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Effect of Dietary Benzoic Acid on Beneficial Microflora and Immune Response in the Intestine of Weaning Pigs

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We evaluated the effect of dietary supplements with benzoic acid on intestinal beneficial bacteria concentration and immune response of weaning pigs. Supplementation with benzoic acid at 0.5% or control diet for 35 days resulted in a higher *Lactobacillus casei* concentration in the cecum. Supplementation with benzoic acid at 0.5% increased concentration of *L. plantarum* in the cecum. Pigs with the control diet and 0.5% benzoic acid had significantly increased concentration of *B. subtillis* in the cecum compared to the antibiotic group, while the concentration of *B. subtillis* in the rectum increased in pigs given 0.3 and 0.5% benzoic acid ($p<0.05$). Compared with the control group, the level of interleukin-1β mRNA showed a significant decrease in the proximal small intestine in pigs fed diets supplemented with benzoic acid at 0.5% or antibiotic. Feeding 0.5% benzoic acid resulted in a marked reduction in the expression of IL-6 mRNA in the middle small intestine ($p<0.05$). Supplementation with benzoic acid at 0.5% or antibiotic resulted in a lower level of tumor necrosis factor-mRNA in the middle intestine. Up to 0.5% benzoic acid may be included in weaning diets for improvement of intestinal beneficial bacteria, thus modulating genes of pro-inflammatory cytokines in the gastrointestinal tract.

Key words : Benzoic acid, weaning pig, health beneficial bacteria, proinflammatory cytokine mRNA, immune response

Introduction

The gastrointestinal tract (GIT) of adult animals is colonized by various diverse microorganisms. Control of immune reaction in the GIT depends on intestinal microorganisms, which curbs proliferation of pathogenic bacteria through bacterial antagonism and makes intestinal epithelial cells to generate various materials in order to control immunocompetence [14]. Young animals take time to develop both intestinal microorganisms and their immune system in the GIT. Furthermore, they experience dramatic changes in the composition of colony of intestinal microorganisms during 7-14 days after weaning [6]. Pie et al. [15] conducted a study on activation of GIT immune system during weaning and suggested that weaning might be related with early response in gene expression of inflammatory cytokines in the GIT.

Several studies have been reported that use of dietary supplements might help inhabitation of microorganisms in the GIT, thus influencing development of intestinal immune system [7,10,19]. Konstantinov et al. [10] reported that use of fermentable carbohydrates in weaning pig diets stimulated lactobacilli with a concomitant suppression of Clostridium-like species. Furthermore, Isolaruri et al. [7] reported that use of probiotics might stimulate immune system by control of cytokine production, thus alleviating intestinal inflammatory responses.

Dietary supplementation with organic acid has been reported to reduce post weaning diarrhea and improve growth performance in pigs [8,9]. Generally, organic acid reduces gastric pH and helps process of digestion of protein, thus inhibiting proliferation of intestinal pathogenic bacteria [17]. Benzoic acid, a weak organic acid, is used as food and feed preservative. It was reported that number of intestinal Gram negative bacteria reduced when benzoic acid was supplemented as an alternative to antiobiotic growth promoters for weaning pigs [5,21]. However, little research has been done about effect of benzoic acid on inhabitation of beneficial bacteria through inhibiting proliferation of pathogenic bacteria in the GIT by using benzonic acid in feed. Therefore, this study was to investigate the effect of benzoic acid on pop-

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ulations of specific beneficial bacteria, thus, improve the immune response through modulation of gene expression of proinflammatory cytokines in the GIT.

Materials and Methods

Animals and experimental design

A total of 32 crossbred (Landrace x Yorkshire x Duroc) pigs were used. The pigs were weaned at 24 days of age with an average initial body weight of 6.62 kg (SD±0.47). Using a randomized complete block design that was replicated three times, the animals were allocated for 35 days into four experimental treatment groups based on their initial body weight and gender.

