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Effect of Supplementing Acidifiers and Organic Zinc in Diet on Growth Performances and Gut Conditions of Pigs

[1]Piya Lampromsuk, [1]Chaiyapoom Bunchasak, [1]Chanwit Kaewtapee,
[2]Suriya Sawanon and [1]Theerawit Poeikhampha
[1]Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand
[2]Department of Animal Science, Faculty of Agriculture at Kamphaensean,
Kasetsart University, Nakhon Pathom, Thailand

Abstract: One hundred and twenty piglets (body weight~7.24 kg, weaned at 24 day of age) were divided into 3 groups with 10 replication of 4 piglets in a completely randomized design. There were 3 feeding consisted; phase I (24-31 days), phase II (32-45 days) and phase III (46-73 days). The diets were divided into 3 treatments by adding 2, 300 ppm of zinc oxide in phase I to II (T1), adding 500 ppm of zinc acetate in phase I to II and plus 3,000 ppm of sodium benzoate based acidifier in all phases (T2) and by adding 500 ppm of zinc acetate in phase I to II and plus 3,000 ppm of formic acid based acidifier in all phases (T3). The results indicated that replacing zinc oxide (T1) by zinc acetate in phase I to II and plus sodium benzoate based acidifier in all phases (T2) significantly increased final body weight, body weight gain ($p<0.05$) and slightly improved FCR ($p>0.05$) while feed consumption was not significantly influenced by all dietary treatments. Moreover, the dietary treatments did not affect to intestinal pH, bacteria population and volatile fatty acids in the caecum of pigs. In term of morphology of absorptive cells, the supplementing zinc acetate in phase I to II and plus sodium benzoate based acidifier in all phases (T2) significantly increased villous height in jejunum and ileum ($p<0.01$), villous width in ileum ($p<0.01$) and crypt dept in jejunum ($p<0.01$) compared to T1 and T3. It can be concluded that supplemental organic zinc at 24-45 days of ages and plus several organic acids as acidifiers (sodium benzoate based acidifier) at 24-73 days of ages in diet may the growth performance via mechanism of nutrients absorption improvement.

Key words: Zinc oxide, zinc acetate, acidifiers, growth performance, pigs

INTRODUCTION

During the post weaning period (4-5 weeks of age), piglets are susceptible to pathogenic microorganism by various factors such as new grouping, change feed and new management. These induced gastrointestinal pathologies and increased occurrence of diarrhea (Tzipori *et al*., 1980; Barnett *et al*., 1989; Wallgren and Melin, 2001). This problem has been resolved by the use of antibiotic in feed to control microbial populations which has been shown to improve the growth performance of weanling pigs (Hays, 1978; Zimmerman, 1986).

Zinc oxide also clearly prevents and reduces the occurrence of pathogenic during post weaning period (Poulsen, 1995). The pharmacological level (2, 000-3, 000 ppm) of zinc oxide was an effective means of treating diarrhea (Kavanagh, 1992). However, these concentrations are quite high and induce some environmental concerns (Kavanagh, 1992; Edwards and Baker, 1999). Due to high bioavailability of organic zinc (such as zinc acetate; Zn $(O_2CCH_3)_2$), it has been considered to be an effective alternative zinc oxide source (Hahn and Baker, 1993) and shown more production performance of laying (Idowu *et al*., 2011) and lambs (Abdelrahman *et al*., 2003). Generally, organic acids increase feed digestion in piglets and suppress the concentration of coliform populations in the gastrointestinal tract (White *et al*., 1969; Thomlinson and Lawrence, 1981; Ravindran and Kornegay, 2006). Among organic acids supplementation, mixed acidifiers (combined acids) have shown better positive effects on growth performance of broiler (Denli *et al*., 2003) and piglets after weaning than single acid (Walsh *et al*., 2007; Hardy, 2002).

Therefore, the objectives of the present study were to evaluate the effectiveness of organic zinc and combined acidifiers supplementation in diet on growth performance, volatile fatty acids, gut condition and microbial population of pigs.

Corresponding Author: Theerawit Poeikhampha, Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand Tel: +66-0-2579-1120

MATERIALS AND METHODS

This study was conducted at Animal Research Farm, Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Experimental animals were kept, maintained and treated in adherence to accepted standards for the humane treatment of animals.

