Slov Vet Res 2018: 55 (1): 43-53
DOI 10.26873/SVR-397-2017
UDC 636.4.083.14.09:615.284:577.27

Original Research Article

MODULATION OF SYSTEMIC INNATE AND ADAPTIVE IMMUNE PARAMETERS IN WEANED PIGS BY SINGLE ORAL APPLICATION OF IMMUNOBIOTICS

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Abstract: The aims of this study were to evaluate the effects of peroral treatment of 4-week old pigs at weaning (Day 0) with a single dose of levamisole (LEVA) or polyoxyethyle-polyoxypropylene (POE-POP), well known immunobiotics (IBC) and promising alternatives to dietary antibiotic growth promoters (AGP), on systemic innate and adaptive immunity by determining phagocytosis (PHC) and microbicidity (MBC) efficiency of their monocytes (MO) and granulocytes (GR), changes of serum C-reactive protein (CRP) and haptoglobin (HpG) profiles and kinetics of $CD4^+CD8^+$ T cells expression during 5 weeks following the treatments. Levels of CRP were decreased by either POE-POP or LEVA ($P<0.05$) at Day 7 and 21, respectively. LEVA-treated pigs had increased levels of HpG ($P<0.05$) at Day 14 and 21, whereas POE-POP-treated pigs had decreased and increased levels ($P<0.05$) at Day 7 and 21, respectively. Both IBC stimulated *in vitro* PHC of GR (from Day 7 to 35) and MBC of MO (at Day 35) from treated pigs ($P<0.05$). MO from POE-POP-treated pigs exhibited increased PHC ($P<0.05$) at Day 35, whereas GR from LEVA- or POE-POP-treated pigs showed increased MBC ($P<0.05$) at Day 7 and 35, respectively. The pigs treated by LEVA or POE-POP had higher proportions of $CD4^+CD8^+$ T cells ($P<0.05$) from Day 14 to 35 or Day 28 to 35, respectively. Tested IBC showed capability of stimulating particularly cellular components of nonspecific and specific immunity during early postweaning period before pigs reach adult immunocompetence values, and thus could be considered as potent immunostimulators in swine production.

Key words: synthetic immunobiotics; innate/adaptive immunity; weaned pig

Introduction

In swine production, at weaning pigs are immunologically immature and exposed to major stressful events, making them highly sensitive and susceptible to digestive disorders or enteric infections (1,2,3). At this time, the development of both innate and adaptive systemic and particularly local immunity (at gut mucosal surfaces) is critical in preventing the potential harmful effects of intestinal pathogens. Substantial research has been conducted over the past three decades to evaluate the potential antimicrobial agents for replacement of the in-feed antibiotics used as performance enhancers in swine production. This initial experience of an in-feed antibiotic growth promoters (AGP) ban in 1986 indicated that there was a reduced growth and increased morbidity/mortality in weaned pigs, which emphasized the therapeutic use of antibiotics in farms (4). The total ban of AGP in the EU since 2006 has had a serious impact on both performance and health status of weaned pigs underlining the need to develop alternative strategies (5). Since then, an intensive amount of research has been focused on

Received: 4 April 2017
Accepted for publication: 20 June 2017

the development of alternatives to AGP to maintain swine health and performance and many excellent reviews have been published on this subject (6,7,8). More recently, the focus has been directed to non-antibiotic approach offered by the use of immunobiotics (IBC), *i. e.* viable microorganisms (probiotics or microbiotics), their bioactive components (prebiotics), plant/fungi extracts (phytobiotics/fungibiotics), animal products or by-products (zoobiotics) and clay minerals (zeolites) as well as by a variety of other substances of natural (10,11,12,13) synthetic origin (14,15). Many of them have beneficial immunomodulatory properties and ability to prevent or reduce severity of systemic and local (intestinal) inflammatory disorders by stimulating non-specific innate and specific adaptive immunity in pigs.