The experimental treatments were as follows: Control (basal diet); PC (basal diet + Avilmycine 0.12%); V3 (basal diet + benzoic acid 0.3%); and V5 (basal diet + benzoic acid 0.5%) (Tables 1). Benzoic acid (VevoVitall®, DSM Nutritional Products) was added at a rate 30 and 50 g per kg diet at the expense of corn. The basal diet was formulated with corn and soybean meal, and it was provided in a two-phase feeding program (Table 2). All diets were met or exceeded NRC (1998) [7] recommendations. The pigs were placed in an experimentally controlled room with a slotted floor and received the diets *ad libitum* for 35 days. On reaching an average body weight of 18±2.1 kg the pigs were transferred to metabolic cages. They were fasted for at least 15 hours and then given exactly 400 g of the diet 3 hours before being killed. This amount of food was consumed by all pigs within 1 hour of being feeding.

pH Values of stomach and urine

A total of 32 weaning pigs were sacrificed at day 35 postweaning. The gastrointestinal tract and the urinary bladder were removed. The latter was cut open, the urine was collected into a glass beaker, and the pH was immediately measured with a pH meter (Φ 5 Series Benchtop Meter, Beckman Coulter, USA). The gastrointestinal tract was divided into the stomach and intestine, and the total contents of stomach were collected and homogenized. An aliquot of digesta sample was immediately centrifuged at 3,400 g for 10 minutes and the pH of the supernatant was determined using the pH meter described above.

Table 1. Composition of antibiotic and benzoic acid for composite

	Treatment			
	Control	PC[1)]	V3[2)]	V5[3)]
Antibiotic (%)	-	0.1		
Benzoic acid (%)	-	-	0.3	0.5

[1)]PC: control diet+Avilamycine
[2)]V3: control diet+Benzoic acid 0.3%
[3)]V5: control diet+Benzoic acid 0.5%

Table 2. Diet compositions in control group (g/1,000 g diet)

Ingredients	Phase I	Phase II
Corn	334.0	466.3
Soybenan meal (44% crude protein)	230.2	236.7
Pepsoygen	154.1	111.4
Whey powder	50.5	51.1
Dried skim milk	30.9	90.0
Lactose	166.0	16.9
Soy oil	6.5	11.3
Mono calcium phosphate	11.2	10.5
Limestone	10.5	0.6
DL-Methionie	0.9	1.2
Vitamin premix[1)]	1.2	1.0
Mineral premix[2)]	1.0	2.0
Salt	2.0	1.0
Choline-Cl	1.0	-
Antibiotics	-	-
Benzoic acid	-	-

[1)]Supplied per kilogram of complete diet: Vitamin A, 16,000 IU; Vitamin D_3, 3,200 IU; Vitamin E, 35 IU; Vitamin K_3, 5 mg; Riboflavin, 6 mg; Calcium pantothenic acid, 16 mg; Niacin, 32 mg; d-Biotin, 128 μg; and Vitamin B_{12}, 20 μg.
[2)]Supplied per kilogram of diet: 281 mg Fe, 288 mg Cu, 143 mg Zn, 49 mg Mn, 0.3 mg I, and 0.3 mg Se.

Microbiological techniques

To allow analysis of microfloral populations, digesta samples from the cecum, colon and rectum were collected immediately frozen in liquid nitrogen, and stored at -80°C until analysis. *Lactobacillus casei* AB 102854 and *Lactobacillus plantarum* AB 102857 were grown in MRS broth (Becton Dickinson, Sparks, MD) at 37°C whilst *Bacillus subtillus* DQ 112340 was cultured in LB broth (Difco, USA) at 37°C. The chromosomal DNA of the pure-cultured cells and intestinal samples was extracted by the G-spin™ Genomic DNA Extraction Kit (Intron, Korea) and the Ultra Clean™ Fecal DNA Kit (MoBio™, USA), respectively. Species-specific primers for each bacterium were designed to anneal to the 16s rRNA (Table 3). The PCR conditions were as follows: pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. The cycles were repeated 30 times and a final extension was given at 72°C for 7 minutes. The species-specific-

Table 3. Species-specific primers for real time PCR

Bacterial species	Items	Oligonucleotide sequences
Lactobacillus casei	Forward	CCGTCACACCATGAGAGTTT
	Reverse	CCTTGTTACGACTTCACCCT
Lactobacillus plantarum	Forward	CCCGTCACACCATGAGAGAGTT
	Reverse	GGCTACCTTGTTACGACTTC
Bacillus subtillis	Forward	GTGCAGAAGAGGAGAGTG
	Reverse	TCAGCGTCAGTTACAGAC