Animal and dietary treatments: In this study, 60 of castrated and 60 female crossbred piglets (Landrace x Large white x Duroc) with an average initial body weight 7.24±0.08 kg, weaned at 24±1 day of age were used. The piglets were divided into 3 experimental groups; each group consisted of 10 replicates of 4 pigs each. Piglets were treated in nursery housing on fully slat floor that control environment by evaporative cooling system. Each pen (1.2 m^2) was equipped with a five-hole automatic feeder and two nipples water to allow *ad libitum* consumption of water and feed. The experimental period was divided into 3 phases; phase I (24-31 days), phase II (32-45 days) and phase III (45-73 days). Piglets were weighed at initiation and the end of experiment to allow calculation of weight gain, feed consumption and feed conversion ratio.

Table 1: Ingredient and chemical composition of experimental diets

Item	Phase I	Phase II	Phase III
Ingredients (kg)			
Broken rice	33.07	39.20	35.52
Corn	-	8.34	20.18
Soy bean oil	3.39	5.90	4.64
Soy bean meal	10.00	26.81	21.69
Full fat soybean	32.83	5.00	10.00
Fish meal	1.50	2.00	3.00
L-Lysine	0.15	0.34	0.42
DL-Methionine	0.13	0.22	0.22
L-Threonine	0.03	0.13	0.16
Sweet whey	15.00	8.00	-
MDCP 21%	2.07	2.01	2.07
Calcium carbonate	0.67	0.82	0.81
Salt	0.16	0.23	0.31
Tested product	0.50	0.50	0.50
[1]Premix	0.50	0.50	0.50
Dietary composition (%)			
Energy, kcal of ME/kg	3,450.00	3,450.00	3,400.00
Protein	22.00	20.00	20.00
Fat	9.98	8.00	8.00
Calcium	1.00	1.00	1.00
Available phosphorus	0.55	0.50	0.50
Lysine	1.40	1.40	1.40
Methionine+cystine	0.84	0.84	0.84
Threonine	0.91	0.91	0.91

[1]Premix: Vitamix ST® consist of vitamin A: 2 MIU, D3: 0.4 MIU, E: 4,000 IU, K3: 0.3 g, B1: 0.2 g, B2: 1 g, B6: 0.4 g, B12: 0.004 g, Pantothenic acid: 1.8 g, Nicotinic acid: 3 g, Biotin: 0.02 g, Copper: 30 g, Manganese: 10 g, Zinc: 20 g, Iron: 30 g, Iodine: 0.1 g, Cobalt: 0.04 g, Selenium: 0.06 g, Flavour: 0.5 g, Feed preservative: 0.15 g and Carrier added to 1.00 kg premix

All nutrients were formulated to meet or exceeded the standard of NRC (1998) (Table 1). A total of 100 ppm of Tiamulin was added in all diet and 200 ppm of amoxicillin was added in phase I of diets. In this study, each phase was divided into 3 treatments according to the supplementations of zinc oxide (T1), zinc acetate (Kemira's zinc acetate, Kemira Asia Pacific Pte. Ltd., Singapore) plus sodium benzoate based acidifier (Kemira Pro GIT SB5, Kemira Asia Pacific Pte. Ltd., Singapore; consists benzoic acid, formic acid, lactic acid and citric acid) (T2) and zinc acetate plus formic acid based acidifier (Kemira Pro GIT SF1, Kemira Asia Pacific Pte. Ltd., Singapore; consists acetic acid, formic acid, lactic acid and citric acid) (T3). The distributions of tested substances are shown in Table 2. Benzoic and formic acid based acidifiers contain approximately 6 and 20% of calcium, respectively. Therefore, limestone was added to balance calcium ratio in all diets.

Samples collections: At the end of the trial, 10 pigs from each treatment were exsanguinated and the digesta was taken, stored for further analysis. The pH in each part of gastrointestinal track including stomach, duodenum, jejunum, ileum, caecum, colon and rectum was directly measured with a pH meter (IQ Scientific Instruments, Carlsbad, CA, USA). The small intestine tissue samples were collected of duodenum, jejunum and ileum tissues for determination of morphology.

