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Exhibit Differential Functions of Various Antibiotic Growth Promoters in Broiler Growth, Immune Response and Gastrointestinal Physiology

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Abstract: Six-hundred forty broilers were allotted into four dietary supplementation of none, 55 ppm bacitracin, 2.5 ppm nosiheptide, or 55 ppm Oxytetracycline (OTC) in a corn-soybean meal basal diet with eight pens in each treatment to investigate the effects of added various Antibiotic Growth Promoters (AGP) in diet on growth performance, immune response and gastrointestinal physiology. Data showed that nosiheptide supplementation but not bacitracin or OTC increased feed efficiency in starter period. The nosiheptide decreased the small intestine weight, IgA-positive cells along the intestinal tract and ileal mucosal secretory IgA (sIgA) concentration but bacitracin and OTC increased the IgA-positive cells in ileum. The nosiheptide supplementation group also elevated ileal sucrase and maltase activities and reduced the *Lactobacillus* counts in rectal contents as compared to the other treatment groups. In conclusion, the enhanced disaccharidase activity, diminished rectal *Lactobacillus* counts and reduced immune activated status are possibly the important factors for the improvement of feed efficiency of broilers on dietary nosiheptide supplementation from days 0-21.

Key words: Antibiotic growth promoters, broiler, immune response, microflora, small intestine

INTRODUCTION

Antibiotic Growth Promoters (AGP) have been widely used in animal production last for 50 years. Feeding broilers with AGP has been documented to increase 3.3-8.0% of gain and improve approximately 3% of Feed Efficiency (FE) (cited in Kim *et al*., 2005). So feeding GPA has been well established and become an integral part of nutritional strategies for broilers feeding. But recent study has proven the existence of pathogenic bacteria resistant to AGP in chickens (Saleha *et al*., 2009) and reduces the use of AGP has been curtailed by legislation. However, the acting mechanisms of AGP remain to be explored in order to develop the alternative AGP for poultry.

At least 4 major mechanisms have been proposed to explain AGP-mediated growth enhancement include 1) inhibit endemic subclinical infection, thus reduce the metabolic costs of the immune system; 2) reduce growth-depressing metabolites (such as ammonia and bile degradation products) produced by microflora; 3) reduce nutrients used by microbes and 4) enhance uptake and absorption of nutrients due to thinner intestinal wall in AGP-fed animals (Dibner and Richards, 2005; Miles *et al*., 2006; Niewold, 2007). Nevertheless, different AGP do not accomplish this domination through the same mechanism (Ferket, 2004). Therefore, the aim of this experiment was to study the different effects among bacitracin, nosiheptide and Oxytetracycline (OTC) on the growth, immune responses and intestinal microflora in broilers during the starter period.

MATERIALS AND METHODS

Animal treatments and management: Six-hundred forty 1-day-old commercial Arbor Acre broilers were randomly allocated into floor pens (4 m^2/pen) with 8 pen/treatment and 20 chicks of equal gender ratio in each pen. The experiment period lasted for 21 days. Birds were fed experimental basal diet with supplementation of no AGP (control), 55 ppm bacitracin, 2.5 ppm nosiheptide, or 55 ppm OTC and water was provided *ad libitum*. The basal diet (Table 1) was formulated to meet the nutrients requirements recommended by NRC (1994) and contained 21.68% CP and 3,070 kcal/kg of ME. This experiment was performed at National Ilan University with prior approval from its Animal Protocol Review Committee.

Birds were reared on the new rice hulls litter under continuous lighting throughout the feeding period. The environmental temperature was maintained at 21.1-25.4°C and heat was supplied during the first 2 weeks for chicks. All chicks were immunized with Infectious Bronchitis (IB) and Newcastle Disease (ND[1]) vaccines on day 4 and day 14, respectively. Feed intake and body weight were measured weekly and mortality was recorded daily. On day 21, one male and one female bird per pen were randomly selected and blood samples were taken via the wing veins to measure serum constituents and antibody titers. Birds were then sacrificed by cervical dislocation and the spleen as well as the contents from the rectal contents were collected to measure splenocyte proliferation and to enumerate rectal microflora.

