# Effect of Supplementation of *Bacillus subtilis* LS 1-2 Grown on Citrus-juice Waste and Corn-soybean Meal Substrate on Growth Performance, Nutrient Retention, Caecal Microbiology and Small Intestinal Morphology of Broilers

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**ABSTRACT :** A feeding trial was conducted to investigate the effect of dietary supplementation of *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean substrate on growth performance, nutrient retention, caecal microbial population and intestinal morphology in broilers. Three hundred twenty d-old Ross chicks were randomly allotted to 4 treatments on the basis of BW in a randomized complete block design. Each treatment had 4 replicates of 20 chicks in each. Experimental diets were fed in 2 phases, starter (d 0 to 21) and finisher (d 21 to 35). Dietary treatments were; negative control (NC: basal diet without any antimicrobial), positive control (PC: basal diet added with 20 mg/kg Avilamycin), basal diet added with 0.30% *Bacillus subtilis* LS 1-2 grown on corn-soybean substrate (P1), and basal diet added with 0.30% *Bacillus subtilis* LS 1-2 grown on citrus-juice waste substrate (P2). Overall BW gain, feed intake and FCR were better (p<0.05) in PC, P1 and P2 treatments as compared to NC. Moreover, overall BW gain and FCR in PC and P2 treatments were greater than P1. Retention of CP, GE (d 21, d 35) and DM (d 35) were increased (p<0.05) in treatments PC, P1 and P2 compared with NC. At d 35, caecal *Clostridium* and *Coliform* counts were lower (p<0.05) in treatments PC, P1 and P2 than NC. Moreover, *Clostridium* and *Coliform* counts in treatment PC was lower (p<0.05) than P1. Villus height and villus height to crypt depth ratio in both duodenum and ileum were increased (p<0.05) in treatments PC, P1, P2 as compared to NC. However, retention of nutrients, caecal microbial population and intestinal morphology remained comparable among treatments P1 and P2. It is concluded that *Bacillus subtilis* LS 1-2 inclusion at 0.30% level had beneficial effects on broilers' growth performance, nutrient retention, caecal microflora and intestinal morphology. Additionally, citrus-juice waste can be used as substrate for growth of probiotic *Bacillus subtilis* LS 1-2. (**Key Words :** *Bacillus subtilis* LS 1-2, Citrus-juice Waste, Corn-soybean Meal, Avilamycin, Performance, Broiler)

## INTRODUCTION

The growing prevalence of antibiotic resistance due to its extreme use in poultry production is an emerging public health issue. This results into severe restriction or total ban on the use of antibiotics in animal and poultry industry in many countries. Consequently, an urgent need is felt globally to look for alternatives to antibiotics for maintaining health and performance under commercial conditions. Probiotics, a microbial culture broth containing antimicrobial compounds, are gaining attention as alternative to antibiotics because of their safety and potentials for improving animal health (Fuller, 1989). Several microorganisms, under the name of "probiotics", have been proposed and used in a wide range of field trials, with the objective to improve growth performance (Khaksefidi and Ghoorchi, 2006; Mountzouris et al., 2007; Awad et al., 2009; Shim et al., 2010), nutrient retention (Li et al., 2008; Mountzouris et al., 2010), caecal microbial balance (Teo and Tan, 2007; Mountzouris et al., 2010) and intestinal morphology (Awad et al., 2009, 2010). Due to the numerous applications of probiotics, attempts are being made to screen and identify new probiotic strains with particular characteristics that would be appropriate for specific applications. Among several bacterial species used as probiotics, spore forming *Bacillus spp.* has been

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preferred due to high resistance of its spores to harsh environment and long term storage at ambient temperature (Fuller, 1989; Hong et al., 2005). However, there is very limited information on the effects of *Bacillus subtilis* on nutrient retention, caecal microbial population and intestinal morphology in broilers.

