Dietary ZnO and arginine supplementation on the dynamic change of microbiota, intestinal morphology, and immune function of weaned pigs subjected to heat stress

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Research

Keywords:

Posted Date: April 13th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-21955/v1

Abstract

Background. Weaning stress is an economically important problem in the pigs, and the economic loss of the growth performance reduction is even more critical if the heat stress adds to the weaning stress. The supplementation of ZnO is an effective option in reducing the adverse effects of weaning time. This study aimed to investigate the effect of the L-arginine (Arg) inclusion and different doses of ZnO to determine the best dietary supplementation ratio on growth performance, intestinal microbiota and integrity, and immune status in weaned pigs. A total of 180 weaned pigs (28 day-old) were randomly allotted to six treatments with 6 replicate pens in each treatment and 5 pigs per pen. The dietary treatments were: control diet (Con; with 1.1% Arg and without ZnO supplementation); Con + 2500 ppm Zn as ZnO (P-Zn); Con + 1.6% Arg (ARG); Con + 500 ppm of Zn as ZnO + 1.6% Arg (ZnArg1); Con + 1000 ppm of Zn as ZnO + 1.6% Arg (ZnArg2); P-Zn + 1.6% Arg (ZnArg3).

Results. The overall result showed that the inclusion of ZnArg3 significantly improved the average daily gain compared with the Con treatment. There was a reduction of feed intake in the Con diet compared with the ZnArg3 diet at phase 1 and overall. At phase 1, the weaned pigs in the ZnArg3 and P-Zn groups exhibited the decreased population of *Clostridium* spp. in the ileum compared with those of the Con group. In addition, a lower ileal *Clostridium* spp. population was detected in the ZnArg2 pigs compared with the Con pigs. At phase 2, the colonization of *Clostridium* spp. was higher in the Con and ARG treatments compared with ZnArg3 treatment. The weaned pigs fed the ZnArg1 and ZnArg3 diets showed a greater villus height of duodenum compared with the Con and P-Zn treatments. The count of eosinophil was significantly higher in the Con and ZnArg1 compared with the ZnArg2 and ZnArg3 treatments. The weaned pigs in the Con group showed increased mRNA expression of HSP27 in the liver compared with the P-Zn, ZnArg1, ZnArg2, and ZnArg3 groups. When fed the basal diet, weaned pigs exhibited enhanced mRNA expressions of IL-6 in the muscle compared with the ZnArg3 group. Dietary supplementation with ZnArg2 decreased the mRNA expressions of IFNγ in the muscle compared with the Con group. Supplementation with P-Zn, ZnArg1, ZnArg2, and ZnArg3 exhibited decreased mRNA expressions of TNF-α compared with the Con group. The mRNA gene expressions of IL-4 were decreased in the jejunum of P-Zn, ARG, ZnArg1, ZnArg2, and ZnArg3 weaned pigs compared with the Con group. The jejunum gene expression of TLR4 was upregulated in the Con and ARG treatments compared with the ZnArg1 and ZnArg3. The ZnArg1, ZnArg2, and ZnArg3 treatments showed a lower mRNA expression of TNF-α compared with the Con group.

Conclusion. The Arg supplementation did not improve the growth performance, microbial composition, or immune status of weaned pigs but showed a similar growth performance when supplemented with 500 ppm Zn as ZnO compared with 2500 ppm Zn as ZnO.

Introductions

The health condition of piglets is highly unstable at weaning time due to the stress of changing feed form and reduced feed intake [1-3]. The decrease of voluntary feed intake and being separated from mother

sow increase the stress and may cause dysbiosis due to sudden microbial changes in intestine. Postwening intestinal microbiota dysbiosis is associated with some unstable situation including enteric infectious pathogens growth, leaky gut, and incomplete intestinal integrity [4]. Besides, in hot seasons, weanling pigs are under the negative influence of high temperatures, which can dramatically enhance the adverse effects of weaning stress. The low recovery rate of heat-stressed weanling piglets is mainly due to the increased oxidative damage [5]. These critical conditions impair the immune system function and intestinal mucosal development, making the piglets susceptible to oxidative stress and microbial invasion.

Supplementation of pharmacological doses of ZnO (2500 ppm or over) in the piglet diet is a common practice among feed mills and farmers to control the adverse effects of postweaning diarrhea. There is evidence that high doses of dietary ZnO allow a higher tolerance to pathogen growth in the intestine [6]. Besides, dietary supplementation with high doses of ZnO increases the absorption rate that can enhance the antioxidant status and decrease the inflammatory responses in organs [7]. However, feeding high doses of ZnO results in substantial quantities of Zn excretion and increasing environmental concern. In order to reduce Zn excretion, the use of pharmacological doses of ZnO is banned and a lower concentration of ZnO is recommended in most of the developed countries.

It is well documented that the infectious morbidity attenuates by L-arginine (Arg) as an immune-nutrient factor. The protective role of Arg is related to the protection of intestinal mucosa and the increase in the recovery rate in the intestinal barrier [8, 9]. The nutritional requirement of Arg is highly dependent on environmental and physiological conditions. Therefore, stressors such as microbial change, heat stress, and sepsis dramatically increase the Arg requirements [10-12]. Arginine plays a crucial role in the production of polyamine and NO, which are strong anti-stress and vasodilator factors in mammals [2]. In addition, Arg facilitates the provision of cellular signaling, intestinal recovery, and immunity factors [10, 13]. Therefore, it can be hypothesized that the use of high dietary Arg levels may decrease the ZnO requirement during the stressful condition. Little information is available on the role of Arg and ZnO on oxidative damage of heat-stressed weanling piglets, in particular, that of immunity status, intestinal integrity, and growth performance. In this study, high dietary Arg and different levels of ZnO are applied to evaluate their effects on growth performance, immune status, intestinal microbiota and morphology of weaned pigs.

Materials And Methods

The project underwent proper ethical standards and the experiments (KW-170519-1) were approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea.

