

Supplementation of a supranutritional dose of zinc sulfate and heat killed *Bacillus firmus* in early weanling pig diet: the effect on growth, neutrophil function and inflammatory cytokines

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ABSTRACT

The objective of the study was to investigate the effect of supplementation of a supranutritional dose of zinc sulfate ($ZnSO_4$) and *Bacillus firmus* derived bio-response modifier (BRM) on growth, blood neutrophil functions, pro-inflammatory and anti-inflammatory cytokine responses in early weanling piglets. In total, 45 piglets (age of 19.25 ± 0.84 days) were randomly divided into five groups: I (basal diet only), II (basal diet supplemented with $ZnSO_4$), III (basal diet supplemented with BRM), IV (basal diet supplemented with $ZnSO_4$ plus BRM) and V (basal diet without weaning from dam). The production of myeloperoxidase (MPO) and superoxide anion (O_2^-), and the concentration of transforming growth factor- β 1 (TGF- β 1) were markedly reduced, whereas the concentrations of intercellular adhesion molecule 1 (ICAM1), and monocyte chemoattractant protein 1 (MCP1)

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were significantly ($P < 0.05$) increased in piglets during the post-weaning period. The neutrophil functions did not improve until day 7 of weaning ($P > 0.05$) but a marked increase ($P < 0.05$) in ICAM1, MCP1 and a decrease in TGF- β 1 concentrations were observed up to days 14 and 7, respectively, in piglets of groups II and III. However, the addition of BRM plus ZnSO₄ in the basal diet improved MPO (day 2) and O₂⁻ (day 7) without any significant alterations in growth and cytokine concentrations in group IV piglets. Finally, it is concluded that the addition of BRM plus ZnSO₄ to the diet stimulates the innate immunity of weaner piglets compared to ZnSO₄ or BRM alone. The findings of the study will definitely help to formulate effective dietary management practice for early weanling piglets in swine herds.

Key words: *Bacillus firmus*; cytokine; piglet; weaning; zinc sulfate

Introduction

Early weaning is one of the most important management practices in modern swine husbandry. The young piglets are abruptly forced to adapt to nutritional, immunological and psychological disturbances after weaning. In spite of several advantages, weaning induced physiological stress has some instant negative impacts on the immune system of the body and the structural and functional activities of the intestines. The notable immunological alterations include compromised function of immune cells and up-regulation of pro-inflammatory cytokines, which in turn cause marked changes to the intestinal structure and function, leading to multiple infections and scouring after weaning (CAMPBELL et al., 2013; HU et al., 2013). Such changes are more severe at 2 to 5 days post-weaning, and it takes around 14 days post-weaning to recover (MEI and XU, 2005). The gut health of early weaned piglets is highly regulated by multiple growth factors and cytokines for restoration of damaged intestines (BLIKSLAGER et al., 2007). Among the various regulatory molecules, pro-inflammatory and anti-inflammatory cytokines help to maintain gut/intestinal health in early weaned piglets (CAMPBELL et al., 2013). The pro-inflammatory chemokines, such as intercellular adhesion molecule 1 (ICAM1) and monocyte chemoattractant protein 1 (MCP1), and anti-inflammatory cytokines, such as transforming growth factor- β 1 (TGF- β 1) secreted by the endothelial, and most immune cells, play a vital role in immune function. Up-regulation of ICAM-1 and MCP-1 and down-regulation of TGF- β have been observed in early weaning associated gut inflammatory reactions (WANG et al., 2008; XIAO et al., 2014; LIU, 2015). TGF- β 1 plays an important regulatory role in the post-weaning adaptation process of the intestine of the pig (MEI and XU, 2005). To minimize the health complications, medicated early weaning practices are often advocated in swine herds (CLARK et al., 1994). In that context, antibiotics are often added to the diet of weanling piglets to prevent infections and improve growth during the weaning transition (CROMWELL, 2002). Due to the mammoth demerits, many countries have either banned or are in the process of banning the use of antibiotics in pig diets as a routine method for promoting growth. One of the promising alternatives to antimicrobials could be the use of a bio-response modifier

