthetic), metals/microelements, minerals, nutrients and nutraceuticals (*2*). Recently, strategies aiming at stimulation of natural porcine defenses, maintenance of their systemic/gut health and performance through the use of such substances with bioactive properties have gained increasing interest in research and have been subject of numerous nutritional and immunohematological investigations in pigs (*3-12*). However, the results obtained with majority of these substances remained inconclusive as the final aim of using such in-feed supplements is to promote pig immunity, health and performance. Among those few which have shown to be effective in zootechnology and biomedicine are: (a) a variety of polysaccharides from different natural sources, such as yeast derivatives β-glucans and mannans (*3*) and (b) natural clay minerals, such as aluminosilicate compounds bentonite, kaolin, diosmectite, attapulgite and zeolite (*12*). The main source for in-feed supplement currently used in pig nutrition as a natural additive is mannan-oligosaccharide (MOS) found in large quantities in yeast cell wall (*3, 9*), The most promising results to date have been obtained with montmorillonite, smectite, illite, kaolinite, biotic and zeolite clinoptilolite (CPL) for their ability to replace AGP as feed additives and to maintain swine health and performance (*7, 11*).

Among 140 types of natural zeolites, the best known and the most thoroughly tested for its safety and efficacy as feed additive for animal species of veterinary importance is CPL (*13*). CPL has shown to be effective as growth-promoting (*14*), immunostimulating (*15*) and gut health restoring anti-bacterial (*16*) dietary supplement in pigs. Moreover, CPL is the most widespread compound in the medical market and recently has been approved as feed additive in the EU. The CPL utilization in animal biotechnology and veterinary medicine are as detoxificating, anti-diarrheic, growth-promoting, antioxidant, hemostatic and immunostimulating agent, and in human medicine as an adjuvant in immunodeficiency, oncology (after chemotherapy and radiotherapy) or reducer of radioactive elements (*7, 11, 12*). Additionally, the use of CPL has beneficial effects on reproductive efficiency in swine (*10*). An interesting finding was reported earlier in 1998 by Stojic et al. (*17*) who documented significant increase in IgG absorption in piglets oraly dosed with CPL immediately after partus. Also, the same phenomenon was previously described in calves (*18*).

Although MOS belongs to non-digestable oligosaccharides, and its mode of action differs from other prebiotics, it represents the main source for in-feed supplement currently used in pig nutritional as a natural additive (*3, 9, 10*). MOS offers a novel approach to balance the intestinal microbiota to beneficial direction and thus improve overall gut health and welfare (*19*). Also, dietary MOS maintain the intestinal morphology and absorptive/digestive functions (*20*), stimulate the immune functions and improve disease resistance (*21, 22*) by promoting bacterial antigen presentation facilitating thereby the shift from innate to adaptive immune responses (*23*) in weaned pigs. However, apparently inconsistent data were obtained on growth promoting effect of MOS as feed additive since some studies reported no benefits (*23, 24*), while others have recorded growth rate improvements in weaned pigs (*25, 26*). In addition, improvement in IgG absorption, was recorded in piglets and calves following peroral administration of MOS during the first day of life (*27, 28*).

The objective assessment of alternatives to in-feed AGP in food animals, including swine must impact positively on their health and performance, and on their products in order to be fully accepted by producers, feed compounders and consumers and to be environmental friendly. Regarding the limited reports on potential adverse effects of MOS (*29, 30*) and CPL (*31, 32*, *33*) in-feed supplements, tested in pigs as natural alternatives to AGP in our recent studies (*15, 34*) we have considered that data on their possible negative consequences for pig health and food safety should be evaluated as it has been already performed and presented for synthetic substances with putative immunostimulating properties (*35, 36, 37, 38, 39, 40*).

Namely, in our previous studies dealing with immunobiotics of either natural (for review see (*2*)) or synthetic origin (*35*, *36, 37*, *38*), the most promising results have been obtained with levamisole (LEVA) and polyoxyethylene-polyoxypropylene (POE-POP) as immunomodulators and adjuvants and potential alternatives to AGP in weaned pigs *(39, 40)*. However, data on their potential adverse effects on porcine health status, particularly in terms of changing their hemogram/erythrocyte constants (*41, 42*) and serum biochemistry parameters (*43*) and inducing alterations of the small intestinal histocytology (*38*) are still scarce. Recently obtained results imply that a single *peroral* immunomodulating treatment with either LEVA or POE-POP was not associated with any adverse effects on monitored immunohematological, serum biochemical and gut histocytology parameters, and thus, does not suggest impairment in general health status of weaned pigs due to these treatments (*44*).

Therefore, this research is a continuation of our previous studies which showed that dietary MOS and CPL acted rather as immunostimulators in recruitment of circulationg and intestinal lymphoid cell subsets than as growth promoting feed supplements in weaned pigs (*15, 34*), and was performed to investigate whether or not

these agens may positively influence peripheral blood cellular and humoral parameters in weaners without negatively affecting their hematological, serum biochemical and gut histological homeostasis.

MATERIALS AND METHODS

Pigs. Sixty crossbred pigs (Topigs®) of both sexes (females and castrates) and body weight of approximately 6.8 kg, progeny of six litters (from 3rd parity sows) from a commercial swine farm in eastern Croatia were used. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diets (without antimicrobials or growth promoters) according to rearing technology of the farm. Experimental and animal management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC).

Experimental design and treatments. Weaned pigs were randomly divided into three groups comprising 20 animals each, ear-tagged with numbers 1-20 and kept in the same rearing facility of the farm in the separate pens as described earlier (*40*). After two days of accommodation the experimental pigs were treated as follows: (1) control pigs received standard weaner diets either SO - 0 from Day 0 (or 4-weeks of age) to Day 21 or with SO-1 from Day 22 to Day 35 (or 9-weeks of age) of the experiment, whereas both diets for the principals were supplemented with either (2) 0.2% of MOS (Bio-Mos®, Alltech, Nicholasville, KY, USA) or (3) 0.5% of CPL (Vetamin®, Panaceo, Austria) as detailed earlier (*38, 40*). The experiment was conducted throughout a period of 35 days and 7 pigs (marked with numbers 1-7) per group were sampled for peripheral blood at seven day intervals starting at Day 0 before the treatment. At either Day 0 or Day 35 of the experiment 5 pigs per group (marked with numbers 16-20) were euthanatized by intracardial injection of 0.3 mL/kg of T61 preparation (Hoechst®, München, Germany) and sampled for histopathology.

