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High Lysine-Yielding *Bacillus subtilis* as a Promising Alternative to Antibiotic for its Effects on Performance and Immune Responses of Linwu Ducks

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Abstract: This study was conducted to determine the effects on performance and immune responses of Linwu ducks supplemented with high lysine-yielding *Bacillus subtilis*. The 200 and 41 days old female commercial Linwu ducklings were distributed randomly into four groups of 60 ducks each which were further replicated to three groups of 20 each. The ducks were placed on one of four dietary treatments: basal, basal with antibiotic (150 mg Aureomycin whose effective content was 15% per kg feed) and fed with basal diet but deleted lysine (0.15%) and respectively added 5×10^{8} CFU and 5×10^{10} CFU *Bacillus subtilis* per kilogram feed. The trial lasted for 63 days. Average body weight gain for 63 days breeding was observed significantly increase in antibiotic-added group ($p<0.05$) but not showed in supplemented *Bacillus subtilis* groups. It was basically lying in the same level to average feed intake and feed conversion ratio as accessed from each group. Mortality experienced pronounced recession with *Bacillus subtilis* or Aureomycin addition. Breast meat, leg meat and liver relative weigh were greatly improved by fed with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* supplemented diet, although, dressing percentage, semi-eviscerated percentage, eviscerated percentage and percentage of abdominal fat were unaffected. No marked significance was observed in serum profile, except for serum Total Protein (TP) increasing as the consequence of 5×10^{10} CFU kg^{-1} *Bacillus subtilis* supplement. Supplement 5×10^{10} CFU kg^{-1} *Bacillus subtilis* also increased crude protein proportion in meat. Amino acid percentages in meat varied differently between experimental treatments. Additionally, the results in the current research revealed that diet supplemented with *Bacillus subtilis* tended to markedly suppress IL-2 both in spleen and thymus compared with control. Dietary treatment with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* addition or Aureomycin addition down regulated IL-18 in spleen when compared with control. Treatments did not induce any significant effects on *INF-α*, *INF-γ*, *IL-1* and *IL-10* gene expression both in thymus and spleen. It was concluded that dietary supplementation with these high lysine-yielding *Bacillus subtilis* showed promising effects as alternatives for antibiotics due to it had a favor to growth performance and slaughter performance and depression on inflammatory cytokines overproduction which was stimulated by pathogens.

Key words: *Bacillus subtilis*, performance, immune, Linwu ducks, China

INTRODUCTION

Antibiotics were conventionally used in animal feeds as growth promoters and for therapeutic purposes. But approvals for the inclusion of non-therapeutic antibiotics in poultry feed were fast disappearing worldwide due to fear of Antibiotic Resistance (AR) in bacteria, a major threat to human health which had emerged in the last

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few decades as a consequence of the selective pressure exerted by the widespread use of antibiotics (Dahiya *et al*., 2006). Moreover, raising of concerns about potential environmental hazards and food safety problems further increased the pressure to reduce the use of a wide range of antibiotics in animal production.

Strategies to promote growth performance and control disease in the absence of antibiotic presently centered upon dietary supplement with probiotics, prebiotics, organic acids, enzymes, plant extracts and hen egg antibodies (Dahiya *et al*., 2006). *Bacillus subtilis* as a kind of probiotic had been lately reported as a promising candidate replacement for antibiotics, essentially because of its amenability to improvement of feed utilization, modulation of intestinal microflora, enhancement of immune responses and antagonism to pathogens (Dahiya *et al*., 2006). Furthermore, *Bacillus subtilis* was known for environment friendly and could be resistant to high temperature and high pressure when extensively used as feed additives (Teo and Tan, 2006). However, an unambiguous application of probiotics in poultry nutrition was still far from being possible. This was mainly due to different strains of *Bacillus subtilis* equipped with each specific character and propensity, rendering the responses of livestock and poultry to different *Bacillus subtilis* supplement differ both quantitatively and qualitatively.

In the previous research, one strain of *Bacillus subtilis* charactering by high Lysine (Lys)-yielding was isolated from pig ileum digesta and then through UV mutagenesis with lysine analogues S-2-aminoethyl-L-cysteine for resistance screening. The mutant strain had good stability and could produce lysine 40.51 mg L^{-1} in Luria-Bertani liquid culture medium supernatant, 22.58% more than the original strain of 33.05 mg L^{-1}. As was well-known, lysine under many practical feeding conditions was as the second limiting amino acid in poultry diets (Dean, 1986). Dietary Lys deficiency limited protein synthesis and impaired the immune response in chickens (Konashi *et al*., 2000). It had been shown that Lys deficiency compromised antibody response and cell-mediated immunity of chickens (Chen *et al*., 2003). On the contrary, high dietary lysine level could improve breast meat yield during the starter period as confirmed by many studies in starter chicks (Xie *et al*., 2009).

Although, these results emphasized the essential health protecting role of Lys and it could be assumed that an extra dietary Lys supply supported the defence reactions and growth performance, improving Lys in the diet by the manner of supplement foregoing high lysine-yielding *Bacillus subtilis* was barely tested and scarcely known *in vivo* to the knowledge, especially as an additive of duck feed. In present study, dietary administration of high lysine-yielding *Bacillus subtilis* or Aureomycin were undertook to test to generate comparable results on performance and immune responses of Linwu ducks in order to further evaluate whether the *Bacillus subtilis* was a suitable alteration.