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Table 1: Ingredients and nutrient composition of basal diet

Item	Starter
Ingredient (%)	
Corn, ground	51.81
Soybean meal, solv. (CP 44%)	39.98
Soybean oil	4.43
DL-methionine	0.20
Choline-chloride (50%)	0.05
Limestone, pulverized	1.60
Dicalcium phosphate	1.33
Salt	0.25
Vitamin premix[a]	0.10
Trace mineral premix[b]	0.10
Coccidostat[c]	0.05
Antibiotic or corn starch[d]	0.10
Calculated nutrient composition	
ME (kcal/kg)	3,070.00
Methionine + Cystine (%)	0.88
Measured nutrient composition	
Crude protein (%)	21.68
Lysine (%)	1.48
Calcium (%)	1.01
Available phosphorus (%)	0.66

[a]Premix provided per kg of complete diet: vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 10 IU; vitamin K_3, 2 mg; riboflavin, 1.5 mg; pyridoxine, 3.0 mg; pantothenic acid, 15 mg; niacin, 30 mg; cyanocobalamin, 7 μg and folic acid, 900 μg.
[b]Premix provided per kg of complete diet: Cu, 10 mg; Zn, 45 mg; Fe, 80 mg; Mn, 55 mg; Se, 0.1 mg and I, 0.3 mg.
[c]Premix provided per kg of complete diet: Salinomycin 60 mg.
[d]Antibiotics were included at the expense of corn starch, antibiotic supplemented with bacitracin 55 ppm, nosiheptide 2.5 ppm and oxytetracycline 55 ppm

Preparation and chemical analysis of samples: All chemicals (unless otherwise noted) were obtained from Sigma[2]. Blood samples were coagulated at 4°C overnight and then centrifuged at 2,000 x g for 15 min to obtain serum. Proventriculus was removed immediately after killing. The intestinal tract samples from the jejunum (approximately 2 cm below the duodenal loop to Meckle's divertivulation) and the ileum (between Meckle's divertivulation and 2 cm above the ileocecal junction) were then longitudinally opened, flushed with a ice-cold Phosphate Buffer Solution (PBS) and blotted to eliminate excess fluid. The gastrointestinal tract and bursa of *Fabricius* were weighed and presented on a relative basis (g/kg of body weight). The dissected intestinal samples were fixed with 4% formaldehyde for villus-crypt morphology and immunohistochemistry evaluation. Mucosal digestive enzyme activities, protein content and secretory IgA (sIgA) were also assayed.

Small intestine morphology and digestive enzyme activity analysis: The intestinal samples were embedded in paraffin, sectioned at 3 μm thicknesses and stained with hematoxylin and eosin following a light microscopy examination[3]. The average villus height and crypt depth were determined from 10 well-oriented and intact villi in a blind fashion. Intestinal IgA-positive cells were identified using the enzyme-linked immuno-peroxidase technique. Tissue sections were blocked with 10% normal goat serum diluted in tris-buffer saline for 30 min and incubated overnight with the primary antibody at 4°C[4]. After being incubated with a streptavidin-biotinylated-horse radish peroxidase chromogen kit[5], cells were counted separately in the lamina propria of 6 well-oriented and intact villi in a blind fashion.

Intestinal mucosa was homogenized in saline with 0.1% triton X-100 and the homogenates were assayed for enzyme activities. Pepsin (EC 3. 4. 23. 1) and disaccharidase activities were determined as previously described by Rick and Fritsch (1974) and Dahlqvist (1964), respectively. Protein concentrations of mucosa samples were measured using a commercial bicinchoninic acid protein assay kit[6].

Serum constituents, mucosal sIgA and splenocyte proliferation measurement: Serum anti-IB and anti-ND titers were determined using commercial ELISA kit[7] and the haemoagglutination inhibition method (Alexander *et al*., 1998). Mucosa sIgA concentration was determined with a commercial ELISA kit[4]. Splenocyte proliferation was measured by incorporating ^{3}H-thymidine into cells as previously described in Lee *et al*. (2003). Briefly the splenocytes were counted and adjusted cell density to 5 x 10^6 cells/mL. One hundred microliter of cell suspension and 100 μL RPMI 1640 (added 5% FBS) with 25 μg/mL concanavalin (Con A) were added and incubated at 37°C with 5% CO_2 incubator. After 48 h incubation, 0.5 μCi/well ^{3}H-thymidine[8] was pulsed for 18 h and was terminated by harvesting cells onto glass fiber filters using an automatic cell harvester[9]. Stimulation index was calculated as counts per minute (cpm) of radioactivity in the presence of a mitogen/cpm of radioactivity and in the absence of mitogen as a basal treatment.

Enumeration of rectal bacteria: The rectal content was immediately transferred into sterile tubes containing 10 mL of a PBS. The suspension was poured into serial 10-dilutions after it was homogenized and centrifuged by the technique developed by Miller and Wolin (1974). *Coliform*, *Lactobacillus* and *Entercoccus* counts were enumerated on *Coliform* agar[10], Rogosa SL agar, or bile esculine agar[11] at 37°C for 24-48 h, respectively.

Statistical analysis: Data were analyzed as a completely randomized design using the GLM procedure of SAS software for ANOVA and means were separated using Duncan's multiple range test and least square means (SAS, 1999) with pen as the experimental unit. Survival rate was subjected to arcsine transformation for analysis. A P-value of <0.05 was considered significant unless otherwise stated (Duncan, 1955).