Animals and Experimental Design. A total of 180 weaned pigs (28 day-old; Landrace × Yorkshire × Duroc; initial BW: 10.45 ± 0.03 kg) of mixed sex were randomly allotted to six treatments with 6 replicate pens in each treatment and 5 pigs per pen. The dietary treatments were: control diet (Con; with 1.1% Arg and

without ZnO supplementation); Con + 2500 ppm Zn as ZnO (P-Zn); CON + 1.6% Arg (ARG); Con + 500 ppm of Zn as ZnO + 1.6% Arg (ZnArg1); Con + 1000 ppm of Zn as ZnO + 1.6% Arg (ZnArg2); P-Zn + 1.6% Arg (ZnArg3). All diets (Table 1) met or exceeded the nutrient requirements according to the NRC (2010). The crude protein, ether extract, lysine, methionine, arginine, calcium, and phosphorus content of the diets were analyzed by methods of AOAC [14]. The treatment diets were fed in a meal form in 2 phases (d 0 to 7, phase ; and d 8 to 14, phase). This experiment was conducted at the facility of Kangwon National University farm and the piglets were housed in slotted and concrete floor pens with a pen size of 1.90 m × 3.0 m. All pens were equipped with a self-feeder and nipple drinker to allow ad libitum access to feed and water. Individual weanling piglet weight and feed intake from each pen were recorded at the beginning of the experiment and at the end of every phase to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F). All the weaned pigs were subjected to a mild heat stress condition at 35° C.

Microbial analyses. To study the effects of dietary treatments on small intestinal microbiota, representative piglets from each group (2 piglets per replicate; one male and one female) reflecting the average BW of the pen were selected and sacrificed by electrocution at d 14 and 28 of each phase. The digesta from the ileum and was collected in sterile plastic bottles for microbial analysis. The samples collected for microbial analysis were immediately placed on ice until analyses were conducted. The ileal digesta sample (one gram) was mixed with 9 ml peptone broth (1%) and the homogenized (Becton, Dickinson and, Franklin Lakes, NJ, USA). In the next step, serially 10-fold dilutions in the pellets were used for Viable counts of bacteria. To determine the total anaerobic bacteria (Tryptic soy agar), *Lactobacillus* spp. (using MRS agar + 0.200 g/l NaN3 + 0.500 g/l L-cystine hydrochloride monohydrate), *Bifidobacterium* spp. (MRS-NPNL: MRS agar + nalidixic acid, paromomycin + neomycin sulphate + lithium chloride), *Clostridium* spp. (TSC agar) and coliforms (violet red bile agar) were used. The gas pack anaerobic system (BBL, No. 260678, Difco, Detroit, MI, USA) was used for preparing anaerobic conditions. The tryptic soy agar, MRS agar, and violet red bile agar were purchased from Difco Laboratories (Detroit), and TSC agar (CM0589) was purchased from Oxoid (Hampshire, UK). The bacterial concentrations were transformed (log) before statistical analysis.

Small intestinal morphology. The sacrificed pigs (two pigs per pen) were subjected to use for the morphological test. The intestinal morphology test was performed according to the procedure described by Hosseindoust et al., [15]. In short, for each intestinal sample, three cross-sections were prepared after staining with azure A and eosin using standard paraffin-embedding procedures. A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. The measurement of villus height was measured from the tip of the villi to the villus–crypt junction, while the crypt depth was defined as the depth of the invagination between adjacent villi and villus width was measured till the mid of the villus. All morphological measurements (villus height and crypt depth) were made in 10-μm increments using an image processing and analysis system (Optimus software version 6.5, Media Cybergenetics, North Reading, MA, USA).

RNA Extraction and Real-time PCR of organ samples. Total RNA was isolated from the Jejunum (50 mg), livers (50 mg) and spleens (100 mg) samples using Trizol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instruction. Extracted RNA was quantified to 1 μg/μl and cDNA synthesis was conducted using the Improm-II Reverse transcription system (Promega, Fitchburg, USA) and PCR was performed using Mx3000P real-time PCR (Stratagen, USA). The results were expressed as a relative expression by using the delta-delta method. The primers of interleukin-4 (IL-4), interleukin-6 (IL-6), interferon-γ (IFNγ), heat shock protein-27 (HSP27), toll-like receptor-4 (TLR4), and tumor necrosis factor-α (TNF-α) were presented in Table 2. In this process, the house-keeping gene, β-actin was introduced to adjust the quantity of input cDNA to maintain the role in internal control [16]. A total of 20 μL reaction system included 10 μL SYBR Premix Ex Taq, 0.8 μL of forward and reverse primer (10 μM), 0.4 μL ROX Reference Dye II (50×), 2.0 μL cDNA template, and 6 μL dd H_2O. Cycling conditions were as followed: 30 s at 95 °C, 40 cycles of denaturation step at 95 °C for 3 sec, 60 °C annealing step for 34 s and a 72 °C extension step for 15 s.

Blood parameters. The blood samples were collected from 2 piglets per pen on the last day of each phase. Five mL of EDTA-treated (Becton Dickinson, Franklin Lakes, NJ) and not-treated blood samples were collected from the jugular vein and stored on ice for immediate hematological analysis. The EDTA-treated blood was diluted by Natt-Herrick solution and mixed for 15 mins for white blood cells (WBC), red blood cells (RBC), lymphocytes, and monocytes were analyzed using Hemavet Multispecies Hematology Systems (Scientific Inc., Oxford, CT). The rest of the blood samples were centrifuged at 1,500 rpm for 20 minutes in a centrifuge, and plasma was separated and used for cortisol analysis. Cortisol was analyzed using an ELISA kit (ADI-900-70; Enzo Life Sciences, Farmingdale, NY).

Statistical Analysis. The data were analyzed as a completely randomized design using the GLM procedure of SAS (SAS Inst. Inc., Cary NC). The pen was the experimental unit for growth performance and feed intake, whereas individual piglet was an experimental unit for the microbial test, intestinal morphology, blood parameters, and gene expression analyses. The Turkey multiple range tests were applied for Treatment means separation by at $P < 0.05$ statistical level.

Results

Growth performance

The results of growth performance are shown in Table 3. There was no difference in the ADG of pigs in the first and second phases. The overall result showed that the inclusion of ZnArg3 significantly improved ($P < 0.05$) the ADG compared with the Con treatment. There was a reduction of ADFI in the Con diet compared with the ZnArg3 diet in phase 1 and overall. The gain to feed ratio was not affected by the treatments.