The tissue samples were cleaned in phosphate buffer saline and fixed in 10% neutral buffer formalin 24 h. The specimen were embedded in Paraffin box and sliced to approximately 5 μm with a microtome, stained with hematoxylin-eosin and mounted on slides. Villous high, crypt depth and villous/crypt ratio were measure by light microscope in accordance with Nunez *et al*. (1996).

The two fraction fresh digesta samples were collected from caecum for determination of volatile fatty acids and microbial shedding (*Escherichia coli* and *Lactobacillus* sp.). In first fraction, the samples were mixed with hydrochloric acid (HCl: 6 M) at a ratio of 5:1 (w/w) to stop microbial activity. The specimen were centrifuged in microcentrifuge tubes (14, 000 rpm, 10 min,

Table 2: The distribution of zinc oxide, zinc acetate, sodium benzoate based acidifier and formic acid based acidifier in diets (tested product, ppm)

	Phase I			Phase II			Phase III		
Item	T1	T2	T3	T1	T2	T3	T1	T2	T3
Zinc oxide	2,300	-	-	2,300	-	-	-	-	-
Zinc acetate	-	500	500	-	500	500	-	-	-
Sodium benzoate based acidifier	-	3,000	-	-	3,000	-	-	3,000	-
Formic acid based acidifier	-	-	3,000	-	-	3,000	-	-	3,000

4°C) and 1 mL of the supernatant was transferred to microcentrifuge tubes and kept at -20°C. The concentration of volatile fatty acids (acetic, propionic, butyric and total acids) in the specimen was determined by a gas chromatography (Shimadzu Model GC-2010 High-end, Shimadzu, Kyoto, Japan) in accordance with Biagi *et al.* (2006).

In second fraction, the procedures used for plating *E. coli* and *Lactobacillus* sp. were adapted from Franklin *et al.* (2002) and De Man *et al.* (1960), respectively as follows: a 5 g subsample was mixed into 45 mL of peptone broth, serially 10 fold diluted with peptone broth and used to inoculate at 3 dilutions (10^{-5}, 10^{-6}, 10^{-7}) and in duplicate at each dilution in Mac-Conkey agar plates (Difco Laboratories, Detroit, MI) for *E. coli* isolation with spread plate technique and were incubate for 24 h at 37°C in aerobic condition. After removal from the incubator, *E. coli* colonies were immediately determined per plate by counting. For *Lactobacillus* sp. used to inoculate at 3 dilutions (10^{-5}, 10^{-6}, 10^{-7}) and in duplicate at each dilution in De Man, Rogosa and Sharpe (MRS) agar plates (Difco Laboratories, Detroit, MI) and were incubated for 24 h at 41.5°C in anaerobic jar and after removal from the incubator, *Lactobacillus* sp. colonies were immediately determined per plate by counting.

Statistical analysis: All data were analyzed by ANOVA using the GLM procedure of SAS (SAS, 1988). Initial body weight was used as the covariant for the analysis of growth performance. The least square mean of each treatment was compared using least significant difference test. The means of bacteria count, pH of diet and the gastrointestinal tract, volatile fatty acids and small intestinal morphology were compared using the Duncan's multiple range tests (Duncan, 1955) for each variable according to the following model:

$$Y_{ij} = \mu + A_i + \epsilon_{ij}$$

where, Y_{ij} is the observed response, A_i is the effect of diet and ϵ_{ij} is experimental error; $\epsilon_{ij} \sim NID(0, \delta^2)$. All statistical analyses were computed in accordance with the Steel and Torrie (1980).