ity of the primers was tested by nPCR. For this 2 μl of template DNA, 1 μl of each primer (5 pmole/μl), and 16 μl of i-MasterMix™ (Intron, Korea) were mixed and nPCR was performed using the T-gradient™ (Biometra, Germany) method. p-Gem Easy Vector™ (Promega, USA) was used for the transformation of Novablue Competent Cell® (Novagen, USA) and the required DNA fragments for PCR detection according to the manufacturer's manual. Transformed colonies were grown on LB broth at 37°C in aerobic conditions and plasmid DNA was extracted into single tubes using DNA-Spin™ plasmid DNA purification kit (Intron, Korea). Extracted plasmid DNA was used as a standard for quantitative real-time PCR after serially diluted 10-fold serial dilutions. Genomic DNA from intestinal digesta samples was used as templates for PCR amplification. The amplification was performed in a 20 μl final volume containing 10 μl iQ™ SYBR® Green PCR Supermix (Bio-Rad, USA), 1 μl of each primer (10 pM), 6 μl of sterile water and 2 μl DNA template. PCR reactions consisted of initial denaturing cycles at 95°C for 5 minute, followed by 40 cycles of annealing and further extension steps. Fluorescence was quantified during the annealing step and product formation was confirmed using the melting curve analysis approach (55-95°C).For both primer sets, a standard curve was generated with serially diluted plasmid DNA and serial concentrations were plotted against the C_t value. The C_t value represents the threshold cycle or the PCR cycle at which an increase in fluorescence from SYBR Green, generated via the binding of SYBR Green to double-stranded DNA, can first be deleted above a baseline value (derived from samples without the DNA template). The iCycler® iQ real-time PCR Detection System (Bio-Rad, USA) then generated a standard curve vs. log DNA concentration for all standards and determined the DNA concentration of unknowns samples by interpolation.

Reverse-transcription PCR detection of cytokine mRNA and quantification of PCR products

The intestinal tract was removed after death by exsanguinations. The mesentery was sampled using scissors and the small intestine was placed on a table and divided into three parts of equal length. Tissue segments of 20 cm length were collected from the middle of each part and flushed with cold saline to remove luminal contents. Subsamples (1 cm) of the segments were collected frozen in liquid nitrogen, and stored at -80°C for cytokine mRNA analysis.

Samples from each intestinal segment were placed in 1 mL of Extract-all (Eurobio, Korea), frozen in liquid nitrogen, and stored at -80°C before mRNA analysis. The samples were homogenized using a Qiashredder spin column (Qiagen, CA). Total RNA was extracted using RNasy mini kit columns (Qiagen, CA) following the manufacturer's recommendations. The RNA was eluted in a final volume of 40 ul and total RNA was quantified using a spectrophotometer at a wave length of 260 nm. The purity was assessed by determining the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). All samples had a ratio in the range 1.7 to 1.9.

For reverse transcription, 1 ug of total RNA was used in final volume of 40 ul, containing 0.5 ug oligo dT, RT buffer (1x), 10mM dithiothretol, 10mM dNTP, and 10 unit Murine Leukemia Virus (M-MLV) reverse transcriptase (Gibco BRL, Grand isaland, NY). Reverse transcription was carried out at 37°C for 50 min, and 70°C for 15 min, and stored at 4°C to generate cDNA from total RNA, using T-Gradient Therrmoblock (Biometra, Gottingen, Germany). Specific primers for β-actin, IL-1β, IL-6, and TNF-α were designed and generated for real time PCR (Table 4). β-actin was also subjected with each cytokine primer as housekeeping gene to normalize results.

Quantitative analysis of IL-1β, IL-6, TNF-α, and β-actin was performed using real time PCR. Real time PCR was performed with iQ™ SYBER® Green Supermix (Bio-Rad Laboratories, Inc., CA) using the MyiQ™ Single Color real-time PCR Detection System (Bio-Rad Laboratories, Inc., CA) according to the manufacturer's instructions. The PCR thermal cycling was started with 1 cycle at 94°C for 5 minute;

Table 4. Sequence of PCR of specific cytokines for quantitative real time PCR

Cytokines	Items	Oligonucleotide sequences
β-actin	Forward	CCATCTACGAGGGGTACG
	Reverse	ACAGCTTCTCCTTGATGTCC
IL-1β	Forward	GCACCCAAAACCTTGACCTC
	Reverse	TTGCCACAATCACAGACACC
IL-6	Forward	CCGGACAAAACTGAAGAACT
	Reverse	GATTGAACCCAGATTGGAAG
TNF-α	Forward	CACCACGCTCTTCTGCCTACTG
	Reverse	CGACGGGCTTATCTGAGGTTTGAG

Table 5. Effect of benzoic acid supplementation on the pH of stomach digesta and urine

Samples	Treatment			
	Control	PC	V3	V5
Stomach	2.90±0.25[1)]	2.37±0.29	2.67±0.27	2.12±0.212
Urine	7.32±0.21[a]	7.85±0.11[a]	6.85±0.28[b]	7.04±0.32[b]

Control, PC, V3, V5 were referred to the Table 1.
[1)]Mean±SD.
[a-b]Different superscript letter are significantly different at $p<0.05$.