Sampling. Blood samples (10 mL) were collected from *v. cava cranialis* using vacutainers (Beckton Dickinson, Plymouth, UK) and separated in two alliquots, one (2 mL) in the plastic tubes with disodium EDTA (Sigma, St. Louis, USA) as an anticoagulant (1 mg/mL) for hematology and one (8 mL) in the glass tubes for serum biochemistry. Immediately following euthanasia at either 4 or 9 weeks of age (at Day 0 and Day 35 of the experiment, respectively), the specimens (1 cm) of mid jejunum and ileum and a part (0.5 cm) of mesenteric lymph node (MLN) were taken from each of 5 pigs per group, and fixed in 10% paraformaldehyde - phosphate buffered saline (PBS) solution (pH 7.2) for 24 hours until used for histopathology analysis.

Hematological analyses. Total leukocyte count was determined using an automated counter (System 9120, Serono Baker, Pennsylvania, USA). The blood smears were prepared and stained according to May Grűnwald-Giemsa technique, examined by a microscope Olympus BX 41 under an immersion magnification in order to determine the differential blood counts. The relative ratio of leukocyte subpopulations (lymphocytes, neutrophils and eosinophils) was acquired in relation to their total counts. An approxymate value of total serum globulins (Glb) was obtained after determining the values of total proteins and albumin with original reagent kits from Olympus Diagnostica using an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostic, Hamburg, Germany) and by subtracting the value for albumin from that obtained for total serum proteins. The numbers of erythrocytes and thrombocytes as well as the levels of hemoglobin and hematocrtit were determined by the standard methods using an automated counter Serono Baker System 9120 (Pennsylvania, USA).

Serum biochemistry analyses. The serum profiles of hepatic enzymes: alkaline phosphatase (ALP), alpartate aminotransferase (AST), alanine aminotransferase (ALT), namma glutamyl transferase (GGT) and creatinine kinase (CK) and metabolites (glucose, creatinine, urea, total cholesterol, total triglycerides, total proteins and albumin) were determined according to the standard methods using original reagent kits from Olympus Diagnostica (OSR6204 for ALP, OSR6509 for AST, OSR6507 for ALT, OSR6520 for GGT, OSR6279 for CK, OSR6521 for glucose, OSR6578 for cratinine, OSR6534 for urea, OSR6516 for total cholesterol, OSR61118 for total triglycerides, OSR6532 for total proteins and OSR6502 for albumin) using an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostic, Hamburg, Germany).

Histolopathological examination. After fixation in 10% paraformaldehyde - PBS solution the specimens of jejunum, ileum and MLN were processed for histpathological examination by dehydration in 70% and 96% ethanol for 1 hour and by two immersions into 100% ethanol for 1 hour. The specimens were incubated overnight in chlorophorm at 56 °C and then tranferred into the mixture of chlorophorm and paraplast (1:1) at 56 °C for 1 hour. Further, they were, measured twice into paraplast (paraplast I and paraplast II) embedding medium (Sigma, Deisenhofen, Germany) for 1 hour at 56 °C. After cooling at room temperature the paraplast-embedded specimens were cut by the microtome (Reichert-Jung, Germany) into 5-6 µm thick serial sections and floated on a water bath heated to approximately 50 °C. The selected sections were picked up with the 2% APES (3-aminopropyl-triethoxysilane, Sigma, St. Louis, USA) in acetone precoated slides and dried horizontally on a warming tray overnight at 37 °C. The sections were dewaxed in xylene (twice or 10 minutes), hydrated in graded alcohol solutions (for 5 minutes n 100%, 96%, 80% and 70% ethanol) and immersed in distilled water**.** After rinsing in distilled water (twice for 5 minutes), the sections were immersed into hemalaun (for 10 minutes), rinsed in a tap water and immersed into eosin

Table 1. *Cellular and molecular hematological parameters of weaned pigs fed diet supplemented with MOS or CPL from Day 0 (4-weeks of age) to Day 35 (9-weeks of age)*

Parameter/Group	Day of the experiment						Pooled SEM
	0	7	14	21	28	35	
Leukocytes (x10^9/L)							
Control	20.45	22.49	23.97	25.12	25.51	26.98	2.56
MOS	23.27	27.57	27.41	24.26	29.03	29.30	2.74
CPL	17.10	18.67	20.37	24.11	25.89	33.51	2.32
Lymphocytes (%)							
Control	49.16	49.95	56.09	56.43	58.87	58.34	1.94
MOS	51.00	49.67	51.29	58.29	53.00	52.67	5.37
CPL	51.14	47.67	57.50	45.57[a]	58.71	53.43	4.46
Neutrophils (%)							
Control	49.03	45.77	44.70	41.68	38.04	39.14	4.17
MOS	45.14	48.33	46.57	41.14	44.43	45.00	5.41
CPL	45.43	50.17	40.17	50.86[a]	37.71	44.43	4.68
Eosinophils (%)							
Control	3.22	3.97	3.07	2.01	4.95	4.87	0.55
MOS	4.00	1.50	3.00	1.00	2.40	2.33	0.68
CPL	4.00	2.33	2.33	3.67[a]	4.50	2.00	0.70
Total Glb (g/L)							
Control	24.96	26.88	26.67	29.58	29.05	30.18	2.15
MOS	27.57	28.10	28.40	30.28	31.99	34.38	2.70
CPL	25.41	23.93	28.28	30.45	28.41	32.92	1.87

Data are expressed as mean ± SEM; groups comprised 7 pigs each. Means that differ significantly from the control group are marked with a superscript[a] (P<0.05).

(for 2 minutes). Then the sections stained with hemalaun-eosin (HE) were again rinsed in distilled water (twice for 5 minutes) and dehydrated in graded alcohol solutions (70%, 80%, 96% and 100% ethanol), enlightened by short immersing into xylene and embedded in the Canada balsam. The sections of the specimens sampled were dried and examined for histopathological changes under a light microscope with built-in camera (Nikon Microphot – FXA, Japan). The selected areas of each tissue specimen section were photographed.

Statistical analysis. Numerical data were analyzed by the Student's *t* test for dependent samples, because there were only three groupus of pigs (*i.e.* the control group fed standard weaner diets and two principal groups fed these diets supplemented with either MOS or CPL) using the StatisticaSixSigma software (StatSoft, Inc.). Significance of differences between the values obtained for pigs in the control group and those obtained in the pigs treated with either of supplements applied were considered as significant at P<0.05 and lower values.

RESULTS

Hematological parameters

None of cellular and molecular hematological parameters tested in weaned pigs were influenced by MOS supplementation during 5 weeks of the experiment (Table 1).