RESULTS

The growth performance of pigs are shown in Table 3. The results indicated that adding 500 ppm of zinc acetate in phase I to II of diets plus 3, 000 ppm of sodium benzoate based acidifier in all phases (T2) significantly increased final body weight and body weight gain ($p<0.05$). The final body weight was 34.67 kg in T2 compared to 32.42 kg in T1 (adding 500 ppm of zinc oxide in phase I to II of diets) and 32.90 kg in T3 (adding 500 ppm of zinc acetate in phase I to II of diets plus formic acid based acidifier in all phases). However, feed consumption of pigs were not significantly different. In addition, the supplementation of zinc acetate in phase I to II of diets plus sodium benzoate based acidifier or formic acid based acidifier in all phases (T2 or T3) slightly improved feed conversion ratio of pigs although statistical significance was not found ($p = 0.25$). During phase I to III, the final body weight increased 6.94% when replaced 2, 300 ppm of zinc oxide in phase I to II of diets with 500 ppm of zinc acetate plus sodium benzoate based acidifier (benzoic acid, formic acid, lactic acid and citric acid) in all phases. Consequently, feed conversion ratio was slightly improved compared to zinc oxide supplementation.

Gastrointestinal pH and bacteria population in the caecum of pigs are shown in Table 4. The pH in gastrointestinal tract of piglets was not significantly influenced by the experimental diets. Similarly, some

Table 3: The growth performance of pigs fed the experimental diets

Item	T1	T2	T3	p-value
Final body weight (kg/pig)	32.42±0.64	34.67±0.70	32.90±0.67	0.04*
Body weight gain (kg/pig/day)	0.51±0.01	0.56±0.01	0.52±0.01	0.04*
Feed consumption (kg/pig/day)	0.90±0.02	0.96±0.02	0.91±0.03	0.26
Feed conversion ratio	1.77±0.03	1.71±0.02	1.73±0.02	0.25

n = 120 and the data was covariant statistically analyzed, Values represent an average±SE, *Significant

Table 4: Gastrointestinal pH and Bacterial population in the caecum (CFU mL^{-1}) of pigs fed the experimental diets

Item	T1	T2	T3	p-value
Gastrointestinal pH				
Stomach	3.53±0.29	4.10±0.28	4.39±0.33	0.14
Duodenum	5.57±0.08	5.65±0.11	5.52±0.15	0.75
Jejunum	6.35±0.11	6.36±0.14	6.37±0.09	0.99
Ileum	6.42±0.18	6.60±0.09	6.63±0.07	0.46
Caecum	5.82±0.09	5.78±0.05	5.87±0.06	0.66
Colon	6.23±0.07	6.06±0.10	6.14±0.08	0.35
Rectum	6.23±0.06	6.19±0.10	6.33±0.07	0.43
Bacterial population in the caecum (CFU mL^{-1})				
E. coli	5.08±0.17	5.07±0.17	5.07±0.24	1.00
Lactobacillus sp.	7.02±0.08	6.94±0.09	6.76±0.12	0.19

n = 10 per treatment and values represent an average±SE

Table 5: Concentration of volatile fatty acids in caecum of pigs fed the experimental diets

Item (mmol l^{-1})	T1	T2	T3	p-value
Acetic acid	14.05±1.44	13.62±0.80	15.34±1.89	0.70
Propionic acid	16.40±1.44	16.66±1.76	15.79±2.04	0.94
Butyric acid	3.75±0.37	4.00±0.70	3.54±0.61	0.85
Valeric acid	0.66±0.11	0.76±0.34	0.55±0.15	0.80
Total volatile fatty acid	34.86±2.60	31.53±4.49	35.22±4.07	0.75

n = 10 per treatment and values represent an average±SE

Table 6: Villous heights, crypt depth (μm) and the villous height to crypt depth ratio of pigs fed the experimental diets

Item	T1	T2	T3	p-value
Villous height				
Duodenum	541.38±10.63	559.74±11.92	505.67±11.25	<0.01*
Jejunum	446.25±10.20	489.99±9.44	435.92±8.85	<0.01*
Ileum	307.38±6.89	330.22±5.39	305.35±6.84	<0.01*
Crypt depth				
Duodenum	350.26±11.52	337.05±7.55	370.46±10.26	0.05*
Jejunum	241.03±6.17	284.52±7.33	243.24±7.32	<0.01*
Ileum	219.62±6.91	216.49±5.39	197.99±5.74	0.03*
Villous height to crypt depth ratio				
Duodenum	1.70±0.07	1.73±0.05	1.44±0.05	<0.01*
Jejunum	1.93±0.06	1.82±0.06	1.88±0.05	0.33
Ileum	1.50±0.05	1.61±0.04	1.65±0.05	0.11
Villous width				
Duodenum	138.06±3.65	140.12±4.06	146.14±4.46	0.36*
Jejunum	123.30±3.00	129.27±2.86	117.92±3.36	0.03*
Ileum	114.70±2.98	125.96±2.75	113.68±3.05	<0.01*

n = 100 per treatment and values represent an average±SE, *Significant

bacteria populations (*E. coli* and *Lactobacillus* sp.) in the caecum of pigs also were not significantly affected by dietary treatments.