Table 2: The effects of AGP on growth performance of broilers

Treatments	Control	Bacitracin	Nosiheptide	OTC†	SEM†
Body weight (g/bird)					
Day 0	41.60	41.13	41.59	41.67	0.30
Day 21	820.60	833.70	837.60	826.20	7.60
Daily gain (g/day)	37.10	37.74	37.91	37.36	0.36
Feed intake (g/day)	50.64	50.71	50.31	50.13	0.44
FE (gain: feed/g: g)	0.733[a]	0.744[ab]	0.754[b]	0.745[ab]	0.005
Survival ratio (%)	98.30	96.44	96.90	98.24	1.31

[a,b]Means within the same row not bearing the same superscripts differ significantly ($p<0.05$).
†OTC and SEM was the abbreviation of oxytetracycline and pooled standard error of mean, respectively.

Table 3: The effects of AGP on the small intestinal structure of broilers

Treatments	Control	Bacitracin	Nosiheptide	OTC†	SEM†
Small intestine (g/kg BW)	37.26[ab]	36.56[a]	35.88[a]	39.65[b]	0.93
Jejunum					
Villus height (μm)	1064.00	946.00	977.00	1095.00	47.70
Crypt depth (μm)	93.00	87.00	79.00	96.00	5.00
Crypt/villus	0.09	0.09	0.08	0.08	0.01
IgA-positive cells (No.)	11.66[ab]	11.24[b]	9.18[b]	14.60[a]	1.06
Ileum					
Villus height (μm)	591.00	702.00	585.00	575.00	56.00
Crypt depth (μm)	74.00	78.00	65.00	69.00	3.50
Crypt/villus	0.12	0.12	0.11	0.12	0.01
IgA-positive cells (No.)	4.24[b]	7.40[a]	2.46[c]	6.74[a]	0.55

[a,b,c]Means within the same row not bearing the same superscripts differ significantly ($p<0.05$).
†OTC and SEM was the abbreviation of oxytetracycline and pooled standard error of mean, respectively

RESULTS

Growth performance: Table 2 presents the effects of AGP on growth performance of broilers. Diet supplemented with nosiheptide improved FE ($p<0.05$). Dietary AGP supplementation did not affect the body weight, feed intake, or survival rate during the feeding period. The survival rate was 97.47% from days 0-21, which was similar between both genders. Dietary treatments failed to affect the weights of bursa of *Fabricius*. The values of bursa of *Fabricius* were 1.11, 1.43, 1.38 and 1.34 g/kg body weight in control, bacitracin, nosiheptide and OTC groups, respectively.

Small intestinal structure: Table 3 presents the effects of AGP on the small intestinal structure of broilers. Nosiheptide or bacitracin supplementation decreased small intestine weight of broilers as compared with the OTC supplementation ($p<0.05$). However, there were not significantly different between AGP and control group. The nosiheptide supplementation also tended to cause a decrease of ileal crypt depth of broilers on day 21 ($p<0.07$). However, dietary treatments did not affect the villus height, crypt depth, or crypt depth/villus height of jejunum and ileum of broilers. Significant differences in the numbers of IgA-positive cells in the jejunum and ileum were observed among the treatment groups. Nosiheptide or bacitracin supplementation decreased numbers of IgA-positive cells in the jejunum as compared to OTC group. However, these AGP groups were not different with the control group. The nosiheptide supplementation decreased numbers of IgA-positive cells in the ileum of broilers whereas bacitracin showed the elevated manner. Figure 1 illustrates the effects of AGP on intestinal mucosal sIgA concentration of broilers. Compared with bacitracin, nosiheptide decreased ileal mucosal sIgA concentration of broilers ($p<0.05$). However, dietary treatments did not affect the mucosal sIgA concentration at the jejunum. Dietary treatments also did not affect serum titer of anti-ND and anti-IB, or splenocyte proliferation (data not shown).