At Day 21 of the experiment the pigs fed CPL supplement had significantly lower proportion of lymphocytes (P<0.05), but higher proportion of neutrophils and eosinophils (P<0.05). Although not significant an age-dependent increase in the proportions of leukocytes and level of Glb was observed in the pigs from all three groups, reaching peak values at the end of the experiment (Table 1).

Neither MOS nor CPL affected the values of red blood picture parameters, indicating that they did not cause any harmful side effects on hemogram and erythrocyte constants during the observation period of 35 days (Table 2).

Serum biochemistry parameters

The serum levels of hepatic enzymes with the exception of significantly lower value of CK (P<0.01) in the pigs fed CPL supplemen at Day 35, were not changed during the experimental period (Table 3).

Interestingly, much lower values of ALT were recorded in both groups of principals (MOS; P<0.05 and CPL; P<0.01) at Day 0 prior to feeding with supplements. At Day 28 of the experiment the pigs fed CPL supplement had significantly lower serum level of total proteins (P<0.05) but higher levels of urea (P<0.05) and creatinine (P<0.01) at Day 35 of the experiment (Table 4).

Table 2. *Red blood picture parameters of weaned pigs fed diet supplemented with MOS or CPL from Day 0 (4-weeks of age) to Day 35 (9-weeks of age)*

Parameter/Group	Day of the experiment						Pooled SEM
	0	7	14	21	28	35	
Erythrocytes (x10^{12}/L)							
Control	5.24	5.76	5.51	5.34	6.09	5.57	0.29
MOS	4.81	5.82	5.61	4.89	5.53	6.01	0.22
CPL	5.00	5.81	5.28	4.96	5.43	6.15	0.17
Hemoglobin (g/L)							
Control	91.41	99.82	93.35	94.77	108.9	99.02	5.96
MOS	83.57	98.33	95.43	91.14	102.7	109.4	4.58
CPL	87.29	97.29	88.83	89.00	97.42	113.4	3.36
Hematocrit (L/L)							
Control	0.30	0.31	0.27	0.30	0.31	0.32	0.01
MOS	0.25	0.30	0.29	0.26	0.31	0.33	0.01
CPL	0.27	0.30	0.27	0.26	0.29	0.34	0.01
MCV (f/L)							
Control	54.70	53.06	51.95	54.83	54.96	56.14	1.57
MOS	51.49	52.33	52.50	53.94	55.70	55.56	1.41
CPL	53.86	51.99	51.62	52.94	53.57	54.94	1.31
MCHC (g/L)							
Control	327.1	323.7	327.8	340.1	330.0	326.9	5.91
MOS	339.3	324.7	324.1	347.0	333.6	328.1	5.78
CPL	324.4	322.9	327.0	339.0	336.0	336.3	5.04

Data are presented as mean ± SEM; groups comprised 7 pigs each.

Table 3. *Serum levels of hepatic enzymes in weaned pigs fed diet supplemented with MOS or CPL from Day 0 (4-weeks of age) to Day 35 (9-weeks of age)*

Parameter/Group	Day of the experiment						Pooled SEM
	0	7	14	21	28	35	
ALP (U/L)							
Control	302.5	226.4	231.3	230.9	237.9	279.4	41.54
MOS	191.3	213.0	252.4	318.0	381.1	291.3	40.78
CPL	315.6	210.7	214.4	322.1	289.0	218.6	43.73
AST (U/L)							
Control	33.1	49.6	49.7	49.7	42.6	46.9	6.97
MOS	35.4	44.3	72.3	42.6	59.7	50.1	12.49
CPL	24.9	40.0	52.1	42.9	37.4	34.6	6.21
GGT (U/L)							
Control	48.1	33.8	36.5	32.0	38.6	35.7	5.91
MOS	47.1	34.3	37.3	36.7	43.4	40.0	5.84
CPL	35.4	28.8	33.3	33.3	41.7	43.6	4.62
ALT (U/L)							
Control	41.7	30.3	34.5	39.3	41.3	50.8	3.85
MOS	27.0[b]	27.9	29.4	34.4	40.3	47.4	2.88
CPL	30.4[a]	26.5	32.6	36.7	49.7	48.1	3.15
CK (U/L)							
Control	237.5	1489	736.0	977.9	2011	2089	678.0
MOS	246.0	948.0	2318	685.4	1731	1117	835.49
CPL	257.7	1097	2069	1210	656.1	384.6[b]	450.04

Data are presented as mean ± SEM; groups comprised 7 pigs each. Means that differ significantly from control group are marked with a superscript [a] (P<0.05) and [b] (P<0.01).

Table 4. *Serum levels of metabolites in weaned pigs fed diet supplemented with MOS or CPL from Day 0 (4-weeks of age) to Day 35 (9-weeks of age)*

Parameter/Group	Day of the experiment						Pooled SEM
	0	7	14	21	28	35	
Urea (mmol/L)							
Control	2.98	5.04	5.11	5.22	4.41	3.22	0.31
MOS	8.69	5.44	5.88	4.86	4.16	4.30	1.48
CPL	2.86	6.06	5.82	5.45	4.78	5.49[a]	0.53
Cholesterol (mmol/L)							
Control	1.96	1.80	1.79	2.33	2.47	2.67	0.20
MOS	2.47	1.74	1.80	2.38	2.53	2.75	0.17
CPL	2.21	1.42	1.85	2.33	2.77	3.31	0.22
Creatinine (µmol/L)							
Control	124.0	109.8	105.4	95.07	92.43	82.04	4.63
MOS	146.3	106.4	90.43	88.86	93.14	95.86	7.78
CPL	121.6	111.3	105.7	94.57	104.3	106.0[b]	5.23
Triglycerides (mmol/L)							
Control	0.49	0.62	0.54	0.59	0.71	0.69	0.17
MOS	0.63	0.61	0.63	0.77	0.65	0.83	0.05
CPL	0.39	0.52	0.55	0.63	0.69	0.87	0.10
Glucose (mmol/L)							
Control	6.26	5.56	5.38	5.90	5.05	6.03	0.27
MOS	5.60	5.97	6.04	5.24	6.14	5.90	0.38
CPL	6.93	6.00	6.05	5.70	6.41	5.37	0.26
Total proteins (g/L)							
Control	57.21	52.59	55.07	56.71	64.13	59.22	1.98
MOS	67.71	53.71	51.71	53.29	57.86	58.57	3.01
CPL	56.86	49.33	53.29	53.29	55.29[a]	63.57	1.92
Albumin (g/L)							
Control	33.07	26.63	24.27	28.44	29.05	26.95	1.58
MOS	33.33	26.28	23.61	24.88	27.57	26.59	1.25
CPL	31.45	25.40	24.24	22.83	26.87	30.66	1.68

Data are presented as mean ± SEM; groups comprised 7 pigs each. Means that differ significantly from control group are marked with a superscript [a] (P<0.05) and [b] (P<0.01).

