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Original article

Dietary supplementation with mannan oligosaccharide and clinoptilolite modulates innate and adaptive immune parameters of weaned pigs

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Abstract

The aim of this study was to assess modulatory effects of dietary supplements mannan oligosaccharide (MOS) and clinoptilolite (CPL) as potential alternatives to antibiotic growth promoters (AGP) given to 4-week old pigs at weaning (Day 0) on their innate/adaptive immunity by determining: alterations in C-reactive protein (CRP) and haptoglobin (HpG) serum levels, efficiency of blood monocytes (MO) and neutrophilic granulocytes (GR) for *in vitro* phagocytosis (PHC)/microbicidity (MBC) and proportion of extrathymic double positive CD4 CD8 ($CD4^+CD8^+$) T cells throughout 35 days of the study. Neither MOS nor CPL changed the serum concentrations of CRP, whereas that of HpG was significantly increased in the CPL supplemented pigs ($p<0.05$) at Day 35. Activity of PHA of GR was significantly increased by both dietary supplements ($p<0.05$) from Day 7 to Day 35. Also, the GR from pigs fed with both supplements had significantly increased MBC at Day 7 ($p<0.05$), but at Day 35 such an increase was observed only for CPL. The *in vitro* PHC/MBC of MO did not change in either group of supplemented pigs. The pigs supplemented with MOS had a significantly higher proportion of $CD4^+CD8^+$ T lymphocytes at Day 28 ($p<0.05$). Although both supplements showed a promising ability to stimulate rather innate than adaptive cellular immunity, it does not appear that any solely applied natural substance such as MOS or CPL in the current study could be a competitive alternative to conventional AGP for improving health and promoting growth in weaned pigs.

Key words: mannan oligosaccharide, clinoptilolite, innate/adaptive immunity, weaned pigs

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Introduction

Recently, increased sanitary problems in intensive production of food animals have been overcome by adding sub-therapeutic doses of antibiotic growth promoters (AGP) to enhance production efficiency, particularly in swine production systems, where their efficiency in increasing growth rate, improving feed utilization and reducing mortality from clinical disease is well documented (Cromwell 2002). Consumers, especially in developed countries, are becoming increasingly concerned about drug residues in meat and other food animal products (Vondruskova et al. 2010). As a result, many countries have banned or are banning the inclusion of antibiotics in swine diets as a routine means of growth promotion. The ban of AGP in the EU goes back to 2006, but has only recently been implemented in the USA as a result of a new Food and Drug Administration (FDA) Veterinary Feed Directive (VFD 2017). Many substances of natural origin are studied as potential alternatives to AGP and some of them have been shown to be capable of reducing the harmful effects of early weaning of pigs and to protect growing pigs against enteric infections (Šperanda and Valpotić 2012, Thacker 2013, Papatsiros et al. 2013, Dhama et al. 2015, Laurino and Palmieri 2015). Promising results have been obtained with mannanoligosaccharide (MOS), particularly in diets for weaned pigs as a nutraceutical/immunomodulator (NC/IM) and for sows as an IM able to increase level of colostral (passive) immunity (Czech et al. 2010). Moreover the obtained results suggest that dietary MOS enhances disease resistance in young pigs by promoting antigen presentation, thus facilitating the shift from an innate to an adaptive immune response (Halas and Nochta 2012). The mode of action of dietary MOS has been shown to be such that it may affect: (i) the intestinal microbiota by attaching to the enteric pathogens in the gut lumen and thus preventing their attachment to the enterocytes) and thereby reducing their colonization, (ii) the morphology of gut mucosal surfaces by increasing the villous height: crypt depth ratio and thus the digestibility of nutrients, and it may (iii) stimulate the systemic and local (intestinal) immunity in pigs (Nochta et al. 2009, Halas and Nochta 2012, Spring et al. 2015, Valpotić et al. 2016a). In addition, beneficial effects have been obtained with natural clay minerals, zeolites (Laurino and Palmieri 2015), among which clinoptilolite (CPL) is the best known as a zootechnical/biomedical feed ingredient widely used in farm animal nutrition as a candidate agent to replace AGP (Papatsiros et al. 2013) due to its unique anti-bacterial properties, safety and efficacy as a dietary supplement in swine (Thacker 2013, Subramaniam and Kim 2015); clinoptilolite has been shown to ameliorate mycotoxicosis, maintain gut health and improve immunity and growth performance in weaned pigs (Vondruskova et al. 2010, Valpotić et al. 2016b). Natural CPL is crystalline, hydrated aluminosilicate clustered to form a skeletal structure comprising multiple nanopores of different sizes act as molecular sieves modulating unique catalytic properties, such as absorption of gases and water molecules, facilitation of ion exchange and act as molecular sieves with long-term chemical and biological stability (Mumpton and Fishman 1977, Pavelic and Hadzia 2003). However, it has been recently recognized that previously observed positive biological effects of some zeolites, such as CPL might be partially ascribed to their ortho-silicic acid-releasing properties as a major form of bioavailable silicon for both animals and humans (Munjas Jurkić et al. 2013). With growing knowledge of the porcine immune system and understanding that its specific immunomodulation, such as vaccination, is limited to a single antigen, it has been established that non-specific immunomodulation implies more generalized stimulus in immune responsiveness of both innate and adaptive immunity, resulting in augmented host reactivity to a great variety of microbial antigens (Dhama et al. 2015). Although further research is still needed in this field, as previously mentioned in numerous excellent reviews alternative methods have been identified to effectively control bacterial infections in swine, particularly enteric infections of weaned pigs, using dietary supplementation with a broad spectrum of immunomodulatory substances (Gallois et al. 2009), as competitive alternatives to conventional AGP.