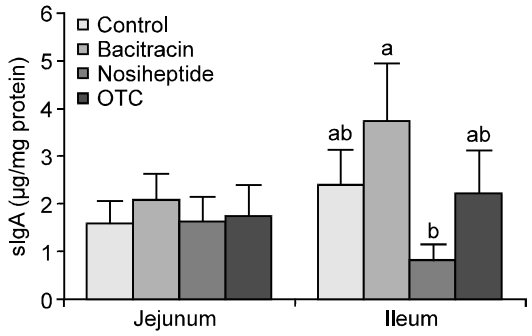

Fig. 1: The effects of AGP on intestinal mucosal sIgA concentration of broilers. The data were shown as the means±SE (n = 16/treatment).
[a,b]Bar with different small letters shown significant differences ($p<0.05$). OTC and sIgA was the abbreviation of oxytetracycline and secretory IgA, respectively

Digestive enzymes and rectal microflora: Table 4 presents the effects of AGP on the digestive enzyme activities of gastrointestinal tract in broilers. OTC

Table 4: The effects of AGP on digestive enzyme activities of gastrointestinal tract in broilers

Treatments	Control	Bacitracin	Nosiheptide	OTC†	SEM†
Proventriculus					
Pepsin#	503.50[ab]	437.30[a]	444.20[a]	519.90[b]	23.35
Jejunum					
Sucrase	80.46	87.00	94.83	72.09	6.83
Maltase	527.90	533.90	593.90	498.90	27.86
Ileum					
Sucrase	46.04[a]	54.80[a]	87.46[b]	57.45[a]	5.97
Maltase	440.30[a]	452.60[a]	622.80[b]	489.80[a]	31.33

[a,b]Means within the same row not bearing the same superscripts differ significantly ($p<0.05$).
†OTC and SEM was the abbreviation of oxytetracycline and pooled standard error of mean, respectively.
#All of the enzyme activities are expressed as μmol/mg protein/min.

Table 5: The effects of AGP on rectal microflora of broilers

Treatments	Control	Bacitracin	Nosiheptide	OTC†	SEM†
Coliform (Log_{10} cfu/g)	6.38	6.33	6.53	6.10	0.14
Enterococci (Log_{10} cfu/g)	6.32	6.28	6.26	6.31	0.14
Lactobacillus (Log_{10} cfu/g)	6.41[a]	6.23[a]	5.33[b]	6.24[a]	0.17

[a,b]Means within the same row not bearing the same superscripts differ significantly ($p<0.01$).
†OTC and SEM was the abbreviation of oxytetracycline and pooled standard error of mean, respectively

supplementation increased the proventriculus pepsin activity as compared to both bacitracin or nosiheptide groups, where nosiheptide increased ileal sucrase and maltase activities as compared to the other groups ($p<0.05$). Table 5 presents the effects of AGP on rectal microflora of broilers. Nosiheptide decreased about 1 log of the numbers of *Lactobacillus* ($p<0.01$) but did not affect the count of *Coliform* or *Enterococci*.

DISCUSSION

Bacitracin and nosiheptide, a family of polypeptide antibiotics, have the gram-positive anti-bactericidal activity and are widely used as AGP in broiler production. Oxytetracycline is broadly applied for the prevention of respiratory diseases or bacterial diarrhea in broilers. The nosiheptide supplementation was more effective than bacitracin and OTC in the enhancement of feed utilization at starter period in the present study. Our data agreed with previous reports about nosiheptide in broilers (McGinnis *et al*., 1978). However, using new litter and low feeding density in this study may be resulted in less improvement of growth performance than those of previous studies (cited in Kim *et al*., 2005). In this study, nosiheptide supplementation was observed to reduce the *Lactobacillus* count without depress the FE of broilers; this may be due to the gastrointestinal tract microflora competing with the host for nutrients and forming organic acids which account for up to 2% of ME intake (Jorgensen *et al*., 1996; Miles *et al*., 2006). In addition, AGP supplementation also decreases the hydrolysis of conjugated bile salts, hence increase fat absorption and further elevate FE (Guban *et al*., 2006).

AGP supplementation also affects the morphology and functions of the small intestine and nutrient utilization of broilers. Dietary supplementation of nosiheptide could change the gut structure and functions including reduced gut weight and increased ileal digestive enzyme activities. Such findings were in accordance with some beneficial effects of AGP feeding reported previously, such as improving of various nutrients absorption can be expected from the thinner gut wall and increase of digestive enzyme activity (Miles *et al*., 2006; Dibner *et al*., 2007).

Broilers fed with AGP, the data showed the highest mucosal sIgA in OTC diet, the lowest in nosiheptide diet and the intermediate in control. The results were consistent with the previous reports (Arias and Koutsos, 2006). These results might suggest that OTC elevated the immune activated status of broilers. However, the nosiheptide decreased the activated status. Reduced immune activity consequently increased anabolic activity of broilers (Klasing, 1998). Therefore, diet supplemented with nosiheptide decreased the immune activated status and contributed to the increase of feed utilization in this study.

Conclusion: Nosiheptide promoted feed utilization in the starter period of broilers whereas did not produce a similar result with bacitracin or OTC. Moreover, the enhanced disaccharidase activities, diminished microflora counts and the reduced immune activated status in the small intestine may play mediatory roles in the improvement of FE from days 0-21 by the nosiheptide supplementation.

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