(BRM) and micronutrients. The beneficial effects of a microbial derived BRM have been reported in neonatal swine health (SCHMIED et al., 2012). The peptidoglycan layer of the cell wall of *Bacillus firmus* has a significant role in immunomodulation (LAWRENCE and NAUCIE, 1998). The cell wall components of the *Bacillus* organism have been reported to stimulate both innate and adaptive immunity both in laboratory animals and human beings (PROKESOVA et al, 1994; ZANVIT et al, 2010). Among the micronutrients, zinc is a vital essential nutrient for many physiological functions of swine (RICHARDS et al., 2010). It has been reported that weaned pigs often suffer from zinc deficiency and this disturbs their innate immunity and increases susceptibility to pathogens (LI et al., 2006; RINK and GABRIEL, 2000; DAVIN et al., 2013). Therefore, supplementation of supranutritional doses of zinc is often practised to improve the health and performances of piglets during the critical period of post-weaning (NATIONAL RESEARCH COUNCIL, 1998; BRITISH SOCIETY OF ANIMAL SCIENCE, 2003). The beneficial effects of supranutritional doses of zinc and BRM have been evaluated separately in weaned piglets in this study. Here, we hypothesized that the combined effects of supranutritional doses of zinc and BRM might have a beneficial effect on the innate immune response in early weanling piglets. Therefore, an attempt has been made to assess the synergistic effect of supranutritional doses of zinc and BRM on performance, the antimicrobial defence of peripheral leukocytes, and pro-and-anti-inflammatory cytokines in early weanling piglets.

Materials and methods

Care and use of animals. The study was conducted on early weanling cross-bred piglets (Landrace × indigenous) from the institute's Swine Production Farm, and the protocol of the experiment was approved by the committee for the purpose of control and supervision of experiments for animals (CPCSEA), India.

Procurement of B. firmus and preparation of biological response modifier (BRM). Freeze-dried *B. firmus* was procured from the Microbial Type Culture Collection and Gene Bank (MTCC No: 7531), Institute of Microbial Technology, Chandigarh, India. The culture was revived in nutrient broth and 11.0 gram of bacterial mass was produced by sub culturing. The total viable bacterial count was measured by the standard plate count method and the total bacterial cells were done by the nephelometry turbidity method. In the current study, the total viable bacterial count was 10^{10} colony-forming units (cfu)/mL and the total cell count was 10^{12} /mL. The bacterial mass was heat killed at 100 °C for 30 minutes; non-viability of bacteria was confirmed by a sterility test. The killed bacterial mass was delipided by chloroform-ethanol (2:1) treatment, freeze dried and stored at -20 °C (LOMAKOVA et al., 2006). The delipidated *B. firmus* was termed as the bio-response modifier (BRM).

Diets and experimental protocol. All diets were based on a dry, basal diet and its composition is depicted in Table 1. The basal diet was formulated according to the NRC (1998), to fulfill the standard nutritional requirement of piglets weighing 4-6 kg. Additional benefits were evaluated after inclusion of BRM and higher Zn nutritional supplementation in the basal diet. The second diet was Zn-enriched and consisted of the control diet mixed with 2000 mg ZnSO₄/ kg of feed. The third diet was a BRM-enriched diet that consisted of the control diet mixed with 100 mg BRM/ kg of feed, and the fourth diet was a Zn plus BRM-enriched diet that consisted of the control diet mixed with both 2000 mg ZnSO₄ and 100 mg BRM/ kg of feed.

Table 1. Ingredients and chemical composition of basal diet

Item	Basal diet
Ingredient (% as fed basis)	
Wheat bran	16.00
Crushed maize	46.00
Deoiled soya bean cake	30.00
Fish meal	6.00
Vitamin and mineral mixture*	1.20
Common salt	0.80
Chemical composition (% dry matter basis)	
Crude protein (CP)	23.95
Digestible energy (kcal/kg)	3400.00
Organic matter	92.73
Ether extract	2.40
Crude fiber	4.79
Nitrogen free extract	61.57
Total ash	5.2

*Per kilogram vitamin and mineral premix contained following compositions: Vitamin A 700000 IU; Vitamin D3 70000 IU; Vitamin E 250 mg; Nicotinamide 1000 mg; cobalt 150 mg; Iodine 325 mg; Iron 1500 mg; Magnesium 6000 mg, Potassium 1000 mg, Sodium 5.9 mg; Sulfur 0.72%; Zinc 9600 mg and Calcium 25.5% and Phosphorus, 12.75%.