Today, when the use of AGP is abandoned in animal production, there is intensive search for alternative strategies to control and prevent losses among weaned pigs, particularly due to enteric infections. These strategies include both natural (16,17,18,19,20) and synthetic IBC (21,22,23,24,25) preparations with antimicrobial detoxifying activities, as well as antioxidant, immunostimulatory and growth-promoting characteristics. However, the vast majority of these compounds produce inconsistent results and rarely equal AGP in their effectiveness. Therefore, it would appear that research is still needed in this area as the adequate alternative to AGP is not available as yet.

Considering our previous research on modulation of porcine immunity, health and growth benefits, the most promising results to date have been obtained with the synthetic IBC, levamisole (LEVA) and polyoxyethyle-polyoxypropylene (POE-POP) copolymers. The major positive effects of LEVA (23,24,25,26,27) and POE-POP (22,24) were to help weaned pigs to develop “appropriate”, but not over excessive active immune responses which may result in growth retardation in developing pigs during early postweaning period. They showed to act as the agents capable of stimulating components of the specific adaptive and nonspecific innate immunity, by increasing recruitment of either circulating $CD45^+$ lymphoid cells, $CD4^+$, $CD8^+$ T and $CD21^+$ B cells or intestinal $CD45RA^+$ naïve lymphoid cells, respectively, in immunologically immature early weaned pigs (24,25), and at the same time neither of them induced any detrimental effects on their haematological, serum biochemistry and gut histocytological homeostasis (29). Actually, this would be a logical continuation and essential supplementation of our aforementioned studies regarding the impact of LEVA and POE-POP simultaneously on immunity and on performance and health of weaned pigs which should be more largely documented as their immunomodulatory effects are also expected in these components of natural host defences.

Thus, the objectives of this study were to evaluate immunostimulatory effects of LEVA and POE-POP as potential alternatives to dietary AGP on components of porcine: (1) innate immunity such as blood phagocytic cells, such as monocytes (MO) and neutrophilic granulocytes (GR) and serum acute phase proteins (APP), such as C-reactive protein (CRP) and haptoglobin (HpG) profiles by establishing either their capability of phagocytosis (PHC) and microbicidity (MBC) or kinetics of their responses, respectively, and (2) adaptive immunity by determining changes in the proportion of extrathymic CD4CD8 double positive T lymphocytes in 28 days old weaned pigs during 35 days following the treatment with a single peroral dose of the IBC tested.

Materials and methods

Ethical and welfare approval

The experimental pigs were kept in facilities approved by the Croatian Association for Accreditation of Laboratory Animal Care, and in accordance with current regulations and standards issued by the Croatian Ministry of Agriculture. Experimental and management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). The current study was supported by the grant no. 053-0532265-2255 from the Ministry of Science, Education and Sport of Croatia on January 2nd 2007.

Pigs

Sixty crossbred pigs (Topigs®) of both sexes (females and castrates) and body weight of 7.21±0.17 kg, progeny of six litters (from 3rd

parity sows) from a commercial swine farm in the eastern Croatia were used. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diet (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).

Study design and treatments

The pigs were randomly divided into three groups comprising 20 animals each, ear-tagged with numbers 1-20 and kept in the separate pens (20 animals in each) of the same rearing facility under the same microclimatic conditions as previously described (25). After two days of accommodation at 28 days of age or Day 0 of the experiment, the pigs were perorally (*p.o.*) treated with a single dose of 10 mL as follows: (1) the controls received saline only, while the principals were treated with either (2) LEVA (Nilverm®, Pliva, Zagreb, Croatia) with 2.5 mg/kg body weight of the drug or (3) POE-POP (CytRx, Atlanta, GA, USA) with 2.5 mg/mL of the copolymer preparation as detailed earlier (24,25). The experiment was conducted throughout 35 days and was terminated at 63 days of age of the pigs, and during that period 7 pigs per group were sampled for peripheral blood at 7 day intervals starting at Day 0 before the treatments.