Table 6. Effect of benzoic acid supplementation on the Ct value of *Lactobacillus casei* in the intestinal digesta

Samples	Treatment			
	Control	PC	V3	V5
Cecum	19.50[b]±0.48[1)]	20.11[ab]±0.73	21.58[a]±0.75	19.14[b]±0.59
Colon	17.89±0.23	17.76±0.47	18.49±0.68	17.74±0.53
Rectum	19.35±0.57	18.15±0.44	18.12±0.19	18.52±0.37

Control, PC, V3, V5 were referred to the Table 1.
[1)]Mean±SD
[a-b]Different superscript letter are significantly different at $p<0.05$.

40 cycles for at 94°C for 45 seconds, at 61°C for 30 seconds, and at 72°C for 20 seconds; 1 cycle at 95°C for 1 minute; 1 cycle at 55°C for 1 minute; 81 cycles at 55°C for 10 seconds.

The purity of PCR product was verified by generating melting curve at the end of the thermal cycling. Relative level of mRNA was analyzed by the comparative C_t method. All C_t values of each sample were normalized to that of β-actin, being used for statistical analysis. The relative amounts of target genes were calculated after normalization to a reference (β-actin) with the arithmetic formula of Kenneth and Thomas.

Statistical analyses of data on pH, microbial populations, and proinflammatory cytokine mRNA expression levels in intestine were performed with ANOVAs using the General Linear Model procedure of the SAS (SAS Inst., Inc., Cary, NC) software. Comparison between treatments was subsequently conducted using the least-squares or Duncan methods. The variance is expressed as the standard error of the mean (SEM) and a *P* value of less than 0.05 was considered to be statistically significant.

Results

pH of stomach digest and urine

The effect of intake of benzoic acid on the pH of the stomach digesta and urine is shown in Table 3. The pH of the stomach digesta was not significantly influenced by the dietary treatments. The pH of the urine was lower in pigs fed benzoic acid supplement at 0.3 and 0.5% than those given the control and antibiotic diet ($p<0.05$).

Effect of benzoic acid intake on intestinal microflora

The effect of intake of benzoic acid on intestinal microflora is presented in Tables 4, 5, and 6. Pigs administrating control diet and 0.5% benzoic acid of diet at 35 days had significantly the lowest Ct values of *L. casei* in the cecum

Table 7. Effect of benzoic acid supplementation on the Ct value of *Lactobacillus plantarum* in the intestinal digesta

Samples	Treatment			
	Control	PC	V3	V5
Cecum	19.62^{a}±0.79$^{1)}$	17.05^{bc}±0.35	18.39^{ab}±0.61	16.47^{c}±0.43
Colon	17.53±0.22	17.06±0.95	18.06±0.80	16.50±0.35
Rectum	22.37^{a}±0.57	20.23^{b}±0.27	19.66^{b}±0.31	19.50^{b}±0.28

Control, PC, V3, V5 were referred to the Table 1.
$^{1)}$Mean±SD
$^{a-d}$Different superscript letter are significantly different at $p<0.05$.

Table 8. Effect of benzoic acid supplementation on the Ct value of *Bacillus subtillis* in the intestinal digesta

Samples	Treatment			
	Control	PC	V3	V5
Cecum	23.34^{b}±0.52$^{1)}$	25.28^{a}±1.23	24.22^{ab}±0.43	22.32^{b}±0.59
Colon	21.01±0.22	19.95±1.08	21.44±0.76	21.31±0.47
Rectum	17.97^{a}±0.28	17.61^{ab}±0.49	16.66^{b}±0.36	16.77^{b}±0.39

Control, PC, V3, V5 were referred to the Table 1.
$^{1)}$Mean±SD
$^{a-b}$Different superscript letter are significantly different at $p<0.05$.