MATERIALS AND METHODS

Feeding procedures and experimental protocol: The 200 and 41 days old female commercial Linwu ducklings (one of the popular and conventional ducks in China which had been domesticated in rural villages for a long time) were individually identified with wing tags and were randomly allotted to four groups separately representing four different treatments. Every group contained three replicates and 20 birds each. Each group was placed in a separate room in an infectious disease containment facility where all rooms were light temperature air flow and traffic-controlled. One group served as a control, only receiving basal diet and its composition was shown in Table 1 which refered to for the nutritional needs of meat duck and lysine requirement of Linwu duck (Lin *et al*., 2014). One group served as a positive control group namely antibiotic group consisting of the basal diet supplemented with 150 mg kg^{-1} Aureomycin whose effective content was 15%. The remaining two groups served as probiotic treated groups in which 5×10^{8} and 5×10^{10} CFU kg^{-1} *Bacillus subtilis* mixed per kilogram feed were given, respectively beginning on day 1 and continuously until the end of the experiment. The trial divided two phase, the starter period (1-28 days) and grower period (29-63 days). During these periods, the ducks were raised with water and feed allowed *ad libitum*. The temperature was kept at 33°C from 1-3 days of age and then it was reduced gradually to room temperature until 21 days of age. Group feed intake and body weight were recorded weekly from the first. All the pens were checked for mortality twice a day and feed intake and feed/gain were all corrected for mortality.

Serum biochemical parameter: At the end of the experiment, blood samples were collected by Vena brachialis puncture under the wing from 9 birds randomly chosen from each treatment and plasma was prepared and stored at -20°C for determination of various biochemical parameters by using biochemical analyzer (Beckman CX4).

Slaughter performance and meat chemical composition: At the age of 63 days, birds were denied access to feed for 6 h before slaughter but water was still free in this period. After being deprived of feed for 6 h, three ducks selected randomly from each replicate were weighed and

Table 1: Composition of starter and grower diets (on as-fed basis)

	Percent (%)			Nutrient level (%)	
Ingredients	Starter (1-28 days)	Grower (29-63 days)	Items	Starter (1-28 days)	(29-63 days)
Corn	45.00	65.66	ME (MJ kg^{-1})	12.52	16.07
Wheat middling	20.84	8.00	CP	15.10	15.62
Soybean meal	14.77	6.95	EE	1.93	1.91
Rice bran	5.00	6.12	Ash	7.12	6.20
Rapeseed meal	5.00	5.00	Ca[2]	0.90	0.70
Cottonseed meal	4.08	4.00	TP[2]	0.77	0.71
$CaHPO_4$	1.76	1.52	AP[2]	0.40	0.40
Limestone	1.18	1.00	Lys	1.06	0.85
Premix[1]	1.00	0.80	Met	0.48	0.29
Soybean oil	0.69	0.62	Met+Cys	0.62	0.44
NaCl	0.33	0.23	-	-	-
DL-Met	0.20	0.05	-	-	-
Lys	0.15	0.05	-	-	-

[1]In the starter diet, the premix provided the following per kg of diets: VA: 950 IU; VB_1: 0.2; VB_2: 0.5; VB_6: 0.3; VB_{12} and 0.0015 mg; VD_3: 240 IU; VE: 1.3 IU; VK_3: 0.3 mg; biotin: 0.008 mg; folic acid: 0.1 mg; D-pantothenic acid: 1.0 mg; nicotinic acid: 2.6 mg; Cu (as copper sulfate): 0.008 mg; Fe (as ferrous sulfate) 0.052 mg; Mn (as manganese sulfate) 0.188 mg; Zn (as zinc sulfate) 0.111 mg; I (as potassium iodide) 1.31 mg; Se (as sodium selenite) 0.2 mg; In the grower diet, the premix provided the following per kg of diets: VA: 380 IU; VB_1: 4.0, VB_2: 0.02; VB_5: 40; VB_6: 0.12; VB_{12} and 0.0006 mg; VD_3: 96 IU; VE: 0.52 IU; VK_3: 0.12; biotin: 0.0032; folic acid: 0.04; D-pantothenic acid: 0.4; nicotinic acid: 1.04; Cu (as copper sulfate) 0.0064 mg; Fe (as ferrous sulfate) 0.0416; Mn (as manganese sulfate) 0.1504 mg; Zn (as zinc sulfate) 0.0888 mg, I (as potassium iodide) 0.40 mg and Se (as sodium selenite) 0.16 mg.
[2]Ca, TP and AP were calculated values and others were measured values

humanely euthanized by carbon dioxide asphyxiation. The birds were subsequently scalded, defeathered and carcasses were eviscerated manually. The yields of dressed carcass weight, eviscerated weight, all eviscerated weight, thymus, spleen, liver, abdominal fat, breast meat (including pectoralis major and pectoralis minor) and leg meat (including thigh and drumstick) were weighted and evaluated. What should be addressed was breast meat and leg meat was all skinless and boneless. Amino acid composition was determined as previously described (Sklan and Halevy, 1984).