Blood sampling

The blood samples (10 mL) of 7 out of 20 pigs (ear-tagged with numbers 1-7 in order that same animals were sampled at each sampling day) from each group were taken ba *vv. cava cranialis* puncture using vacutainers (Beckton Dickinson, Plymouth, UK) and separated in three aliquots into either glass tubes (2 mL) with disodium EDTA (Sigma, St. Louis, MO, USA) as an anticoagulant (1 mg/mL) for flow cytometry analysis of extrathymic CD4CD8 double positive T cells or plastic heparinized tubes (2 mL) for the *in vitro* testing of MO/GR capability of PHC/MBC as well as into glass tubes (6 mL) without anticoagulant (Becton Dickinson, Rutherford, NJ, USA) for serum APP (CRP and HpG) determination.

Flow cytometry analysis

Peripheral blood lymphoid cells were isolated by Histopaque (specific density 1.077 g/mL; Sigma, Deisenhofen, Germany) density gradient centrifugation as detailed earlier (30). 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 a 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, a 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 (30). The fluorescence of the mAb-labelled porcine lymphoid cells was quantified using 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 (31). Only cells with a light scatter characteristics of lymphoid cells were analysed. More than 95% of such cells were $CD45^+$ for this lymphoid cell marker. The total proportion of T lymphocytes was calculated from the two-colour staining by the addition of $CD4^+ + CD8^+ + CD4^+CD8^+$ cells (32). The proportion of double positive $CD4^+CD8^+$ cells as a percentage of the total $CD4^+$ cell subset was calculated with the following formula: $(CD4^+CD8^+/(CD4^+CD8^+ + CD4^+))*100 = CD4^+CD8^+(\%)$ according to Zuckermann and Husmann (33).

Monocyte/granulocyte isolation and phagocytosis/microbicidity assays

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 (34). Leukocyte rich supernatant was collected from plasma, washed trice in the medium 199 (MEM, minimal essential

medium, Institute of Immunology, Zagreb, Croatia) and 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. Then supernatants were discarded and nonadherent cells were washed with MEM heated at 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 yeast *Saccharomyces cerevisiae* 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. Then MEM was discarded and the chambers were covered by a cover slide and examined with 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 ingested yeast cells/total number of yeast cells x 100, *i. e.* the ingestion index (ii), where the ii is the number of ingested yeast cells/ number of phagocytes. 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 percentage were the % of MBC is the number of ingested dead yeast cells/total number of ingested yeast cells x 100, *i. e.* the digestion index (di).

Determination of serum acute phase proteins

Serum was separated from blood cells by centrifugation at 1200 g for 15 min, divided in two 0.5 mL aliquots and stored at -20 °C until analysed. The Tridelta Phase™ 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, the 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 colour development changed to yellow by addition of a stop solution was measured by a microtiter plate reader 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 is 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 Phase™ Range Haptoglobin Assay (Tridelta Development Ltd., Maynooth, County Kildare, Ireland) according to the manufacturer's instructions. Briefly, the HpG measurement is based on the fact that the peroxidase activity of free haemoglobin is inhibited at a low pH level (3 to 4). The haemoglobin binds to HpG in blood serum and at a low pH level it preserves the peroxidase activity of bound haemoglobin. Thus, the peroxidase activity of bound haemoglobin is directly proportional to the amount of HpG present in the serum sample. The absorbance of the samples was measured on an automated analyser Olympus AU 400 (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.

Statistical analysis

As the pigs were of a same breed, weighed approximately 7.20 kg at the weaning age of 26 days. Blood samples of the 7 pigs in each group were used as pooled sample. Data were analysed by Student's *t* test for independent samples using the StatisticaSixSigma software (StatSoft, Inc.). Each of two treated groups were compared with the control group of pigs without testing the time points within one group. Graphs were made using statistical Software SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with module PROC SGPLOT. The differences between obtained values for treated groups and the control group of pigs were considered significant at $P<0.05$ or lower.