Economy of probiotic products depends upon method of fermentation and type of substrate used for fermentation. Several studies have reported that probiotic produced by solid substrate fermentation (SSF) is cost-effective and environment-friendly (Adams et al., 2002; El-blendary, 2006; Shim et al., 2010). Cost of production of probiotics can be reduced by use of agro-industrial by-products as a substrate for the growth of probiotic microbes. Citrus-juice waste (CJW) is a by-product of juice industry having characteristics required for its use as substrate for the growth of probiotics during fermentation (Contreras Esquivel et al., 1999). World production of citrus fruits is near 90 million tones per year (Marin et al., 2007). Most of these fruits is squeezed to juice and by-product including peel, segment membrane, and other parts are considered as CJW (Willkin et al., 2007), which contain high amount of pectin. But due to high processing cost, use of CJW as animal feed is restricted, so most of CJW is dumped into ocean. *Bacillus spp.* is well known for their ability to produce pectinase, by using pectin in citrus peel as the sole carbon source. At the same time, citrus and soy pectin have a structural similarity. It was anticipated that *Bacillus* strains isolated from soybean paste would be able to use the pectin in citrus peel as a sole carbon source (Matsumoto et al., 2000). By taking advantage of this, CJW can be utilized as substrate for the growth of probiotic, *Bacillus subtilis*. This can help to improve the economy of probiotic production and control environmental pollution. Therefore, objectives of this study were to investigate the effect of *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean substrate on growth performance, nutrient retention, caecal microbiology and intestinal morphology in broiler chickens.

## MATERIALS AND METHODS

The protocol for this experiment was approved and birds were cared according to the guidelines of the Institutional Animal Care and Use Committee of Kangwon National University, Chunchon, Republic of Korea.

### Preparation of probiotic products

Probiotic microbe, *Bacillus subtilis* LS 1-2 isolated from natto (fermented soy bean) was maintained in the laboratory at -80°C as stock culture. A culture broth (CB) medium containing 6% corn steep liquor, 4% molasses, 0.3% yeast extract, 0.5% $KH_2PO_4$ and 0.25% $K_2HPO_4$ in distilled water was prepared and autoclaved before being used. Two ml of stock culture was added to 2 L of autoclaved CB and incubated at 37°C at pH 7.0 for 48 h. The final microbial count of *Bacillus subtilis* LS 1-2 used as starter was $10^9$cfu/ml.

The *Bacillus subtilis* LS 1-2 grown on CB was used as starter to produce probiotic product by solid substrate fermentation (SSF). Citrus-juice waste and corn-soybean meal were used as substrates and water was added to maintain a 30% moisture level followed by pasteurization. Then the substrates (13 kg) were inoculated with 2 L of starter and fermented for 7 d at 32°C and at pH 7.0. After 7 d of fermentation the microbial biomass was dried in a forced-air drying oven at 40°C for 72 h. The chemical composition and microbial count of probiotic product (*Bacillus subtilis* LS 1-2) grown on citrus-juice waste and corn-soybean meal substrate is presented in Table 1.

### Birds, diets and management

Three hundred twenty d-old Ross broilers were obtained from a local hatchery and randomly allotted to 4 treatments on the basis of initial BW in a randomized complete block design. Each treatment had 80 broilers arranged in 4 replicates of 20 broilers each. Dietary treatments were; negative control (NC: basal diet without any antimicrobial), positive control (PC: basal diet added with 20 mg/kg Avilamycin), basal diet added with 0.30% *Bacillus subtilis* LS 1-2 grown on corn-soybean meal substrate (P1) and basal diet added with 0.30% *Bacillus subtilis* LS 1-2 grown on citrus-juice waste substrate (P2). In present study we supplemented 0.30% *Bacillus subtilis* LS 1-2 because efficacy for most probiotics in animals could be demonstrated with the daily intake of $10^8$ and $10^9$ microorganisms (Patterson and Burkholder, 2003; Shim et al., 2010). The inclusion of probiotic product at 0.30% of the diet resulted in the total microbial count more than $10^9$ cfu/kg diet. Basal diet was in mash form and was formulated for starter (d 0 to 21) and finisher (d 21 to 35) periods and its composition is shown in Table 2. The antibiotic (Avilamycin) was added to the diet at the expense of corn, whereas probiotic products were added to the starter and finisher diets by equally replacing corn and