Microflora composition

The ileal microbial population is shown in Table 4. There was no difference in the population of Total anaerobic bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., and Coliforms between the treatments. At phase 1, the weaned pigs in the ZnArg3 and P-Zn groups exhibited the decreased population of *Clostridium* spp. in the ileum compared with those of the Con group ($P < 0.01$). In addition, a lower ileal *Clostridium* spp. population was detected in the ZnArg2 pigs compared with the Con pigs. At phase 2, the colonization of *Clostridium* spp. was higher in the Con, and ARG treatments compared with ZnArg3 treatment.

Intestinal morphology

As shown in Table 5, when the weaned pigs fed the ZnArg1 and ZnArg3 diets the villus height of Duodenum was increased compared with the Con and P-Zn treatments. There was no difference in villus height in the jejunum and ileum. In addition, the crypt depth and villus height to crypt depth ratio were not affected by supplementing Arg or ZnO in the diet.

Blood parameters

The results of the blood parameters are shown in Table 6. The number of WBC and RBC was not affected by the treatments. The number of Lymphocytes was not affected at d 7, however, there was a lower number of lymphocyte in the treatments ZnArg1, ZnArg2, and ZnArg3 compared with the Con pigs at d 14. There was no difference between the treatments for the number of monocytes, however, eosinophil number was significantly higher in the Con and ZnArg1 compared with the ZnArg2 and ZnArg3 treatments. Plasma cortisol was not affected by the treatments.

Gene expression in the organs

The weaned pigs in the Con group showed an increased mRNA expression of HSP27 in the liver compared with the P-Zn, ZnArg1, ZnArg2, and ZnArg3 groups ($P < 0.05$, Figure 1). An increased gene expression of TLR4 was observed in the ARG pigs compared with ZnArg3 pigs. There were no differences between the treatments in the gene expression of IL-4, IL-6, IFNγ, and TNF-α in the liver. When fed the basal diet, weaned pigs exhibited enhanced mRNA expressions of IL-6 in the muscle compared with the ZnArg3 group ($P < 0.05$, Figure 2). Dietary supplementation with ZnArg2 decreased the mRNA expressions of IFNγ in the muscle compared with the Con group ($P < 0.05$). Supplementation with P-Zn, ZnArg1, ZnArg2, and ZnArg3 exhibited decreased mRNA expressions of TNF-α compared with the Con group ($P < 0.05$). There were no differences between the treatments in the gene expression of IL-4, HSP27, and TLR4. The mRNA gene expressions of IL-4 were decreased in the jejunum of P-Zn, ARG, ZnArg1, ZnArg2, and ZnArg3 weaned pigs compared with the Con group (Figure 3; $P < 0.05$). The ZnArg1, ZnArg2, and ZnArg3 treatments showed a lower mRNA expression of TNF-α compared with the Con group. The gene expression of TLR4 in the jejunum was upregulated in the Con and ARG treatments compared with the ZnArg1 and ZnArg3. The treatments did not significantly impact the mRNA expression of IL-6, IFNγ, and HSP27 in the jejunum.

Discussion

The supplementation of pharmacological doses of ZnO is routinely considered in weaned pigs diet to alleviate diarrhea incidence and reduce the growth depression during the weaning period [6, 17]. In our study, the response to ADG and ADFI in pigs supplemented with ZnArg3 was increased relative to the Con pigs, however, there were no differences between the Arg and ZnO supplemented treatments. Compromising the growth performance in the Con pigs could be resulting from the insufficient nutrients intake, as shown by the significantly reduced ADFI in the Con pigs. The finding that supplementing 2500 mg/kg of Zn as ZnO showed no growth benefit compared with 500 or 1000 mg/kg Zn as ZnO was in contrast to previous researchers, who reported a significant difference between 2500 mg/kg ZnO and levels less than 1000 mg/kg [18]. It is believed that the lower doses of ZnO (below 1000 ppm) is not effective in the performance of weaned pigs [6, 19]. Our results showed that there was a comparable ADG between the ZnArg1, ZnArg2, and ZnArg3. It seems that the positive anti-stress effects of dietary Arg mediated the difference between ZnArg3 and ZnArg1 treatments. Anti-stress effects of Arg may have a complementary role for the pharmacological dose of ZnO effects in case of decreasing the stress, as Arg activates the production of nitric oxide and peroxynitrite [20]. However our results reject the hypothesis of direct effects of Arg in pathogen killing [21], as the number of coliforms and clostridia in the intestine was not affected in Arg-supplemented weaned pigs. Therefore, the intracellular role of Arg can be more highlighted than extracellular roles, and only the pharmacological dose of ZnO (2500 ppm) showed a definite effect in controlling *Clostridium* spp. The decreased villus height in the Con pigs may be another reason to explain the lower growth performance, which is indirectly linked to the anti-pathogenic properties of ZnO.

During weaning stress, the growth performance of pigs followed by controlling diarrhea becomes a priority. Thus, the microbial population plays an important role in the performance of pigs by eliminating the opportunistic pathogens. Moreover, it is widely accepted that the intestine mucosal integrity generally is associated with the intestinal microbiota. This interaction might be of importance when animals have to struggle with the change of diet form and facing a severe change in the microbial biomass and composition in the intestine. Although in the current study the absorption of Zn and the concentration of ZnO in the digesta was not studied, we did compare the microbiota composition in different phases. A lower number of *Clostridium* spp. was detected in the ZnArg3 pigs compared with the Con pigs, however, the lower ZnO supplementation (ZnArg1 and ZnArg2) did not show a definite effect on *Clostridium* spp. colonization. Hosseindoust et al., [22] and Yusof et al., [7] reported that the antimicrobial activity of ZnO against coliforms makes it an ideal candidate to control the microbiota, however, they did not report any change in the number of *Clostridium* spp. In another study, the population of coliforms and *Clostridium* spp. was linearly decreased in the ileum and colon of weaned pigs when the dose of ZnO increased from 500 ppm to 2500 ppm [6]. It is possible to speculate that influences on the intestinal microbiota might not be modified by the doses lower than the pharmacological recommendation for ZnO. Also, the addition of an anti-stress nutritional item such as Arg did not aid the antimicrobial effects. This result disagrees with the report of Ren et al. [23], who supplemented a high dose of Arg into the diet of mice to manipulate the microbial change in favor of beneficial bacteria including *Lactobacillus* spp. It is crucial to investigate

whether Arg alone or a combination with ZnO in lower doses, will influence the weaned pig's microbiota and immune status.

