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Expression Profiling of Cytokine-related Genes in the Small Intestine of Healthy Pre and Post-weaned Piglets Administered Orally with Probiotic and Zinc

Arup Kalita[1], Manmath Talukdar[2], Pranab Chandra Kalita[1], Nagendra Nath Barman[3], Parimal Roychoudhury[4], Om Prakash Choudhary[1], Probal Jyoti Doley[1]

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ABSTRACT

Background: The present study was designed to evaluate alterations in the immunological status of the small intestine after dietary inclusion of probiotic and zinc in pre and post-weaned piglets.

Methods: For the present study, 18 healthy Large White Yorkshire (LWY) piglets, irrespective of sex, were divided into three different age groups [pre-weaning (20 days old, n = 3), weaning (30 days old, n = 3) and post-weaning (60 days old, n = 3)]. The piglets were weaned at 28 days of age. They were divided into control group (C) fed with basal diet and treatment group (T) fed with combined probiotic and zinc oral supplement along with the basal diet. A probiotic mixture consisted of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium longum* (1.25×10^9cfu/d for 10 days) along with zinc supplement (ZnO @ 2000 ppm/d for 10 days) administered orally to the treatment group of piglets.

Result: The relative expression of TNF-α, IL-1β and IL-6 genes was significantly higher ($P<0.05$) in the treatment group of piglets as compared to the control group. However, the expression of IL-8, IL-12p40 and IL-18 genes were significantly lower ($P<0.05$) after oral administration of probiotic and zinc to the piglets.

Key words: Cytokine gene, Piglet, Probiotic, Small intestine, Zinc.

INTRODUCTION

Cytokines are small peptide molecules mainly derived from lymphocytes and macrophages (Priyanka *et al*., 2019). These cytokines not only play a central role in immune cell response but also participate in the maintenance of tissue integrity. Changes in the cytokine network in the gut of the piglet may be expected at weaning because abrupt changes in dietary and environmental factors lead to important morphological and functional adaptations in the gut.

Probiotics are viable microorganisms that enhance barrier function, modulation of the mucosal immune system, production of antimicrobial agents, enhancement of digestion and absorption of food and alteration of the intestinal microflora (Jean *et al.,* 2003). Zinc is an important trace element that decreased the incidence of nonspecific post-weaning diarrhea in nursery pigs (Poulsen, 1995).

Therefore, the present study was undertaken to evaluate and compare the effect of probiotic and zinc on proinflammatory cytokine gene expression in the intestine between the control and treatment group of piglets throughout the pre and post-weaning period.

MATERIALS AND METHODS

The present study was conducted in the pig farm of the College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl, Mizoram, India, during the year 2017 to 2019. The Institutional Animal Ethics Committee (IAEC) ethically approved the animals used for the experiment vide Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/490 dated 09.08.2017. The experimental animals were consisted of 18 (eighteen) healthy Large White Yorkshire (LWY) piglets, irrespective

[1]Department of Veterinary Anatomy and Histology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl-796 015, Mizoram, India.
[2]Department of Veterinary Anatomy and Histology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.
[3]Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.
[4]Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl-796 015, Mizoram, India.

Corresponding Author: Om Prakash Choudhary, Department of Veterinary Anatomy and Histology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl-796 015, Mizoram, India. Email: dr.om.choudhary@gmail.com

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of sex at different stages of development as age-group of 20, 30 and 60 days. Each of 6 (six) numbers of these piglets was selected from 3 (three) sows. The piglets were divided into control group (C) fed with basal diet and treatment group (T) fed with combined probiotic and zinc oral supplement along with the basal diet. A mixture of probiotic consisted with *Lactobacillus acidophilus* (650 million), *Lactobacillus rhamnosus* (400 million) and *Bifidobacterium longum* (200 million) @ 1.25×10^9 CFU/day (Liu *et al.,* 2014) and ZnO @ 2000 ppm/day (Case and Carlson, 2002) were administered orally to the treatment group of piglets from birth to 10 days of age. The piglets of the control group were given the same volume of sterilized saline solution.

The experimental animals were sacrificed at day 20, 30 and 60 from both the groups by anesthetizing with diazepam @ 2 mg/kg body weight followed by ketamine @ 10 mg/kg body weight intravenously and then exsanguinated the animals. After sacrifice, the parts of the small intestine were dissected out as per the method described by Habel (1964). Tissue samples were taken immediately after sacrifice from the duodenum (5 cm caudal to the pylorus), jejunum (In the middle of the jejunum) and ileum (5 cm cranial to the ileocaecal valve).