**Table 1.** Chemical composition and microbial counts (cfu/g) of probiotic products

| Item | Probiotic CSM[1] | Probiotic CJM[2] |
|---|---|---|
| pH | 6.12 | 6.38 |
| DM (%) | 93.16 | 92.76 |
| CP (%) | 32.15 | 29.34 |
| *Bacillus subtilis* (cfu/g) | $1.0 \times 10^8$ | $2.8 \times 10^8$ |

[1] Probiotic CSM: *Bacillus subtilis* 1-2 grown on corn-SBM medium.
[2] Porbiotic CJM : *Bacillus subtilis* 1-2 grown on citrus juice waste medium.

soybean meal. All the nutrients met or exceeded the nutrient requirements as recommended by NRC (1994) as shown in Table 2.

The birds were housed in rice hull-covered floor pens. Each pen was provided with a self-feeder and hanging bell drinker to allow free access to feed and water. The house temperature was maintained at 34°C for the first 5 d and then gradually reduced according to normal managemental practices, until a temperature of 23°C was achieved. Lighting was provided for 23 h/d.

**Sampling and measurements**

The birds were weighed individually and pen feed

**Table 2.** Ingredient and chemical composition of basal diets (as-fed basis)[1]

|  | Starter (d 0 to 21) | Finisher (d 21 to 35) |
|---|---|---|
| Ingredient (%) | | |
| Corn | 49.25 | 51.66 |
| Wheat | 2.00 | 5.00 |
| Fish meal | 2.00 | - |
| Soybean meal (45%) | 34.18 | 31.07 |
| Corn gluten | 8.00 | 8.00 |
| Animal fat | 1.52 | 1.26 |
| DL-methionine (88%) | 0.24 | 0.14 |
| Choline-chloride (50%) | 0.10 | 0.10 |
| Tri-calcium phosphate | 1.61 | 1.57 |
| Limestone | 0.60 | 0.70 |
| Salt | 0.30 | 0.30 |
| Mineral premix[2] | 0.10 | 0.10 |
| Vitamin premix[3] | 0.10 | 0.10 |
| Calculated chemical composition | | |
| ME (kcal/kg) | 3,200 | 3,200 |
| CP (%) | 22.00 | 20.00 |
| Ca (%) | 1.00 | 0.90 |
| Av. P (%) | 0.45 | 0.40 |
| Lys (%) | 1.25 | 1.11 |
| Met+Cys (%) | 0.95 | 0.80 |
| Thr (%) | 0.86 | 0.78 |
| Trp (%) | 0.28 | 0.25 |

[1] Dietary treatments were NC = Negative control, basal diet without antibiotic and probiotic; PC = Positive control, basal diet added with 20 mg/kg Avilamycin, P1 = basal diet added with 0.30% *Bacillus subtilis* grown on corn-soybean meal medium; P2 = basal diet added with 0.30% *Bacillus subtilis* grown on citrus-juice waste medium. Avilamycin was added to the starter and finisher diets at the expense of corn, and probiotic products were added to the starter and finisher diets by equally replacing corn and soybean meal.
[2] Supplied per kilogram of diet: 45 mg Fe, 0.25 mg Co, 50 mg Cu, 15 mg Mn, 25 mg Zn, 0.35 mg I, 0.13 mg Se.
[3] Supplied per kilogram of diet: 9,000 IU vitamin A, 1,800 IU vitamin $D_3$, 30 IU vitamin E, 1.5 mg vitamin $K_3$, 1.5 mg vitamin $B_1$, 5 mg vitamin $B_2$, 4 mg vitamin $B_6$, 0.025 mg vitamin $B_{12}$, 15 mg pantothenic acid, 35 mg niacin, 0.15 mg biotin, 0.65 mg folic acid, 12 mg antioxidant.