In the present study, the combination of heat stress and weaning challenge in untreated pigs caused severe morphological disruption of villus that was shown by a decreased villus height in the duodenum of the Con pigs compared with ZnArg1 and ZnArg3 pigs. Intriguingly, the villus height in the jejunum and ileum remained unchanged. This result was surprising as the authors had hypothesized that the flow of ZnO to the lower sections of the intestine and its antimicrobial effects would have enhanced the villus height in the distal sections of the intestine. Kim et al., [6] reported a greater villus height in the duodenum and ileum of weaned pigs by the supplementation of a pharmacological dose of ZnO into the diet. The reduced villus crypt structure alteration including villus height and crypt depth, crypt hyperplasia, and generally intestinal structural injury occurs mostly during the weaning [24]. The dietary Arg only numerically improved the villus height. The role of different doses of Arg on intestinal morphology should be further studied since the weaning time is the most critical period.

Gene

In the present study, the supplementation of ZnO remarkably decreased the mRNA expression levels of HSP27 that was involved in liver inflammation. The association between HSP expression and stress was previously reported in other studies [2, 4]. The low gene expression of HSP27 in the liver and a tendency for lower blood cortisol in Zn-supplemented diets may indicate a lower stress level, however, the pigs fed high dietary Arg did not show any improvement in reducing the stress level, although Arg is reported to be an anti-stress or immunomodulatory factor by the activation of ornithine decarboxylase and generating polyamines [10]. In addition, the reports related to the influence of dietary Arg on the TLR gene expression in weanling pigs during heat stress is scanty. In this study, a high level of dietary Arg increased the gene expression of TLR4 in the jejunum and liver. These findings may be explained by the result of a microbial test wherein dietary Arg showed significantly higher *Clostridium* spp. population compared with ZnO-supplemented treatments in the small intestine. A lower number of *Clostridium* spp. was detected in the ZnArg3 pigs compared with the Con pigs, which in turn caused a decrease in TLR4 expression in the jejunum. The pathogenic microbes are recognized by the innate immune system through the stimulation of TLRs. The TLR4 is a type of pattern recognition receptors in epithelial cells that have been identified as the main pathway on the innate immune system for recognition of lipopolysaccharide (LPS), a component of the bacterial cell wall [25]. The over-expression of the TLR signaling pathway may be responsible for the stress-induced suppression of the immune system. Interestingly, our result supports the idea that the cytokines production through TLR4 signaling among ZnO-supplemented treatments by increasing the gene expression of IL-4 in the jejunum and IL-4 and TNF-α in the liver. Dietary ZnO beneficially affected the bacterial communities and mRNA expression of inflammatory cytokines and TLR4.

A large body of evidence has shown the up-regulation gene expression of pro-inflammatory within a short time of weaning [24, 26, 27]. In the present study, the expression of TNF-α was decreased in the jejunum

of the ZnO-supplemented weaned pigs. Intestinal inflammation occurs in pigs after weaning. The high inflammation induces an elevated specific secretion of cytokines from intestinal lymphocytes [28]. The current study showed a lower expression of IL-4 in the jejunum and a lower count of lymphocytes. IL-4 is known as B lymphocyte growth promoter and can linearly modulate the production and development of B and T lymphocytes [29]. In contrast, Han et al [10] reported that the content of IL-2 and IFNγ in the serum were increased in cyclophosphamide-challenged weaned piglets. The over-production of TNF-α and IL-6 compromise the intestinal epithelial permeability, resulting in increased pathologic opening in the tight junction [18]. The extra-cellular pathogens such as intestinal microbes secrets IL-4, IL-10, and IL-13 to activate the immune system and produce strong antibody-mediated responses [30]. In agreement, it has long been known that the IL-6 concentration increases in stressful conditions and during acute inflammation [31]. The increased gene expression of TNF-α, IL-6, IFNγ were noticed in clostridium-challenged [32] or salmonella-challenged pigs [33]. It has been reported that some useful bacteria flora downregulate the gene expression of inflammatory cytokines [34]. In addition, the higher number of eosinophil in the Con pigs may be responsible for higher gene expression of cytokines. It has been reported that the eosinophils increase the gene expression of IL-1, IL-4, IL-6, and TNF-α [35]. As the increase of pro-inflammatory cytokines adversely affects the development of intestinal epithelium, controlling the concentration of intestinal inflammatory cytokines and intestinal integrity is crucial to alleviate intestinal disorders at the weaning time.

Conclusions

The growth performance, intestinal microbiota, microbial composition, and immune status of weanling pigs was improved by the ZnO supply but not by the supplementation of Arg. However, the heat-challenged weaned piglets fed diets supplemented with Arg with low ZnO (500 ppm) content expressed similar growth performance, intestinal microbiota, intestinal integrity, as those fed diets with Arg with high ZnO (2500 ppm) content. Therefore, it can be postulated that the dietary Arg alone can only be moderately effective during the weaning stress but not practically applicable without dietary ZnO supplementation.