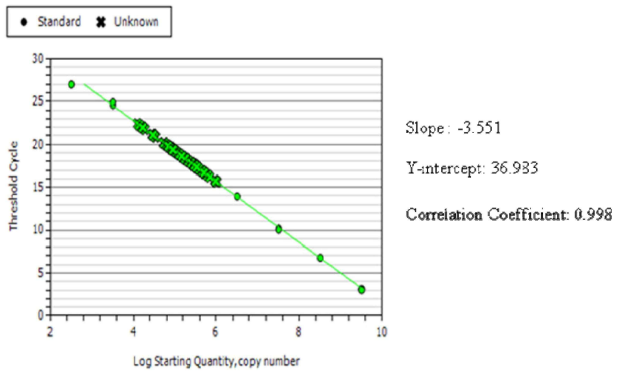

Fig. 1. Standard calibration and results of real-time PCR for microbial population of *Lactobacillus casei*. The regression equation obtained from calibration and correlation coefficients (R^2) was expressed.

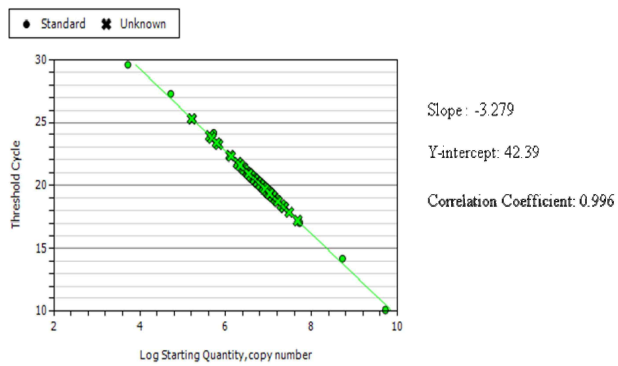

Fig. 2. Standard calibration and results of real-time PCR for microbial population of *Lactobacillus plantarum*. The regression equation obtained from calibration and correlation coefficient (R^2) was expressed.

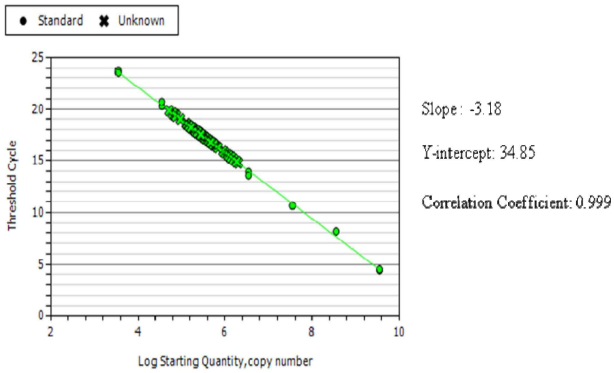

Fig. 3. Standard calibration and results of real-time PCR for microbial population of *Bacillus subtilis*. The regression equation obtained from calibration and correlation coefficient (R^2) was expressed.

among treatments ($p<0.05$). In Fig. 1, a strong linear relation ($R^2 \geq 0.99$) between the Ct values and the $\log_{10}$ of the input number of copies are presented. Pigs administrating 0.5% benzoic acid of diet had a significantly the lowest Ct values of *L. plantarum* in the cecum among treatments ($p<0.05$). The Ct values of *L. plantarum* in the rectum were significantly lower in pigs fed antibiotic and benzoic acid than those given the control diet ($p<0.05$). Pigs administrating control diet and 0.5% benzoic acid of diet had significantly lower Ct values of *B. subtillis* in cecum ($p<0.05$). The Ct values of *B. subtillis* in rectum were significantly lowest in pigs fed 0.3 and 0.5% benzoic acid ($p<0.05$). In Fig. 2 and 3, the square regression coefficients (R^2) of *L. plantarum* and *B. subtillis* assay

