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Evaluation of Multi-microbial Probiotics Produced by Submerged Liquid and Solid Substrate Fermentation Methods in Broilers

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ABSTRACT : Two experiments were conducted to evaluate multi-microbe submerged liquid (SLF) and solid substrate (SSF) fermented probiotic products in broilers. The SLF and SSF probiotics were comprised of *Lactobacillus acidophilus* (1.1×10^9 and 4×10^8 cfu/g), *Bacillus subtilis* (1.1×10^9 and 4.8×10^9 cfu/g), *Saccharomyces cerevisiae* (1.5×10^7 and 1.0×10^4 cfu/g) and *Aspergillus oryzae* (2.6×10^7 and 4.3×10^7 cfu/g), respectively. In Exp. 1, 640 day-old Ross chicks were allotted to 4 treatments, each comprising 4 replicates (40 chicks/replicate). The basal diet was prepared without any antimicrobials (negative control, NC), and 20 mg/kg avilamycin (positive control, PC), 0.3% SLF and 0.3% SSF probiotics were added to the basal diets as treatments. Birds fed PC and SSF diets showed improved ($p<0.001$) overall weight gain and F/G than birds fed NC and SLF diets; whereas, birds fed SLF diet had better weight gain and F/G than birds fed NC diet. Retention of CP was higher ($p<0.05$) in birds fed the SSF diet than birds fed PC, SLF and NC diets. Birds fed the SLF diet tended to have higher ($p<0.10$) cecal total anaerobic bacteria than birds fed PC and NC diets; whereas, lesser cecal coliforms were noticed in birds fed PC, SLF and SSF diets than birds fed the NC diet. In Exp. 2, 640 day-old Ross chicks were randomly allotted to 4 treatments in a 2×2 factorial arrangement. Each treatment had 4 replicates (40 chicks/replicate). Two different multi-microbe probiotic products (0.3% SLF or SSF) each with two different antibiotics (10 mg/kg colistin, or 20 mg/kg avilamycin) were used as dietary treatments. Birds fed the SSF diet had greater weight gain ($p<0.001$), better F/G ($p<0.05$), greater retention of energy ($p<0.001$) and protein ($p<0.05$), and lesser cecal *Clostridium* (d 35) than birds fed SLF diet. Birds fed the colistin-supplemented diet had less ($p<0.01$) cecal coliforms when compared with birds fed the avilamycin diet. Additionally, birds fed the avilamycin diet had greater energy retention ($p<0.05$) than birds fed the colistin diet. Thus, the results of this study suggest the multi-microbe probiotic product prepared by a solid substrate fermentation method to be superior to the probiotic product prepared by submerged liquid fermentation; moreover, feeding of probiotics with different antibiotics did not elicit any interaction effect between probiotic and antibiotic. (**Key Words** : Broilers, Multi-microbial Probiotics, Fermentation Methods, Performance, Nutrient Retention, Cecal Microflora)

INTRODUCTION

Probiotics are live microbial feed supplements beneficially affecting the host animal by improving their intestinal microbial balance. Probiotics have been used as a substitute for antibiotics in animal diets and also as an aid in the colonization of normal microflora in newly-hatched chicks (Fuller, 1989). Use of probiotics as a feed additive in poultry has been extensively reviewed by a number of researchers (Jernigan et al., 1985; Barrow, 1992; Jin et al., 1997).

Most research findings on probiotics have demonstrated the use of monostrain or multistrain probiotic microbes belonging to the same species or genus (Timmerman et al., 2004). According to Sanders and Veld (1999), the positive effects of probiotics are influenced by the genera, species and strain of probiotic microbes. Moreover, Sanders and Veld (1999) further suggested that the use of multistrain and multispecies probiotics might be more effective than monostrain probiotics. However, there have been no attempts to develop multi-microbe probiotic products.

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Previous studies have reported improved performance in pigs fed diets supplemented with combination of probiotics and antibiotics (Pollman et al., 1980; Stavric and Kornegay, 1995). Moreover, Nousiainen and Setala (1993) suggested that the probiotic microbes may be more easily established in the digestive tract of animals if the natural flora is weakened by the use of antibiotics.