Thus, the aims of this study were to assess the immunomodulatory effects of dietary supplements MOS and CPL as potential alternatives to AGP applied to 4-week old pigs at weaning (Day 0) on their innate/adaptive immunity by determining alterations in: (1) C-reactive protein (CRP) and haptoglobin (HpG) serum levels, (2) efficiency of blood monocytes (MO) and granulocytes (GR) for *in vitro* phagocytosis (PHC)/microbicidity (MBC) and (3) patterns of extrathymic double positive CD4 CD8 ($CD4^+CD8^+$) T cell proportions throughout 35 days of the study.

Materials and Methods

Pigs. Sixty crossbred pigs (Topigs®), females and castrates, weighing on average 7.15 ± 0.16 kg, the progeny of six litters from 3rd parity sows reared at a commercial pig farm in eastern Croatia were used. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diet (with-

out antimicrobials or growth promoters) according to the 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).

Study design and dietary regimes. The pigs were randomly divided into three groups comprising 20 animals each, ear-tagged and marked with numbers 1-20, and kept in the same rearing facility of the commercial farm in separate pens (20 animals in each) located in near proximity to each other so the infection might easily spread from one pen to another. The experimental pigs were kept under the same environmental conditions, and thus all three groups had similar potential for exposure to infectious agents present in such an environment during the experimental period. After two days of accommodation at 28 days of age (Day 0 of the study) the pigs were treated as follows: (1) control pigs received standard phase 1 weaner diet from Day 0 to Day 21 and phase 2 diet from Day 22 to Day 35 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 (Valpotić et al. 2017). The experiment was conducted throughout a period of 35 days and 7 pigs (marked with numbers 1-7 in order that the same animals were sampled at each sampling day during the study) per group were sampled for peripheral blood at seven day intervals starting at Day 0 before the dietary regimes were applied.

Monitoring of microclimatic parameters. Throughout the study period, microclimate parameters, air temperature (°C), relative humidity (%) and airflow rate (m/s), and air concentrations of noxious gases, carbon dioxide (vol. %) and ammonia (ppm), were monitored in the weaner unit using digital portable devices (Testo and Drager, Germany). Microclimate parameters and gaseous air contamination were measured at several sites in the unit, in weaner the biozone, in order to obtain average representative values.

Blood sampling. At the same weekly intervals blood samples (10 mL) of 7 out of 20 pigs from each group were collected by *v. cava cranialis* puncture using vacutainers (Beckton Dickinson, Plymouth, UK) and separated into three aliquots, either in glass tubes (2 mL) with disodium EDTA (Sigma, St. Louis, USA) as an anticoagulant (1 mg/mL^{-1}) for flow cytometry analysis of $CD4^+CD8^+$ T cells or in plastic heparinized tubes (2 mL) for the *in vitro* testing of MO/GR capability of PHC/MBC as well as in glass tubes (6 mL) without anticoagulant (Becton Dickinson, Rutherford, NJ, USA) for serum APP (CRP and HpG) determination.

Determination of serum levels of acute phase proteins (APP). Serum was separated from blood cells by centrifugation at 1200 g for 15 min, divided in two aliquots of 0.5 mL and stored at -20°C until analyzed. The Tridelta PhaseTM Range porcine CRP kit (Tridelta Development Ltd., Maynooth, County Kildare, Ireland) was used as a solid phase in sandwich immunoassay. The serum samples, including standards of known CRP content, were added to microtiter plates in order to bind to coated microwells. After washing to remove any unbound material, horse radish peroxidase (HRP) labeled anti-porcine-CRP antibody was added to each well and incubated for 45 min. The microtiter plates were washed again to remove any unbound material and tetra methylbenzidine (TMB) substrate solution was added and the plates were incubated for 20 minutes at room temperature. The intensity of blue color development changed to yellow by addition of a stop solution was measured using a BioTek Absorbance Microplate Reader EL x 808 (BioTek Instruments, Inc., Highland Park, VT, USA) and optical density was measured at 450 nm. The intensity of obtained absorbance for each well was proportional to the concentration of CRP in the tested serum sample. A standard curve, prepared from 7 standard dilutions in duplicates, was used for calculating the concentration of CRP in porcine serum. The serum concentration of porcine HpG was quantified spectrophotometrically using commercial reagent from PhaseTM Range Haptoglobin Assay (Tridelta Development Ltd., Maynooth, County Kildare, Ireland) according to the manufacturer's instructions. The HpG measurement is based on the fact that the peroxidase activity of free hemoglobin is inhibited at a low pH level (3 to 4). The hemoglobin binds to HpG in blood serum and at a low pH level it preserves the peroxidase activity of bound hemoglobin. Thus, the peroxidase activity of bound hemoglobin is directly proportional to the amount of HpG present in the serum sample. The absorbance of the samples was measured on an Olympus AU 400 automated analyzer (Olympus diagnostica, Hamburg, Germany) at 600 nm. A calibration curve was prepared from five standard dilutions in duplicates in order to facilitate calculation of HpG concentration in porcine serum.