Cross-bred piglets (Landrace × indigenous) from the institute's experimental swine production farm were used in the present study. The piglets and dam were kept in the same farrowing pens with a concrete floor, and piglets were allowed to suck the dam's milk *ad libitum* until weaning. The overall management practices were identical for all piglets. A total of 45 cross-bred piglets (Landrace × indigenous), of both sexes, aged 19.25 ± 0.84 days, weighing 4-6 kg body weight, were randomly separated and assigned

to the control (group I), ZnSO₄ (group II), BRM (group III) and ZnSO₄ plus BRM (group IV) groups. Each group consisted of nine piglets, with individual piglets being used as the experimental unit. Another group, called Group V, consisting of nine piglets was kept with the dam and served as the unweaned control group. It received only the control diet. Feeding schedule was initiated 7 days before weaning and continued up to 14 days post-weaning. The piglets were fed twice a day, with free access to water and feed. The piglets received no medication during the study period. The health of the piglets was examined daily by expert veterinarians, for any signs of disease or stress. The piglets were weighed 7 days before weaning (-7 day) and 14 days post-weaning.

Assessment of peripheral neutrophil functions.

Collection of blood samples. The blood samples (approximately, 6.0 mL) were collected by venipuncture of the anterior vena cava into K3ethylenediaminetetraacetic acid (K3EDTA)-coated vacutainer tubes. Blood samples were collected before initiation of treatment (-7 day) and thereafter on days 2, 7 and 14 after weaning. After collection of blood, 2.0 mL blood was used for neutrophil isolation, and the rest of the samples were immediately centrifuged at 200 g for 10 min to separate plasma and stored at -20 °C until analysis.

Isolation of neutrophil. The neutrophils from the blood were separated following the method described by FIELD et al. (1985). Briefly, 2.0 mL blood samples were mixed with 2.0 mL phosphate buffered saline (PBS, 0.0132M, pH 6.8 ± 0.1) and centrifuged at 1000 g for 15 min at room temperature to obtain packed red cells. The red cells were lysed by addition of 12 mL distilled water and the isotonicity was restored after 45 sec by addition of 6 mL phosphate buffered 2.7% NaCl, and thoroughly mixed. After centrifugation at 125 g for 10 min, the supernatant was removed and the cell pellet, consisting primarily of neutrophils, was re-suspended in Hanks Balanced Salt Solution (HBSS). The viability of cells was checked by the trypan blue exclusion technique, and purity was determined by differential leukocyte count. The cell numbers were adjusted to 1×10⁶/mL.

MPO activity. The concentrations of MPO in neutrophils were measured following the standard procedure (BRETZ and BAGGIOLINI, 1974), and enzyme concentration was calculated by using the molar extinction coefficient for oxidized *O*-dianisidine (Sigma, USA). The optical density (O.D.) value was measured at 560 nm in the spectrophotometer. The molar extinction coefficient for oxidized *O*-dianisidine was assayed with known amounts of glucose oxidase-peroxidase assay (20,040 ± 400).

O₂⁻ production. The O₂⁻ production of isolated neutrophils stimulated by phorbol-12-myristate-13-acetate (PMA, 5 µg/mL, Sigma, St Louis, MO, USA) was measured according to the method described by NAGAHATA et al. (1986). Briefly, the reaction mixture, consisting of 200 µL of nitroblue tetrazolium solution (0.1% NBT in Earle's Balanced Salt Solution), 200 µL of 1×10⁶ neutrophil/mL and 25 µg of PMA was prepared,