Submerged liquid fermentation (SLF) involves growth of microbes in an aqueous medium and solid substrate fermentations (SSF) are characterized by the growth of microorganisms on moist solid substrates in the absence of free flowing water (Mitchell and Lonsane, 1992). Currently, an SLF process is being employed for the production of probiotics (Patel et al., 2004); while SSF are used in biotech industrial process such as production of enzymes (Battan et al., 2006), organic acids (Lu et al., 1998), and bio-pesticides (El-bendary, 2006). The potential advantages of SSF over SLF are simple culture facilities, relatively low investment, and higher production of biomass containing microbial metabolites with less waste output (Badu and Satyanarayana, 1995). Hu et al. (2008) used a mixed culture comprising *Lactobacillus fermentum*, *S. cerevisiae* and *B. subtilis* for fermenting compound feed by SSF and reported high counts of lactic acid bacteria and reduced enterobacteria in the fermented feed. Moreover, when SSF feed was included in the diet of growing-finishing pigs their performance and nutrient digestibility was similar to pigs fed antibiotics (Hu et al., 2008).

Therefore, the objectives of this study were to evaluate multi-microbe probiotics prepared by SLF and SSF methods and to investigate the effect of feeding multi-microbe probiotic products in combination with different antibiotics (colistin, Co, and avilamycin, Av) in the diet of broilers.

MATERIALS AND METHODS

Probiotics production and preparation

Probiotic microbes *Lactobacillus acidophilus* (KNU No. 31), *Saccharomyces cerevisiae* (KNU No. 55), *Bacillus subtilis* (KNU No. 42), *Aspergillus oryzae* (KNU No. 48) were maintained in the laboratory as mother cultures. 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.

In the SLF method, 2 L of autoclaved CB was inoculated with 2 ml of mother culture of each microbe separately and subjected to fermentation for 48 h. *L. acidophilus* and *B. subtilis* were incubated at 37°C at pH 7.0, whereas *S. cerevisiae* and *A. oryzae* were incubated at 32°C at pH 4.0. The microbes grown on CB were directly sprayed on 13 kg of corn-soybean meal (1:1) used as carrier, followed by drying at 40°C for 72 h. This was termed the SLF probiotic product and was composed of 1.1×10^9 cfu/g *L. acidophilus*, 1.1×10^9 cfu/g *B. subtilis*, 1.5×10^7 cfu/g *S. cerevisiae* and 2.6×10^7 cfu/g *A. oryzae* (Table 1).

Table 1. The number of microflora population of submerged liquid and solid substrate fermented probiotics

Microbe (cfu/g)	Probiotic products	
	SLF	SSF
Lactobacillus acidophilus	1.1×10^9	4.0×10^8
Bacillus subtilis	1.1×10^9	4.8×10^9
Saccharomyces cerevisiae	1.5×10^7	1.0×10^4
Aspergillus oryzae	2.6×10^7	4.3×10^7

The microbes grown on CB were used as starter to produce probiotic product by the SSF method. Corn and soybean meal (1:1) was used as the substrate and water was added to maintain a 30% moisture level followed by pasteurization. Then the substrate (13 kg) was inoculated with 2 L of starter and fermented for 7 days. The conditions maintained during fermentation for different microbes were as follows: *L. acidophilus* starter+5 L CB at 37°C and pH 6.8; *B. subtilis* starter+5 L water at 37°C and pH 7.0; *S. cerevisiae* starter+5 L CB at 32°C and pH 4.0; *A. oryzae* starter+5 L water at 32°C and pH 4.0. After 7 days fermentation, the microbial biomass was dried at 40°C for 72 h and mixed to obtain the SSF probiotic product. The microbial count in the SSF probiotic product was 4.0×10^8 cfu/g *L. acidophilus*, 4.8×10^9 cfu/g *B. subtilis*, 1.0×10^4 cfu/g *S. cerevisiae* and 4.3×10^7 cfu/g *A. oryzae*.

Experimental design, birds and diets

In Exp. 1, 640 broilers (Ross, day-old), were randomly allotted to 4 treatments. Each treatment had 4 replications with 40 broilers in each replicate. Dietary treatments were: negative control (NC, diet without antibiotic), positive control (PC, diet added with 20 mg/kg avilamycin), and diets added with 0.3% SLF and 0.3% SSF probiotic products.