The concentrations of volatile fatty acids in caecum of pigs fed the experimental diets are shown in Table 5. Dietary treatments did not significantly influence to the concentrations of acetic acid, propionic acid, butyric acid, valeric acid and total volatile fatty acids ($p<0.05$).

The villous heights, crypt depth and the villous height to crypt depth ratio of small intestinal tract of piglets fed the experimental diets are shown in Table 6. In duodenum segment, zinc oxide (T1) and zinc acetate plus sodium benzoate based acidifier (T2) had significantly higher villous height than zinc acetate plus formic acid based acidifier (T3) ($p<0.01$). T2 significantly increased villous height in jejunum and ileum ($p<0.01$) compared to those other groups. On the other hand, T2 had significantly lower crypt depth in duodenum than T3 ($p<0.05$) and inversely significant highest in jejunum segment ($p<0.01$) while the T3 showed lowest of crypt depth ($p<0.05$). Moreover, T2 significantly increased villous width in jejunum ($p<0.05$) and highly significant increased in ileum ($p<0.01$). In term of villous height/crypt depth ratio, piglets fed dietary T3 had the lowest value.

DISCUSSION

Although, the supplementation of high level of zinc (3,000 ppm as zinc oxide) improved growth performance (Mei *et al*., 2009) and widely recommended (Case and Carlson, 2002), but NRC (1998) recommended only 100 ppm of zinc for nursery pig, commercially. This study clearly indicates that using zinc acetate in phase I to II of diets plus combined acidifiers (sodium benzoate or formic acid based acidifiers) in all phases can reduce dosage of zinc supplementation (zinc oxide) in phase I to II of diets. The results support several investigators who reported that organic source (zinc acetate) was more effective than inorganic source (zinc oxide) due to higher bioavailability (Carlson *et al*., 1998; Katouli *et al*., 1999; Mavromichalis *et al*., 2000). Since the combined acidifiers were also added to the diets, the improvement of growth performance may come from the need to explain in detail. In addition, the adding of mixed organic acid may increased growth performance of piglets similarly as study of Celik *et al*. (2008) and Khosravi *et al*. (2012) directly supplemented organic acid in diet of broilers and observe growth performance of broilers.

The ineffectiveness of dietary treatments on gastrointestinal pH may be caused by intestinal biostatics on acids-base balance (Fuller and Perdigon, 2003). In piglets, Kaewtapee *et al*. (2010) directly added organic acid in drinking water of piglets and could not find or did not observe changing of gastrointestinal pH. Accordingly, this was similar to what have found in broiler and mice (Abdel-Fattah *et al*., 2008).

In commercial, losses of piglet as a result of diarrhea are often seen. Using combined acidifiers as feed additive possible to control the population of intestinal microorganisms and promote the growth of beneficial

bacteria such as lactic acid bacteria and may protect pigs from diarrhea. Combined acidifiers may be utilized by lactic acid bacteria such as *Lactobacillus* sp. and *Bifidobacterium* sp. (Asano *et al*., 1994), thus the population of intestinal lactic acid bacteria could be increased. Acidifiers as organic acid can be dissolved and entering the cell in the undissociated form and dissociating in the more alkaline cell interior causing acidification of the cytoplasm and inhibition of cell metabolism of pathogenic microorganism (Lueck, 1980), subsequent reducing the frequency of post weaning diarrhea and improving growth performance in piglets without any effect on feed consumption (Davis *et al*., 2002; Knarreborg *et al*., 2002; Mroz *et al*., 2002; Taube *et al*., 2009). In this study, the dietary treatments (zinc oxide, sodium benzoate based acidifier and formic acid based acidifier) did not influence any acidity in gastrointestinal tract and intestinal microorganisms. The reasons of this phenomenon may be given as (1) this study was conducted under well managed conditions and less of stress, thus incidence of diarrhea was not found and/or (2) dietary zinc may enhance the growth through mode of action within cellular metabolism than the effects in gut ecology and physiology (Case and Carlson, 2002).