intake was noted at the end of each phase to calculate BW gain and feed conversion ratio (FCR) for starter and finisher phases. Two digestibility trials were conducted during the last week of starter and finisher phases to determine retention of DM, CP and GE. From d 14 (starter) and d 28 (finisher) onwards, 2 birds from each replicate were allocated to individual cage (one bird/cage), to facilitate collection of excreta samples. The starter and finisher diets containing 0.25% chromic oxide as an indigestible marker were fed from d 14 and d 28 onwards during each phase of digestibility trials. Excreta samples (about 50 g/d per bird) were collected from each bird for 48 h. Then excreta samples collected for 2 d were pooled and dried using a forced-air drying oven at 60°C and stored for the analysis of DM, CP and GE. Additionally, 8 birds per treatment (2 birds from each pen) were slaughtered at the end of each phase (d 21 and d 35) to study the microflora in caecal contents. The samples of caecal contents were collected in sterile plastic bottles and were immediately placed on ice until the analysis was conducted later on the corresponding day. The samples of intestinal segment (d 35) were also collected from the region of duodenum, jejunum and ileum and after removal of its contents flushed with physiological saline and submerged in a fixative solution (0.1 M collidine buffer, pH 7.3) containing 3% glutaraldehyde, 2% para-formaldehyde and 1.5% acrolein and then brought to laboratory to study morphological changes.

**Chemical and microbial analyses**

Dry matter and CP analysis of experimental diets and excreta samples were done according to the AOAC International (1995) methods. Gross energy of experimental diets and excreta was measured by a bomb calorimeter (Model 1216, Parr Instrument Co., Molin. IL), while chromium was measured with an atomic absorption spectrophotometer (Model AA-680G, Shimadzu, Japan) according to the procedure of Fenton and Fenton (1979).

The caecal microflora was analyzed by using culture technique as described previously (Choi et al., 2009). The microbial groups analyzed were total anaerobic bacteria (Tryptic soy agar), *Bifidobacterium spp.* (MRS agar+0.02% $NaN_3$+0.05% L-cystine hydrochloride monohydrate), *Clostridium spp.* (Tryptose sulphite cycloserine agar) and coliforms (violet red bile agar). The microbial populations were log transformed before statistical analysis. The microbiological assay of probiotic (*Bacillus subtilis* LS 1-2) products was also carried out by using plate count agar (No. 247940, Difco, Detroit, MI) technique.

**Small intestine morphology**

Three cross-sections for each intestinal sample were prepared after staining with azure A and eosin using standard paraffin embedding procedures. A total of 10 intact,

**Table 3.** Effects of supplementation of antibiotics and *Bacillus subtilis* LS 1-2 on growth performance in broilers during starter (d 0-21), finisher (d 21-35), and overall period (d 0-35)

| | NC | PC | P1 | P2 | SEM[3] | p-value |
|---|---|---|---|---|---|---|
| Starter (d 0-21) | | | | | | |
| Weight gain (g) | 614[c] | 657[a] | 645[b] | 650[ab] | 4.49 | <0.001 |
| Feed intake (g) | 988[b] | 1,009[a] | 1,002[ab] | 1,005[ab] | 3.23 | 0.095 |
| FCR | 1.61[a] | 1.53[b] | 1.55[b] | 1.55[b] | 0.01 | 0.002 |
| Finisher (d 21-35) | | | | | | |
| Weight gain (g) | 1,037[d] | 1,170[a] | 1,103[c] | 1,130[b] | 12.84 | <0.001 |
| Feed intake (g) | 2,007[c] | 2,121[a] | 2,037[b] | 2,060[b] | 11.59 | <0.001 |
| FCR | 1.93[a] | 1.81[c] | 1.85[b] | 1.82[bc] | 0.01 | <0.001 |
| Overall (d 0-35) | | | | | | |
| Weight gain (g) | 1,651[d] | 1,827[a] | 1,748[c] | 1,779[b] | 16.96 | <0.001 |
| Feed intake (g) | 2,994[c] | 3,130[a] | 3,039[b] | 3,065[b] | 14.16 | <0.001 |
| FCR | 1.81[a] | 1.71[c] | 1.74[b] | 1.72[c] | 0.01 | <0.001 |