In Exp. 2, 640 chicks (Ross, day-old), were randomly allotted to four treatments in a 2×2 factorial arrangement. Each treatment had 4 replications, comprising 40 birds per replicate. The factorial arrangement was comprised of probiotic products prepared by two different fermentation methods (0.3% SLF and 0.3% SSF) each with two different antibiotics (10 mg/kg colistin, Co and 20 mg/kg avilamycin, Av).

In both experiments the treatment starter and finisher diets were fed from d 0 to 21 and d 22 to 35, respectively. The antibiotics (Co and Av) were added to the diets at the expense of corn, whereas the probiotic products (SLF and SSF) were added to the diets by equally replacing corn and

Table 2. Ingredient and chemical composition of the basal diets used during Experiments 1 and 2

Ingredients (%)	Experiment 1[1]		Experiment 2[2]	
	Starter	Finisher	Starter	Finisher
Corn	55.68	58.34	51.78	60.52
SBM (44%)	26.19	21.43	30.29	23.26
Wheat	2.00	5.00	-	-
Corn gluten meal	7.00	8.00	8.50	8.00
Fish meal (55%)	2.00	-	-	-
Soybean oil	3.65	3.86	5.30	4.50
Tricalcium phosphate	1.84	1.66	-	-
Dicalcium phosphate	-	-	1.85	1.62
Limestone	0.75	0.75	1.32	1.24
Salt	0.30	0.03	0.30	0.30
L-lysine HCl (78%)	0.11	0.22	0.05	0.15
$_{DL}$-Methionine (50%)	0.18	0.03	0.21	0.01
Choline chloride (50%)	0.10	0.13	0.20	0.20
Vitamin premix[3]	0.10	0.13	0.10	0.10
Trace mineral premix[4]	0.10	0.15	0.10	0.10
Chemical composition				
ME (kcal/kg)	3,200	3,200	3,200	3,200
CP (%)	22.00	20.10	23.10	20.10
Ca (%)	1.00	0.90	1.00	0.90
Avail. P (%)	0.45	0.40	0.45	0.40
Lysine (%)	1.10	1.00	1.10	1.00
Methionine (%)	0.50	0.39	0.51	0.38

[1] Dietary treatments in Exp. 1 were NC (negative control, diet without antimicrobials), PC (positive control, diet added with 20 mg/kg avilamycin), SLF (diet added with 0.3% probiotic prepared by submerged liquid fermentation), and SSF (diet added with 0.3% probiotic prepared by solid substrate fermentation). Antibiotics were added to the basal diets at the expense of corn, while the probiotic products were added to the basal diets by equally replacing corn and SBM.

[2] Dietary treatments in Exp. 2 were diets added with 0.3% probioitc products (SLF or SSF) each with different antibiotics (10 mg/kg colistin, Co; or 20 mg/kg avilamycin, Av). Antibiotics were added to the basal diets at the expense of corn, while the probiotic products were added to the basal diets by equally replacing corn and SBM.

[3] Supplied per kg 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, 3 mg vitamin B_6, 0.025 mg vitamin B_{12}, 15 mg pantothenic acid, 35 mg niacin, 0.15 mg biotin, 1 mg folic acid.

[4] Supplied per kg diet: 56 mg Fe, 56 mg Cu, 70 mg Zn, 84 mg Mn, 1.4 mg I, 0.07 mg Co, 0.2 mg Se, 0.15 mg Cr.

soybean meal (Table 2). All the nutrients met or exceeded the nutrient requirements as recommended by National Research Council (1994).

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

Measurements and sampling

The birds were weighed individually and pen feed intake was noted at the end of each phase to calculate BW gain and feed/gain ratio (F/G) for starter and finisher phases. A nutrient retention trial was conducted during the last week of the finisher phase. From d 28 onwards, 2 birds from each replicate were allocated in individual cages (one bird/ cage), to facilitate collection of excreta samples. The finisher diets containing 0.25% chromic acid as an indigestible marker were fed from d 28 onwards. Excreta samples (about 100 g/d per bird) were collected from each bird for 48 h. Then excreta samples collected for 2 d were pooled by bird and dried using a forced-air drying oven at 60°C and stored for further analysis.