and the final volume (440 μL) was adjusted with Earle's solution. After incubating at 37 $^{\circ}\text{C}$ for 30 min, the reaction was stopped by adding 3.0 mL of 0.5 (N) Hydrochloric acid (HCl), and centrifuged at 200 g for 5 min. The cells were washed twice with 3.0 mL of 0.5 (N) HCl and resuspended in 3 mL of dimethyl formamide (DMF). Formazone was extracted by heating in a boiling water bath for 10 min and 2 mL of 10 (N) potassium hydroxide (KOH) was added. The mixture was centrifuged (200 g for 5 min.) at room temperature, and the optical density (OD) of the DMF layer was read at 710 nm using DMF as a blank. Delta OD (ΔOD) was calculated by subtracting resting OD from the OD stimulated by PMA. The results were expressed as $\text{OD}/1 \times 10^6$ neutrophils /30 min in 3 mL of DMF.

Measurement of ICAM-1, MCP-1 and TGF- β 1 in plasma. The concentrations of ICAM-1 (ELISA kit for Intercellular Adhesion Molecule 1, Porcine (Pig), SEA548Po, Cloud-Clone Corp. USA, Uscn Life Science Inc.), MCP-1 (ELISA kit for Monocyte Chemotactic Protein 1, Porcine (Pig), SEA087Po, Cloud-Clone Corp. USA, Uscn Life Science Inc.) and TGF- β 1 (Quantikine ELISA Kit, TGF- β 1 Immunoassay, MB100B, R & D System. Inc) in the plasma were measured using separate commercially available ELISA kits according to the manufacturer's instructions.

All the estimations described above were carried out before treatment (-7 day) and thereafter on days 2, 7 and 14 of weaning.

Statistical analysis. The data were analyzed using the statistical package SAS v 9.3 (SAS Institute, Inc. 2011, Cary, NC, USA) to observe the effect of the independent diet variable on various neutrophil functions and pro-and anti-inflammatory cytokine markers. The data were taken repeatedly at different time intervals from each animal and analyzed using repeated measure ANOVA, with animal as the subject and period as the repeated measurement. For analyzing the effect of diets on myeloperoxidase activity, superoxide production and concentration of ICAM1, MCP1 and TGF- β 1, repeated measures ANOVA were applied using the following general linear model:

$$Y_{ijk} = \mu + G_i + ID(G_i) + D_j + (GD)_{ij} + e_{ijk}$$

Where

Y_{ijk} = observed value of the response variable for i -th group at j -th day

μ = general mean effect

G_i = i -th group effect

$ID(G_i)$ = animal within group (random term)

D_j = effect of the j -th day

e_{ijk} = error term

Multiple comparisons between groups, days and the interaction effects of i-th group and j-th day were performed using the Tukey HSD test at 5% level of significance. An individual pig was considered the experimental unit. A probability level (P) of 0.05 was selected as the statistical selection limit for all tests. The results are expressed as means and SEM.

Results

Health condition and body weight of piglets in response to diet supplementation. Clinically no illness was observed during the trial period, including post-weaning scours. The weight gain of piglets was calculated by taking weight values after 14 days of diet supplementation and subtracting the corresponding weight of each piglet at day -7. The average weight gain during the 21 days study was 2.62 ± 0.28 kg, 2.26 ± 0.40 kg, 2.74 ± 0.25 kg, 3.07 ± 0.15 kg and 2.57 ± 0.17 kg for groups I, II, III, IV and V respectively. Weanling piglets supplemented with either ZnSO₄ (P = 0.41), BRM (P = 0.79) or ZnSO₄ plus BRM (P = 0.24) did not exhibit a statistically significant increase in body weight relative to piglets in the control diet groups.

Effect on peripheral neutrophil functions. A marked reduction of O₂⁻ production and MPO activity was observed in weanling piglets (group I) compared to unweaned piglets (group V) receiving only the basal diet. Group II piglets (zinc enriched diet) did not exhibit any improvement in O₂⁻ and MPO production in neutrophils on day 7 (P<0.05) and day 2 (P<0.05) of weaning, respectively, but an improvement was observed on day 14 for O₂⁻ and day 7 onwards for MPO production. There was a significant improvement in O₂⁻ and MPO production by neutrophils in group II piglets (BRM enriched diet) on day 7 of weaning. The ZnSO₄ plus BRM supplementation (group IV) did not alter MPO production statistically in piglets from the pre-weaning to the post-weaning period. Although O₂⁻ production significantly dropped on day 2 of weaning (P<0.05) an improvement was noted on day 7 and 14 of weaning. No statistically significant interaction (P<0.01) was observed between diet and O₂⁻ (P = 0.779) and MPO (P = 0.978) production by neutrophils (Table 2).