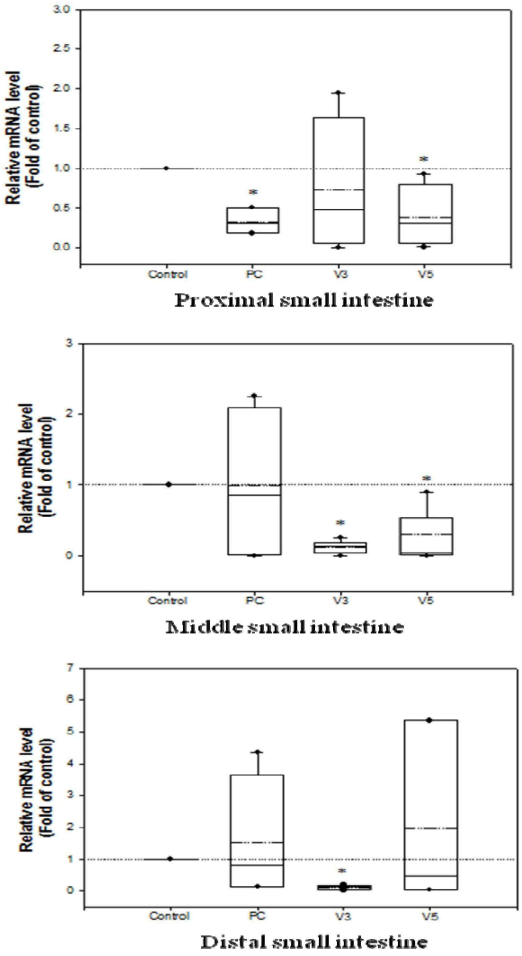

Fig. 4. Effect of benzoic acid supplementation on the relative abundance of IL-1β mRNA in the intestine of pigs. Data are presented by box and whisker plots; the box indicates the range between the 25th and 75th percentiles, and the straight and dotted lines in the box show the median and mean values, respectively. The whiskers represent the distribution of values, and the black dots show the minimal and maximal numbers. * $p<0.05$ compared with control.

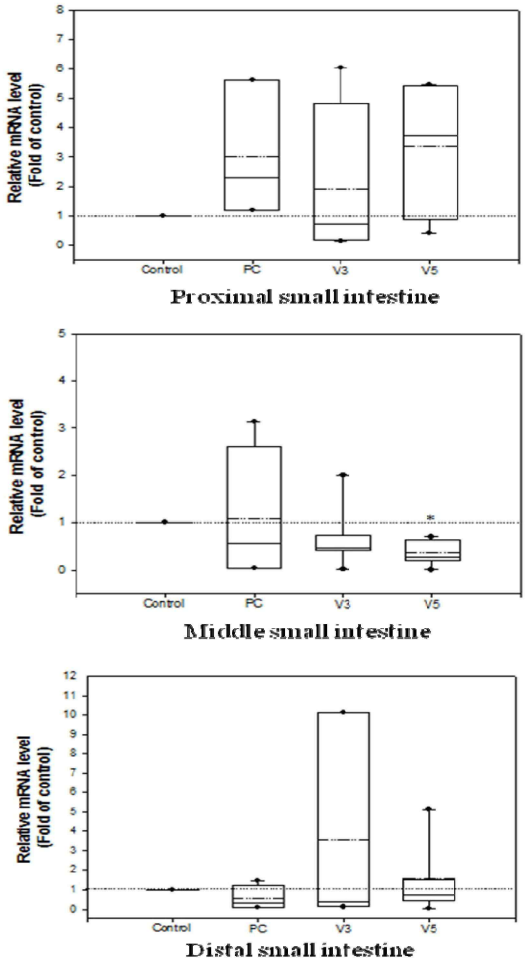

Fig. 5. Effect of benzoic acid supplementation on the relative abundance of IL-6 mRNA in the intestine of pigs. Data are presented by box and whisker plots; the box indicates the range between the 25th and 75th percentiles, and the straight and dotted lines in the box show the median and mean values, respectively. The whiskers represent the distribution of values, and the black dots show the minimal and maximal numbers. * $p<0.05$ compared with control.

were 0.997 and 0.998, respectively. In this study, examination of specific beneficial bacteria was designed using absolute quantification of 16S rRNA and real-time PCR. Quantification can be performed relative to an external standard curve, using serial dilutions of an external standard for generation of a standard curve of Ct (threshold cycle) against the initial target copy numbers. The calculation of copy numbers of unknown samples can be made according to the linear regression of the standard curve; the y-intercept indicates the sensitivity and the slope indicates amplification efficiency.

Effect of benzoic acid intake on intestinal proinflammatory cytokine mRNA expression

The effect of intake of benzoic acid on intestinal proinflammatory cytokine mRNA expression in intestine is shown in Fig. 4, 5, and 6. Pigs administrating antibiotic diet and 0.5% benzoic acid had significantly lower in IL-1β mRNA expression in the proximal small intestine compared to control groups ($p<0.05$). The IL-1β mRNA expression in the middle region of the small intestine were significantly lower in pigs fed benzoic acid supplement than those given the control diet ($p<0.05$). Furthermore, a decrease in IL-1β