Expression analysis of cytokine genes by real-time qPCR

In the present investigation, the cytokine mRNA expression was done as follows:

RNA extraction and cDNA synthesis

At necropsy, the different segments of the small intestine were sliced at 1 cm thickness. The slices were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction and cDNA synthesis was performed. Approximately 100 mg of tissue was homogenized for RNA extraction. The total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) following the manufacturer's recommendation. The integrity of extracted RNA from each sample was analyzed by confirmed by agarose gel electrophoresis. The quantity of total RNA was estimated using a NanoDrop spectrophotometer (ND-2000C, Thermo Fisher Scientific, Wilmington, DE). The purity of RNA was assessed by determining the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). RNA samples with a ratio of 1.8 to 2 were only considered for further experiments. Total RNA from each group was reverse transcribed into cDNA in a 20 µL reaction mixture using Maxima H minus First Stand cDNA synthesis kit (Fermentus) according to the supplier's instruction. cDNA was synthesized using 250 ng of template RNA, uniformly for all samples and oligo $(dT)_{12\text{-}18}$ primers.

Quantification of cytokine gene expression by real-time qPCR

The expression level of transcripts genes TNF-α, IL-1β, IL-6, IL-8, IL-12 p40 and IL-18 and beta-actin (housekeeping gene) were measured by Lightcycler 480 real-time PCR instrument with software version 1.5. Each sample was tested in triplicate in Lightcycler®Multiwell plate 96 (Roche Diagnostics, Mannheim, Germany). The oligonucleotides for qPCR (Table 1) were designed based on prior sequence information from other animals and BLAST analysis against the *sus scrofa* genome. The reaction mixture (10 µL total volume) comprised of 2 µL cDNA, 5 µL 2× SYBR green master mix (Thermo Scientific, Lithuania, EU), 0.5 µM primer pairs and 2 µL PCR-grade water. A no-template control reaction (NTC) was included in each assay. The thermocycling conditions employed for all the genes were: preincubation at 95°C for 10 min followed by 40 cycles of 30 sec at 95°C, 30 sec at 59°C and 30 sec at 72°C. After the amplification, a melting peak analysis with a temperature gradient of 0.1°C sec^{-1} from 65°C to 97°C was performed to confirm the PCR amplification specificity, contamination and absence of primer-dimers. Relative quantification of a target gene was done by comparing the expression level of reference gene beta-actin as per the method described by Livak and Schmittgen (2001).

Table 1: List of genes and sequences of the primers used for real-time PCR.

Gene product[a]	Primer		Product size
	Direction[b]	Sequence (5′→3′)	
TNF-α	F	GGCCCAAGGACTCAGATCAT	105
	R	GCATACCCACTCTGCCATTG	
IL-1β	F	TGAATTCGAGTCTGCCCTGT	194
	R	AGTCCCCTTCTGTCAGCTTC	
IL-6	F	ACCGGTCTTGTGGAGTTTCA	88
	R	TAATCTGCACAGCCTCGACA	
IL-8	F	GTGATTGAGAGTGGACCCCA	103
	R	CCTTCTGCACCCACTTTTCC	
IL-12 p40	F	GCCAAGGTTACATGCCACAA	89
	R	ACAGATGCCCATTCACTCCA	
IL-18	F	CTGCTGAACCGGAAGACAAT	193
	R	TCAAACACGGCTTGATGTCC	
BACT	F	CCCTGGAGAAGAGCTACGAG	156
	R	CGTCGCACTTCATGATGGAG	