Abbreviations

ARG: arginine supplementation

TNF: tumor necrosis factor

IL: interleukin

INF: interferon

HSP: heat shock protein

TLR: toll like receptor

LPS: lipopolysaccharide

ADG: average daily gain

ADFI: average daily feed intake

G:F: gain to feed ratio

WBC: white blood cells

RBC: red blood cells

P-Zn: 2500 ppm of Zn as ZnO

ZnArg1: 500 ppm of Zn as ZnO + 1.6% Arg

ZnArg2: 1000 ppm of Zn as ZnO + 1.6% Arg

ZnArg3: 2500 ppm of Zn as ZnO + 1.6% Arg

NO: nitrogen oxide

Declarations

Acknowledgments

This work was supported by Grant No. PJ014796012020 from Rural Development Administration, Republic of Korea

Author Contributions

All authors contributed to this work. Conceived and designed the experiments: S.Y.Yoon., S.J.Sa. Performed the experiments: S.Y.Yoon., H.S.Ko. Analyzed the data: G.W.Kim, J.S.Kim Interpreted the data: J.W.Choi, J.S.Kim Wrote the paper: H.S.Ko and J.S.Kim.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The protocol for the experiment was approved and laying hens were cared according to the guidelines of the Institutional Animal Care and Use Committee of Kangwon National University (KW-170519-1), Chuncheon, Republic of Korea.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Formula and chemical composition of basal diets (as-fed basis)

Treatments	CON	P-Zn	ARG	ZnArg1	ZnArg2	ZnArg3
Zinc oxide (ppm)	0	2500	0	500	1000	2500
Arginine (%)	1.1	1.1	1.6	1.6	1.6	1.6
Corn	49.79	49.48	49.29	49.23	49.17	48.98
SBM dehulled	17.20	17.20	17.20	17.20	17.20	17.20
Fish Chile	3.00	3.00	3.00	3.00	3.00	3.00
Whey (Korea)	10.00	10.00	10.00	10.00	10.00	10.00
Lactose	6.00	6.00	6.00	6.00	6.00	6.00
SDPP	4.00	4.00	4.00	4.00	4.00	4.00
Sugar	4.00	4.00	4.00	4.00	4.00	4.00
Soy Oil	3.00	3.00	3.00	3.00	3.00	3.00
$_{L}$-Lysine 78%	0.09	0.09	0.09	0.09	0.09	0.09
$_{DL}$-Methionine 100%	0.06	0.06	0.06	0.06	0.06	0.06
$_{L}$-Tryptophan 10%	0.18	0.18	0.18	0.18	0.18	0.18
Arg (100%)	-	-	0.50	0.50	0.50	0.50
Limestone	1.1	1.1	1.1	1.1	1.1	1.1
MCP	0.82	0.82	0.82	0.82	0.82	0.82
Salt	0.43	0.43	0.43	0.43	0.43	0.43
ZnO 78%	0	0.31	0	0.06	0.12	0.31
Vitamin premix[1]	0.11	0.11	0.11	0.11	0.11	0.11
Mineral premix[2]	0.22	0.22	0.22	0.22	0.22	0.22
Total	100	100	100	100	100	100
Chemical composition (%)						
MO	9.71	9.71	9.71	9.71	9.71	9.71
CP	18.99	18.99	18.99	18.99	18.99	18.99
Crude fat	5.06	5.06	5.06	5.06	5.06	5.06
Crude fiber	1.19	1.19	1.19	1.19	1.19	1.19
Ash	5.20	5.20	5.20	5.20	5.20	5.20
Ca	0.85	0.85	0.85	0.85	0.85	0.85
P	0.68	0.68	0.68	0.68	0.68	0.68
Lys	1.20	1.20	1.20	1.20	1.20	1.20
Met	0.39	0.39	0.39	0.39	0.39	0.39

Met_Cys	0.73	0.73	0.73	0.73	0.73	0.73
Thr	0.78	0.78	0.78	0.78	0.78	0.78
Trp	0.24	0.24	0.24	0.24	0.24	0.24
Arg	1.10	1.10	1.60	1.60	1.60	1.60
Zn	0.077	2.5	0.077	0.5	1.0	2.5

Abbreviations: DDGS, Dried distiller's grains with solubles; TCP, Tricalcium phosphate; ME metabolizable energy; CP, Crude protein; Av, Available.

[1]Supplied per kilogram of diet: 16,000 IU vitamin A, 3,000 IU vitamin D_3, 40 IU vitamin E, 5.0 mg vitamin K_3, 5.0 mg vitamin B_1, 20 mg vitamin B_2, 4 mg vitamin B_6, 0.08 mg vitamin B_{12}, 40 mg pantothenic acid, 75 mg niacin, 0.15 mg biotin, 0.65 mg folic acid.

[2]Supplied per kilogram of diet: 45 mg Fe, 0.25 mg Co, 50 mg Cu, 15 mg Mn, 25 mg Zn, 0.35 mg I, 0.13 mg Se.

Table 2. Primers

Gene	Primer sequence(5___>3)
β-actin_F	CAACACAGTGCTGTCTGGTGGTA
β-actin_R	ATCGTACTCCTGCTTGCTGATCC
IL4_F	TGTGCCCACGCTGTGCTTACA
IL4_R	CTTGTGGCAGTGCTGGCTCTCC
IL6_F	AGAAATCCCTCCTCGCCAAT
IL6_R	AAATAGCGAACGGCCCTCA
IFNγ_F	CTGAAGAACTGGACAGAGAG
IFNγ_R	CACCAGCTTCTGTAAGATGC
HSP27_F	GGAGATCACCGGCAAACACG
HSP27_R	CCTCCACTGTCAGCATCCCA
TLR4_F	GTCTCTCCTTCCTTACCTGCTGTTC
TLR4_R	AGGAGGAGAAAGACAGGGTAGGTG

Abbreviations: IL, Interleukin; IFN, Interferon; HSP, Heat shock protein; TLR, Toll like receptor; TNF, Tumor necrosis factor.