ICAM-1, MCP-1 and TGFβ1. The mean concentrations of ICAM-1 and MCP-1 were markedly (P<0.05) increased, whereas the mean TGFβ1 concentration was significantly (P<0.05) decreased in the post-weaning period in the weaned piglets (group I). The mean ICAM-1 and MCP-1 concentrations remained persistently elevated, and the mean TGFβ1 concentration remained persistently lower in the post-weaning period in comparison with pre-weaning value in the zinc-enriched diet supplemented weanling piglets (group II). In the BRM supplemented piglets, the average ICAM-1, TGFβ1 and MCP-1 concentrations reached towards pre-weaning values on days 14 and 7, respectively. Interestingly, the ICAM-1, MCP-1 and TGFβ1 concentrations did not differ significantly from the pre-

weaning to the post-weaning period in ZnSO₄ and BRM supplemented piglets (group IV). Statistically no significant interaction was found between diet and concentrations of ICAM-1 (P = 0.40), MCP-1 (P = 0.60) and TGFβ1 (P = 0.21) (Table 3).

Table 2. Changes in the mean values of superoxide anion (O₂⁻; ΔOD/1×10⁶cells/3 mL DMF) and myeloperoxidase (MPO, μmoles/1×10⁶ cells) production by peripheral blood neutrophils in response to supplementation with ZnSO₄ (group II), BRM (group III), ZnSO₄ plus BRM (group IV) and in control piglets (groups I and V).

Groups	-7 day	2 day	7 day	14 day	T	S	T×S
O ₂ ⁻ (ΔOD/ 1×10 ⁶ cells/3 mL DMF)							
Gr. I	0.230 ± 0.027 ^{a,A}	0.105 ± 0.011 ^{b,A}	0.145 ± 0.012 ^{b,A}	0.133 ± 0.021 ^{b,A}	<0.001	<0.001	0.77
Gr. II	0.228 ± 0.024 ^{a,A}	0.112 ± 0.012 ^{b,A}	0.144 ± 0.019 ^{b,A}	0.215 ± 0.036 ^{a,A}			
Gr. III	0.232 ± 0.025 ^{a,A}	0.146 ± 0.029 ^{b,AB}	0.164 ± 0.033 ^{a,A}	0.204 ± 0.034 ^{a,A}			
Gr. IV	0.234 ± 0.016 ^{a,A}	0.193 ± 0.026 ^{b,B}	0.198 ± 0.035 ^{a,A}	0.223 ± 0.033 ^{a,A}			
Gr. V	0.232 ± 0.025 ^{a,A}	0.205 ± 0.025 ^{a,AB}	0.215 ± 0.027 ^{a,A}	0.204 ± 0.034 ^{a,A}			
MPO (MPO, μmoles/ 1×10 ⁶ cells)							
Gr. I	3.64 ± 0.41 ^{a,A}	1.94 ± 0.37 ^{b,A}	2.20 ± 0.30 ^{b,A}	2.51 ± 0.31 ^{b,A}	<0.001	<0.001	0.97
Gr. II	3.58 ± 0.38 ^{a,A}	2.02 ± 0.21 ^{b,A}	2.40 ± 0.39 ^{a,A}	2.63 ± 0.29 ^{a,A}			
Gr. III	3.66 ± 0.35 ^{a,A}	2.44 ± 0.37 ^{b,AB}	2.81 ± 0.34 ^{a,A}	2.85 ± 0.32 ^{a,A}			
Gr. IV	3.64 ± 0.52 ^{a,A}	3.38 ± 0.47 ^{a,B}	3.43 ± 0.48 ^{a,A}	3.61 ± 0.50 ^{a,A}			
Gr. V	3.72 ± 0.62 ^{a,A}	3.18 ± 0.63 ^{a,B}	3.72 ± 0.62 ^{a,A}	3.23 ± 0.35 ^{a,A}			