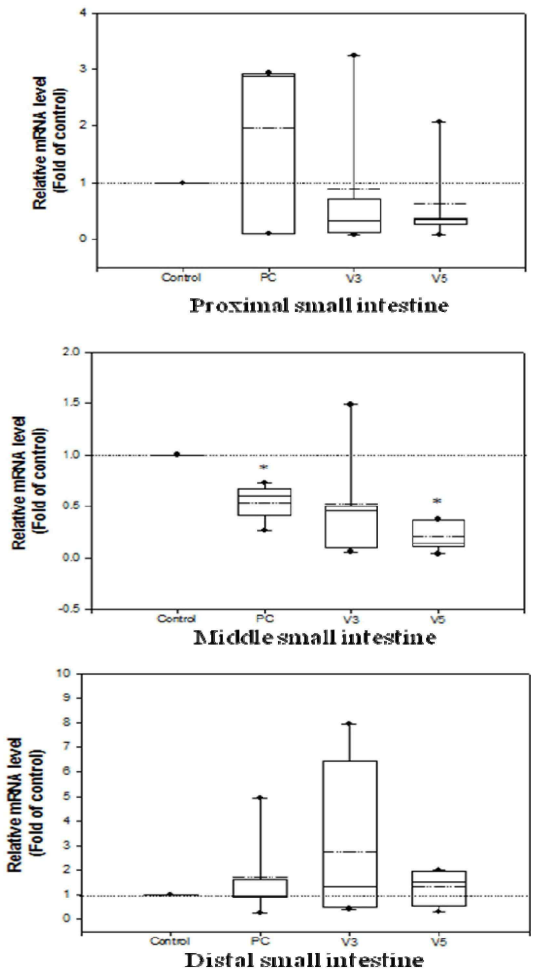

Fig. 6. Effect of benzoic acid supplementation on the relative abundance of TNF-α mRNA in intestine of pigs. Data are presented by box and whisker plots; the box indicates the range between the 25th and 75th percentiles, and the straight and dotted lines in the box show the median and mean values, respectively. The whiskers represent the distribution of values, and the black dots show the minimal and maximal numbers. * $p<0.05$ compared with control.

mRNA expression was observed in the distal small intestine of pigs administrating 0.3% benzoic acid ($p<0.05$). Feeding 0.5% benzoic acid resulted in a marked reduction in the expression of IL-6 mRNA in the middle small intestine ($p<0.05$). The TNF-α mRNA expression in the middle small intestine was significantly lower in pigs fed antibiotic and 0.5% benzoic acid supplement than those given the control diet ($p<0.05$).

Discussion

The intestinal microorganisms take time to form stable community after birth. Its colonization depends on host origin and other physiological factors. The anaerobic and facultative anaerobic bacteria grow rapidly from germ-free state in the GIT on birth [5]. Several studies were reported that bifidobacteria was not detected in suckling pigs, while lactobacilli had a predominant member of the small intestinal bacteria. Weaning is the most stressful time in the life of a pig and outbreaks of diarrhea [13,16]. During the weaning period, the young pigs cope with rapidly changing microbita. In study of Ewing and Cole [3], most of lactobacilli and beneficial bacteria decrease in times of stress, while pathogenic bacteria such as coliform increase.

The use of dietary supplements may facilitate development of immune system in the GIT by influencing on colonization microbita. Also, dietary modulation of the GIT microbita can result in an enhancement of colonization resistance against potential pathogens [10,11]. Dietary organic acids have been used as feed additives in piglet nutrition to lower gastric pH and enhance proteolytic digestion, thus controlling the growth of pathogenic bacteria [17,18]. Benzoic acid is used as a food and feed preservative due to the its ability to induce a reduction of dietary pH and buffering capacity. In the present study, dietary supplementation with benzoic acid did not influence gastric pH. However, the addition of benzoic acid to the diet resulted in reduced urinary pH. This finding is in agreement with that of Kluge et al. [9], who reported that inclusion of benzoic acid did not affect pH of digesta in the stomach, but significantly reduced urinary pH. The latter effect is due to excretion of hippuric acid as a result of the conjugation of benzoic acid with glycine and this reduced urinary pH.