Results

Performance and health status of pigs

Pigs treated with POE-POP and LEVA had a significant increase in live body weight, improved feed efficiency and average daily gain compared to the control pigs at the end of the trial. Both treatments positively influenced overall health status by reducing the incidence and severity of diarrhea and mortality which were documented in our previous studies (24,25).

Serum levels of CRP and HpG

The changes in APP profiles (CRP and HpG) in the serum of pigs treated with LEVA or POE-POP during 5 weeks following the treatment are presented in Figure 1 and Figure 2. None of IBC tested increased the concentration of serum CRP in treated pigs in comparison to the values obtained in the control pigs during the experimental period (Figure 1). However, the levels of CRP were significantly decreased in the pigs treated with either POE-POP or LEVA (P<0.05) at Day 7 and Day 21, respectively.

In the pigs that received LEVA significantly increased levels of serum HpG where recorded at Day 14 and Day 21 (P<0.05) of the experiment as compared to the respective values obtained in the control pigs (Figure 2). The values of HpG in the serum of POE-POP-treated pigs where either significantly decreased at Day 7 or increased at Day 21 (P<0.05, respectively).

Capability of phagocytosis and microbicidity of granulocytes and monocytes

Both IBC tested stimulated phagocytic activity of peripheral blood neutrophilic GR as demonstrated by the *in vitro* assays (Figure 3).

Thus, LEVA and POE-POP exhibited such stimulatory effects by significantly increasing phagocytic capability (ii) of GR from treated pigs at Days 7, 14, 28 and 35 as compared to those in the control pigs during the same period of the experiment. Also, the GR from pigs treated with either LEVA or POE-POP had significantly increased MBC at Day 7 and Day 35 (P<0.05), respectively (Figure 4).

The PHC of MO was significantly increased in the pigs treated with POE-POP (P<0.05) at Day 35 (Figure 5). However, the MBC of MO was significantly increased by both IBC (P<0.05) at Day 35 (Figure 6).

Proportion of CD4/CD8 double-positive T cells

The pigs treated with either LEVA or POE-POP had significantly higher proportions of peripheral blood $CD4^+CD8^+$ T lymphocyte than the control pigs from Day 14 to Day 35 and from Day 28 to Day 35 (P<0.05), respectively (Figure 7).

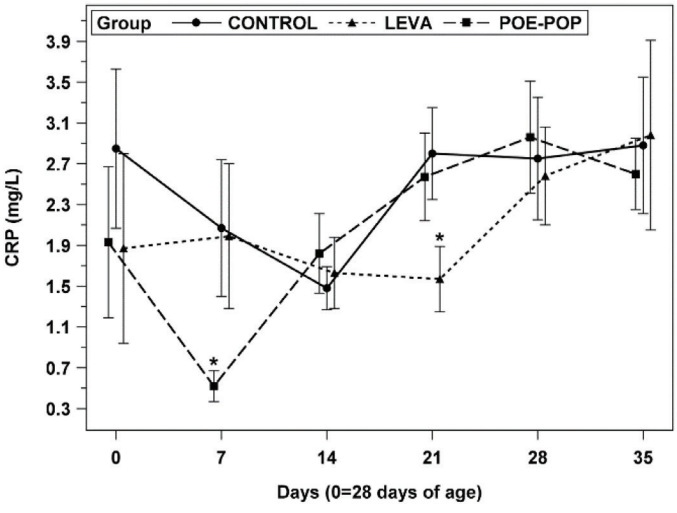

Figure 1: Concentration (mg/L) of C-reactive protein (CRP) in serum (Mean ± SEM) of weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at P<0.05 from the control values