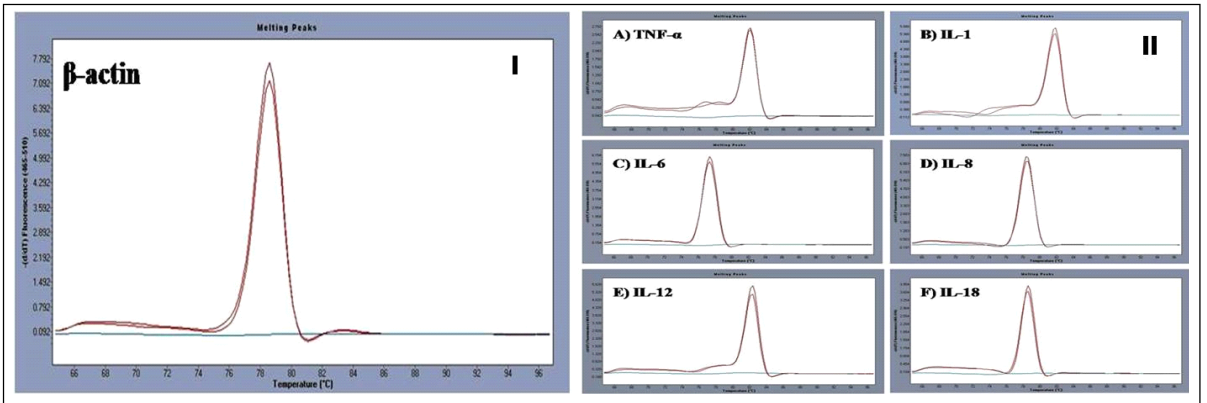
The data obtained were analyzed using the statistical package SPSS version 20.

RESULTS AND DISCUSSION

The relative expression of six cytokine genes (TNF-α, IL-1β, IL-6, IL-8, IL-12p40 and IL-18) in the small intestinal tissue of control and treated piglets were quantified and compared by real-time qPCR at different age-groups. The cycle threshold (Ct) or crossing point (Cp) values, the level of the fluorescence signal that reflects a statistically significant increase over the calculated baseline signal, for all the amplification curves during the real-time PCR reaction was recorded and used for the calculation of mean fold change. Relative quantities of cytokine mRNA were normalized to beta-actin and all reactions were made in triplicates using samples derived from three biological repeats. Melting curve analysis did not yield any non-specific peak from each primer set tested (Fig 1). Additionally, every PCR product generated a prominent band with an expected size in the gel electrophoresis analysis (Fig 2). These indicated that non-specific amplification with the primer sets tested was not detected in the real-time PCR analysis. Results are reported in terms of fold increase in cytokine transcripts at different age-groups of control and treatment group piglets.

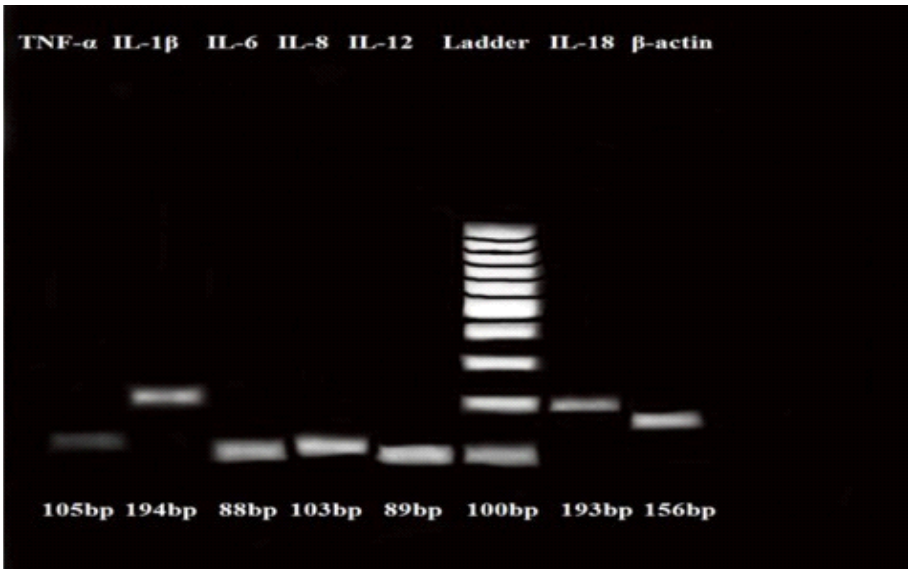
TNF-α expression

The result of the present investigation showed significantly higher ($P<0.05$) TNF-α gene expression in all the segments of the small intestine of all age groups of treated piglets (1.5 to 3-fold) than the control group of animals (Fig 3A).



A) TNF-α, B) IL-1β, C) IL-6, D) IL-8, E) IL-12p40 and F) IL-18. After amplification, a melting peak analysis with a temperature gradient was performed to confirm the PCR amplification specificity.