RNA extraction and quantitative real-time PCR: Total RNA was extracted using Trizol following the manufacturer's protocol. For each sample, 5 mg RNA was further purified using a Nucleospin RNA II kit (Clontech). Reverse transcription was performed using a SuperScript II Reverse Transcriptase kit (Invitrogen) and Random Primers (Invitrogen) following the manufacturer's protocol. Quantitative PCR was performed in a final volume of 25 mL using 100 ng cDNA, 400 nM each primer and 12.5 mL iTaq SYBR Green Supermix with ROX (Bio-Rad). The primers used were listed in supplementary Table 2 (available in JGV Online). Quantitative PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following program: initial denaturation for 2 min at 95°C followed by 40 cycles of 15 sec at 98°C and 1 min at 60°C and a melt-curve analysis. To rule out genomic contamination, control PCRs were performed in the absence of reverse transcriptase. Normalized expression was then calculated as target gene R0/GAPDH R0 for each sample.

Dates analysis and statistic: Dressing carcass weight, eviscerated weight, all eviscerated weight were present as a rate relative to live B.W. Abdominal fat rate was calculated by dividing abdominal fat weight by all eviscerated weight together with abdominal fat weight. Breast meat and leg meat rate were directly calculated by dividing them all eviscerated weight, respectively. Immune organ index expressed as a percentage relative to all eviscerated weight. Standard deviation, minimum, maximum and mean values were calculated using the software package SPSS 18.0 for Windows (SPSS Inc., Chicago, IL). Correlations between variables ($p<0.05$) were determined by correlation analyses using Pearson's linear correlation coefficient with SPSS 18.0, the results expressed in such a form: mean±standard error.

Table 2: Sequences of primers used for real-time PCR

Items	Primers sequences
IFN-α	Forward: 5'-TCCACCTCCTCCAACACCTC-3'
	Reverse: 5'-TGGGAAGCAGCGCTCGAG-3'
IFN-γ	Forward: 5'-GCTGATGGCAATCCTGTTTT-3'
	Reverse: 5'-GGATTTTCAAGCCAGTCAGC-3'
IL-1β	Forward: 5'-TCGACATCAACCAGAAGTGC-3'
	Reverse: 5'-GAGCTTGTAGCCCTTGATGC-3'
IL-2	Forward: 5'-GCCAAGAGCTGACCAACTTC-3'
	Reverse: 5'-ATCGCCCACACTAAGAGCAT-3'
IL-8	Forward: 5'-GCAGGCACACAGACTCTGAA-3'
	Reverse: 5'-TTGGCCAGAATTGCCTTTAC-3'
IL-10	Forward: 5'-GCTGTCACCGCTTCTTCACCT-3'
	Reverse: 5'-GGCTCACTTCCTCCTCCTCATC-3'
IL-18	Forward: 5'-AAATCCTCCATCGCTTCCTT-3'
	Reverse: 5'-TTTCCCGTGTTCTTCCTCAC-3'
β-actin	Forward: 5'-GATGTGGATCAGCAAGCAGGAGT-3'
	Reverse: 5'-GGGTGTGGGTGTTGGTAACAGT-3'

RESULTS AND DISCUSSION

The effects of experimental treatments on the growth performance of Linwu ducks were presented in Table 3. Average body weight gain for 63 days breeding was observed significantly increase in antibiotic-added group ($p<0.05$) but not showed in supplemented with

Table 3: The effects on the performance of Linwu ducks during starter and grower period

Items	Control	Aureomycin	Probiotic I	Probiotic II
Birth weight (g)	42.10±1.020	39.83±0.640	39.90±0.98	41.83±0.740
63 days live weight (g)	1397.04±16.12^{b}	1464.20±14.98^{a}	1416.68±3.99^{b}	1420.06±16.56^{ab}
Average body weight gain (g day^{-1})	21.85±0.250^{b}	22.97±0.250^{a}	22.21±0.07^{ab}	22.23±0.260^{ab}
Average feed intake (g day^{-1})	91.68±1.540	88.67±1.070	90.60±2.72	89.22±2.220
Feed/Gain	4.20±0.120	3.86±0.010	4.08±0.11	4.02±0.150
Mortality (%)	23.33±1.670^{a}	5.00±2.890^{b}	10.00±2.89^{b}	3.33±1.670^{b}

Table 4: The effects on slaughter performance of Linwu ducks (unit: %)

Items	Control	Aureomycin	Probiotic I	Probiotic II
Dressing percentage	90.08±0.31	91.49±0.66	90.22±0.420	90.36±0.23
Semi-eviscerated percentage	82.03±0.27	82.96±0.67	82.67±0.120	82.38±0.47
Eviscerated percentage	71.85±0.80	73.25±0.55	72.90±0.290	72.93±0.93
Percentage of abdominal fat	1.83±0.39	1.48±0.23	1.47±0.190	1.43±0.03
Breast muscle rate	9.73±0.30^{b}	10.67±0.37^{ab}	11.63±0.500^{ab}	12.48±1.02^{a}
Leg muscle rate	8.74±0.41^{b}	11.03±0.34^{ab}	10.11±0.129^{ab}	12.98±0.23^{a}
Liver rate	20.6±0.050^{b}	21.70±0.64^{ab}	21.70±0.570^{ab}	25.00±1.88^{a}
Thymus rate	4.80±0.78	3.90±0.82	5.80±0.340	5.40±0.16
Spleen rate	0.89±0.07	0.90±0.07	1.00±0.050	0.90±0.11

Probiotic I: diet supplemented with *Bacillus subtilis* 5×10^{8} CFU kg^{-1}; Probiotic II: diet supplemented with *Bacillus subtilis* 5×10^{10} CFU kg^{-1}; values in the same row not sharing a common superscript differ significantly ($p<0.05$)

Bacillus subtilis group. It was basically lying in the same level to average feed intake and feed conversion ratio as accessed from each group. Mortality experienced pronounced recession with *Bacillus subtilis* or Aureomycin addition ($p<0.05$).