Table 3. Effect of heat stress on body weight (BW) average daily gain (ADG), average daily feed intake (ADFI), feed efficiency (gain:feed) of weanling piglets fed diets supplemented with or without zinc and arginine

Treatments	Con	P-Zn	ARG	ZnArg1	ZnArg2	ZnArg3	SEM	*P-value*
Zinc oxide (ppm)	0	2500	0	500	1000	2500		
Arginine (%)	1.1	1.1	1.6	1.6	1.6	1.6		
Intial BW, kg	10.42	10.46	10.44	10.48	10.49	10.42	0.11	0.995
7 days BW, kg	12.37	12.53	12.57	12.67	12.66	12.72	0.1	0.191
14 days BW, kg	14.45	14.71	14.78	15.03	15.00	15.16	0.25	0.396
ADG								
0 to 7 d	278	296	305	313	310	329	17.11	0.429
8 to 14 d	297	311	316	338	335	348	27.98	0.8
0 to 14 d	288^{b}	304^{ab}	310^{ab}	325^{ab}	322^{ab}	340^{a}	10.94	0.034
ADFI								
0 to 7 d	458^{b}	476^{ab}	482^{ab}	507^{ab}	489^{ab}	533^{a}	16.36	0.048
8 to 14 d	514	525	512	532	539	560	23.67	0.718
0 to 14 d	486^{b}	501^{ab}	497^{ab}	520^{ab}	514^{ab}	547^{a}	13.44	0.050
Gain:feed ratio								
0 to 7 d	0.607	0.623	0.631	0.615	0.634	0.617	0.03	0.985
8 to 14 d	0.578	0.591	0.614	0.627	0.619	0.620	0.04	0.934
0 to 14 d	0.592	0.606	0.624	0.625	0.626	0.623	0.05	0.780

Table 4. Effect of heat stress on intestinal microbiota of weanling piglets fed diets supplemented with or without zinc and arginine

Treatments	Con	P-Zn	ARG	ZnArg1	ZnArg2	ZnArg3	SEM	*P-value*
ZnO (ppm)	0	2500	0	500	1000	2500		
Arg (%)	1.1	1.1	1.6	1.6	1.6	1.6		
d 7								
Total anaerobic bacteria	8.84	8.89	8.86	8.73	8.82	8.69	0.12	0.838
Bifidobacterium spp.	7.66	7.72	7.71	7.63	7.74	7.8	0.15	0.975
Lactobacillus spp.	8.63	8.66	8.64	8.52	8.6	8.62	0.07	0.725
Clostridium spp.	8.61^{a}	8.23^{b}	8.51^{ab}	8.38^{abc}	8.34^{bc}	8.19^{b}	0.06	0.001
Coliforms	8.39	8.23	8.27	8.21	8.22	8.21	0.05	0.115
d 14								
Total anaerobic bacteria	8.61	8.64	8.65	8.55	8.61	8.77	0.1	0.731
Bifidobacterium spp.	7.47	7.6	7.52	7.49	7.42	7.66	0.13	0.835
Lactobacillus spp.	8.38	8.39	8.33	8.28	8.29	8.45	0.08	0.588
Clostridium spp.	8.54^{a}	8.24^{ab}	8.41^{a}	8.27^{ab}	8.34^{ab}	8.03^{b}	0.07	0.003
Coliforms	8.32	8.25	8.33	8.24	8.29	8.2	0.04	0.128

Table 5. Effect of heat stress on intestinal morphology in weanling piglets fed diets supplemented with or without zinc and arginine

Treatments	Con	P-Zn	ARG	ZnArg1	ZnArg2	ZnArg3	SEM	*P-value*
Zinc oxide (ppm)	0	2500	0	500	1000	2500		
Arginine (%)	1.1	1.1	1.6	1.6	1.6	1.6		
Villus height (VH, μ)								
Duodenum	528^{b}	550^{b}	567^{ab}	606^{a}	587^{ab}	676^{a}	27.94	0.014
Jejunum	459	508	495	532	501	583	28.74	0.095
Ileum	391	408	432	438	433	453	15.33	0.101
Crypt depth (CD, μ)								
Duodenum	341	333	315	328	334	346	15.22	0.768
Jejunum	243	265	283	280	260	271	18.37	0.665
Ileum	235	254	253	253	256	253	11.71	0.576
VH/CD								
Duodenum	1.56	1.67	1.82	1.84	1.79	1.97	0.11	0.21
Jejunum	1.91	1.92	1.84	1.93	1.97	2.17	0.14	0.658
Ileum	1.69	1.62	1.67	1.77	1.79	1.8	0.11	0.804

Table 6. Effect of heat stress on blood characteristics in weanling piglets fed diets supplemented with or without zinc and arginine

Treatments	Con	P-Zn	ARG	ZnArg1	ZnArg2	ZnArg3	SEM	*P-value*
Zinc oxide (ppm)	0	2500	0	500	1000	2500		
Arginine (%)	1.1	1.1	1.6	1.6	1.6	1.6		
WBC, 10^3/ul								
Day 7	9.4	9.78	9.82	10.09	10.23	10.12	0.3	0.435
Day 14	13.74	16.11	15.68	16.53	16.15	16.09	1.43	0.777
RBC, 10^6/ul								
Day 7	7.46	7.72	7.7	7.92	7.6	7.85	0.3	0.906
Day 14	7.93	8.17	7.11	7.51	7.06	7.72	0.33	0.132
Lymphocytes, %								
Day 7	74.13	71.56	72.54	70.97	70.72	70.66	1.07	0.192
Day 14	77.94^{a}	71.54^{ab}	71.03^{ab}	68.65^{b}	69.36^{b}	69.45^{b}	1.87	0.016
Monocytes, %								
Day 7	3.95	3.3	3.18	3.12	3.07	3.03	0.32	0.353
Day 14	4.6	4.51	4.43	3.85	3.28	4.23	0.35	0.101
Eosinophil, %								
Day 7	2.1	2.32	2.13	1.98	2.07	2.13	0.15	0.766
Day 14	3.57^{a}	2.78^{ab}	2.95^{ab}	3.40^{a}	2.52^{b}	2.63^{b}	0.24	0.021
Cortisol, ug/dL								
Day 7	5.17	4.8	4.58	4.12	4.37	4.43	0.33	0.30
Day 14	5.39	4.53	4.88	4.62	4.11	4.2	0.24	0.086