Monocyte/granulocyte phagocytosis/microbicidity assays *in vitro*. Peripheral blood leukocytes (MO and GR) were isolated from heparinized venous blood using Ficoll-Hypaque 1077 (Sigma, St. Louis, MO, USA) density gradient centrifugation at 1500 x g for 30 minutes at 4°C and their *in vitro* capabilities of PHC (cell ingestion) and MBC (cell digestion)

were assessed as described earlier (Patterson-Delafield and Lehrer 1978, Lukač et al. 1994). Leukocyte rich supernatant was collected from plasma, washed twice in medium 199 (minimal essential medium, MEM, Institute of Immunology, Zagreb, Croatia) and the concentration of obtained leukocytes was adjusted to 1 x 10^6/mL. The suspension of isolated cells was divided into aliquots of 0.25 mL, placed into small chambers (1.5 cm in diameter) and incubated at 37°C with 5% CO_2 in the air for 30 min. Supernatants were then discarded and nonadherent cells were washed with MEM heated to 37°C. The adherent cells *i. e.* phagocytes (MO and GR) remained in the chambers and 0.25 mL of suspension comprising 40 x 10^6/mL of viable cells (at least 99%) of *Saccharomyces cerevisiae* yeast was added to each chamber. The chambers were incubated for 30 min, washed, and the cells were stained with 0.05% acridine orange (Sigma, St. Louis, USA) in MEM for 1 min. The MEM was then discarded and the chambers were covered with cover slide and examined with a fluorescence microscope at 800 x magnification. At least 100 of either GR or MO with phagocytosed yeast cells were counted. The obtained results were expressed as percentage of the cells that phagocytosed, where the % of PHC capability is presented as the number of phagocytosed cells/total number of cells x 100, *i. e.* the ingestion index (ii), where the ii is the number of phagocytosed yeast cells/number of phagocytes. The capability of intracellular killing of yeast cells was determined based on differentiation between dead (stained red) and live (stained green) yeast cells, and was expressed as a percentage were the % of MBC is the number of phagocytosed dead yeast cells/total number of phagocytosed yeast cells x 100, *i. e.* the digestion index (di).

Analysis of $CD4^+CD8^+$ T lymphocytes by flow cytometry. Peripheral blood lymphoid cells were isolated by Histopaque (specific density 1.077 g/mL; Sigma, Deisenhofen, Germany) density gradient centrifugation as detailed earlier (Terzić et al. 2002). Murine monoclonal antibodies (mAbs) specific for porcine surface phenotypic markers CD4 (clone 74-12-4, isotype IgG2b) and CD8 (clone 76-2-11, isotype IgG2a) conjugated to either Pe/Cy5® or phycoerythrin (Abcam, Cambridge, UK), respectively, and an mAb to porcine CD45 (clone K252-1E4, isotype IgG1) conjugated to FITC (AbD Serotec, Kidlington, Oxford, UK) were utilized for flow cytometric (FCM) analysis to study identification/quantification patterns of respective peripheral blood lymphoid cell subsets. Briefly, single cell suspensions (100 µL) were prepared in triplicates (comprising 10 000 cells each) and incubated with mAbs (50 µL) and processed as previously described (Terzić et al. 2002). The fluorescence of the mAb-labelled porcine lymphoid cells was quantified using an EPICS-XL Coulter flow cytometer (Coulter Electronics, Hialeah, FL, USA). The isotype-matched mouse immunoglobulins were used to detect a nonspecific fluorescence in the control cell suspensions as described previously (Valpotić et al. 1994). Only cells with a light scatter characteristic of lymphoid cells were analyzed. More than 95% of such cells were identified as $CD45^+$ leukocytes. The total proportion of T lymphocytes was calculated from the two-color staining by the addition of $CD4^+$ + $CD8^+$ + $CD4^+CD8^+$ cells (Zuckermann and Gaskins 1996). The proportion of $CD4^+CD8^+$ cells as a percentage of the total $CD4^+$ cell subset was calculated using the following formula: (% $CD4^+CD8^+$ x 100)/(% $CD4^+CD8^+$ + $CD4^+$) according to Zuckermann and Husmann (1996).

Statistical analysis

As the pigs were of a same breed and a similar body weight at the weaning age of 26 days they were considered as dependent samples. Numerical data were analyzed by Student's *t* test for dependent samples using the StatisticaSixSigma software (StatSoft, Inc.). Each treated group was compared with the control group at 7-day intervals during 5 weeks of the study. Graphs were made using the statistical Software SAS 9.4 package (Statistical Analysis Software 2002-2012 by SAS Institute Inc., Cary, USA). Significance of differences between the treated groups and control group of pigs were considered as significant at $p<0.05$ and lower values.

Results

Microenvironmental conditions in rearing facility. Average values of microclimate parameters and noxious gas air concentrations determined in the weaner unit during the study period were as follows: air temperature 25.69°C, relative humidity 56.62%, airflow rate 0.13 m/s, carbon dioxide 0.12 vol.% and ammonia 4.50 ppm levels.