As described in Table 4, there was barely response to the supplement with antibiotic and probiotic on dressing percentage, semi-eviscerated percentage, eviscerated percentage and percentage of abdominal fat but birds fed the diet with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* increased breast and leg muscles proportion. Relative liver weight (g 100 g^{-1} of B.W.) was significantly increased by diet with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* ($p<0.05$) while relative thymus and spleen weight were not significantly influenced by dietary addition of Aureomycin and *Bacillus subtilis*.

Considering that supplement with these high lysine-yielding *Bacillus subtilis* had possibility to make difference on meat composition profile, researchers also took a test on the containing of each chemical constitution on breast and leg muscle. As described in Table 5 and 6, breast meat showed significantly higher values for moisture content in control. Diet with antibiotic and high lysine-yielding *Bacillus subtilis* did exert no influence on the contents of fat and ash both at breast and leg muscle but on crude protein when *Bacillus subtilis* reaching to 5×10^{10} CFU kg^{-1} additive amount which might be partly responsible for higher breast and leg muscle rates. Besides, supplemented with Aureomycin and *Bacillus subtilis* resulted in significant alterations on amino acid constitution. With dietary accretion of Aureomycin, birds' breast muscle display pronounced decline on Ser, Gly, Ala and Try but companied with growth on Cys, Met, Arg and His. Interestingly, no

Table 5: The effects on the chemical component on breast muscle of Linwu ducks

Items	Control	Aureomycin	Probiotic I	Probiotic II
Moisture (%)	74.48±0.36^{a}	72.92±0.45^{b}	72.90±0.22^{b}	72.74±0.25^{b}
Fat (%)	1.65±0.06	2.47±0.39	2.11±0.25	1.83±0.21
Crude protein (%)	88.51±1.00^{b}	88.98±0.28^{b}	89.53±0.16^{ab}	90.57±0.27^{a}
Ash (%)	8.02±0.97	7.77±0.20	8.80±0.28	7.87±0.74
Asp	8.25±0.04^{ab}	8.12±0.08^{b}	8.31±0.09^{ab}	8.38±0.05^{a}
Thr	4.08±0.01	3.99±0.06	4.05±0.04	4.10±0.04
Ser	3.27±0.02^{a}	3.09±0.02^{b}	3.20±0.02^{a}	3.29±0.06^{a}
Glu	13.20±0.03^{b}	13.10±0.03^{b}	13.60±0.10^{a}	13.50±0.03^{a}
Gly	3.70±0.05^{b}	3.64±0.05^{c}	4.12±0.03^{a}	3.86±0.01^{b}
Ala	5.40±0.01^{a}	5.15±0.05^{b}	5.47±0.11^{a}	5.40±0.04^{a}
Cys	0.67±0.02^{b}	0.85±0.03^{a}	0.72±0.03^{b}	0.70±0.03^{b}
Val	4.21±0.01	4.06±0.01	4.15±0.09	4.21±0.01
Met	2.14±0.02^{b}	2.59±0.11^{a}	2.50±0.01^{a}	2.40±0.10^{a}
Ile	4.28±0.01^{ab}	4.20±0.01^{b}	4.28±0.07^{ab}	4.35±0.03^{a}
Leu	8.21±0.03	7.98±0.12	8.09±0.23	8.15±0.17
Tyr	3.19±0.01^{a}	3.10±0.01^{b}	3.22±0.02^{a}	3.21±0.02^{a}
Phe	3.83±0.01	3.69±0.01	3.71±0.07	3.75±0.05
Lys	7.69±0.02	7.61±0.01	7.74±0.10	7.76±0.05
His	2.60±0.05^{b}	2.79±0.01^{a}	2.53±0.07^{b}	2.60±0.03^{ab}
Arg	5.84±0.01^{b}	5.96±0.01^{a}	5.96±0.05^{a}	5.81±0.05^{b}

Probiotic I: diet supplemented with *Bacillus subtilis* 5×10^{8} CFU kg^{-1}; Probiotic II: diet supplemented with *Bacillus subtilis* 5×10^{10} CFU kg^{-1}; values in the same row not sharing a common superscript differ significantly ($p<0.05$)

significant alteration of amino acids was observed on leg muscle by diet supplemented with Aureomycin. When ducks fed with 5×10^{8} CFU kg^{-1} *Bacillus subtilis* there were dramatically augments on Glu, Gly, Met and Arg on breast muscle compared with control and increase on Ser, Glu, Gly, Ala and Tyr compared with Aureomycin treatment. Fed on diet with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* only demonstrated increase on Glu when it compared with control but Asp, Ser, Glu, Gly, Ala, Ile and Tyr when it compared with Aureomycin treatment. On breast mucle, fed on diet with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* enhanced increase on Val and Ile compared with both control and Aureomycin treatment.