On d 21 of the experimental feeding and at the end of experiment (d 35), 8 birds per treatment (2 birds from each pen) were slaughtered to study microflora of cecal contents (Exp. 1 and 2) and ileal amino acid digestibility (Exp. 2). Immediately after slaughter, the digestive system was excised. Samples of cecal contents were aseptically collected and a weighed amount was suspended in sterile phosphate buffer solution containing cysteine (0.05% wt/vol), and used for enumeration of bacteria populations.

The chyme from the terminal ileum was collected and stored in a sterilized plastic bottle in an icebox and freeze-dried for amino acid analysis.

Chemical and microbial analyses

Analysis of the experimental diets and excreta was done according to the methods of AOAC (1990). Gross energy was measured by a bomb calorimeter (Model 1216, Parr Instrument Co., Molin. IL), and chromium was determined with an automated spectrophotometer (Shimadzu, Japan), according to the procedure of Fenton and Fenton (1979). Following acid hydrolysis in 6 N HCl at 105°C for 24 h, amino acid concentrations of diets and ileal contents in Exp. 2 were analyzed by HPLC (Waters 486, USA). Sulfur-containing amino acids were analyzed after cold performic acid oxidation (Moore, 1963) overnight with subsequent hydrolysis.

The microbiological assay of probiotic products was also carried out by culturing techniques. *L. acidophilus* was enumerated using MRS agar added with 0.02% NaN_3 and 0.05% L-cystine hydrochloride monohydrate, *B. subtilis* by using plate count agar, *S. cerevisiae* and *A. oryzae* by potato dextrose agar. The cecal microflora was analyzed as described previously (Choi et al., 2009). The microbial groups analyzed were total anaerobic bacteria (Tryptic soy agar), *Bifidobacterium spp.* (MRS agar), *Lactobacillus spp.* (MRS agar+0.02% NaN_3+0.05% L-cystine hydrochloride monohydrate), *Clostridium spp.* (TSC agar) and coliform bacteria (Voilet red bile agar). The anaerobic conditions during the assay of total anaerobic bacteria, *Bifidobacterium* spp. and *Clostridium spp.* were created by using a gaspak anaerobic system (BBL, Difco, Detroit, MI). The microbial populations were log transformed before statistical analysis.

Statistical analysis

Data generated in both experiments was subjected to statistical analysis using the GLM procedure of SAS (1996) statistical software package. Pen was the experimental unit for analysis of all the parameters. The data in Exp. 1 were analyzed by using one way-ANOVA and when significant differences were noted, means were compared using Duncan's multiple range test. In Exp. 2, the data was analyzed as a 2×2 factorial design contrast. The model contained main effects of probiotic, antibiotic and probiotic ×antibiotic interaction. The values of $p<0.05$ were considered to be statistically significant; whereas, $p<0.10$ was considered as a tendency.

RESULTS

Experiment 1

Growth performance : During the starter period, birds fed the PC diet showed better F/G ($p<0.01$) than birds fed NC and SLF diets, while birds fed the SSF diet had better F/G than birds fed the NC diet (Table 3). During finisher and overall periods birds fed PC, SSF and SLF diets showed higher weight gain ($p<0.001$) and improved F/G ($p<0.05$) when compared with birds fed the NC diet. Feed intake during the finisher period was greater in birds fed the PC diet compared with birds fed the NC diet. Additionally, greater overall weight gain and better F/G was recorded in birds fed the SSF diet when compared with birds fed the SLF diet.