T - treatment, S - sampling time, T×S - treatment × sampling time. Superscripts with small letters (a, b) indicate comparison between the days (P<0.05), mean ± SE differ significantly (P<0.05)) from pre-weaning baseline value (day-7). Superscripts with capital letters (A, B) indicate comparison between the groups (P<0.05), mean ± SE differ by P<0.05. Group or day with common letters do not differ significantly at P<0.05.

Table 3. Changes in the mean concentration of ICAM⁻¹ (ng/dL), MCP⁻¹ (ng/mL) and TGF β1 (pg/mL) in blood plasma in response to supplementation with ZnSO₄ (group II), BRM (group III), ZnSO₄ plus BRM (group IV) and in control piglets (groups I and V).

Groups	-7 day	2 day	7 day	14 day	T	S	T×S
ICAM-1 (ng/dL)							
Gr. I	739.25 ± 58.22 ^{a,A}	1112.57 ± 25.64 ^{b,A}	1076.56 ± 28.94 ^{b,A}	1078.66 ± 34.53 ^{b,A}	<0.001	<0.001	0.40
Gr. II	717.01 ± 89.42 ^{a,A}	1091.24 ± 33.17 ^{b,A}	1032.13 ± 37.45 ^{b,A}	967.05 ± 61.66 ^{b,AB}			
Gr. III	713.85 ± 73.03 ^{a,A}	962.83 ± 67.07 ^{b,A}	966.46 ± 62.14 ^{b,B}	769.10 ± 54.45 ^{a,B}			
Gr. IV	726.43 ± 92.79 ^{a,A}	770.43 ± 81.38 ^{a,B}	783.28 ± 117.76 ^{a,BC}	762.65 ± 75.30 ^{a,B}			
Gr. V	720.36 ± 97.50 ^{a,A}	762.80 ± 92.28 ^{a,B}	728.54 ± 86.80 ^{a,C}	763.13 ± 91.95 ^{a,B}			
MCP-1 (ng/mL)							
Gr. I	1.71 ± 0.19 ^{a,A}	2.46 ± 0.14 ^{b,A}	2.66 ± 0.28 ^{b,A}	2.57 ± 0.18 ^{b,A}	<0.001	<0.001	0.60
Gr. II	1.66 ± 0.12 ^{a,A}	2.52 ± 0.28 ^{b,B}	2.49 ± 0.22 ^{b,A}	2.68 ± 0.33 ^{b,A}			
Gr. III	1.708 ± 0.21 ^{a,A}	2.51 ± 0.15 ^{b,B}	1.98 ± 0.30 ^{a,A}	1.92 ± 0.25 ^{a,A}			
Gr. IV	1.71 ± 0.23 ^{a,A}	1.86 ± 0.22 ^{a,AB}	1.77 ± 0.42 ^{a,A}	1.78 ± 0.29 ^{a,A}			
Gr. V	1.77 ± 0.12 ^{a,A}	1.79 ± 0.25 ^{a,AB}	1.72 ± 0.36 ^{a,A}	1.85 ± 0.25 ^{a,A}			
TGF β1 (pg/mL)							
Gr. I	119.36 ± 4.24 ^{a,A}	72.34 ± 4.92 ^{b,A}	78.52 ± 10.86 ^{b,A}	97.17 ± 8.05 ^{b,A}	<0.001	<0.001	0.21
Gr. II	115.36 ± 6.56 ^{a,A}	88.10 ± 7.42 ^{b,AB}	84.33 ± 7.19 ^{b,A}	89.39 ± 7.89 ^{a,A}			
Gr. III	117.75 ± 9.27 ^{a,A}	85.28 ± 9.04 ^{b,AB}	90.30 ± 9.82 ^{b,AB}	108.17 ± 2.98 ^{a,A}			
Gr. IV	120.42 ± 3.80 ^{a,A}	104.79 ± 5.26 ^{a,BC}	111.92 ± 5.39 ^{a,BC}	106.22 ± 8.41 ^{a,A}			
Gr. V	118.20 ± 6.63 ^{a,A}	114.00 ± 5.96 ^{a,C}	116.84 ± 5.56 ^{a,C}	110.63 ± 4.36 ^{a,A}			