Biochemical parameters provided valuable knowledge about physiological reactions occurring reflecting to the changing feedstuff and allowed us to have a better understanding on the effect of dietary on growth performance, production trait and immune reaction. Therefore, biochemical parameters were analyzed at the end of the experiment and summarized in Table 7. Except for Total Protein (TP), bare significance affected by either of the antibiotic or probiotic accretion diets were noted in Glucose, Albumin, Urea Nitrogen (UN), Triglyceride (TG), Cholesterol (CHO), Low-Density Lipoprotein (LDL) and High-Density Lipoprotein (HDL). Additionally, enzymes involving in material metabolism such as Glutamic-Pyruvic Transaminase (GPT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Lactic Dehydrogenase (LDH), Creatine Kinase (CK) and Gamma-Glutamyl Transpeptidase (GGT) showed no statistical significance among treatments.

Mortality experienced pronounced recession with *Bacillus subtilis* or Aureomycin addition. However, thymus and spleen relative to body weight combining with albumin in serum did not vary statistically among experimental treatments. The effects of treatments on immune related inflammatory cytokines in thymus and spleen were tested by RT-qPCR and showed in Table 8 and 9, respectively. In thymus, supplemented with *Bacillus subtilis* tended to markedly suppressed IL-2 compared with control and dampened IL-8 and IL-18 in comparison with Aureomycin treated group. Similarly, feeding *Bacillus subtilis* resulted in a marked reduction in *IL-2* gene expression in spleen compared with control. Differently, IL-8 did not show pronounced influencetion among treatments and treatment with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* addition as well as treatment with Aureomycin down regulated IL-18 compared with control.

Besides, these reatments did not induce any significant effect on *INF-α*, *INF-γ*, *IL-1* and *IL-10* gene expression both in thymus and spleen.

Poultry diet supplement with antibiotics bear the risk of increasing antibiotic-resistant pathogens. As an alternative replacement on post-antibiotics era, probiotics were supposed to be one of the most promising candidates, probiotics like *Bacillus subtilis* maintained a better microbial environment in the digestive tract of birds by suppressing the growth of harmful micro-organismin in gut thus improving the feed digestibility and efficient of utilization of feed which resulted in a better performance of animal. Also evidences accumulated

Table 6: The effects on the chemical component on leg muscle of Linwu ducks

Items	Control	Aureomycin	Probiotic I	Probiotic II
Moisture (%)	74.60±0.10	73.78±0.10	73.82±0.25	73.66±1.18
Fat (%)	2.73±0.33	3.87±0.26	3.02±0.22	3.56±0.84
Crude protein (%)	87.21±0.27^{b}	87.89±0.45^{b}	88.40±0.65^{ab}	89.91±0.77^{a}
Ash (%)	6.09±0.17	6.51±1.00	7.06±0.30	6.19±0.37
Asp	7.97±0.14	7.91±0.03	8.10±0.11	8.09±0.02
Thr	3.96±0.05	3.90±0.03	4.00±0.07	3.97±0.03
Ser	3.14±0.06	3.10±0.03	3.16±0.05	3.07±0.03
Glu	13.00±0.28	12.70±0.12	13.15±0.20	13.10±0.03
Gly	3.48±0.04	3.45±0.04	3.51±0.06	3.55±0.05
Ala	5.15±0.02	5.06±0.03	5.17±0.07	5.11±0.04
Cys	0.80±0.03	0.84±0.03	0.70±0.01	0.76±0.08
Val	3.93±0.05^{b}	3.91±0.01^{b}	3.96±0.05^{ab}	4.06±0.01^{a}
Met	2.50±0.13	2.56±0.12	2.45±0.06	2.48±0.25
Ile	4.11±0.07^{b}	4.13±0.06^{b}	4.16±0.05^{ab}	4.33±0.01^{a}
Leu	7.84±0.11	7.86±0.12	7.84±0.12	7.97±0.05
Tyr	3.24±0.06	3.27±0.01	3.28±0.06	3.17±0.06
Phe	3.63±0.03	3.67±0.04	3.63±0.06	3.73±0.01
Lys	7.48±0.10	7.43±0.05	7.53±0.12	7.62±0.06
His	2.40±0.04	2.33±0.02	2.43±0.02	2.34±0.08
Arg	5.56±0.07	5.49±0.07	5.61±0.08	5.65±0.06

Probiotic I: diet supplemented with *Bacillus subtilis* 5×10^{8} CFU kg^{-1}; Probiotic II: diet supplemented with *Bacillus subtilis* 5×10^{10} CFU kg^{-1}; values in the same row not sharing a common superscript differ significantly ($p<0.05$)