Figures

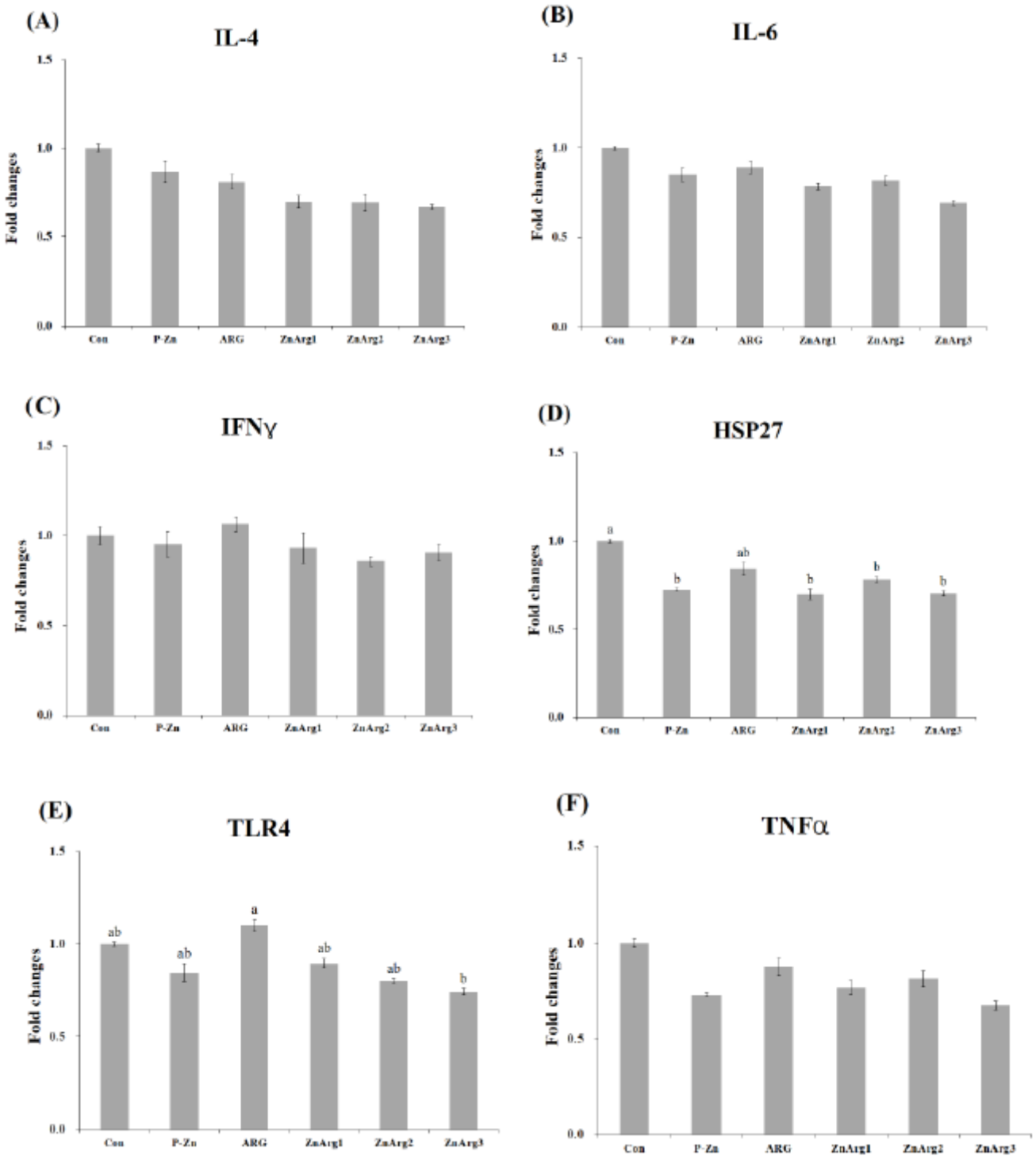

Figure 1

. Relative expression of IL 4, IL 6, IFNγ, HSP27, TLR4 and TNFα in the liver of weanling piglets . Abbreviations: IL, Interleukin; IFN, Interferon; HSP, Heat shock protein; TLR, Toll like receptor; TNF, Tumor necrosis factor. Error bars represent standard e rror of means. Bars with different letters (a~b) differ significantly across all 4 treatment groups (P <0.05).