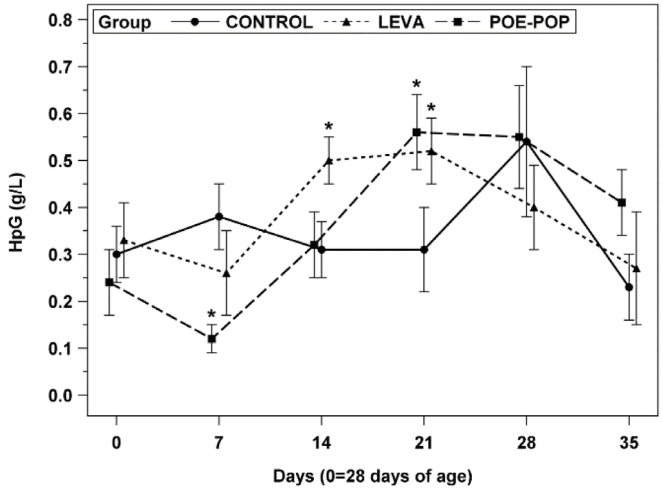

Figure 2: Concentration (g/L) of haptoglobin (HpG) in serum (Mean ± SEM) of weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

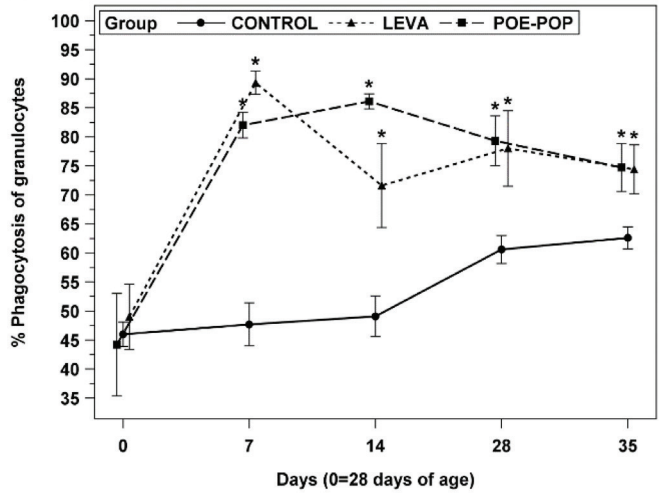

Figure 3: *In vitro* capability of phagocytosis (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (aLEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

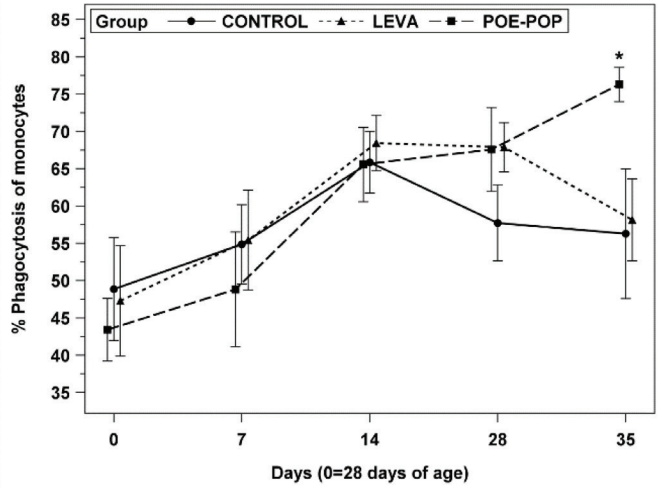

Figure 4: *In vitro* capability of microbicidity (%) of peripheral blood neutrophilic granulocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