Concentrations of CRP and HpG in serum. Serum alterations in levels of CRP and HpG in pigs fed diets supplemented with CPL or MOS during 5 weeks following the treatment are presented in Table 1 and 2. Neither CPL nor MOS changed serum concentrations of CRP in supplemented pigs as compared with those recorded in the control pigs during the experimental period (Table 1). The concentration of HpG was significantly increased in the pigs fed diet supplemented with CPL ($p<0.05$) at Day 35 of the experiment (Table 2). Interestingly, a much higher

Table 1. Concentration (mg/L) of C-reactive protein (CRP) in serum (Mean ± SEM) of weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each.

Group/days	Serum level of CRP (Mean ± SEM mg/L) in pigs during the experiment					
	0	7	14	21	28	35
Control	2.85 ± 0.78	2.07 ± 0.67	1.48 ± 0.21	2.80 ± 0.45	2.75 ± 0.60	2.88 ± 0.67
MOS	3.80 ± 1.08	1.78 ± 0.65	1.39 ± 0.23	1.97 ± 0.27	2.02 ± 0.41	2.91 ± 0.33
CPL	2.40 ± 1.15	3.03 ± 0.94	1.71 ± 0.60	1.85 ± 0.40	1.53 ± 0.38	1.63 ± 0.23

Table 2. Concentration (g/L) of haptoglobin (HpG) in serum (Mean ± SEM) of weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each.

Group/days	Serm level of HpG (Mean ± SEM g/L) in pigs during the experiment					
	0	7	14	21	28	35
Control	0.30 ± 0.06	0.38 ± 0.07	0.31 ± 0.06	0.31 ± 0.09	0.54 ± 0.16	0.23 ± 0.07
MOS	0.53 ± 0.08*	0.39 ± 0.06	0.40 ± 0.03	0.38 ± 0.07	0.48 ± 0.17	0.22 ± 0.04
CPL	0.46 ± 0.09	0.36 ± 0.08	0.36 ± 0.08	0.28 ± 0.05	0.39 ± 0.10	0.72 ± 0.10*

* Significantly different from the control group ($p<0.05$)

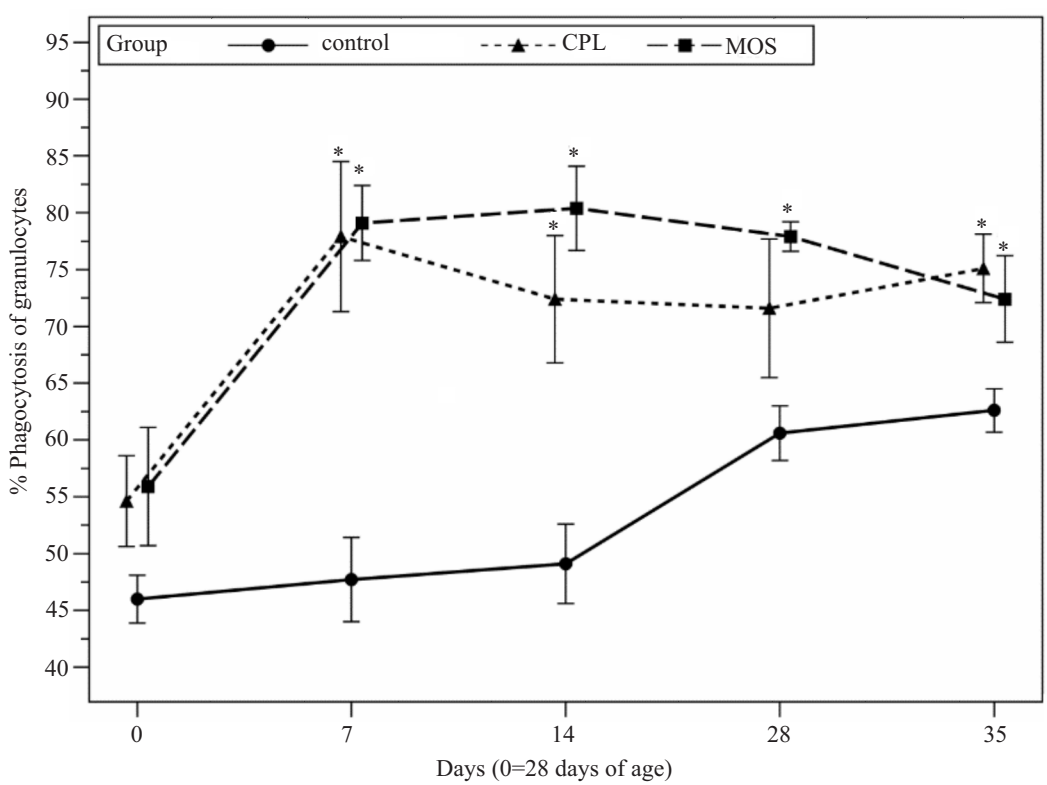

Fig. 1. *In vitro* capability of phagocytosis (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each. Values marked with an asterisk differ significantly at $p<0.05$ from control values.