T - treatment, S - sampling time, T×S - treatment × sampling time. Superscripts with small letters (a, b) indicate comparison between the days (P<0.05), mean ± SE differ significantly (P<0.05) from pre-weaning baseline value (day-7). Superscripts with capital letters (A, B, C) indicate comparison between the groups (P <0.05), mean ± SE differ by P<0.05. Group or day with common letters do not differ significantly at P<0.05.

Discussion

Early weaned piglets are more prone to multiple infections due to compromised immunity, resulting in high morbidity and mortality in the post-weaning period. Therefore, boosting the innate immunity of weaned piglets in this critical period would be a favorable option to minimize the undesirable stress conditions. In the present study, we found the decreased functional capacity of peripheral blood neutrophils after the early weaning of piglets, and the findings are in accordance with previous workers (WATTRANG et al., 1998; HAN et al., 2014). In the current study, although the supranutritional level of ZnSO₄ supplementation did not improve the O₂⁻ production and MPO activity of peripheral neutrophils until day 7, a significant enhancement was noted on day 14 of weaning. Zinc is an essential micronutrient having an important role in revitalizing impaired immune functions (RINK and GABRIEL, 2000). It has been reported that supranutritional supplementation of zinc enhances non-specific innate immunity through natural killer cell activity, chemotaxis, phagocytosis, generation of the oxidative burst, and cytokine response in newly weaned piglets (KIM et al., 2009; LIU et al., 2014). In our study, improvement of the neutrophil function might have been due to the immunomodulatory potential of zinc sulfate. The *B. firmus* derived BRM supplementation significantly improved the O₂⁻ production and MPO activity of neutrophils from day 7 onwards. The enhanced activity of neutrophil function indicates the immunomodulatory potential of *B. firmus* derived BRM. LOMAKOVA et al. (2006) reported that delipidated *B. firmus* (DBF) activates peritoneal macrophages in mice through cytokine activity. Further, DBF has been reported to be a very potent adjuvant to stimulate innate immunity after intranasal immunization with inactivated influenza virus type A in a mouse model (ZANVIT et al., 2010).

The ICAM-1 and MCP-1 are pro-inflammatory chemokines and TGF- β is an anti-inflammatory cytokine. In the present study, the persistent elevation of ICAM-1 and MCP-1 concentration and the marked (P<0.05) decrease in TGF β 1 concentration in piglets during the post-weaning period might be due to inflammatory responses (LIU, 2015; XIAO et al., 2014). Although we did not measure the concentrations of ICAM-1, MCP-1 and TGF- β in the intestinal compartment, the alteration of these molecules in the peripheral circulation might be due to systemic inflammatory reaction following early weaning (McCRACKEN et al., 1999; GAO et al., 2011). Alterations in cytokines due to gut starvation affect systemic inflammatory responses besides the mucosal immune system. The upregulation of pro-inflammatory cytokines increases intestinal epithelial permeability and regulates the immune functions, whereas anti-inflammatory cytokines protect against intestinal inflammation by preserving the barrier function (HOWE et al., 2005; AL-SADI et al., 2009; CAMPBELL et al., 2013). Moreover, activation of the immune system in piglets during weaning causes gut upset and reduced feed intake. Here,