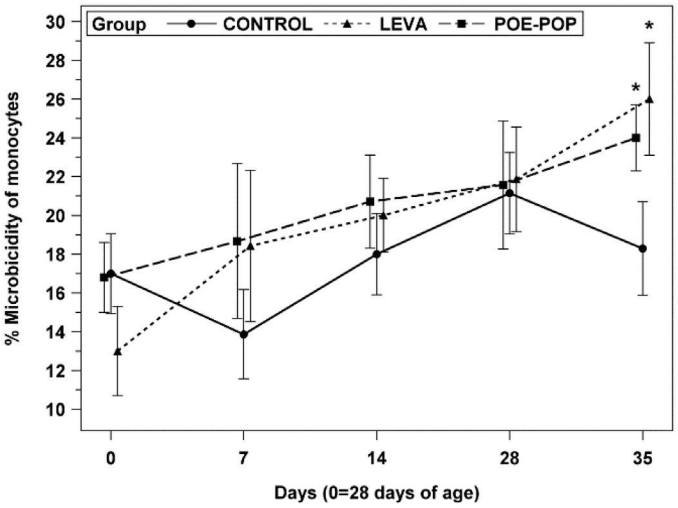

Figure 5: *In vitro* capability of phagocytosis (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

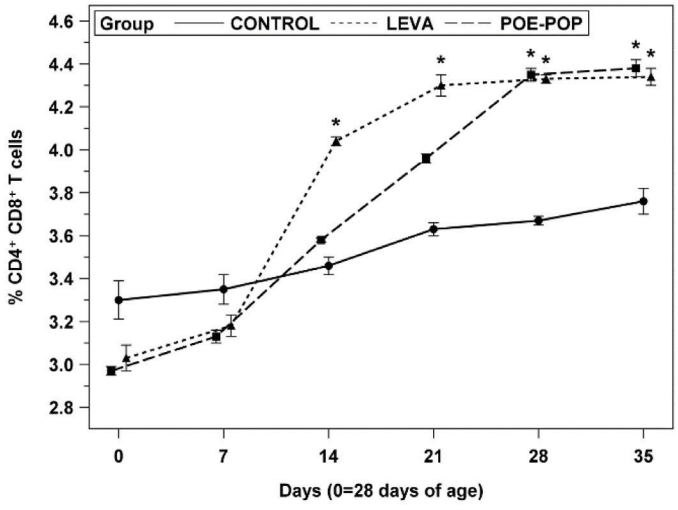

Figure 6: *In vitro* capability of microbicidity (%) of peripheral blood monocytes (Mean ± SEM) in weaned pigs per orally treated with a single dose of 10 mL of either levamisole (LEVA) or polyoxyethylene-polyoxyprpylene (POE-POP) at Day 0 (or 4-weeks of age) during 5 weeks following the treatment. Control pigs received 10 mL of saline only; groups comprised 7 pigs each. The values marked with asterisk differ significantly at $P<0.05$ from the control values

Discussion

In the present study, early responses of serum CRP and HpG in 4-weeks old weaned pigs induced by synthetic IBC either LEVA or POE-POP (at weaning or Day 0) were monitored (from Day 7 to Day 35 following the treatments) in order to obtain data on their potential modulating effects on porcine APP. Since there are no similar studies dealing with the effects of LEVA and/or POE-POP on porcine APP levels as far as we know, only comparison of obtained values for CRP and HpG with normal and acute ranges of these parameters in young pigs were logical and could be practical (35). Namely, the effects of IBC tested may influence normal (5-30 mg/L and 0.01-2.20 g/L) and acute (50-750 mg/mL and 3.00-8.00 g/L) ranges of either CRP or HpG levels, respectively, as important markers of diseases, health and welfare in pigs (36). Although, both IBC either decreased CRP or increased HpG serum levels, between Day 7 and Day 21 of the experiment, they did not affect normal ranges of their concentrations, which is a favourable outcome of our research. Particularly, in the cases of nonspecific immune responses to infective agents, inflammation, trauma and stress-induced conditions (37), exogenous manipulations with porcine innate immune system components such as CRP and HpG as performed with LEVA and POE-POP in the current study, may disturb rapid and significant changes in their serum concentrations and consequently abrogate their protective efficiency and value as diagnostic and prognostic indicators

of disturbed physiological homeostasis and health status of pigs (37). This is particularly true for weaned pigs during early postweaning period when they are exposed to a variety of pathogenic challenges (such as infectious diseases in field conditions), inflammatory lesion following natural injuries (such as tail and ear bites), acute inflammatory responses due to autoimmune or other disorders (such as arthritis and ulcerated umbilical hernia) and/or to stressors (38,39). Beside, these biological functions of CRP in not fully immunocompetent weaned pigs, its crucial role is in linking nonspecific innate immunity and specific adaptive immunity by interacting with specific receptors on phagocytic cells to mediate PHC or induce the release of anti-inflammatory cytokines (40).