Apparent nutrient retention : The apparent retention of DM, Ca, and P did not differ among the dietary treatments (Table 4). Birds fed probiotic diets (SSF and SLF) had greater retention of CP ($p<0.01$) when compared with birds

Table 3. Performance of broilers fed multi-microbe SLF and SSF probiotic products (Exp. 1)[1]

Item	NC	PC	SLF	SSF	SEM[2]
Starter (d 0 to 21)					
Weight gain (g)	622	653	641	648	6.84
Feed intake (g)	1,041	1,001	1,050	1,027	11.78
F/G	1.67[a]	1.53[c]	1.64[ab]	1.59[bc]	0.02
Finisher (d 22 to 35)					
Weight gain (g)	1,005[c]	1,135[a]	1,048[b]	1,102[a]	13.85
Feed intake (g)	1,954[b]	2,060[a]	1,967[b]	2,011[ab]	14.96
F/G	1.94[a]	1.82[c]	1.88[b]	1.82[c]	0.02
Overall (d 0 to 35)					
Weight gain (g)	1,627[c]	1,788[a]	1,688[b]	1,750[a]	18.12
Feed intake (g)	2,994	3,061	3,018	3,038	16.60
F/G	1.84[a]	1.71[c]	1.79[b]	1.74[c]	0.02

[a, b, c] Means within a row without a common superscript are significantly different ($p<0.05$).

[1] Dietary treatments were: NC (negative control, diet without antimicrobials), PC (positive control, diet added with avilamycin), SLF (diet added with probiotic prepared by submerged liquid fermentation) and SSF (diet added with probiotic prepared by solid substrate fermentation). Each mean represents 4 pens.

[2] Standard error of the means.

Table 4. Apparent nutrient retention (%) in broilers fed multi-microbe SLF and SSF probiotic products (Exp. 1)[1]

Item	NC	PC	SLF	SSF	SEM[2]
DM	80.26	80.12	81.39	82.24	0.60
CP	67.03[c]	68.31[bc]	70.12[b]	72.70[a]	0.66
GE	77.26	78.11	79.36	80.42	0.52
Ca	36.64	38.56	40.65	38.64	0.70
P	35.44	35.39	35.43	36.78	0.96

[a, b, c] Means within a row without a common superscript are significantly different ($p<0.05$).

[1] Dietary treatments were: NC (negative control, diet without antimicrobials), PC (positive control, diet added with avilamycin), SLF (diet added with probiotic prepared by submerged liquid fermentation) and SSF (diet added with probiotic prepared by solid substrate fermentation). Each mean represents 4 pens with 2 birds in each.

[2] Standard error of the means.

fed the NC diet; additionally, birds fed the SSF diet had higher CP retention than birds fed PC and SLF diets.

Cecal microflora : The population of different microbes in the cecal contents at d 21 did not differ among the dietary treatments (data not shown), but on d 35 the population of total anaerobic bacteria tended to be higher ($p<0.10$) in birds fed the SLF diet when compared with birds fed NC and PC diets (Table 5). Birds fed PC, SSF and SLF diets had lower ($p<0.01$) cecal coliform populations than birds fed the NC diet.

Experiment 2

Growth performance : Birds fed SSF diets had greater weight gain during starter ($p<0.05$), finisher ($p<0.01$) and overall periods ($p<0.001$) and had better overall F/G ($p<0.05$) ratio than birds fed SLF diets (Table 6). During the

Table 5. Microbial population (log_{10} cfu/g) in cecal contents in broilers fed multi-microbe SLF and SSF probiotic products (Exp. 1)[1]

Item	NC	PC	SLF	SSF	SEM[2]
Day 35					
Total anaerobic bacteria[3]	8.98	8.93	9.81	9.13	0.14
Bifidobacterium spp.	8.65	8.52	8.88	8.76	0.18
Lactobacillus spp.	8.68	8.17	8.79	9.09	0.14
Clostridium spp.	7.82	7.34	7.56	7.43	0.08
Coliforms	8.43[a]	7.37[c]	7.91[b]	7.54[bc]	0.13

[a, b, c] Means within a row without a common superscript are significantly different ($p<0.05$).

[1] Dietary treatments were: NC (negative control, diet without antimicrobials), PC (positive control, diet added with avilamycin), SLF (diet added with probiotic prepared by submerged liquid fermentation) and SSF (diet added with probiotic prepared by solid substrate fermentation). Each mean represents 4 pens with 2 birds in each.

[2] Standard error of the means. [3] $p<0.10$.