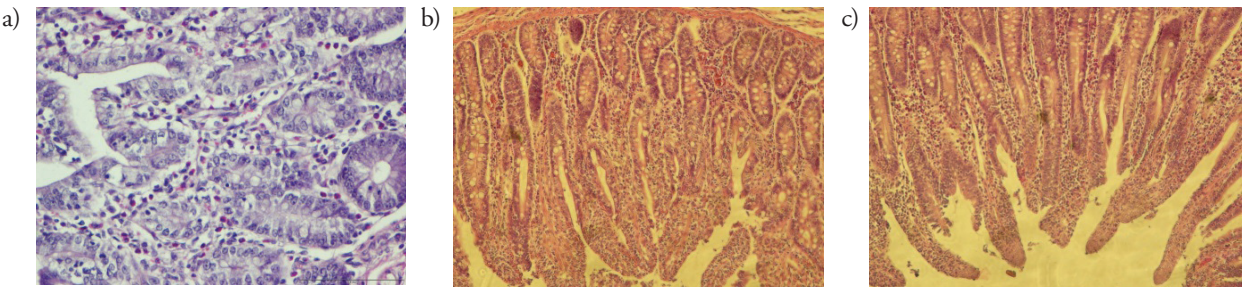

Figure 1. *Mid jejunum of pigs from the control group (a) and from the principal groups fed diet supplemented with either MOS (b) or CPL (c) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the experiment. HE, x 10.*

Histological findings

A moderate apical necrosis of villous epithelim was visible in the mid jejunum of the pig from the control group (Figure 1a).

Also, a mild atrophy of crypts and vast infiltration of mononuclear leukocytes (MNL), predominantly natural killer (NK) cells and globular leukocytes (GL) within the lamina propria (LP) were observed. A mild oedema of submucosis with hyperemic blood vessels could be noticed. In the mid jejunum of the MOS supplemented pig the villi tended to amalgamate and became shorter and desquamation of epithelium in some places were visible (Figure 1b). A vast infiltration of MNL with less numer-

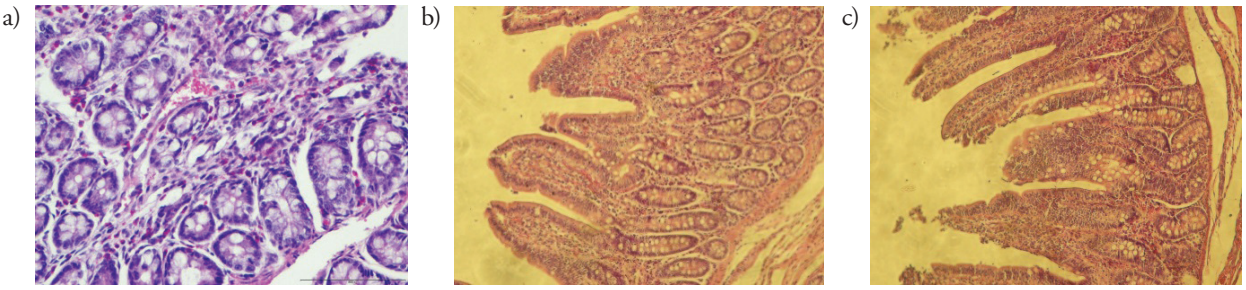

Figure 2. *Mid ileum of 9-week-old pigs from the control group (a) and from the principal groups fed diet supplemented with either MOS (b) or CPL (c) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the experiment. HE, x 4, x 10 or x 20, respectively.*

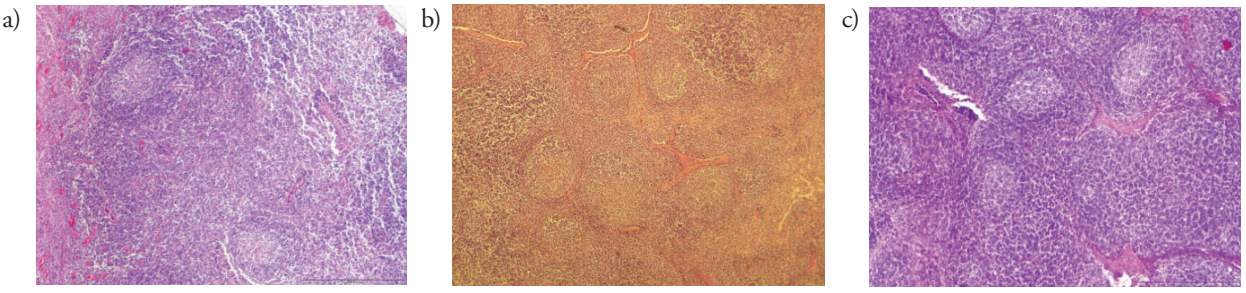

Figure 3. *Lymphoid follicules in the MLN of 9-week-old pigs from the control group (a) and from the principal groups fed diet supplemented with either MOS (b) or CPL (c) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the experiment. HE, x 4.*

ous GL was observed in LP and follicular lymphoid hyperplasia could be noticed within submucosae. Similarly strong infiltration of MNL with more numerous GL and mild infiltration of polymorphonuclear leukocytes (PMNL) were observed within jejunal LP of the pig fed CPL supplement (Figure 1c). A moderate focal desquamation of epithelium with mildly oedematous mucosae were noticed in the mid ileum of the pig from the control group (Figure 2a).

Also, a vast infiltration of MNL with moderate number of GL were observed within LP. In ileal Peyer's patches (PP) granulomatous follicular inflammation with moderate infiltration of eosinophils and multitude of NK cells were visible. A milder focal oedema of ileal LP with intensive infiltration of MNL and NK cells were observed in the pig treated with dietary MOS (Figure 2b). Less intensive follicular hyperplasia of PP was also visible. In the CPL supplemented pig an intensive infiltration of MNL with moderate number of GL in LP and follicular hyperplasia within submucosae of ileum were noticed (Figure 2c). In the mesenteric lymph node (MLN) of the pig from the control group clearly visible a dense accumulation of lymphocytes and rather prominent sinus histiocytosis were recorded (Figure 3a).