Table 7: The effects on the serum biochemical parameters

Items	Control	Aureomycin	Probiotic I	Probiotic II
GLU (mmol L^{-1})	11.74±0.4800	11.90±0.330	11.19±0.540	12.08±0.630
UN (mmol L^{-1})	0.92±0.0420	0.91±0.057	0.86±0.074	0.79±0.035
TG (mmol L^{-1})	0.73±0.0800	0.75±0.080	0.78±0.120	0.84±0.170
CHO (mmol L^{-1})	5.09±0.2900	5.09±0.020	5.12±0.270	5.48±0.210
LDL (mmol L^{-1})	0.99±0.1000	1.02±0.060	1.07±0.120	1.08±0.130
HDL (mmol L^{-1})	7.80±1.6200	10.22±0.170	6.93±0.200	7.28±1.390
TP (g L^{-1})	32.14±1.0100^{b}	33.74±0.140^{ab}	33.91±1.340^{ab}	34.37±1.280^{a}
ALB (g L^{-1})	12.06±0.2800	13.63±1.430	13.09±0.410	13.61±1.060
ALP (U L^{-1})	196.67±3.3400	184.11±12.84	187.11±13.09	164.33±8.000
GPT (U L^{-1})	26.89±2.6300	30.67±2.190	25.22±2.350	32.78±3.820
AST (U L^{-1})	33.56±5.3900	36.22±7.670	28.67±5.510	33.22±4.950
LDH (U L^{-1})	494.22±76.600^{ab}	505.83±63.30^{ab}	364.61±2.190^{b}	622.50±43.54^{a}
CK (U L^{-1})	460.89±127.90	321.83±63.77	408.06±67.53	287.67±46.74
GGT (U L^{-1})	3.35±0.3800	4.34±0.600	4.33±0.690	4.01±0.470

Glucose (GLU), Total Protein (TP), Albumin (ALB), Urea Nitrogen (UN), Triglyceride (TG), Cholesterol (CHO), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), Glutamic-Pyruvic Transaminase (GPT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Lactic Dehydrogenase (LDH), Creatine Kinase (CK) and Gamma-Glutamyl Transpeptidase (GGT). Probiotic I: diet supplemented with *Bacillus subtilis* 5×10^{8} CFU kg^{-1}; Probiotic II: diet supplemented with *Bacillus subtilis* 5×10^{10} CFU kg^{-1}; values in the same row not sharing a common superscript differ significantly ($p<0.05$)

Table 8: The effects on gene expressions of inflammatory cytokines in thymus

Items	Control	Aureomycin	Probiotic I	Probiotic II
IFN-α	1.29±0.32	1.40±0.38	0.76±0.10	0.73±0.11
IFN-γ	0.69±0.13	1.16±0.20	1.08±0.33	1.11±0.36
IL-1β	1.42±0.39	1.14±0.63	0.68±0.16	1.24±0.32
IL-2	1.24±0.21^{a}	1.03±0.14^{ab}	0.80±0.12^{b}	0.69±0.04^{b}
IL-8	1.30±0.30^{ab}	1.80±0.32^{a}	0.77±0.10^{b}	0.90±0.14^{b}
IL-10	0.58±0.19	0.49±0.12	0.26±0.06	0.41±0.12
IL-18	1.03±0.07^{ab}	1.38±0.22^{a}	0.98±0.10^{b}	0.87±0.12^{b}

Table 9: The effects on gene expressions of inflammatory cytokines in spleen

Items	Control	Aureomycin	Probiotic I	Probiotic II
IFN-α	1.10±0.13	0.95±0.27	1.16±0.21	0.97±0.20
IFN-γ	1.11±0.29	0.93±0.17	1.43±0.19	1.43±0.25
IL-1β	1.22±0.18	0.85±0.21	0.91±0.18	1.19±0.35
IL-2	1.08±0.13^{a}	0.87±0.14^{ab}	0.73±0.06^{b}	0.58±0.08^{b}
IL-8	1.08±0.12	0.69±0.13	0.90±0.17	0.86±0.22
IL-10	1.28±0.16	1.01±0.18	0.82±0.28	0.58±0.30
IL-18	1.03±0.09^{a}	0.46±0.10^{c}	0.78±0.07^{ab}	0.70±0.11^{bc}

Probiotic I: diet supplemented with *Bacillus subtilis* 5×10^{8} CFU kg^{-1}; Probiotic II: diet supplemented with *Bacillus subtilis* 5×10^{10} CFU kg^{-1}; values in the same row not sharing a common superscript differ significantly ($p<0.05$)

suggested *Bacillus subtilis* could produce a broad range of enzymes such as β-glucanases, xylanases, keratinase, alkaline protease, fibrinolytic enzymes which had a favor to improve feed utilization for growth profile (Wang and Shih, 1999; El-Helow and El-Ahawany, 1999; Joo *et al*., 2002; Ko *et al*., 2004; Yuan *et al*., 2005). However, as a novel strain characterized by yielding lysine and first applied in duck additive, high lysine-yielding *Bacillus subtilis* did show marked positive impact on average date gain, average feed intake and feed efficiency in the current research, even though the lysine level of diets supplemented with *Bacillus subtilis* was lower 15% than the other two groups. Thus, it was needed to further detect the fluctuation of intestinal bacteria and digestive enzymes after *Bacillus subtilis* addition in the future research which was believed to give us a clear clue for understanding earlier consequence. What should be noted was mortality experienced pronounced recession as a consequence of *Bacillus subtilis* and Aureomycin supplement which may be associated with *Bacillus subtilis* and Aureomycin which could control or limit the growth and colonization of numerous pathogenic and nonpathogenic species on gut, leading to a low risk for intestinal disease incident.