Table 6. Effects of multi-microbial probiotic products and different antibiotics on growth performance of broilers (Exp. 2)[1]

Probiotic	SLF		SSF		SEM[2]	p-value[3]		
Antibiotic	Co	Av	Co	Av		Probiotic	Antibiotic	Probiotic×Antibiotic
Starter (d 0 to 21)								
Weight gain (g)	583	588	606	599	3.92	0.04	0.89	0.43
Feed intake (g)	925	922	926	926	10.64	0.92	0.95	0.96
F/G	1.59	1.57	1.53	1.55	0.02	0.26	0.98	0.59
Finisher (d 22 to 35)								
Weight gain (g)	873	862	895	913	6.78	<0.01	0.73	0.17
Feed intake (g)	1,614	1,619	1,634	1,652	10.15	0.23	0.60	0.76
F/G	1.85	1.88	1.83	1.81	0.01	0.06	0.83	0.35
Overall (d 0 to 35)								
Weight gain (g)	1,422	1,415	1,466	1,478	8.62	<0.01	0.82	0.41
Feed intake (g)	2,539	2,541	2,560	2,578	13.48	0.33	0.74	0.79
F/G	1.79	1.80	1.75	1.74	0.01	0.03	0.83	0.73

[1] Dietary treatments were diets added with submerged liquid (SLF) or solid substrate (SSF) fermented probiotic products each with different antibiotics (colistin, Co; or avilamycin, Av). Each mean represents 4 pens.

[2] Standard error of the means. [3] Main effect of probiotic (SLF or SSF), antibiotic (Co or Av) and their interaction.

Table 7. Effects of multi-microbial probiotic products and different antibiotics on nutrient retention (%) in broilers (Exp. 2)[1]

Probiotic / Antibiotic	SLF		SSF		SEM[2]	p-value[3]		
	Co	Av	Co	Av		Probiotic	Antibiotic	Probiotic×Antibiotic
DM	77.95	78.15	79.20	78.73	0.24	0.07	0.76	0.48
CP	64.60	65.05	69.64	66.67	0.84	0.04	0.41	0.27
GE	76.63	77.40	79.89	81.52	0.56	<0.01	0.05	0.44
Ca	35.45	35.62	37.15	36.67	0.81	0.45	0.93	0.86
P	43.99	43.69	43.79	43.38	0.85	0.90	0.85	0.98

[1] Dietary treatments were diets added with submerged liquid (SLF) or solid substrate (SSF) fermented probiotic products each with different antibiotics (colistin, Co; or avilamycin, Av). Each mean represents 4 pens with 2 birds in each.
[2] Standard error of the means.
[3] Main effect of probiotic (SLF or SSF), antibiotic (Co or Av) and their interaction.

finisher period, birds fed SSF diets tended to have better F/G ($p<0.10$) than birds fed SLF diets; however, antibiotics had no effect on the performance of birds.

Apparent nutrient retention and ileal amino acid digestibility : Birds fed SSF diets had greater CP ($p<0.05$) and energy ($p<0.001$) retention and tended to have greater DM retention ($p<0.10$) when compared to birds fed SLF diets (Table 7). Antibiotics had no effect on nutrient retention except for greater energy retention recorded in birds fed Av when compared with birds fed Co.

Birds fed SSF diets had higher ($p<0.05$) ileal digestibility of arginine, histidine, and cystine and tended to have higher ($p<0.10$) ileal digestibility of threonine and glutamic acid than birds fed SLF diet (Table 8). However, inclusion of Co or Av had no effect on the ileal amino acid digestibility. Moreover, a significant interaction effect between antibiotic and probiotic was recorded for glutamic acid ($p<0.01$) and tendency towards an interaction effect was observed for cystine ($p<0.10$).

Cecal microflora : No differences in the cecal microbial population were recorded at d 21 (data not shown). At d 35, birds fed SSF diets had lower ($p<0.01$) cecal *Clostridium* population than birds fed SLF diets. Birds fed Co diets had lower ($p<0.01$) cecal coliform population, while birds fed Av diets tended to have lower ($p<0.10$) cecal *Clostridium* population (Table 9).