Also, considerably expressed primary and secondary lymphoid follicles along with a strong follicular hyperplasia as well as a strong sinus eosinophili and infiltration of GL could be observed. A strong follicular hyperplasia with significantly expressed secondary follicles comprising moderate numbers of eosinophils and GL prevalently within subcapsular area of the MLN from the pig fed MOS supplement werenoticed (Figure 3b). A similar finding was recorded in the MLN from the CPL-treated pig as a strong follicular hyperplasias and prominent proliferation of lymphocytes with moderate sinus infiltration of eosinophils and histiocytes could be seen (Figure 3c).

DISCUSSION

The results obtained on dietary MOS or CPL treatments of pigs throughout 5 weeks following weaning did not indicate that these substances adversely affect their overall health status in terms of changes in hematological and serum biochemical parameters as well as in integrity of their intestinal mucosal barrier. Generally, the hematological parameters were only differing slightly from the reference values for young pigs (*45*). Dietary MOS supplementation, at the dose applied in our study, neither influenced the level of total Glb nor counts/proportions of white blood cells (WBC) in weaned pigs. The former finding is in contrast to that reporting that the pigs fed 0.03% of the yeast cell wall extract had higher IgA serum concentrations than their controls (*23*). However, when a dose of 0.3% has been applied differences were nonsignificant, similarly as we have obtained for total serm Igs with a dose of 0.2%. The later fact seems to be inconsistent with our finding of increased proportions of circulating $CD45^+$ lymphoid cells, $CD4^+$ and $CD8^+$ T or $CD21^+$ B cells in pigs fed MOS supplement (*34*). However, it

could be ascribed to short-term alterations in the expression of membrane markers (*i. e.* differentiation antigens) on immunophenotype-specific cell subsets within population of lymphoid cells due to the exposure of pigs to dietary MOS during postweaning immune maturation as suggested by Davis *et al.* (*46*). More evidence has been reported that dietary MOS has both direct and indirect modulatory effects on porcine immune functions by promoting bacterial antigen presentation facilitating thereby the shift from innate to adaptive immune responses (*23*) and on improvement of disease resistance (*21*). Also, MOS improves immune responses by rapidly increased numbers of leukocytes, lymphocytes and neutrophils of nursery pigs 7 days post-infection with porcine reproductive and respiratory syndrome virus (*47*). These discrepancies are probably due to differences in the study design (*32, 47*), the duration of experiment (*21, 23*), breed of the pigs tested (*21, 23, 47*) and particularly dosage of the agent (*32*). In the current study, when pigs were fed with dietay CPL the percentage of lymphocytes decreased and percentages of neutrophils and eosinophils simultaneously increased. However, these changes in systemic immune function were short (recorded only at Day 21 of the experiment) and we assume that could be explained as an indirect response to transient alterations that were occurring in the intestinal immune responsiveness to dietary CPL (*15*). Although the values for total serum Glb were not significantly increased in either MOS- or CPL- treated pigs during the experiment, it is interesting that both were much higher (34.38±2.7 g/L and (32.92±1.87 g/L, respectively) at Day 35 than the reference values (22.4-24.6 g/L) according to Kaneko *et al.* (*48*). Such deviations could be ascribed to the differences in genetic and paragenetic factors between tested pigs.

None of the tested agents affected the values of hemogram and erythrocyte constants indicating that their use in the pig nutrition does not pose a risk for the consumers in this regard. Morever, our data were within range of reference values for weaned pigs (*49*). Regarding dietary MOS supplementation, our results on hematological parameters agreed with those of Grela *et al.* (*29*) who also claimed that the agent did not change hemogram, WBC or leucogram, and that only erythrocyte count was significantly higher at the end of the experiment or Day 84 of life. Interestingly, our values obtained for WBC in the MOS supplemented pigs at the end of the experiment or Day 63 of life were much higher (29.3±2.74 *vs*. 12.3±1.02 x 10^9/L) than that recorded at the nearest day of sampling or Day 56 of the experiment by abovementioned authors (*29*). Conversely, their values for erythrocytes obtained at Day 56 were higher (7.36±0.16 *vs.* 6.01±0.22 x 10^{12}/L) than that recorded by us at Day 63. This is probably due to differences in the breeds of pigs used, the study design and particularly duration of the experiment as we have followed-up the effect of dietary MOS for 35 days, whereas they observed it for 56 days.

The results obtained in the current study for hematological parameters of dietary CPL supplemented pigs partly agree for leukocytes and neutrophils with those obtained by Šperanda *et al.* (*31*), with the exception of higher proportion of lymphocytes recorded either at Day 7 and 8 (47.67±4.46 *vs*. 36.4±3.39%) or at Day 14 (57.5±4.46 *vs*. 40.2±3.39%), respectively, of the experiments. More recently, were reported data on total leukocyte count (30.11±2.29 x 10^9/L) in pigs fed 0.5% of CPL supplement (*14*) similar to that obtained by us (33.51±2.32 x 10^9/L) with the same amount of CPL supplement. Data obtained for hemogram and erythrocyte constants were only consistent with those for erythrocyte counts (*31*), and were much lower for values of hemoglobin either at Day 7 and Day 8 (97.29±3.36 *vs*. 115.2±8.26) or at Day 14 (88.83±3.36 *vs*. 104.6±8.26 g/L), hematocrit (0.30±0.01 *vs*. 0.420±0.02 L/L or 0.27±0.01 *vs*. 0.36±0.02 L/L), MCV (51.99±1.31 *vs*. 66.38±1.27 f/L or 51.62±1.31 *vs*. 63.8±1.31 f/L) or much higher for values of MCHC (322.9±5.04 *vs*. 273.6±10.75 g/L or 327.0±5.04 *vs*. 287.0±10.75 g/L). However, all these deviations were within range of reference values for young pigs (*45*).