It is believed that increasing acids utilizing bacteria increase volatile fatty acids production (Asano *et al*., 1994; Tsukahara *et al*., 2002) and these acids are energy source of intestinal epithelial cell growth (Poeikhampha *et al*., 2007; Poeikhampha and Bunchasak, 2010). Significant enchantment of volatile fatty acids may be produced from lactic acid and acetic acid, since these acids can be the substrates to form volatile fatty acids via activities of lactic acid bacteria such as *Lactobacillus reuteri* and *L. mucosae* and acid-utilizing bacteria such as *Megasphaera elsdenii* and *Mitsuokella multiacida* (Tsukahara *et al*., 2002; Poeikhampha and Bunchasak, 2011). Furthermore, Asano *et al*. (1994) reported that increase intestinal lactic acid bacteria population leading to produce lactic acid and acetic acid and tended to had lower pH in the intestine.

However, supplementation of sodium benzoate based acidifier and formic acid based acidifier not influenced the caecal acetic acid, propionic acid, butyric acid, valeric acid and total volatile fatty acids concentrations. This result is in accordance with the results of gastrointestinal pH (ceacum) and lactic bacteria population in ceacum that are normally involved with concentrations of volatile fatty acids. It would be explained that due to bacteria populations were not influenced by dietary treatments, productions of volatile fatty acids were not changed, therefore, acid-base status in intestine is constant. Moreover, it can also give other explanation that these supplemental organic acids may be dissolved and absorbed in the small intestine and used as energy sources of intestinal epithelial cell growth (Piva *et al*., 1997) and not reach the large intestine (caecum). Another one is volatile fatty acids that produced in caecum may be forced backwards to the small intestine via anti-peristalsis mechanism (Fuller and Perdigon, 2003) and used by small intestine cell.

Factors affecting small intestinal development of pigs includes, stress of weaning, adaptation to solid feed during weaning period, dietary factor and so on (Steven *et al*., 2001). For these reasons, villous atrophy after weaning is caused by an increased rate of cell loss or a reduced rate of cell renewal that associate with activity of enzymes such as lactase and sucrase (Pluske *et al*., 1995). Supplementing sodium benzoate based acidifier increased villous height in duodenum, jejunum and ileum. It is believed that sodium benzoate is directly absorbed in small intestine and stimulates epithelial cell proliferation (Asano *et al*., 1994) and not reached the large intestine where the volatile fatty acids were detected. Unlike formic acid based acidifier may dissolved and entering the cell of bacteria. Additionally, formic acid may disturb the epithelial cell growth, especially in duodenum (Hunter and Segel, 1973; Lueck, 1980). Formic acid based acidifier did not influence the intestinal villous height in jejunum and ileum compared to control.

In this study, sodium benzoate and formic acid based acidifier may directly absorbed in small intestine and not reached to large intestine where the volatile fatty acids and intestinal bacteria were detected thus in this study the concentration of volatile fatty acids and intestinal bacteria not influenced by the treatments compared to control. Supplementation of zinc acetate plus sodium benzoate based acidifier (T2) increased the villous height in duodenum, jejunum and ileum and crypt depth in jejunum and ileum. The function of villous and crypts are important to the production performances of pigs since the portion of the digestive and absorptive capacity of small intestine occurs near and around the villous and crypts (Steven *et al*., 2001), especially in duodenum where the almost of digestive and absorptive activities are functioned (Fuller and Perdigon, 2003). Thus, the improvement of growth performance of piglets by supplementing zinc acetate plus sodium benzoate based acidifier (T2) would mainly came from the increase in intestinal villous surface and the absorptive efficiency of the small intestine. Unlikely, supplementing 2, 500 and 3, 000 ppm of zinc oxide in diet did not give any benefit to intestinal morphology in the duodenum, gut ecology of weaned pigs (Carlson *et al*., 1998; Katouli *et al*., 1999).

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