NC = basal diet without any antimicrobial. PC = basal diet added with 20 mg/kg Avilamycin.
P1 = *Bacillus subtilis* LS 1-2 grown on corn-soybean meal medium. P2 = *Bacillus subtilis* LS 1-2 grown on citrus-juice waste medium.
[1] Standard error of means. [abcd] Values with different superscripts in the same row differ significantly (p<0.05).

well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height was measured from the tip of the villus to the villus crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. All morphological measurements (villus height and crypt depth) were made in 10-μm increments by using an image processing and analysis system (Optimus software version 6.5, Media Cybergenetics, North Reading, MA).

**Statistical analysis**

Data generated in present study were subjected to statistical analysis using the GLM- one-way analysis of variance test using the SAS (1996) statistical software package. The pen was used as the experimental unit for the analysis of growth performance (BW, feed intake and FCR) and nutrient retention parameters, whereas individual broiler was used as experimental unit for analysis of caecal microbial population and intestinal morphology parameters. When significant differences (p<0.05) were noticed among treatment means, they were separated by using Duncan's multiple range tests.

**RESULTS**

**Growth performance**

Broilers BW did not differ between experimental treatments on d 1 (i.e. beginning of experiment). However, during the starter, finisher and overall study period birds fed PC, P1 and P2 diets showed greater BW gain (p<0.001; Table 3) and better FCR (p<0.01) than birds fed NC diet. Whereas, during finisher phase and overall study period

feed intake of bird fed PC, P1 and P2 diets was greater (p<0.001) than birds fed NC diet. Birds fed PC diet showed higher BW gain and feed intake than birds fed P1 and P2 diets during finisher phase and overall study period. Moreover, during overall study period BW gain and FCR of birds fed P2 were better (p<0.05) than birds fed P1 diet.

**Nutrient retention**

Retention of DM (d 35), CP and GE (d 21 and d 35) was improved (p<0.05; Table 4) in birds fed PC, P1 and P2 diets than birds fed NC diet. Whereas, retention of CP (d 21, d 35), and GE (d 35) in birds fed PC diet was higher than P1 diet but remained comparable with birds fed P2 diet. However, nutrient retention of DM, CP and GE remained comparable (p>0.05) among treatment P1 and P2 (d 21 and d 35).

**Microbial population**

The caecal microbial population did not differ (p>0.05: Table 5) between the experimental treatments at d 21. However at d 35, birds fed PC and probiotics (P1 and P2) diets showed decrease (p<0.05) in caecal *Clostridium* and *Coliform* counts as compared to birds fed NC diet. Additionally, at d 35, *Clostridium* and *Coliform* counts of birds fed PC diet was lower (p<0.05) than birds fed P1 and P2 diets. Caecal microbial count of birds fed P1 and P2 diets remained comparable at d 21 and d 35.

**Intestinal morphology**

At d 35, birds fed antibiotic (PC) and probiotics (P1 and P2) diet had increased (linear, p<0.05; Table 6) villus height and villus height to crypt depth ratio in duodenum and ileum as compared to birds fed NC diet. However, duodenal