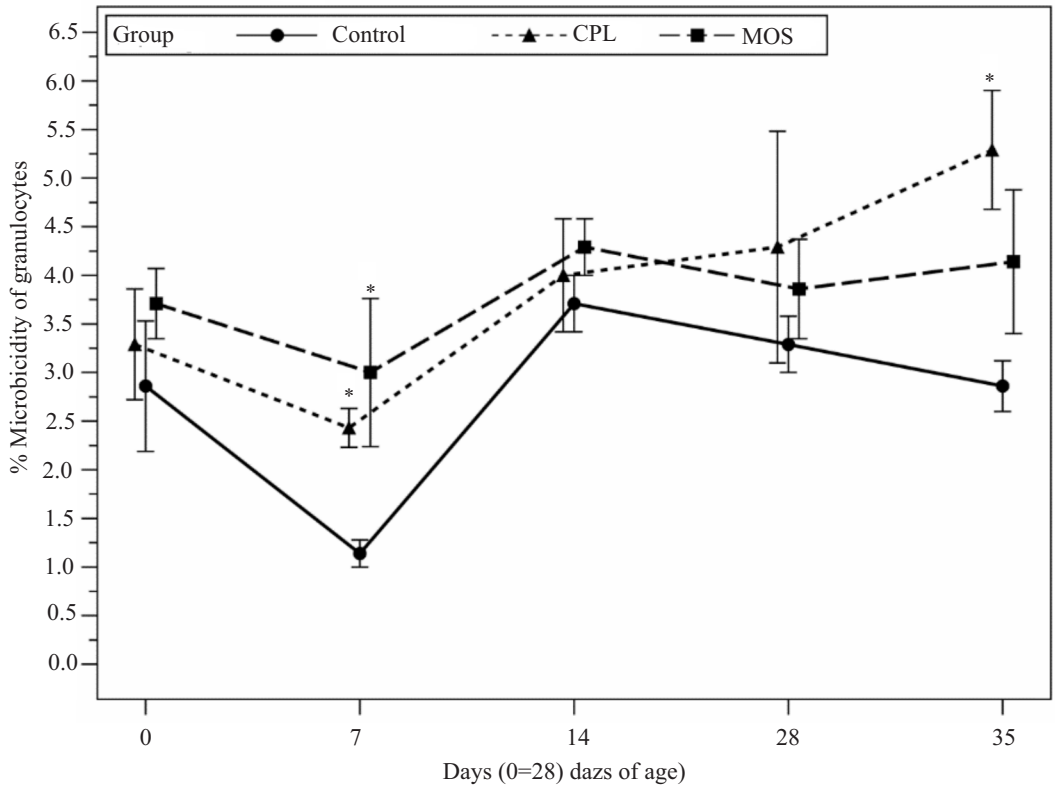

Fig. 2. *In vitro* capability of microbicidity (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each. Values marked with asterisk differ significantly at $p<0.05$ from control values.

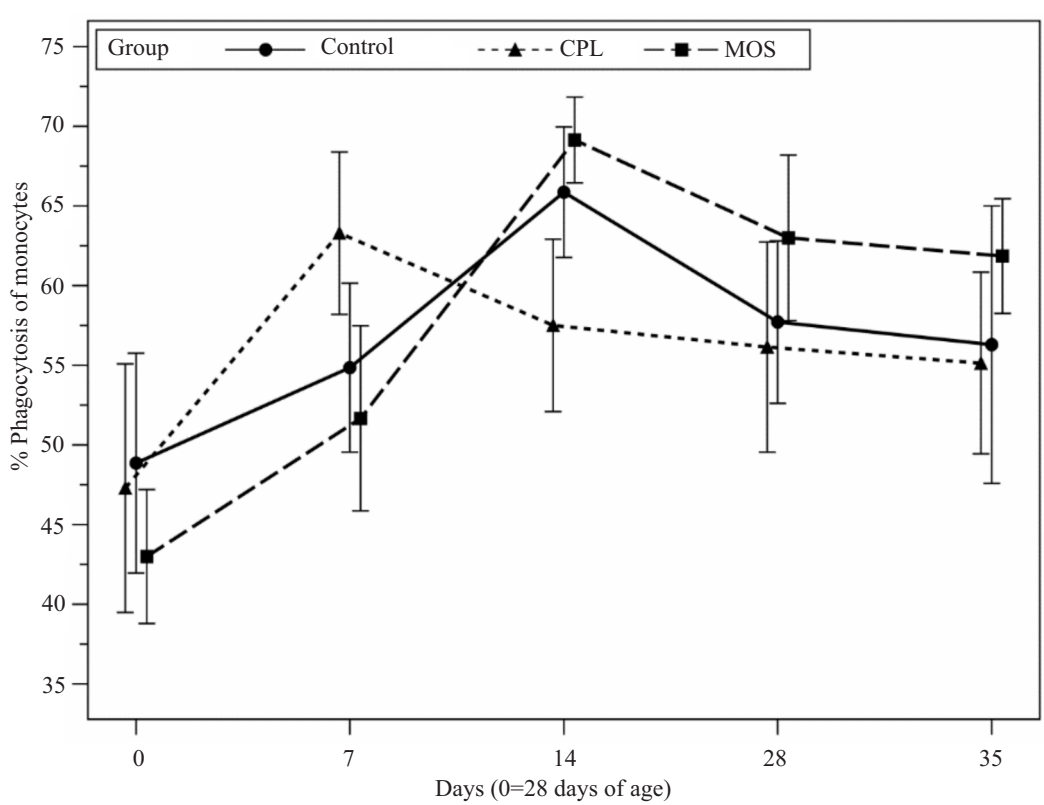

Fig. 3. *In vitro* capability of phagocytosis (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each.