Fig 1: Melting peak analysis I: β-actin gene, II: cytokine gene.



Real-time PCR amplified products of cytokine genes (TNF-α, IL-1β, IL-6, IL-8, IL-12p40 and IL-18) on agarose gel (2%).

Fig 2: Agarose gel electrophoretic profile of different cytokine gene products.

Further, in the duodenum of treated piglets at days 30 and 60, TNF-α gene expression was found to be highly significantly (P<0.01) increased. Azad *et al.* (2018) reported TNF-α secretion with the presence of probiotics like *Bifidobacteria* and *Lactobacilli* as recorded in the present study. The TNF-α, IL-1 and IL-6 cytokines were produced in response to pathogens that could affect B and T cells to induce an adaptive immune response and the gut epithelium formed a link between the innate and adaptive immune systems (Belardelli and Ferrantini, 2002). The result of the present study demonstrated that oral administration of probiotic and zinc increased TNF-α expression, which indicated that probiotic and zinc treatment could improve the mucosal immune activity in piglets.

IL-1β expression

In the present investigation, significantly (P<0.05) higher IL-1β transcripts was observed after oral administration of probiotic and zinc in all age groups of piglets in compared to the control animals (Fig 3B). There was 2 to 3-fold up-regulation of IL-1β gene expression in all treatment groups of piglets. The current observations were in consonance to the findings of Haller *et al.* (2000) and Wang *et al.* (2019). According to them, IL-1β was one of the earliest pro-inflammatory cytokines mainly involved in the initiation and regulation of inflammatory and innate immune responses. Besides, Panja *et al.* (1998) documented that IL-1β was constitutively expressed in freshly isolated intestinal epithelial cells from healthy tissues and played a potential role in epithelial cell turn-over in humans. According to Dinarello (2018), this innate immunity of host defense was manifested by inflammation, but when it became uncontrolled, it might be detrimental to the host. In the present study, the relative up-regulation of IL-1β in the treatment group implies that the innate immune mechanism was up-regulated by the Pathogen-Associated Molecular Patterns (PAMPs), as probiotics being prokaryotic cells and a higher rate of epithelial cell turn-over, especially in higher age-groups.

IL-6 expression

The expression of IL-6 transcripts was found to be increased significantly (P<0.05) in all age-group of piglets fed with probiotic and zinc than the control group (Fig 4A)

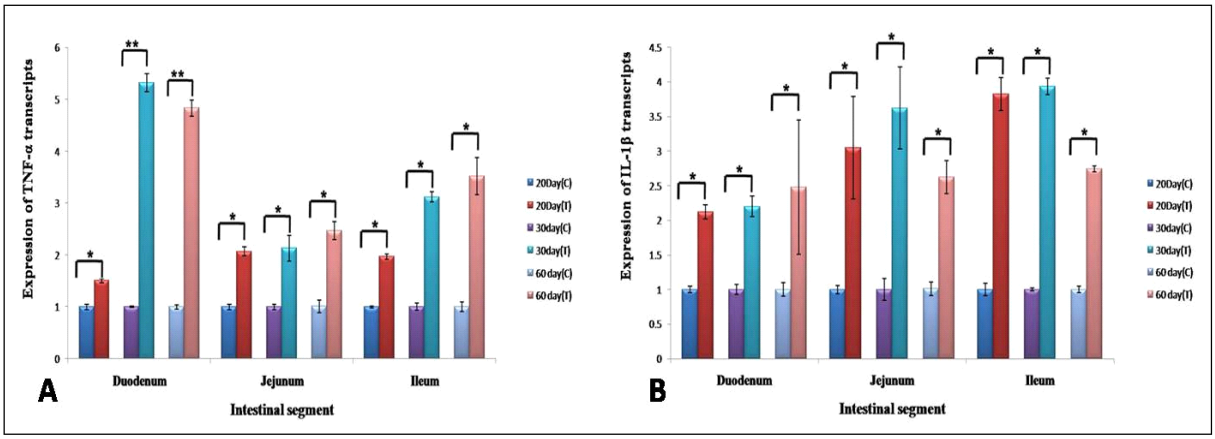


Fig 3: Comparative A) TNF-α and B) IL-1β expressions in the small intestinal segment of control and treatment group piglets (*P<0.05, **P<0.01).