**Table 4.** Effects of supplementation of antibiotics and *Bacillus subtilis* LS 1-2 on apparent nutrient retention (%) in broilers (d 21 and 35)

|  | NC | PC | P1 | P2 | SEM[1] | p-value |
|---|---|---|---|---|---|---|
| d 21 |  |  |  |  |  |  |
| DM | 74.93 | 76.31 | 75.97 | 76.21 | 0.27 | 0.241 |
| GE | 75.86[b] | 78.91[a] | 77.84[a] | 78.39[a] | 0.36 | 0.002 |
| CP | 60.51[c] | 65.19[a] | 62.26[b] | 62.65[b] | 0.52 | 0.002 |
| d 35 |  |  |  |  |  |  |
| DM | 75.16[b] | 79.22[a] | 77.72[a] | 79.17[a] | 0.51 | 0.002 |
| GE | 73.71[c] | 79.33[a] | 76.93[b] | 77.53[ab] | 0.60 | <0.001 |
| CP | 59.56[c] | 64.70[a] | 62.70[b] | 63.67[ab] | 0.54 | <0.001 |

NC = basal diet without any antimicrobial. PC = basal diet added with 20 mg/kg Avilamycin.
P1 = *Bacillus subtilis* LS 1-2 grown on corn-soybean meal medium. P2 = *Bacillus subtilis* LS 1-2 grown on citrus-juice waste medium.
[1] Standard error of means. [abc] Values with different superscripts in the same row differ significantly (p<0.05).

**Table 5.** Effects of supplementation of antibiotics and *Bacillus subtilis* LS 1-2 on caecal microbial populations (Log$_{10}$ CFU/g) in broilers (d 21 and 35)

|  | NC | PC | P1 | P2 | SEM[1] | p-value |
|---|---|---|---|---|---|---|
| d 21 |  |  |  |  |  |  |
| Total anaerobic bacteria | 9.97 | 9.78 | 9.95 | 9.93 | 0.04 | 0.330 |
| *Bifidobacteria spp.* | 8.79 | 8.87 | 9.03 | 8.99 | 0.05 | 0.361 |
| *Clostridium spp.* | 7.53 | 7.07 | 7.42 | 7.36 | 0.07 | 0.108 |
| *Coliforms* | 7.63 | 7.22 | 7.50 | 7.48 | 0.07 | 0.179 |
| d 35 |  |  |  |  |  |  |
| Total anaerobic bacteria | 10.24 | 9.70 | 10.12 | 10.18 | 0.05 | 0.426 |
| *Bifidobacteria spp.* | 9.23 | 9.09 | 9.28 | 9.33 | 0.05 | 0.420 |
| *Clostridium spp.* | 8.07[a] | 7.41[c] | 7.82[b] | 7.69[b] | 0.06 | <0.001 |
| *Coliforms* | 7.93[a] | 7.24[c] | 7.62[b] | 7.52[bc] | 0.08 | 0.002 |

NC = basal diet without any antimicrobial. PC = basal diet added with 20 mg/kg Avilamycin.
P1 = *Bacillus subtilis* LS 1-2 grown on corn-soybean meal medium. P2: *Bacillus subtilis* LS 1-2 grown on citrus-juice waste medium.
[1] Standard error of means. [abc] Values with different superscripts in the same row differ significantly (p<0.05).

**Table 6.** Effects of supplementation of antibiotics and *Bacillus subtilis* LS 1-2 on small intestinal morphology in broilers (d 35)

|  | NC | PC | P1 | P2 | SEM[1] | p-value |
|---|---|---|---|---|---|---|
| Duodenum |  |  |  |  |  |  |
| Villus height (μm) | 1,509[b] | 1,541[a] | 1,534[a] | 1,537[a] | 4.99 | 0.064 |
| Crypt depth (μm) | 364 | 354 | 362 | 357 | 2.03 | 0.368 |
| VH/CD[2] | 4.15[b] | 4.35[a] | 4.24[ab] | 4.31[a] | 0.03 | 0.018 |
| Ileum |  |  |  |  |  |  |
| Villus height (μm) | 536[b] | 562[a] | 557[a] | 560[a] | 3.79 | 0.043 |
| Crypt depth (μm) | 144 | 139 | 140 | 140 | 1.10 | 0.448 |
| VH/CD | 3.76[b] | 4.06[a] | 3.98[a] | 4.00[a] | 0.04 | 0.001 |