Several studies have reported effects of supplementation with organic acids on the pathogenic microflora of weaning piglets. Canibe et al. [1] reported that organic acid inclusion in pig diets reduces the number of *Enterobacteria* in different sections of the gastrointestinal tract. According to Torrallardona et al. [21], supplementation with benzoic acid actually resulted in increased bacterial counts in gastrointestinal tract. Kluge et al. [9] reported that supplementation of benzoic acid did not influence the gastric pH but reduced the number of gram-negative bacteria in the duodedum. This may indicate that benzoic acid could control and limit growth and colonization of pathogenic bacteria. This might be indicated that benzoic acid is a weak acid with a relatively high constant of dissociation and presented in the stomach and small intestine as an undis-

sociated form. The undissociated form of benzoic acid can diffuse passively through the bacterial cell wall, dissociate itself when the pH is above the pKa, and cause a drop in intracellular pH. As a result, enzymatic processes stop, thus cell death occurs [19]. In the present study, supplementation of 5% benzoic acid resulted in an increase in the number of *L.casei, L.plantarum*, and *B.subtilis* in various segment in GIT.

The development of intestinal immune system in pigs takes place over a period of several weeks. However, this development depends on community of intestinal microorganisms in the GIT. Further, activation of the GIT immune system during weaning has been reported [22]. Pie et al. [17] reported early response in gene expression of inflammatory cytokines in the GIT is related with weaning, which might cause anatomical and functional disorders in the GIT. Cytokines play an important role in mediation of immune response to infection. IL-1, IL-6, and tumor necrosis factor (TNF-α) are typical proinflammatory cytokines produced by macrophages and monocytes of the innate immune system as a rapid response against pathogens or other [2,12]. In addition, increased gene expression of inflammatory cytokine genes, including those of IL-1β, IL-6, and TNF-α, is a very important indicator of inflammatory conditions in the intestine. A normal level of expression is found in healthy intestinal epithelial cells; however, this increase is in response to pathogenic bacterial infections. In the current study, weaning piglets fed benzoic acid at a level of 0.5% showed a reduced level of inflammatory cytokine gene expression in the different segments of the small intestine, compared with animals fed the control diet. This finding provides evidence that inclusion of 0.5% benzoic acid creates a hospitable environment for beneficial bacteria in GIT, which ultimately leads to an increase in their population size. This increase may be responsible for preventing production of inflammatory mediators, which could have a negative effect on the integrity of the intestine.

In conclusion, inclusion levels of benzoic acid up to 0.5% can be advantageous as a result of encouraging proliferation of specific beneficial bacteria, thus having a positive impact on gene expression of inflammatory cytokines in the intestine.

Acknowledgment

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초록 : 사료내 벤조산 첨가가 이유돼지의 장내 미생물 균총 및 면역체계에 미치는 영향

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본 연구에서는 이유자돈 사료에 벤조산 첨가가 위장 내 소화물과 방광 내 뇨의 pH, 장내 유익 미생물 균총수 및 장 조직 내 염증성 싸이토카인 유전자 발현양에 미치는 영향을 조사하였다. 자돈기 5주 동안 이유자돈 사료내 벤조산 0.3%과 0.5% 첨가는 자돈 위 내 pH는 감소시키지는 않았으나, 방광 내 뇨의 pH 수치는 유의적으로 감소하는 것으로 나타났다. *Lactobacillus casei* 수는 맹장부위에서 벤조산 0.5% 첨가구와 대조구에서 유의적으로 가장 높게 나타났다. *Lactobacillus plantarum* 수는 맹장부위에서 벤조산 0.5% 첨가구에서 가장 높게 나타났다. 또한, 직장부위에서 *L.plantarum* 수는 대조구를 제외하고 모든 처리구에서 유의적으로 높게 나타났다. *Bacillus subtillis* 수는 맹장부위에서 벤조산0.5% 첨가구와 대조구에서 유의적으로 가장 높게 나타났으며, 직장부위에서는 벤조산0.3%와 0.5% 첨가구에서 가장 높게 나타났다. IL-1β mRNA 유전자 발현 수준은 소장 상단에서는 벤조산 0.5%와 항생제 처리구에서, 소장중부에서는 벤조산 0.3%와 0.5% 처리구에서, 말단부위에서는 벤조산 0.3% 처리구에서 대조구보다 유의적으로 낮게 나타났다. IL-6 mRNA 유전자 발현 수준은 소장중부에서는 벤조산 0.5% 처리구에서 대조구보다 유의적으로 낮게 나타났다. TNF-α mRNA 유전자 발현 수준은 소장 중단에서 벤조산 0.5%와 항생제 처리구에서 대조구 보다 낮게 나타났다. 본 실험을 통하여, 자돈 사료 내 benzoic acid 0.5% 첨가는 뇨의 pH 감소와 장내 유익 미생물 균총 수 조절 및 소장 내 면역체계 반응에 긍정적인 영향을 미치므로 자돈 건강을 향상 할 수 있으리라 사료된다.