Exogenous immunostimulation by IBC, such as LEVA and POE-POP firstly implies stimulation of non-specific innate immunity components, such as tissue macrophages, circulating MO and neutrophilic GR as well as NK-cells and γδ TCR^+ T lymphocytes (21). Accordingly, the current study investigated the impact of either LEVA or POE-POP as a potent IBC for porcine cellular components of specific adaptive immunity (23,25,24, respecitely), on PHC/MBC *in vitro* functions of blood MO and GR in weaned pigs during 5 weeks following the treatments at Day 0 or 4 weeks of age. The PHC function of GR have been stimulated by both IBC throughout 5 weeks following the treatments, whereas only the treatment with POE-POP increased *in vitro* phagocytosis of MO. Our findings are consistent with those reporting that the treatment with LEVA enhanced *in vitro* PHC function of neutrophilic GR, but not of MO in weaned pigs (41). However, since the experimental design of the latter study differ from that in the current study, *i. e.* LEVA was given intramuscularly in daily dose of 2.5 mg/kg of body weight for three consecutive days, this comparison should be taken advisedly. The *in vitro* MBC functions of GR and MO were enhanced equally by both IBC tested, although their stimulating effects were observed at Day 35 only. There are numerous studies similar to the current study performed on natural IBC such as probiotics, prebiotics and phytobiotics (11,12), in order to determine their modulating effects on either PHC or PBL functions of porcine circulating (16) and intestinal phagocytes (17,18,) but data obtained mostly remained inconsistent and inconclusive.

The results of the present study obtained on proportion of peripheral blood CD4/CD8 double positive T cells are consistent with previously reported finding that this subset of lymphocytes increases gradually with age (43). Namely, regardless the treatment applied all three groups of the pigs showed trend of increase in the proportion of this T cell subset with age during rather short period of 5 weeks of the experiment. However, both IBC tested stimulated significantly higher increase of CD4/CD8 double positive T cells from Day 14 to Day 35 (LEVA) and Day 28 to Day 35 (POE-POP) as compared to the values obtained in non-stimulated control pigs. This finding is also in agreement with earlier described functional characteristics of these cells as antigen primed T helper cells with memory/effector phenotype (44). Interestingly, the pigs stimulated with LEVA had increased proportions of circulating CD4/CD8 double positive T cells two weeks earlier than the pigs stimulated with POE-POP. Since both IBC tested were applied *p. o.*, it is very likely that they simultaneously reached the gut-associated lymphoid tissues (GALT) of the treated pigs. Such delay in the response to POE-POP as compared to that of LEVA could be due to differences in their immunogenicity for porcine intestinal CD4/CD8 double positive T cells. As established much earlier their predominant localization in the inductive sites of the GALT, such as Peyers patches, and their immediate migration into the circulation following antigen (or immunogen) stimulation (44) could be also a reason for that. Namely, our previous studies suggesting that LEVA exhibited more rapid and effective immunostimulating properties when used either as mucosal IBC (23,25) or adjuvant for mucosal vaccines (45) than POE-POP did (24,21,22, respectively). As we did not find similar data on modulating effectiveness of LEVA or POE-POP on porcine extrathymic CD4/CD8 double positive T cells we may only quote those found for the other circulating T cell subsets in weaned pigs. More recent studies have shown that POE-POP and, particularly LEVA stimulated almost the same kinetics of recruitment of $CD4^+$ and $CD8^+$ T cells in weaned pigs, *i. e.* from Day 21 to Day 35 (24) or from Day 14 to Day 35 (25), respectively.