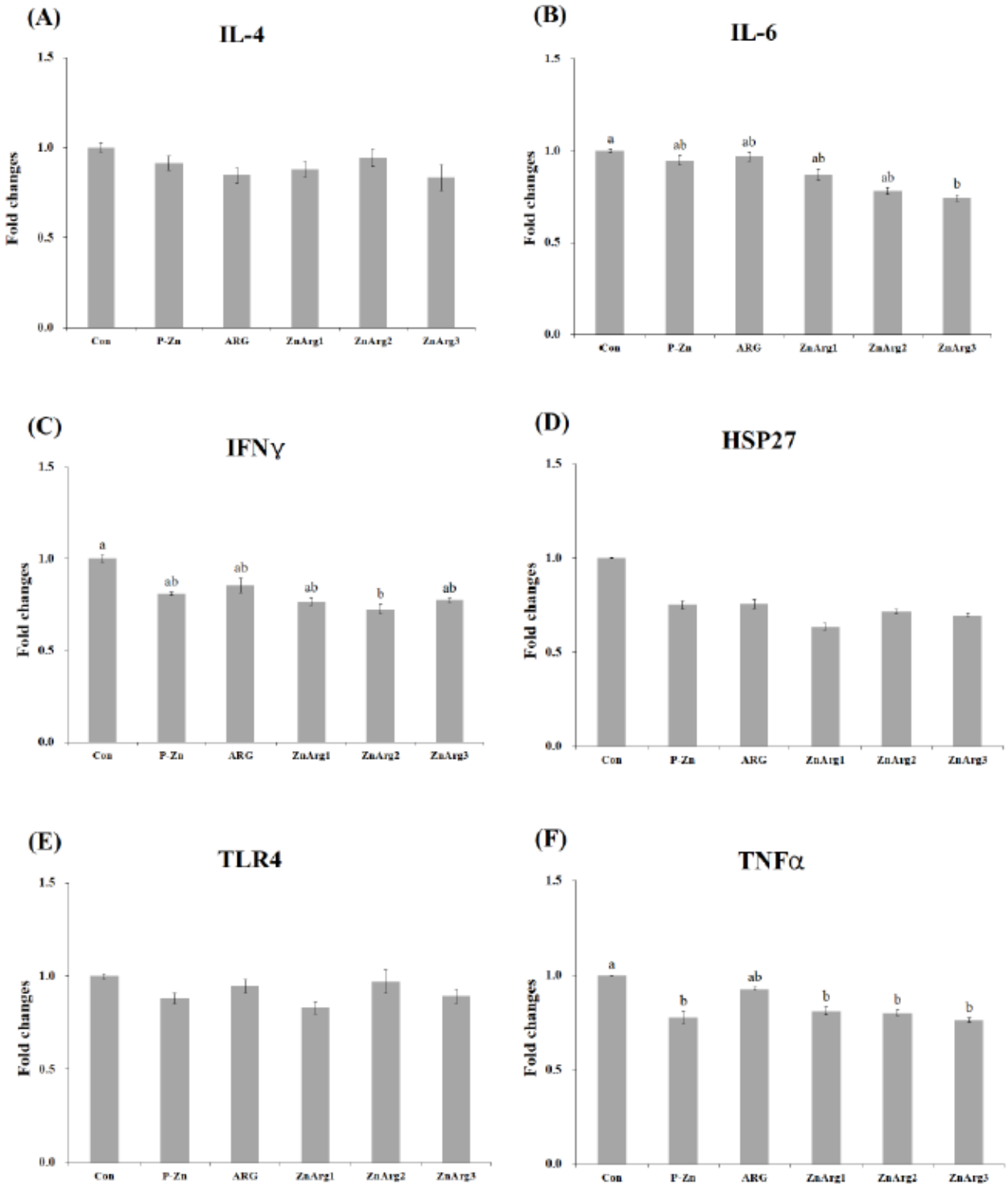

Figure 2

Relative expression of IL-4, IL-6, IFNγ, HSP27, TLR4 and TNFα in the muscle of weanling piglets. Abbreviations: IL, Interleukin; IFN, Interferon; HSP, Heat shock protein; TLR, Toll like receptor; TNF, Tumor necrosis factor. Error bars represent standard error of means. Bars with different letters (a~d) differ significantly across all 4 treatment groups ($P < 0.05$).

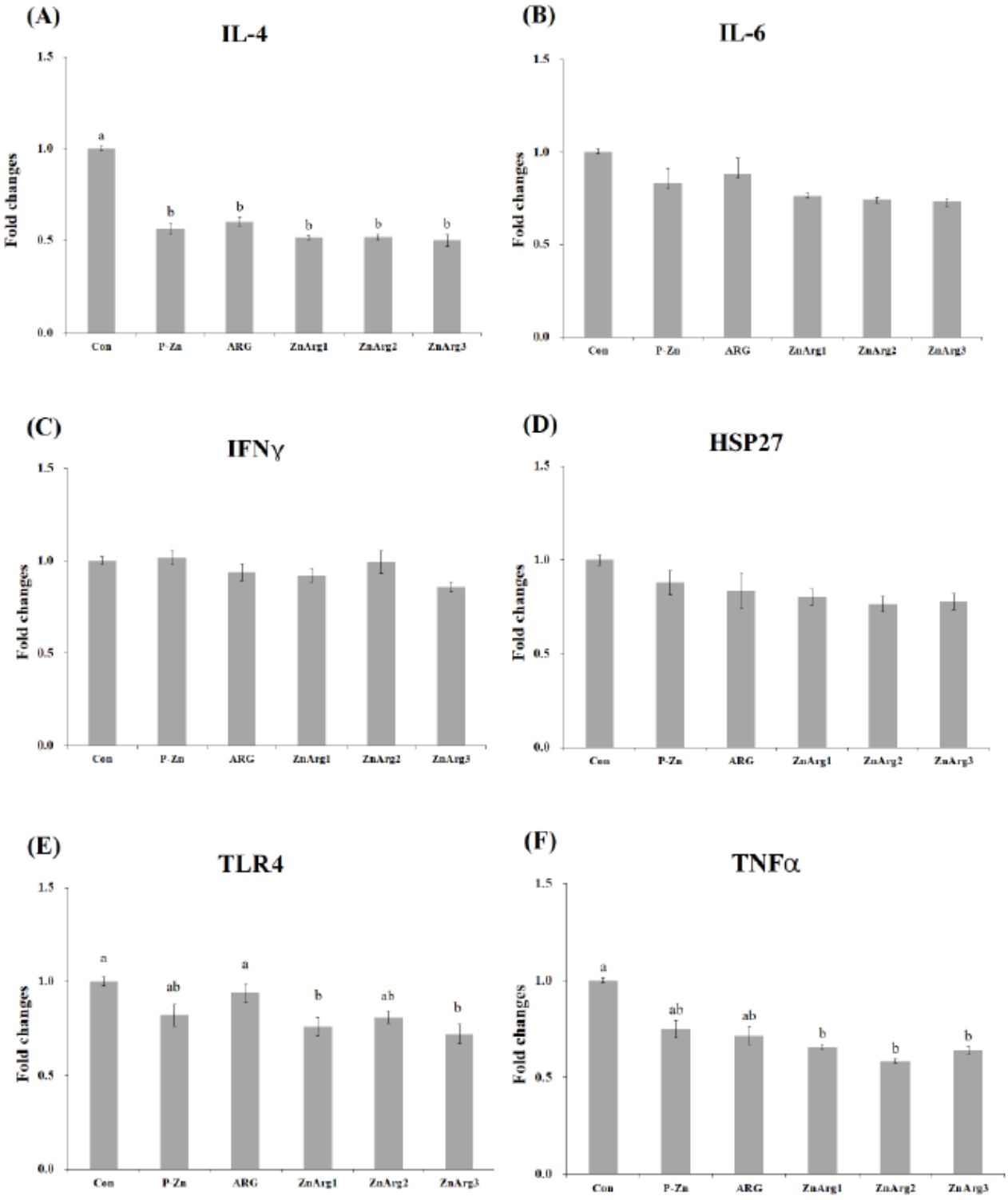

Figure 3

Relative expression of IL-4, IL-6, IFNγ, HSP27, TLR4 and TNFα in jejunum of weanling piglets. Abbreviations: IL, Interleukin; IFN, Interferon; HSP, Heat shock protein; TLR, Toll like receptor; TNF, Tumor necrosis factor. Error bars represent standard error of means. Bars with different letters (a~d) differ significantly across all 4 treatment groups ($P < 0.05$).