Except the minor oscillations, the values of the most biochemical parameters including liver enzymes and metabolites tested, were within normal range for swine and in accordance with age of the pigs (*48, 49*). While the inclusion of MOS in pig diet did not affect values of tested parameters, CPL supplementation decreased serum level of total proteins and CK concentration at Day 28 and 35, respectively, and increased levels of urea and creatinine at Day 35 of the experiment. The other authors reported that dietary MOS partly affected serum levels of metabolites tested by either increasing or decreasing levels of total cholesterol fractions HDL and LDL, respectively, but did not affect the levels of triglycerides and cholesterol (*29*) similarly as we have recorded for the later metabolites in this study. Also, MOS supplementation may decrease HDL level and increase concentration of ALT (*30*), and the later finding is not in agreement with our finding of unaffected value of ALT. However, our data on unaffected levels of urea, cholesterol, triglycerides, glucose, total proteins, albumin, ALP, AST and ALT by dietary MOS supplementation agreed well with these authors. The obtained effects of dietary CPL on serum biochemistry parameters tested in weaned pigs were mostly in contrast to those who reported that the agent decreased total cholesterol (*14, 32*) and urea levels (*14*) while increased triglycerides, AST (*32*) and ALT (*14*). Namely, we have observed unaffected levels of these metabolites and AST or ALT as well as an opposite finding of increased level of urea. There are no data for serum values of GGT and CK obtained in similarly designed trials, and thus we have compared our results with those of the other authors who simultaneously tested antitoxic efficiency of dietary CPL in pigs fed on zearalenone contaminated feed (*31*). Much lower values of GGT were obtained either at Day 7 and Day 8 (28.8±4.62 *vs.* 45.2±5.53 U/L) or at Day 14 (33.3±4.62 *vs*. 47.33±7.14 U/L) of the experiment

in the current study. Conversely, in this study much higher values of CK obtained either at Day 7 and Day 8 (1097±450.04 *vs.* 233.60±321.88 U/L) or at Day 14 (2069±450.04 *vs*. 298.6±321.88 U/L) of the experiment.

The histological features of the small intestinal mucosa in the pigs from the principal groups showed very mild damages and were rather normal for farm pigs which were exposed to the natural gut infections, indicating that tested feed supplements did not induce any additional histopathological changes. Also, there were no any adverse histological changes in the MLN tested following 5 weeks of dietary MOS or CPL supplementation. On the contrary, these supplements provided an immune protection against intraluminal microbiota as it was suggested for MOS (*20, 21, 22*) as well as for CPL (*15, 50, 51*). Such protection of jejunal and ileal mucosal surfaces of the either MOS- or CPL- treated pigs was characterized by more extensive infiltration of lymphoid and myeloid cells as compared to those in untreated control pigs. An increased cellularity of the MLN and infiltration of dispersed and aggregated lymphatic tissues by histiocytes and eosinophils was observed within the small intestinal mucosa. As the mucosal damages were found to be less severe than those observed in the pigs experimentally immunized with attenuated F4ac$^+$non-enterotoxigenic *Escherichia coli* vaccine candidate strain (*52*), we assumed that the dietary supplements tested did not additionally damaged jejunal and ileal mucosa in the treated pigs. However, data on potential adverse effects of MOS or CPL applied as in-feed supplements on porcine general health status, particularly in terms of inducing alterations of gut histology as their firstly expected effects should be in this compartment are still scarce (*23, 31*), and are limited to gut morphology/absorptive function (*20, 23, 25*), beneficial microbiota (*19, 24, 30*) and immunity (*15, 23, 34, 46*).

Since there are no evidences that these compounds will be degraded during their passage through the gastrointestinal tract of treated pigs, the risk for the consumers is also an important aspect. In our recent studies we have followed-up gut health status of weaned pigs fed with either MOS or CPL supplements by daily clinical observation and scoring the incidence/severity of diarrhea, by weekly examination of intestinal microbiota and by monthly checking changes in the intestinal cellular immunity (*15, 34*). However, the literature dealing with the impact of dietary MOS or CPL supplementation on porcine health parameters tested in our current study is very rare, and thus further research are needed, particularly on their pharmacokinetics and physiological homeostasis in food animals.

Considering all provided evidences in this study it can be concluded that dietary MOS or CPL when applied as feed additives in dose of either 0.2% or 0.5%, respectively, did not disturb normal metabolic pathways and systemic and local (intestinal) homeostasis in weaned pigs. Namely, the obtained results imply that MOS (generally) and CPL (regardless sporadically influencing values of very few parameters, but still within the range of reference values) were not associated with adverse effects on a great majority of monitored parameters relevant for general health status of growing pigs.

REFERENCES

1. BLECHA F, CHARLEY B 1990 Rationale for using immunoptentiators in domstic food animals. In: Blecha F, Charley B (eds) Immunomodulation in domestic food animals. Academic Press, San Diego, CA, USA, p 3-19. https://doi.org/10.1016/B978-0-12-039235-3.50007-1
2. ŠPERANDA M, VALPOTIĆ I 2012 Immunoregulation: a proposal for an experimental model. In: Kapur S, Barbosa Portela M (eds) Immunosuppression: Role in health and diseases. InTech, Rijeka, Croatia, p 255-90. https://doi.org/10.5772/29970
3. KOGAN G, KOCHER A 2007 Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livest Sci 109: 161-65. https://doi.org/10.1016/j.livsci.2007.01.134
4. GALLOIS M, OSWALD I P 2008 Immunomodulators as efficient alternatives to in-feed antimicrobials in pig production? Arch Zootechn 11: 15-32.
5. GALLOIS M, ROTHKŐTTER H J, BAILEY M, STOKES C R, OSWALD I P 2009 Natural alternatives to in-feed antibiotics in pig production: can immunomodulators play a role? Animal 3: 1644-61
6. LALLES J P, BOSI P, JANCZYK P, KOOPMANS S J, TORRALLARDONA D 2009 Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. Animal 3: 1625-43
7. VONDRUSKOVA H, SLAMOVA R, TRCKOVA M, ZRALY Z, PAVLIK I 2010 Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned pigs:a review. Vet Med 55: 199-224
8. CHO J H, ZHAO P Y, KIM I H 2011 Probiotics as dietary additive for pigs: a review. J Anim Vet Adv 10: 2127-134. https://doi.org/10.3923/javaa.2011.2127.2134
9. HALAS V, NOCHTA I 2012 Mannan oligosaccharides in nursery pig nutrition and their potential mode of action. Animals 2: 261-74. https://doi.org/10.3390/ani2020261
10. PAPATSIROS V G, KATSOULAS p D, KOUTOULIS K C, KARATZIA M, DEDOUSI A, CHRISTODOULOPOULOS G 2013 Alternatives to antibiotics for farm animals. CAB Reviews 8: 1-15
11. THACKER P A 2013 Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Biotechnol 4: 35-46. https://doi.org/10.1186/2049-1891-4-35
12. LAURINO C, PALMIERI B 2015 Zeolite: "the magic stone"; main nutritional, environmental, experimental and clinical fields of application. Nutr Hosp 32: 573-81
13. EFSA 2013 Panel on Additives and Products or substances used in animal Feed (FEEDAP); Scientific Opinioun on the safety and efficacy of clinoptilolite of sedimentary origin for all animal species. EFSA Journal 11: 3039-52. https://doi.org/10.2903/j.efsa.2013.3039
14. PRVULOVIC D, KOSARCIC S, POPOVIC M, DIMITRIJEVIC D, GRUBOR-LAJŠIĆ G 2012 The influence of hydrated aluminosillicate on biochemical and haematological blood parameters, growth and carcass traits of pigs. J Anim Vet Adv 11: 134-40. https://doi.org/10.3923/javaa.2012.134.140
15. VALPOTIC H, TERZIC S, VINCE S, SAMARDZIJA M, TURK R, LACKOVIC G, HABRUN B, DJURICIC D, SADIKOVIC M, VALPOTIC I 2016c In-feed supplementation of a clinoptilolite favourably modulates intestinal and systemic immu-

nity and some production parameters in weaned pigs. Vet Med-Czech 61: 317-27. https://doi.org/10.17221/175/2015-VETMED