supranutritional zinc supplementation was not able to reduce the ICAM-1 and MCP-1 concentrations in the post-weaning period. However, the concentration of TGF β 1 was significantly ($P<0.05$) increased on day 14 of post-weaning, which might be due to the restoration of the integrity of the intestinal barrier, as TGF- β 1 is believed to play a regulatory role in the post-weaning adaptation process of the intestines in pigs (MEI and XU, 2005). HU et al. (2013) observed increased mRNA levels of TGF- β and IL-10 in high zinc diet supported post-weaning piglets. In BRM supplemented piglets, the ICAM-1 and MCP-1 concentrations initially increased significantly ($P<0.05$) on day 3 post-weaning, and thereafter the values decreased towards the pre-weaning value on days 14 and 7 respectively. The concentration of TGF β 1 improved significantly ($P<0.05$) on day 14 post-weaning. The initially increased concentrations of ICAM-1 and MCP-1 might be due to cytokines secreted from inflammatory cells in response to *B. firmus* derived BRM (TAKAHASHI et al., 1995). The secretion of inflammatory cytokines from immune cells by the *B. firmus* derived BRM/ adjuvant was demonstrated by previous researchers in mouse models (LOMAKOVA et al., 2006; ZANVIT et al., 2010). In the present study, the production of O₂⁻ and MPO by leukocytes, and the concentrations of ICAM-1, MCP-1 and TGF β 1 in the plasma did not differ significantly ($P>0.05$) between the pre- and post-weaning periods in response to combined delipidated *B. firmus* and zinc supplementation in piglets. Peptidoglycan, derived from the cell wall of *Bacillus* spp. has been shown to have immunomodulatory properties, along with zinc, in immune-deficient mice models (HAYHURST et al., 2008 ; RAVLIC-GULAN et al., 2000). The responses of neutrophils and chemokines were much earlier under combined treatment than with ZnSO₄ or BRM alone, and this might be due to synergistic effect or potentiation of the immunostimulant effect of BRM by ZnSO₄.

The results of the present study indicate that supplementation of a supranutritional dose of Zinc sulfate and *B. firmus* derived BRM synergistically stimulated peripheral blood neutrophil functions, anti-inflammatory cytokine, and decreased the proinflammatory cytokines in early weanling piglets. It might be associated with immunomodulation and reduced inflammatory response in the post-weaning period. The findings of the study will definitely help to formulate effective dietary management practices for early weanling piglets in farm conditions.

Conflicts of interest

The authors declare they have no conflict of interest.

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SAŽETAK

Kod rano odbijene prasadi istražen je učinak dodavanja supranutritivnih doza cinkova sulfata ($ZnSO_4$) i modifikatora biološkog odgovora (BRM) izvedenog iz bakterija *Bacillus firmus* na rast, funkcije krvnih neutrofila te proupalni i protuupalni odgovor citokina. Ukupno 45 prasadi (u dobi $19,25 \pm 0,84$ dana) nasumično je podijeljeno u pet skupina: I (samo osnovni obrok), II (osnovnom obroku dodan je $ZnSO_4$), III (osnovnom obroku dodan je BRM), IV (osnovnom obroku dodan je $ZnSO_4$ i BRM) i V (osnovni obrok kod neodbijene prasadi). Kod prasadi su nakon odbića znakovito sniženi proizvodnja mijeloperoksidaze (MPO), superoksidnog aniona (O_2^-) i koncentracija transformacijskog čimbenika rasta- β 1 (TGF- β 1), a koncentracije međustanične adhezijske molecule-1 (ICAM1) i monocitnog kemoatraktanta protein - 1 (MCP1) znakovito su povišene ($P < 0,05$). Funkcije neutrofila nisu poboljšane do 7. dana od odbića ($P > 0,05$), ali kod prasadi iz skupine II. i III. je 14. i 7. dana opaženo znakovito povišenje ($P < 0,05$) ICAM1, MCP1, te sniženje koncentracije TGF- β 1. U prasadi iz skupine IV dodavanje BRM i $ZnSO_4$ u osnovnom obroku poboljšalo je MPO (2. dan) i O_2^- (7. dan), bez znakovitih promjena u rastu i koncentraciji citokina. Na kraju, zaključeno je da dodavanje kombinacije BRM i $ZnSO_4$ u obrok potiče urođenu imunost prasadi što nije slučaj kada se $ZnSO_4$ ili BRM dodaju zasebno. Rezultati ovog istraživanja pomoći će u formuliranju učinkovitog upravljanja hranidbom kod rano odbijene prasadi u krdu svinja.

Ključne riječi: *Bacillus firmus*; citokin; prasad; odbiće; cinkov sulfat