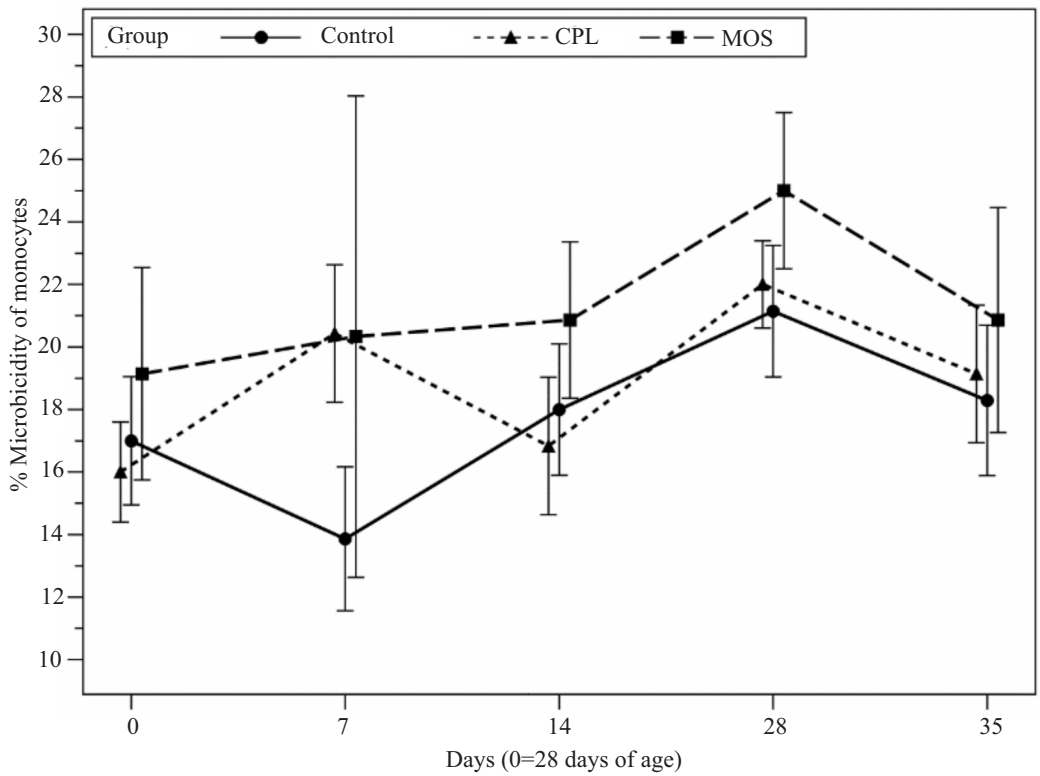

Fig. 4. *In vitro* capability of microbicidity (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each.