16. PAPAIOANNOU D S, KYRIAKIS C S, ALEXOPOULOS C, TZIKA E D, POLIZOPOULOU Z S, KYRIAKIS S C 2004 A field study on the effect of the dietary use of a clinoptilolite-rich tuff, alone or in combination with certain antimicrobials, on the health status and performance of weaned, growing and finishing pigs. Res Vet Sci 76: 19-29
17. STOJIĆ V, GAGRČIN M, FRATRIĆ N, TOMAŠEVIĆ M, KIROVSKI D 1998 The effect of a clinoptiolite mineral adsorbent on colostral immunoglobulin G absorption in newborn piglets. Acta Vet (Beograd) 48: 19-26
18. STOJIĆ V, ŠAMANC H, FRATRIĆ N 1995 The effect of clinoptilolite based mineral adsorber on colostral immunoglobulin G absorption in newborn calves. Acta Vet (Beograd) 45: 67-74
19. CASTILLO M, MARTIN-ORUE SM, TAYLOR-PICKARD JA, PEREZ JF AND GASA J 2008 Use of mannan-oligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: Effects on microbiota and gut function. J Anim Sci 86: 94-101
20. POEIKHAMPHA T, BUNCHASKA C 2011 Effects of sodium gluconate, mannan oligosaccharide and potassium diformate on growth performance and small intestinal morphology of nursery pigs. Asian-Aust J Anim Sci 24: 844-50. https://doi.org/10.5713/ajas.2011.10334
21. YIN YL, TANG ZR, SUN ZH, LIU ZQ, LI TJ, HUANG RL, RUAN Z, DENG ZY, GAO B, CHEN LX, WU CY AND KIM SW 2008 Effect of galacto-mannan-oligosaccharides or chitosan supplementation on cytoimmunity and humoral immunity in early-weaned piglets. Asian-Aust J Anim Sci 21: 723-31
22. ŠPERANDA M, ĐIDARA M, ŠPERANDA T, DOMAĆINOVIĆ M, VALPOTIĆ H, KOVAČEVIĆ J, ANTUNOVIĆ Z, NOVOSELEC J 2008 Hydrolyzed brewery yeast product like immunomodulator in weaned piglets. Archiva Zootechn 11: 52-60
23. SAUERWEIN H, SCHMITZ S, HISS S. 2007 Effects of dietary application of a yeast cell wall extract on innate and acquired immunity, on oxidative status and growth performance in weanling piglets and on the ileal epithelium in fattened pigs. J Anim Physiol Anim Nutr 91: 369-80. https://doi.org/10.1111/j.1439-0396.2006.00663.x
24. WHITE LA, NEWMAN MC, CROMWELL GI AND LINDEMANN MD 2002 Brewers dried yeast as a source of mannan-oligosaccharides for weanling pigs. J Anim Sci 80: 2619-28
25. VAN DER PEET-SCHWERING CMC, JANSMAN AJM, SMIDT H AND YOON I 2007 Effect of yeast culture on performance, gut integrity, and blood cell composition of weanling pigs. J Anim Sci 85: 3099-109. https://doi.org/10.2527/jas.2007-0110
26. ZHAO PY, JUNG JH AND KIM IH 2012 Effect of mannan oligosaccharides and fructan on growth performance, nutrient digestibility, blood profile, and diarrhea score in weaned pigs. J Anim Sci 90: 833-39
27. LAZAREVIĆ M, ŠABANOVIĆ M 2003 Mannan oligosacharides enhance absorption of colostral IgG in newborn calves and piglets. Lucrari Stientificae, Medicina Veterinara 36: 564-68
28. LAZAREVIC M, SPRING P, SHABANOVIC M, TOKIC V, TUCKER A L 2010 Effect of gut active carbohydrates on plasma IgG concentrations in piglets and calves. Animal 4: 938-43. https://doi.org/10.1017/S1751731110000194
29. GRELA E R, SEMENIUK V, CZECH A 2006 Efficacy of fructooligosaccharides and mannanoligosaccharides in piglet diets. Med Wet 62: 762-65
30. REKIEL A, WIECEK J, BIELECKI W, GAJEWSKA J, CICHOWICZ M, KULISIEWICZ J, BATORSKA M, ROSZKOWSKI T, BEYGA K 2007 Effect of addition of feed antibiotic flavomycin or prebiotic BIO-MOS on production results of fatteners, blood biochemical parameters, morphometric indices of intestine and composition of microflora. Arch Tierz, Dummerstorf 50: 172-80
31. ŠPERANDA M, LIKER B, ŠPERANDA T, ŠERIĆ V, ANTUNOVIĆ Z, GRABAREVIĆ Ž, SENČIĆ D, GRGURIĆ D, STEINER Z 2006 Haematological and biochemical parameters of weaned piglets fed on fodder mixture contaminated by zearalenone with addition of clinoptilolite. Acta Vet (Beograd) 56: 121-36. https://doi.org/10.2298/AVB0603121S
32. PRVULOVIĆ D, JOVANOVIĆ-GALOVIĆ A, STANIĆ B, POPOVIĆ M, GRUBOR-LAJŠIĆ G 2007 Effects of a clinoptilolite supplement in pig diets on performance and serum parameters. Czech J Anim Sci 52: 159-64
33. PRVULOVIC D, KOSARCIC S, POPOVIC M, GRUBOR-LAJSIC G, 2009 Effects of hydrated aluminosilicates on growth performance and blood parameters of pigs. Cuban J Agri Sci 43: 59-63
34. VALPOTIĆ H, TERZIĆ S, ANDRIŠIĆ M, VINCE S, SAMARDŽIJA M, ŠPERANDA M, LACKOVIĆ G, HABRUN B, ĐURIČIĆ D, FOLNOŽIĆ I, BACH-BACICH A, VALPOTIĆ I 2016b Impact of dietary mannan oligosaccharide supplementation on peripheral blood and intestinal mucosal immune cells, gut microbiota and productivity in weaned pigs. Acta Vet Brno 85: 267–76. https://doi.org/10.2754/avb201685030267
35. ŠVER L, TRUTIN–OSTOVIĆ K, ŽUBČIĆ D, CASEY T A, DEAN–NYSTROM E A, VALPOTIĆ I 1996 Porcine gut T, B and null/γ δ TCR$^+$ cell quantification in the protective immunity to fimbrial/toxin antigens of *Escherichia coli*. Period Biol 98: 473-8.
36. VALPOTIĆ H, LACKOVIĆ G, TOMLJENOVIĆ A, BOŽIĆ F, POPOVIĆ M, VIJTIUK N, KOVŠCA-JANJATOVIĆ A, GREGURIĆ-GRAČNER G, VALPOTIĆ I 2009a Effect of levamisole on the number of intestinal goblet cells in weaned pigs experimentally vaccinated against colibacillosis. Vet arhiv 79: 543-53
37. VALPOTIĆ H, ŠURAN J, PREVENDAR CRNIĆ A, ŠPERANDA M, ĐIDARA M, VALPOTIĆ I, BOŽIĆ F 2009b Stimulation by levamisole of cel-mediated immunity in weaned pigs. Ital J Anim Sci 8 (Suppl. 3): 122-24. https://doi.org/10.4081/ijas.2009.s3.122
38. VIJTIUK N, ŠVER L, LACKOVIĆ G, POPOVIĆ M, BOŽIĆ F, VALPOTIĆ I 2005 Intestinal immune response of weaned pigs experimentally vaccinated with F4ac$^+$ non-enterotoxigenic strains of Escherichia coli. Acta Vet Brno 74: 595-601. https://doi.org/10.2754/avb200574040595
39. VALPOTIĆ H, MRŠIĆ G, GRŠKOVIĆ B, ŠPOLJARIĆ D, KEZIĆ D, SREČEC S, MATAUŠIĆ-PIŠL M, LACKOVIĆ G, CAPAK D, MIHELIĆ D, VLAHOVIĆ K, VALPOTIĆ I, PIRKIĆ A, ANĐELINOVIĆ D, POPOVIĆ M 2013 Effect of polyoxyethylene and polyoxypropylene nonionic block copolymers on performance and recruitment of immune cell subsets in weaned pigs. Acta Vet Scand 55: 54-62. https://doi.org/10.1186/1751-0147-55-54
40. VALPOTIĆ H, ŠPERANDA M, KOVŠCA-JANJATOVIĆ A, ĐIDARA M, LACKOVIĆ G, BOŽIĆ F, HABRUN B, SREČEC S, MATAUŠIĆ-PIŠL M, VALPOTIĆ I 2014 Levamisole stimulates proliferation of circulating and intestinal immune cell subsets, gut health and performance in weaned pigs. Can J Anim Sci 94: 43-53. https://doi.org/10.4141/cjas2013-097
41. BOŽIĆ F, MRLJAK V 2001 Levamisole modulates the numbers and phagocytic functions of leukocytes in weaned pigs vaccinated with non-enterotoxigenic F4ac$^+$ *Escherichia coli*. Praxis vet 49: 21-30
42. ŠPERANDA M, ĐIDARA M, ŠPERANDA T, DOMAĆINOVIĆ M, VALPOTIĆ H, ANTUNOVIĆ Z 2009 Feed additives potent immunomodulators? Proc 1st Workshop Feed to Food, FP7 REG-