DISCUSSION

The major microbes used as probiotics include

Table 8. Effects of multi-microbial probiotic products and different antibiotics on ileal amino acid digestibility (%) in broilers (Exp. 2)[1]

Probiotic / Antibiotic	SLF		SSF		SEM[2]	p-value[3]		
	Co	Av	Co	Av		Probiotic	Antibiotic	Probiotic×Antibiotic
Essential amino acids								
Arginine	79.68	78.60	80.69	81.14	0.39	0.02	0.65	0.28
Histidine	72.10	71.78	73.90	75.61	0.64	0.03	0.55	0.38
Isoleucine	69.24	69.59	70.54	71.98	0.56	0.12	0.43	0.63
Leucine	81.86	80.19	82.43	82.39	0.48	0.17	0.38	0.41
Lysine	73.09	73.74	74.02	74.67	0.33	0.18	0.34	0.99
Phenylalanine	75.35	75.99	76.28	77.49	0.46	0.22	0.34	0.76
Threonine	59.05	60.52	61.45	62.17	0.58	0.09	0.34	0.74
Valine	69.46	68.74	68.49	69.25	0.35	0.76	0.98	0.34
Methionine	83.82	83.15	83.81	83.91	0.71	0.82	0.86	0.81
Non-essential amino acids								
Alanine	76.58	76.12	76.56	78.07	0.36	0.18	0.46	0.17
Aspartic acid	65.96	65.37	65.60	65.89	0.24	0.88	0.77	0.41
Cystine	59.72	57.27	60.64	66.32	1.24	0.03	0.44	0.07
Glutamic acid	80.87	80.17	80.35	81.89	0.22	0.06	0.18	<0.01
Glycine	57.54	57.57	58.06	59.32	0.51	0.31	0.55	0.57
Serine	69.84	70.38	70.61	68.66	0.63	0.73	0.61	0.37

[1] Dietary treatments were diets added with submerged liquid (SLF) or solid substrate (SSF) fermented probiotic products each with different antibiotics (colistin, Co; or avilamycin, Av). Each mean represents 4 pens with 2 birds in each.
[2] Standard error of the means.
[3] Main effect of probiotic (SLF or SSF), antibiotic (Co or Av) and their interaction.

Table 9. Effects of multi-microbial probiotic products and different antibiotics on cecal microflora (log_{10} cfu/g) of broilers (Exp. 2)[1]

Probiotic / Antibiotic	SLF		SSF		SEM[2]	p-value[3]		
	Co	Av	Co	Av		Probiotic	Antibiotic	Probiotic×Antibiotic
Day 35								
Total anaerobic bacteria	8.37	8.45	8.28	8.64	0.10	0.80	0.31	0.52
Bifidobacterium spp.	6.60	6.69	6.81	6.96	0.09	0.18	0.52	0.87
Lactobacillus spp.	7.97	8.05	8.23	8.36	0.11	0.25	0.65	0.89
Clostridium spp.	6.80	6.63	6.48	6.24	0.08	<0.01	0.06	0.97
Coliforms	7.60	8.03	7.35	7.84	0.10	0.17	0.01	0.87

[1] Dietary treatments were diets added with submerged liquid (SLF) or solid substrate (SSF) fermented probiotic products each with different antibiotics (colistin, Co; or avilamycin, Av). Each mean represents 4 pens with 2 birds in each.
[2] Standard error of the means.
[3] Main effect of probiotic (SLF or SSF), antibiotic (Co or Av) and their interaction.

Lactobacillus, *Saccharomyces*, *Bacillus*, *Streptococcus*, and *Aspergillus* (Tannock, 2001). The success of these microbes in providing beneficial effects to the host depends on their ability to tolerate heat, osmotic stress and oxygen stressors during processing and storage (Ross et al., 2005). For the propagation of probiotics the process of SLF is contemporarily dominant in both research and industry, while SSF has been commonly used in food fermentation to ameliorate the nutritional quality of cereals (Patel et al., 2004). The number of microbes in the probiotics prepared by SLF and SSF methods was similar except for fewer numbers of *S. cerevisiae* in probiotics produced by the SSF method (1.0×10^4 vs. 1.5×10^7, Table 1). These differences in the number of *S. cerevisiae* might be due to the poor heat dissemination through the solid substrate in the SSF process compared with the liquid medium in SLF (Raimbault, 1998).