In slaughter performance, fed on high lysine-yielding *Bacillus subtilis* markedly improved breast meat and leg meat relative weight but not abdominal fat and other carcass trait. It appeared that birds fed on this kind of *Bacillus subtilis* supplemented diets had utilised much more nutrients and energy to deposit breast and leg muscle than muscles in other parts and abdominal fat and it was possible that lysine produced by *Bacillus subtilis* could be partly responsible for this results. As confirmed by many studies on ducks and chickens, a high dietary lysine level could improve breast meat yield (Bastianelli *et al*., 2007; Xie *et al*., 2009). Lys adequacy was crucial to breast meat yield even though body weight was unaffected (Moran *et al*., 1990). Breast meat, as the most valuable part of the poultry carcass was more sensitive to dietary lysine deficiency than other skeletal meats and the higher response to Lys of the pectoralis major could be partly attributed to the type and composition of muscle fiber (Tesseraud *et al*., 1996; Berri *et al*., 2008; Dozier *et al*., 2008). Leclercq (1998) pointed out lysine deficiency specifically reduces pectoralis major growth these muscles had Type IIb fibers. In contrast, proportions of sartorius and anterior latissimus dorsi which contained both type I and IIb fibers were less influenced by lysine. Tesseraud *et al*. (1999) experiment, lysine deficiency decreased the weight of the pectoralis major (to 55% decrease), gastrocnemius (to 45% decrease) and sartorius muscles (to 30% decrease) which indicated that lysine had a greater effect on pectoralis major muscle (a breast muscle) and gastrocnemius (a muscle in leg) (Tesseraud *et al*., 2001).

According to results from the test on chemical composition of breast and leg muscle, *Bacillus subtilis* had positive influence on the content of the protein but ash and fat were unaffected which suggested the accretion of breast and leg muscle weight were mainly derived from the argument of protein. Probiotics might affect the muscle protein mass by influencing on protein metabolism as was reported that a better retention of nutrients especially for nitrogen and improved protein efficiency ratio were observed in probiotic fed birds (Khaksefidi and Ghoorchi, 2006). Additionally, amino acids were major regulators of protein metabolism (Hocquette *et al*., 2007), researchers speculated that lysine synthesis by *Bacillus subtilis* in the study had closely relationship with muscle protein accretion. Previous research illustrated that lysine supplementation in a lysine-deficient diet otherwise balanced in terms of other amino acids had been shown to greatly modify the amounts of protein synthesized and degraded in chickens with a particularly drastic effect observed on breast muscle development (Tesseraud *et al*., 2009). Thus, greater protein deposition was expected to achieve in ducks when the ratio of protein synthesis to protein degradation was maximized by a optimal dietary lysine level (Urdaneta-Rincon and Leeson, 2004).

Given the protein was made up by amino acids, the content increase of protein implied the alteration on amino acides. Researchers therefore, made further determination on the content of each amino acid. In the experiment both *Bacillus subtilis* and Aureomycin played a regulatory

role in amino acid of breast muscle but showed different results. Additionally, consequence made by *Bacillus subtilis* in different additive level also differed. Compared with the breast muscle, muscle from leg showed less variation, only Val and Ile increased were observed in 5×10^{10} CFU kg^{-1} *Bacillus subtilis* supplementation. Unexpectedly, neither breast nor leg muscle showed marked alteration on lysine by fed on diet supplemented with these high lysine yields *Bacillus subtilis*.

Related mechanism which took to elucidate the modifications on carcass amino acid composition was rarely been reported. What researchers knew was that alterations on carcass amino acid proportions were the results of processes of anabolism and catabolism which occurred simultaneously. Lysine as the most abundant amino acid in skeletal muscle proteins was assumed to have close interrelationship with other amino acids (Tesseraud *et al*., 2009). As revealed by Sklan and Noy (2004) when lysine supply limited growth, catabolism of lysine was very low but catabolism of the other amino acids were increased because lysine supply was apparently limiting protein synthesis. In contrast, catabolism of the other amino acids decreased initially with increasing dietary lysine as muscle synthesis increased before decreasing to a plateau whereas lysine catabolism continued to increase linearly with increasing dietary concentrations. Thus, researchers had the reason to believe that a relative increase on other amino acid proportion companied with the increase of lysine produced by *Bacillus subtilis*.

Biochemical parameters usually reflected host physiological status and evaluation of biochemical profile provided valuable information about the metabolic variation to feed stuff (Toghyani *et al*., 2010). Fed on diet supplemented with *Bacillus subtilis* had created a numerical increase on AST and ALT content in plasma but not reached to significant elevation. As was well-known, significant increase in AST and ALT content in plasma were usually considered as indications of liver damage due to the leakage of these enzymes from the liver cytosol into the blood (Ozer *et al*., 2008). However, there was barely a report whether the increase AST and ALT in certain range, below the pathological level was reflect something meaning on metabolic, although AST and ALT were indicated to exert functions with transamination of amino acids and operation of keto acids which were probably fed into Tricarboxylic Acid cycle (TCA) for oxidation or used for other amino acid synthesis (El-Demerdash *et al*., 2004; Ozer *et al*., 2008). In addition, blood UN was known to be correlated with protein catabolism and changes in serum UN concentrations could reflect the whole body status of amino acid metabolism and utilization in animals (El-Demerdash *et al*., 2004). Results presented in the experiment did not show UN elevation on *Bacillus subtilis* treatment group, even displayed a marginal drop which may suggest a potential enhancement on synthesis of amino acids and protein in animals. Strongly supporting this viewpoint, plasma total protein concentrations significantly increased in ducks treated with *Bacillus subtilis*. Liver was the primary source of serum proteins. Total serum protein content may also reflect hepatic protein metabolic status in response to dietary treatments (Khan *et al*., 2006). Thus, dietary supplement of *Bacillus subtilis* may have dietary amino acids for the preferred precursor of hepatic protein synthesis, eventually, improved whole body protein anabolism.