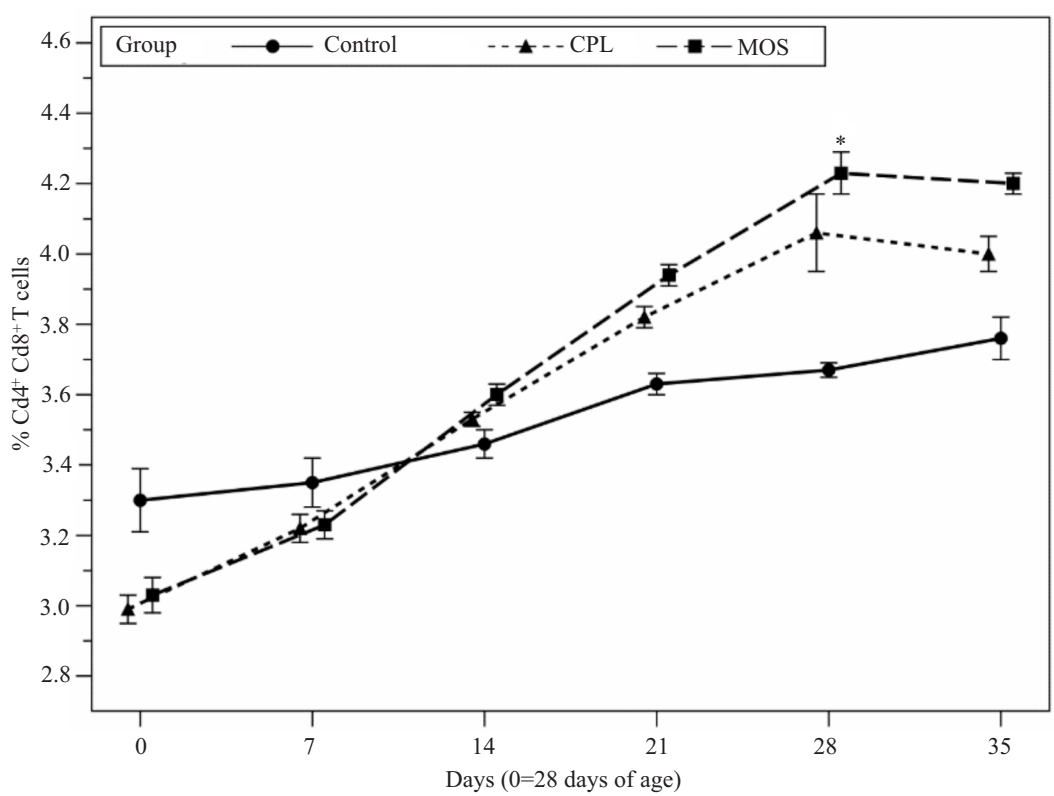

Fig. 5. Proportion (%) of peripheral blood $CD4^+CD8^+$ T cells (Mean ± SEM) in weaned pigs fed diets supplemented with either 0.5% of clinoptilolite (CPL) or 0.2% of mannan oligosaccharide (MOS) from Day 0 (or 4 weeks of age) to Day 35 (or 9 weeks of age) of the study. Control pigs received standard weaner diets; groups comprised 7 pigs each. Values marked with an asterisk differ significantly at $p<0.05$ from control values.

level of HpG was recorded in the pigs from the second principal group on Day 0 ($p<0.05$) prior to the treatment with MOS in relation to the control non-treated pigs.

Phagocytic and microbicidity activity of granulocytes and monocytes. Phagocytic activity of peripheral blood neutrophilic GR was significantly increased by both dietary supplements tested ($p<0.05$) from Day 7 to Day 35, with the exception of a slightly, but not significantly, higher value obtained for CPL at Day 28 of the experiment as demonstrated by the *in vitro* assays (Fig. 1). Also, the GR from pigs fed with either CPL or MOS supplements had significantly increased MBC at Day 7 ($p<0.05$), but at Day 35 of the experiment such an increase was observed only for CPL (Fig. 2). However, the *in vitro* PHC and MBC of MO were not changed in the pigs fed with either CPL or MOS as compared to the values obtained for the control non-treated pigs (Fig. 3 and Fig. 4).

Proportion of $CD4^+CD8^+$ T lymphocytes. The pigs supplemented with MOS had significantly higher proportions of peripheral blood $CD4^+CD8^+$ T lymphocytes than the control pigs at Day 28 ($p<0.05$) (Fig. 5).

Discussion

The values of all microclimate parameters and noxious gas air levels measured in the weaner unit throughout the study period were within the optimal range recorded previously (Ostović et al. 2015). Therefore, their potential adverse effects on the health and welfare of weaned pigs as well as on interference with the study results were ruled out. In the current study, serum levels of CRP were not affected in pigs fed by either MOS or CPL supplements throughout the 5 weeks following their weaning at 4 weeks of age, suggesting that both supplementations did not alleviate the level of this APP tested as a marker of the inflammatory response associated with the stress of weaning, which is partly in agreement with an earlier finding that α-1-acid glycoprotein serum concentration was not altered by dietary mannans (Davis et al. 2004). Conversely, the same authors reported that phosphorylated mannans decreased blood neutrophil/lymphocyte ratio could thus moderate such a detrimental inflammatory response accompanying weaning. Interestingly, serum HpG concentration was substantially increased in CPL-fed pigs after 5 weeks of supplementation at Day 35, which could be ascribed to their response to either harmless dietary antigens and commensal bacteria, or to potential pathogens as suggested by Stokes et al. (2004) who stated that pigs kept in intensive farming systems reach immunocompetence comparable to that of adult pigs by 7-9 weeks of age. Since there are no similar studies dealing with the effects of CPL on porcine APP levels as far as we know, only comparison of the obtained values for either CRP or HpG with their normal (5-30 mg/L and 0.01-2.20 g/L) and acute (50-750 mg/L and 3.0-8.0 g/L) ranges, respectively, in young pigs may be performed. Namely, both tested supplements did not influence the serum level of CRP and the obtained values were within the normal range, which is favorable as it did not reflect an immune response to pathogens, inflammation, tissue injury or stress (Murata et al. 2004). However, our assumption regarding the immunomodulatory effects of MOS was confirmed as the MOS-fed weaned pigs experimentally infected with porcine reproductive respiratory syndrome (PRRS) virus had significantly increased serum levels of HpG as well as of IL-1β, IL-12 cytokines involved in the innate, T-helper 1, and T-regulatory immune responses which may favorably promote both innate and adapted cell-mediated immunity and suppress the release of pro-inflammatory mediators (Che et al. 2012). Also, the pigs fed MOS and experimentally infected with PRRS virus had decreased TNF-α and increased serum IL-10, CRP and HpG levels as well as decreased numbers of total blood leukocytes and lymphocytes in comparison to those of the noninfected pigs (Che et al. 2011). Regarding the porcine cellular components of non-specific innate immunity tested in this study, such as blood MO and GR, *i. e.* their functional capability, the obtained data show that only GR from either MOS- or CPL-fed pigs had significantly increased PHA/MBC activities, which differ from those obtained for PHA activity of neutrophilic GR as reported earlier (Sauerwein et al. 2007). This discrepancy could be explained by differences in study design such as breed of pigs, dietary regime, yeast cell wall extract (containing only 10% of mannan) applied as feed additive and a shorter period of supplementation. Moreover, the same authors observed that the dietary preparation tested was associated with an increase in serum concentration of HpG reaching maximal values between 1-2 weeks after weaning, which is in contrast to our observation that dietary MOS failed to exert an influence on porcine HpG level. However, even more unfavorable is the fact that MOS (as well as CPL) also failed to influence CRP level in pigs as we recorded, since its crucial role is to link innate and the adaptive immunity in immunologically immature weaned pigs by interacting with specific receptors on phagocytic cells in order to mediate their PHA/MBC activities and to