Although, the IBC from natural sources will be more easily accepted by the consumers, both synthetic IBC tested are shown to be safe for pigs (29) and thus, for food safety they

must show to be effective in their purpose as potential immunostimulators (14,15), namely to act as alternatives to AGP and provide health and performance to pigs. Actually, LEVA and POE-POP showed capability of enhancing either functions or recruitment of cellular components of nonspecific/innate (MO/GR) and specific/adaptive immunity (extrathymic CD4/CD8 double positive T lymphocytes of memory/effector phenotype), respectively, and thereby help pigs to develop "optimal", not over excessive active immune responses during early postweaning period before they reach adult immunocompetence values. Immune activation can be counterproductive in healthy animals and divert nutrients from growth towards defence so measures must be taken to minimise the negative aspects to performance. Proper timing and duration of immunostimulation is essential but the fact that the increased immune response is sometimes associated with decreased performance is a problem that needs addressing. Therefore, it can be concluded that immunostimulatory effects of LEVA and POE-POP (without affecting normal/acute ranges of CRP and HpG serum levels as important markers of health status in pigs) provided a novel perspective for their use during early postweaning period before pigs reach adult immunocompetence values, and thus could be considered as potent immunostimulators in swine production.

Conflict of interest

Authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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VPLIV NA SISTEMSKO PRIROJENO IN PRIDOBLJENO IMUNOST PRI ODSTAVLJENIH PUJSKIH PO ENKRATNEM DODATKU IMUNOBIOTIKOV PREKO PREBAVNEGA TRAKTA

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Povzetek: Namen raziskave je bil ugotoviti vpliv lavamisola (LEVA) in polyoxyethylen-polyoxypropylena (POE-POP), znanih imunobiotikov, ki bi jih lahko uporabljali v prašičereji za boljši prirast namesto antibiotikov, na sistemsko prirojeno in pridobljeno imunost pri odstavljenih, 4 tedne starih pujskih. Vsem živalim smo LEVA in POE-POP dajali preko ust (peroralno) in tedensko v odobju petih tednov dodajanja imunobiotikov ugotavljali stopnjo fagocitoze in protimikrobno aktivnost monocitov in granulocitov, raven serumskega C-reaktivnega proteina (CRP), profil haptoglobina (HpG) ter raven prisotnosti limfocitov T, ki izražajo gena CD4 in CD8 v krvi. Rezultati so pokazali, da je bila raven serumskega CRP znižana pri pujskih, ki so prejemali tako LEVA kot POE-POP 7. in 21. dan raziskave ($p < 0,05$). Pujski, ki so prejemali LEVA, so imeli zvišan HpG 14. in 21. dan raziskave ($p < 0,05$), medtem ko so imeli pujski, ki so prejemali POE-POP, 7. dan raziskave HpG znižan, 21. dan pa zvišan ($p < 0,05$). Oba imunobiotika sta v pogojih in vitro dvignila stopnjo fagocitoze pri granulocitih in protimikrobno aktivnost monocitov na 35. dan raziskave ($p < 0,05$). Pujski, ki so prejemali en ali drugi imunobiotik, so imeli 35. dan raziskave višje vrednosti limfocitov T, ki so izražali CD4 in CD8, od vrednosti na 14. ali 28. dan raziskave ($p < 0,05$). Rezultati raziskave torej kažejo, da sta oba imunobiotika pozitivno vplivala na izbrane kazalce delovanja imunskega sistema in sta potencialno zanimiva za uporabo v prašičereji za spodbujanje delovanja imunskega sistema in s tem boljši prirast prašičev.

Ključne besede: odstavljeni pujski; umetni imunobiotiki; prirojena imunost; pridobljena imunost;