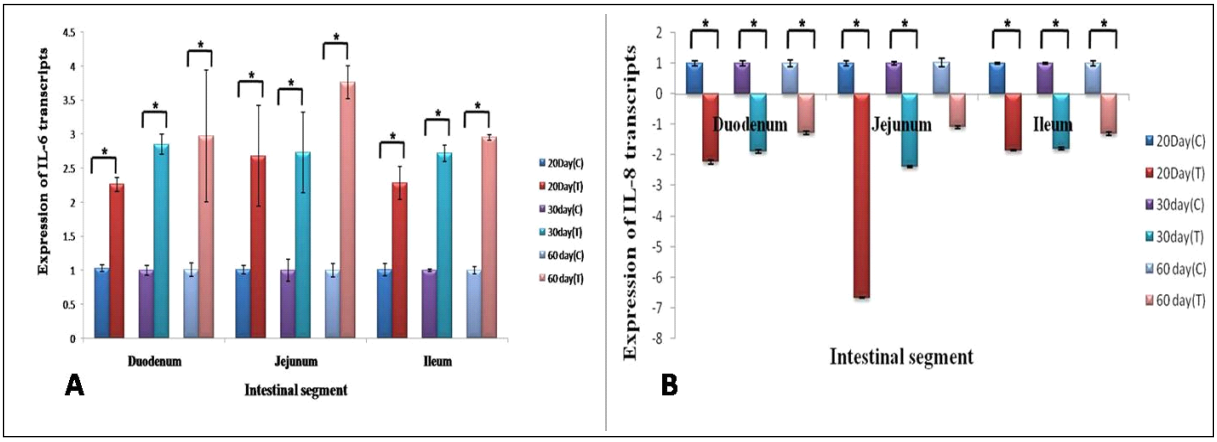


Fig 4: Comparative A) IL-6 and B) IL-8 expressions in the small intestinal segment of control and treatment group piglets (*P<0.05).

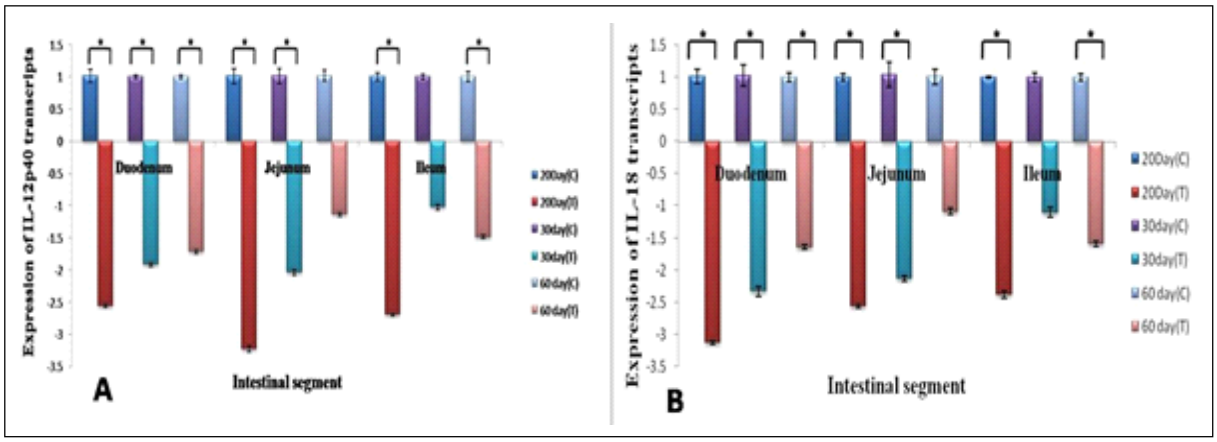


Fig 5: Comparative A) IL-12p40 and B) IL-18 expressions in the small intestinal segment of control and treatment group piglets (*P<0.05).

under study. There was 2-fold up-regulation of this gene in all treated animals except in jejunum at day 60 with 3-fold up-regulation. The higher expression of the IL-6 gene after dietary inclusion of probiotics was also reported earlier in chicken, mice and pigs (Lee *et al.,* 2010; Lemme-Dumit *et al.,* 2018; Laskowska *et al.,* 2019), respectively. The result of the present study could be correlated to a previous report about other probiotic strains, *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506, which induced IL-6 gene expression in porcine intestinal epithelial cells (Villena *et al.,* 2014). Lemme-Dumit *et al.* (2018) documented that an increased level of IL-6 could be related to the enhancement of IgA producing B-cell population and the IL-10 production. In the same piglets, a separate immunofluorescence study was done and the results showed an increased number of IgA B-cell populations in the treatment group of piglets (Kalita *et al*., 2020). In the current study, the increased expression of IL-6 transcripts recorded in the treated piglets might beneficially modulate host natural immune responses.