induce the release of anti-inflammatory cytokines (Du Clos and Mold 2001). It is probably likely that such a finding could be responsible for reduced PHA/MBC functions of blood MO observed with both tested supplements. Namely, it has been previously reported that an enhanced phagocytic activity of macrophages residing in intestinal *lamina propria* was observed in weaned pigs fed a diet with mannans (Davis et al. 2004). The results of the present study obtained for proportion of porcine peripheral blood $CD4^+CD8^+$ T cells are consistent with a previously reported finding that this subset of lymphocytes increases gradually with the age of pigs (Zuckermann 1999). This trend was, however, disrupted at Day 28 of the study when MOS-fed pigs had a significantly higher proportion of $CD4^+CD8^+$ T cells as compared to the values obtained in non-stimulated control pigs. This finding is also in agreement with previously described functional characteristics of these cells as antigen primed T helper cells with memory/effector phenotype (Zuckermann and Husmann 1996). As we did not find similar data on the modulating effects of MOS or CPL on porcine $CD4^+CD8^+$ T cells we may only quote those found for the other circulating T cell subsets in weaned pigs. In an extensive study Nochta et al. (2009) found that dietary MOS enhanced both specific cellular (by *in vitro* reactivity of peripheral blood lymphocytes to common mitogens) and humoral immune responses (by detection of neutralizing antibodies following immunization with inactivated Aujeszky's disease virus vaccine) in weaned pigs. A similar study has shown that the $CD4^+CD8^+$ ratio and proliferation of these T cell subsets were increased in early weaned (2-week old) pigs fed a diet supplemented with live yeast preparation for 3 weeks as compared to that of controls at day 7 of the experiment (Jiang et al. 2015). More recent studies showed that both CPL and, particularly MOS stimulated almost the same kinetics of recruitment of either $CD4^+$ (from Day 21 to Day 35 and from Day 28 to 35) or $CD8^+$ (from Day 21 to Day 28) T cells in weaned pigs (Valpotić et al. 2016a,b), respectively. However, as for numerous substances tested as a potential alternative to AGP, the influence of dietary MOS and CPL on porcine systemic immunity, but more importantly on local (intestinal) immunity is not always reliable. Also, their effects on gut health and growth performance were not consistent, and should be further investigated. Accordingly, our previous studies were focused on establishing immunostimulatory potentials of MOS and CPL on specific cellular systemic and local gut mucosal immunity in weaned pigs (Valpotić et al. 2016a,b) and to clearly state the relationships between immunomodulation responses to gut health and growth performance. The objective of introducing such bioactive substances in pig diets is to make them more sustainable regarding safety for animals and consumers (Valpotić et al. 2017), as well as to document their anti-microbial potential in improving general health status and production efficiency. Regarding the aforementioned studies on MOS and CPL potentials as NCs/IMs and alternatives to AGP, and their substantial effects on the defensive capability of the porcine mononuclear phagocytic system, the importance of APP as markers of disrupted homeostasis and the significance of $CD4^+CD8^+$ T lymphocytes as antigen primed cell subset, these components of innate/adaptive immunity have also been assessed by us in order to obtain additional information regarding their efficiency in promoting "desired" functions of nonspecific/specific protective immunity in immunologically immature young pigs.

Based on a substantial body of literature on this issue, it appears that the observed beneficial effects of natural alternatives to conventional antimicrobials that have played a major role for almost 70 years in development of the pig industry (Cromwell 2002) have shown inconsistent results regarding growth performance and their influence on pig immunity remains inconclusive (Gallois et al. 2009). Although, the majority of these natural substances, particularly dietary immuno-oligosaccharides (Spring et al. 2015) and activated zeolites (Vondruskova et al. 2010, Subramaniam and Kim 2015) showed promising results regarding their impact on the modulation of immunity, gut health and physiology and development of the intestinal mucosal immune system in young pigs, it does not appear that any one natural remedy could be a sole alternative to AGP in swine feeding operations. Although both dietary supplements applied in this study showed an ability to stimulate molecular and cellular components of the innate and adaptive immunity tested, it is not likely that either MOS or CPL when solely applied could completely replace conventional AGP in improving immune functions, maintaining gut health and promoting the growth of weaned pigs. However, further research is needed in this field, alternative methods have been identified and non-antibiotic bioactive substances have been tested in effectively controlling bacterial infections in food animals (Allen et al. 2014). All of the alternative substances tested as potent NCs and IMs pose no known threat to animal and human health, and probably a combination of the most potent, and at the same time synergistic, can lead to the adequate replacement of antibiotics in pig production. With further research it is highly likely that a technologically applicable, cost and health effective alternative to feed AGP could and will be found.

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