POT-3 and 13th Symp Feed Technology, Lević J (ed) Institute for Food Technology, Novi Sad, Serbia, pp 57-65

43. BILANDŽIĆ N, MITAK M, ŠIMIĆ B 2010 Levamisole increases leukocyte count and immunoglobulin levels in young boars. Slov Vet Res 47: 13-19
44. VALPOTIĆ H, BARIĆ-RAFAJ R, MRLJAK V, ŠPERANDA M, GRABAREVIĆ Ž, SAMARDŽIJA M, FOLNOŽIĆ I, ĐURIČIĆ D, HARAPIN I, VINCE S, VALPOTIĆ I 2016a The influence of immune modulation with levamisole and polyoxyethylene-polyoxypropylene copolymers on hematological and biochemical parameters and intestinal histomorphology of weaned pigs. Vet. arhiv 86: 667-83
45. THORN C E 2000 Normal hematology of the pig. In: Schalm's Veterinary Hematology II, Pub. (Feldman, B. F., J. G. Zinkl, N. C. Jain, eds) Lippincott, Williams, Willkins, Philadelphia, SAD, pp 1089-95
46. DAVIS ME, MAXWELL CV, ERF GF, BROWN DC AND WISTUBA TJ 2004 Dietary supplementation with phosphorylated mannans improves growth response and modulates immune function of weanling pigs. J Anim Sci 82: 1882-91
47. CHE TM, JOHNSON RW, KELLEY KW, VAN ALSTINE WG, DAWSON KA, MORAN CA AND PETTIGREW JE 2011 Mannan oligosaccharide improves immune responses and growth efficiency of nursery pigs experimentally infected with porcine reproductive and respiratory syndrome virus. J Anim Sci 89: 2592-602
48. KANEKO J J, HARVEY J W, BRUSS M L 2008 Clinical biochemistry of domestic animals. 6th ed, Academic Press, San Diego, CA, USA, pp 883-88
49. FRIENDSHIP R M, LUMSDEN J H, McMILLAN I, WILSON M R 1984 Hematology and biochemistry reference values for Ontario swine. Can J Comp Med 48: 390-93
50. ISLAM M M, AHMED S T, KIM S G, MUN HS, YANG C J 2014 Dietary effect of artificial zeolite on performance, immunity, fecal microflora concentration and noxious gas emission in pigs. Ital J Anim Sci 13: 830-35
51. HU C H, XIAO K, SONG J, LUAN Z S 2013 Effects of zinc oxide supported on zeolite on growth performance, intestinal microflora and permeability, and cytokines expression of weaned pigs. Anim Feed Sci Technol 181: 65-71
52. VIJTIUK N, ĆURIĆ S, LACKOVIĆ G, UDOVIČIĆ I, VRBANAC I, VALPOTIĆ I 1995 Histopathological features in the small intestine of pigs infected with F4ac+ non-enterotoxigenic or enterotoxigenic strains of *Escherichia coli*. J Comp Pathol 112: 1-10. https://doi.org/10.1016/S0021-9975(05)80085-4