IL-8 expression

The expression of the IL-8 gene in the current study was significantly (P<0.05) down-regulated (1.08 to 6.6 folds) in the treatment group of piglets at different ages than the control animals except in the jejunum of day 60 treatment group (Fig 4B). The results of the present investigation were similar to the earlier study by Llewellyn and Foey (2017), who described the inhibitory effect of *Lactobacillus* and *Bifidobacterium longum* on IL-8 expression in porcine intestinal epithelial cells. Bai *et al.* (2004) documented that TNF-α could induce epithelial cells to secrete IL-8, which had leukocytes chemotactic and stimulatory properties. However, *Bifidobacterium longum* and *Lactobacillus bulgaricus*, inhibited the secretion of IL-8 in HT29 cells when stimulated with TNF-α one hour after co-culture with the two probiotic strains. It indicated that these strains could trigger anti-inflammatory pathways within the gut epithelium. The significant down-regulation of IL-8 gene expression recorded in the treatment group piglets of the present study might be correlated with decreased pathological inflammatory reactions in the gut.

IL-12p40 expression

In the current study, the expression of IL-12p40 transcripts was found to be down-regulated significantly (P<0.05; 1.03 to 3.2 folds) in all age-group of piglets fed with probiotic and zinc than the control animals (Fig 5A). However, at day 60 of jejunum and day 30 of ileum of treated piglets, there was no significant difference in the expression of this gene compared to control animals. The down-regulation of the IL-12p40 gene after probiotic treatment was also reported by Ng *et al.* (2010) and Bermudez-Brito *et al.* (2015) in humans. The role of IL-12p40 in antibacterial, antiviral and antitumor activities was also reported by Kinjo *et al.* (2002); Denton *et al.* (2007) and Coughlin *et al.* (1998) in mice, respectively. DCs could directly trigger NK-cell activation to kill viruses, intracellular bacteria and tumors. Kolb-Maurer *et al.* (2003) in humans documented that unstimulated dendritic cells (DCs) generally produced low levels of IL-12 and IL-18 and were characterized by their ability to efficiently take up antigen while their T cell stimulatory function was poor. Down-regulation of IL-12p40 transcripts in the treated piglets of the present study could be correlated with decreased activity of NK cells and $CD8^+$ cytotoxic T lymphocytes, which might be indicative of a comparatively healthy gut in this group of piglets.

IL-18 expression

The IL-18 gene expression was significantly (P<0.05; 1.08 to 3.1 folds) down-regulated in all the piglets of the treatment group of piglets compared to the control group of animals except at day 60 of jejunum and day 30 of the ileum (Fig 5B). Cross *et al.* (2004) and Kling *et al.* (2018) reported suppression of IL-18 expression after the inclusion of probiotic bacteria, which agreed to the present finding. According to Hardy *et al.* (2013), Natural killer cells (NKs) played a vital role in antiviral response and these cells were activated by DCs, which secrete soluble factors, such as IL-12 and IL-18. A decreased level of IL-12 and IL-18 in

the present study indicated healthy intestinal epithelial cells in the gut and provided better immunity to the treatment group of piglets over the control animals.

In the present study, dietary inclusion of probiotic and zinc changed the overall cytokine profile in piglets. Overall, increased expression of TNF-α, IL-1β and IL-6 transcripts in the small intestine of piglets might have resulted in the stimulation of innate immune cells to eradicate microbes, enhancement of IgA^+ cell population and increased epithelial cell turn-over. On the other hand, the down-regulation of IL-8, IL-12 and IL-18 gene expression possibly led to decreased activity of NK cells and $CD8^+$ cytotoxic T lymphocytes, which were an indication of minor infections associated with intracellular pathogens and the presence of healthy intestinal epithelial cells in the gut.

CONCLUSION

From the observations of the present study, it could be concluded that dietary inclusion of probiotic and zinc might be led to immunomodulation in the small intestine of piglets.

Conflict of interest

All authors declare that they have no conflict of interest.

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