In both experiments, birds fed multi-microbe probiotic product prepared by the SSF method showed higher weight gain than birds fed SLF probiotic product. The higher gains in birds fed SSF diets might be due to greater nutrient retention and was also reflected in improved F/G. In Exp. 1, the performance and cecal coliform population in birds fed SSF probiotic product were comparable with birds fed avilamycin. In agreement with the findings of the present study, Mountzouris et al. (2007) reported similar growth-promoting effects among birds fed avilamycin and birds administered a multi-species probiotic product (comprising *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, *Lactobacillus salivarius*) in feed and water. Additionally, in Exp. 1, birds fed probiotic diets (SLF or SSF) had higher overall weight gain and better F/G than birds fed the NC diet. The beneficial effects of probiotics in broilers are in agreement with a number of previous research studies (Owings et al., 1990; Cavazzoni et al., 1998; Kabir et al., 2004; Mountzouris et al., 2007). Moreover, improved performance was observed in broilers offered feed fermented by *Bacillus subtilis var. natto* and *S. cerevisiae* Y10 in a 2-stage fermentation process (Chen et al., 2009).

Probiotics beneficially affect the host animal by improving its intestinal balance (Fuller, 1989), creating gut microecological conditions that suppress harmful microorganisms (Line et al., 1998; Pascual et al., 1999), and by favoring beneficial microorganisms. In line with previous findings, birds fed probiotic diets had lower cecal coliforms (Exp. 1) than birds fed the NC diet. However, there were no differences in the cecal microbial population among birds fed SSF and SLF diets in Exp. 1; whereas, in Exp. 2, birds fed SSF diets had lower cecal *Clostridium spp.* than birds fed SLF diets. This difference may be due to the use of antibiotic in combination with probiotics in Exp. 2.

The benefits of feeding antibiotic growth promoters have been reviewed previously (Ferket, 2004; Dibner and Richards, 2005; Denev, 2006). Avilamycin is an orthosomycin antibiotic produced by *Streptomyces viridochromogenes* and is predominately active against gram-positive pathogenic bacteria (Weitnauer et al., 2001). In Exp. 1, avilamycin was used as an antibiotic in the PC diet and was effective in improving the performance of birds and reducing the population of harmful cecal coliforms. Improved performance of broilers fed avilamycin has been reported previously (Wellenreiter et al., 2000; Denev, 2006; Ohh et al., 2009) and these improvements may be due to the benefits obtained from the antibacterial property of avilamycin. In Exp. 2, avilamycin and colistin were used in combination with probiotics produced by SLF and SSF methods. Colistin is a decapeptide antibiotic produced by *Bacillus polymyxa* and has antibacterial activity mainly against gram-negative bacteria (Ziv, 1981). Thus the choice of antibiotics in Exp. 2 was based on the antibacterial spectra (avilamycin against gram-positive and colistin against gram-negative bacteria). Consequently, the birds fed colistin had lower cecal coliform populations.

Birds fed probiotics produced by the SSF method displayed better growth promoting effects, greater retention of CP (Exp. 1 and 2) and GE (Exp. 2), and ileal digestibility of arginine, histidine and cystine in Exp. 2 than birds fed

SLF probiotics. The better results obtained by feeding probiotics produced by the SSF method might be attributable to the higher production of secondary microbial metabolites during solid fermentation (Graminha et al., 2008). These metabolites include organic acids (lactic acid produced by *Lactobacillus spp.*), enzymes (amylase and protease produced by *A. oryzae* and *Bacillus spp.*), and antimicrobial substances (iturin and surfactin produced by *Bacillus subtilis*) during solid fermentation. However, the SSF fermentation technology still suffers from many technological hurdles due to lack of knowledge about various aspects of the process and lack of adequate fermentors (Robinson et al., 2001), making it difficult to apply for industrial production of probiotics.

Thus the results of the present study suggest that multi-microbe probiotics produced by solid substrate fermentation are superior to probiotics produced by submerged liquid fermentation, while there are no added benefits of feeding probiotics in combination with antibiotics. However, further studies are needed to identify the microbial metabolites produced during fermentation and to develop adequate solid substrate fermentors for industrial production of probiotics.

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