No significant differences were observed in concentration of triglycerides, cholesterol, LDL and HDL in the serum profile. The literature concerning the benefits of probiotic preparation on serum lipid profile had elicited inconsistent and conflicting findings. Triglycerides did not show significant difference by Li *et al*. (2011) when supplemented with *Bacillus subtilis*. Also, no significant differences were observed in triglycerides, HDL, LDL and VLDL levels between treatments with probiotic and control at the age of 21 days broiler age (Ashayerizadeh *et al*., 2011). There was however, a great deal of evidences that probiotics supplementation reduced triglycerides, cholesterol and LDL concentration in serum (Santoso *et al*., 1995; Mohan *et al*., 1996; Kalavathy *et al*., 2003; Panda *et al*., 2006). Several mechanisms of action had been proposed. Probiotic could utilize the cholesterol presented in the gastrointestinal tract for their own metabolism or disintegrating bile salts and de-conjugate production of digestive enzymes (Gilliland *et al*., 1985; Mohan *et al*., 1995; Abdulrahim *et al*., 1996). The usage of probiotics could also reduced pH in the intestinal tract. Solvability of non-conjugate bile acids were lowered at a low pH and consequently they were absorbed less from the intestine and were excreted more in the feaces (Ashayerizadeh *et al*., 2011).

Control of avian diseases and food borne pathogens remained a high priority in the absent antibiotic era (La Ragione and Woodward, 2003). There was a growing body of literature to report that *Bacillus subtilis* modulated the immune responses and exerted profound protective effects against pathogens (La Ragione and Woodward, 2003; Rajput *et al*., 2013). In the research, thymus and spleen relatived to body weight combining with albumin in serum did not vary statistically among experimental treatments although, thymus and spleen relatived to body weight numerically increased in

Bacillus subtilis treatment. In agreement with (Tang *et al*., 2012) research, no differences were found on the relative weights of spleen and thymus by feeding *Bacillus subtilis*-fermented cottonseed meal. Another supportive findings of (Chen *et al*., 2009) described that *Bacillus subtilis* and *Saccharomyces cerevisiae* mixed fermented feed had no significant effect on spleen and thymus relative weight. While by Awad *et al*. (2009) research, the absolute and relative weights of spleen and thymus tended to be significantly greater for the *Bacillus subtilis*-supplemented group.

Researchers subsequently combined RT-qPCR to analyse the expression of a subset of duck genes implicated in the host immune in spleen and thymus which were the main sites to exert immune function. The current findings suggested that *Bacillus subtilis* markedly attenuated the IL-2 expression both in spleen and thymus in comparison with control. However, a contrary tendency that *Bacillus subtilis* could support to enhance the production of inflammatory cytokine IL-2 in serum was showed by Rajput *et al*. (2013) research on Shaoxing duck, another local breed in China. Additionally significant inhibition on IL-10 by Rajput *et al*. (2013) research was not observed in the study. IL-2 was an inflammatory cytokine which played an important role in endorse cell mediated immunity related to Th1 cells (Rajput and Li, 2012). IL-10 was one of the main anti-inflammation factors which was crucial in IL-1β, TNF-α and IL-6. IL-1β and IL-18 were structurally homologous proteins that played critical roles in initiating inflammation (Laurent *et al*., 2001). In the study, IL-1β kept constant both in thymus and spleen but supplemented with 5×10^{10} CFU kg^{-1} *Bacillus subtilis* inhibited the expression of IL-18 in spleen.

Cytokine was produced in response to various infections. Overproduction or aberrant regulation of cytokines may harm the host by inducing tissue injury or alteration of the immune system. Infected poultry obviously increased which could be both an indication of the induction of inflammation and an immune response against the pathogenic bacteria and parasite. In a previous report, a significant enhancement of IL-2 transcript level was observed in the spleen and intestine following primary and secondary infections with *E. acervulina* (Yun *et al*., 2000). IL-1β, IL-6 and IL-8 levels were significantly upregulated in the intestinal tissues and in the livers of the Salmonella infected birds (Kaiser *et al*., 2000; Withanage *et al*., 2004). Hong and Noh (2006) research, transcripts of the pro-inflammatory cytokines IFN-α, IL-1β, IL-6 and IL-17 were increased up to 2020 fold following primary infection of *Eimeria acervulina* and *Eimeria tenella* primary.

Previous studies showed that *Bacillus subtilis* was an effective competitive exclusion agent for use in poultry to control avian pathogenic *Escherichia coli*, *Salmonella enteric*, *Streptococcus* sp. *Campylobacter* sp. and *Clostridium perfringens* in young chickens (La Ragione and Woodward, 2003; Teo and Tan, 2006). Thus, the capable of *Bacillus subtilis* inhibiting pathogens may be effective in controling and suppressing the expression of inflammatory cytokines which was responsed to and influenced by pathogens, resulting in less compromise to host immune systems and health. As a confirmed support, the mortality of duck which fed on *Bacillus subtilis* was markedly lower than control.

CONCLUSION

It was concluded that dietary supplementation of these high lysine-yielding *Bacillus subtilis* showed promising effects as alternatives for antibiotics due to it had favors in growth performance and slaughter performance and depression on inflammatory cytokines overproduction which was stimulated by pathogens.

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