NC = basal diet without any antimicrobial. PC = basal diet added with 20 mg/kg Avilamycin.
P1 = *Bacillus subtilis* LS 1-2 grown on corn-soybean meal medium. P2 = *Bacillus subtilis* LS 1-2 grown on citrus-juice waste medium.
[1] Standard error of means. [2] Villus height to crypt depth ratio.
[abc] Values with different superscripts in the same row differ significantly (p<0.05).

and ileal crypt depth was not affected (p>0.05) by any of the dietary treatments. Duodenal and ileal villus height and villus height to crypt dept ratio remained comparable (p>0.05) among treatment PC, P1 and P2.

## DISCUSSION

The efficiency of probiotics depends upon various factors like, selection of strain, administration level, application method, ability of selected strain to survive at environmental temperature, long term storage and viability, method of fermentation used and type of substrate used for growth of probiotic microbes (Sanders and Veld, 1999; Timmerman et al., 2004, Shim et al., 2010). Among most commonly used microbes as probiotics, *Bacillus subtilis* is spore forming bacteria having resistance to high temperature and harsh storage conditions and generally regarded as safe strain for use as probiotic in poultry production (Fuller, 1989). Several reports indicated favorable results with broilers, layers, and turkey using various strains of *Bacillus* (Tortuero and Fernandez, 1995; Cavazzoni et al., 1998; Fritts et al., 2000; Khaksefidi and Ghoorchi, 2006; Opalinski et al., 2007). The main objectives of this study were to determine effect of *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean meal substrate on broiler nutrition. Antibiotic, Avilamycin was used with the objective of evaluating the potential of *Bacillus subtilis* LS 1-2 as an alternate to antimicrobial growth promoters.

In present study, supplementation of Avilamycin and *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean meal substrate resulted into improved BW gain, feed intake and FCR. Beneficial effects of supplementation of antibiotic and *Bacillus subtilis* are in good agreement with previous studies on broilers (Opalinski et al., 2007; Chen et al., 2009). Similarly, supplementation of various strains of *Bacillus subtilis* reported higher BW gain and better FCR in broilers (Fritts et al., 2000; Khaksefidi and Ghoorchi, 2006). Our results showed that during finisher and overall period, FCR of birds fed *Bacillus subtilis* LS 1-2 grown on citrus-juice waste as substrate were comparable with birds fed Avilamycin. In agreement with present findings, Mountzouris et al. (2007, 2010) reported similar growth promoting effects among birds fed Avilamycin and probiotics product. Previous study in author's lab reported comparable growth performance among birds supplemented with Avilamycin and multi-microbes probiotics product containing *Bacillus subtilis* (Shim et al., 2010). Higher overall BW gain and FCR in birds fed diet supplemented with *Bacillus subtilis* LS 1-2 grown on citrus-juice waste than birds fed diet supplemented with *Bacillus subtilis* LS 1-2 grown on corn-soybean meal substrate might be due to

higher microbial count ($2.8 \times 10^8$ cfu/g vs. $1.0 \times 10^8$ cfu/g) in probiotic grown on citrus-juice waste substrate. Improvement in growth performance and feed efficiency of broiler chickens supplemented with different strains of probiotics (Kabir et al., 2004; Mountzouris et al., 2007; Awad et al., 2009, 2010) are supposed to be induced by the collective effect of probiotic action including the improvement of feed intake and nutrient retention (Shim et al., 2010), maintenance of beneficial microbial population (Fuller, 1989), alteration of bacterial metabolism (Jin et al., 1997), and increased digestive enzyme activity and decreased ammonia production (Jin et al., 2000). In the present study, improved growth performance in birds fed probiotic products and antibiotics might be due to greater nutrient retention and improved gut health. Improvement in FCR in probiotic and antibiotic supplemented birds could be due to increased feed intake in order for the birds to compensate for achieving their growth potential.

In this study, supplementation of probiotic (*Bacillus subtilis* LS 1-2) products and Avilamycin improved retention of DM, CP and GE. In agreement with the findings of present study, Shim et al. (2010) reported higher nutrient retention among birds fed Avilamycin and birds administered probiotic preparation containing *Bacillus subtilis*. Higher nutrient retention in probiotic supplemented groups might be due to modulation of gut environment, improved gut barrier function via competitive exclusion of pathogenic microorganisms, improvement of beneficial intestinal microbial balance and stimulation of mucosal immune system (Farnell et al., 2006; Higgins et al., 2008; Vicente et al., 2008; Mountzoris et al., 2009, 2010). On the other hand, improved nutrient retention with Avilamycin might be due to increased nutrient availability for absorption and birds growth via suppression of growth and metabolic activities of harmful gut microflora with simultaneous alteration intestinal morphology, intestinal epithelium thickness and epithelial cell turnover (Barton, 2000; Miles et al., 2006; Mountzouris et al., 2010; Shim et al., 2010). Improved performance of broilers fed Avilamycin was also reported previously (Wellenreiter et al., 2000; Denev, 2006; Ohh et al., 2009) and these improved performances might be due to the benefits obtained from the antibacterial property of Avilamycin.

In this work, *Bacillus subtilis* LS 1-2 showed potential of reducing harmful microflora like *Clostridium* and *Coliforms* in caecal digesta of broilers at d 35. Other studies in broiler chickens with supplementation of various probiotics have also reported potential of probiotics to suppress pathogenic bacteria and fortification of intestinal microflora with beneficial bacteria (Teo and Tan, 2007; Higginis et al., 2008; Mountzouris et al., 2010). Probiotics beneficially affect the host animal by improving its intestinal balance (Fuller, 1989) and creating gut micro-

ecological conditions that suppress harmful microorganisms like *Clostridium and Coliforms* (Line et al., 1998; Pascual et al., 1999; Shim et al., 2010), and by favoring beneficial microorganisms like *Lactobacillus* and *Bifidobacterium*. In the present experiment, there was no difference in microbial population among birds fed on *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean meal substrate. This indicates that *Bacillus subtilis* LS 1-2 grown on citrus-juice waste substrate have equal efficacy with that grown on corn-soybean meal substrate.

Intestinal morphology including duodenal and ileal villus height and crypt depth as well as villus height to crypt depth ratio are indicative of gut health in broilers. Increased villus height and villus height to crypt depth ratio are directly correlated with an increased epithelial turnover (Fan et al., 1997), and longer villi are correlated with activation of cell mitosis (Samanya and Yamauchi, 2002). Whereas, shortening of villi and deeper crypts lead to poor nutrient absorption, increased secretion in gastrointestinal tract and reduced performance (Xu et al., 2003). In the present study, supplementation of *Bacillus subtilis* LS 1-2 in broiler diets resulted in increased villus height and villus height to crypt depth ratio in duodenum and ileum at d 35. Results obtained in the present study are consistent with Samanya and Yamauchi (2002), who reported increased villus height and villus height to crypt depth ratio of duodenum in birds supplemented with *Bacillus subtilis var. natto*. Awad et al. (2010) reported that supplementation of *Lactobacillus* in broilers' diet increased villus height and villus height to crypt depth ratio in duodenum and decreased ileal crypt depth. Our results provide new information regarding potential of *Bacillus subtilis* LS 1-2 as promoter of improving gastrointestinal morphology in broiler chickens irrespective of type of substrate used for its growth.

Results obtained in present study indicates that *Bacillus subtilis* LS 1-2 inclusion at 0.30% level had beneficial effects on broilers growth performance, nutrient retention, caecal microflora and intestinal morphology. Additionally, citrus-juice waste is equally efficient as corn-soybean meal substrate for growth of probiotic *Bacillus subtilis* LS 1-2.

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