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Effects of dietary *Bacillus amyloliquefaciens* on mucosal immunity, cecal volatile fatty acids and microbial diversity in broiler chickens

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ABSTRACT

This trial was conducted to investigate the adhesion of *Bacillus amyloliquefaciens* to Caco-2 cells, the effects of *Bacillus amyloliquefaciens* on the mucosal immunity, cecal volatile fatty acids and microbial diversity in broiler chickens. Three hundred and sixty 1-d-old Ross 308 chicks were randomly divided into 3 dietary treatments groups, which birds fed with basal diet, basal diet supplemented with colistine sulfate, and basal diet supplemented with *Bacillus amyloliquefaciens*. Polymerase chain reaction denaturing gradient gel electrophoresis was used to analyze the change of cecal microflora, and Gas Chromatography was used to analyze the cecal volatile fatty acids. Data showed that: 1) *Bacillus amyloliquefaciens* had a good adhesion ability to epithelial cells; 2) the supplementation of *Bacillus amyloliquefaciens* significantly increased the concentration of ileal mucosal secretory IgA and interleukin 6, decreased ($P<0.05$) the concentration of tumor necrosis factor-α; 3) resulted in the change of cecal microbial community, higher levels of acetic acid, methylacetic and isovaleric acid in the birds. Thus, we considered that *Bacillus amyloliquefaciens* enhanced the mucosal immunity, increased the cecal concentration of major volatile fatty acids and the diversity microflora community in broilers.

Key words: Bacillus amyloliquefaciens, Broiler chickens, Cecal microflora, Mucosal immunity, Volatile fatty acids.

INTRODUCTION

Since 2006, dietary antibiotics have been banned in the European Union (EU), due to the development of antibiotic resistance and accumulation of harmful drug residues in animal products (Capcarova *et al.*, 2011). Probiotics have been frequently utilized as an important alternative to antibiotics in the production of livestock, which generally includes members of Lactobacillus, Bacillus, Bifidobacterium, Enterococcus, Saccharomyces cerevisiae, Lactococcus, Aspergillus oryzae and Streptococcus (Lee *et al*., 2010; Koli *et al*., 2017). Among them, *Bacillus cereus, Bacillus subtilis*, and *Bacillus clausii* have been widely used in poultry farming (Al-Fataftah *et al.*, 2014; Lee *et al.*, 2010). *Bacillus*-based probiotics have recently shown tremendous promise because of their capacity to form spores, which are known to withstand harsh environmental stress and the influence of pH, nutrients, and other relevant factors in the gastrointestinal tract of animals (Shivaramaiah *et al.*, 2011). *Bacillus*-based probiotics have been shown to modify gut microflora balance (Gangadharan *et al.*, 2008), and enhance the immune response in broilers (Hong *et al.*, 2005). Previously, studies indicated that *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) is closely related to *Bacillus subtilis* that produces various extracellular enzymes including ±-amylases, cellulase, metalloproteases, and proteases, which enhance the digestibility and absorption of nutrients in addition to immune function in the gut (Gracia *et al.*, 2003; Gangadharan *et al.*, 2008). A study conducted by Ahmed *et al*. (2014) showed the concentration of serum IgG and IgA and the emissions of fecal ammonia and hydrogen sulfide were decreased by the dietary *B. amyloliquefaciens* in broilers . Lei *et al*. (2015) indicated that the dietary *B. amyloliquefaciens* improved the growth performance, increased nutrient digestibility and modulated the intestinal microflora in broilers. The objective of the present study was to evaluate the effects of *B. amyloliquefaciens* in comparison to colistine sulfate on the mucosal immunity, the cecal volatile fatty acids (VFAs) and diversity of cecal microflora in broiler chickens.

MATERIALS AND METHOD

All following procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). A total of 360 1-d-old Ross 308 birds were obtained from a local hatchery (Charoen Pokphand Group, Haining, China) and randomly divided into 3 treatments groups, with 6 pens per treatment and 20 birds per pen. Birds treated as control group (Cont) were fed with antibiotic free basal diet; the antibiotic group (Anti) were fed with basal diet, which was supplemented with 20 mg colistine sulfate/Kg; and the *B. amyloliquefaciens* group

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(Baf) were fed basal diet, supplemented with 10^9 cfu *B. amyloliquefaciens*/Kg. The probiotic, *B. amyloliquefaciens* (CGMCC 9384) was provided by Zhejiang Huijia Biological Technology Ltd., Anji, China. After fermentation (37 °C, 48h) and drying, strain was granulated and approximately 1.7×10^{10} CFU/g. Water and feed were provided *ad libitum*. Temperature was kept for 35 °C the first week, and gradually decreased to 25 °C at the rate of 2.5 °C /week. The lighting program used in the study is 23 h of light and 1 h of darkness. Birds were raised in cages and fed for 42 days. The basal diet was based on NRC (1994) requirements; the ingredient and nutrient composition were listed in Table 1.

Caco-2 cells were seeded on 6-well plates (sterile slides on above), incubated for 48h, were washed twice with sterile PBS and plated with *B. amyloliquefaciens* (1×10^6 cfu/mL). Then, the strain-infected Caco-2 cells were incubated for 0.5, 1.0, 1.5 and 2.0 h at 37°C under 5% CO_2 conditions. The count of adhesive *B. amyloliquefaciens* were calculated by selecting the stains attached into 100 cells randomly.

On d 7, 21, and 42, six birds from each treatment group were randomly selected and killed by venipuncture. Ileal segment was aseptically collected from the small intestine. Then, mucosa samples were obtained by scraping with slide and transferred to sterilized tubes, which stored at -80 °C for assessment of cytokines. Cecum was transferred into the sterile containers with gentle squeezing and stored at -80 °C for polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and VFAs analysis. The chicken ELISA tests of interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), secretory IgA(S-IgA) were performed by following the kit's manufacturer's instructions (Cusabio Biotech Co. Ltd., Wuhan, China). The analysis of VFAs was conducted by Headspace Sampler Gas Chromatography (Agilent Technologies, New Castle, DE). One gram cecal content was blended with 6% phosphorous acid (m/v, 1:4), directly determined by Agilent Technologies 6890N Network System (Agilent Technologies, New Castle, DE) equipped with a 30 m × 0.25 mm × 0.25 μm column (DB-FFAP, Agilent Technologies) and a flame ionization detector (FID).

Table 1: Ingredients and nutrient levels of the basal experimental diet[1] (air-dry basis)

Ingredients	Content(%)
Corn	61.50
Soybean meal	27.50
Fish meal[2]	5.00
Soybean oil[3]	2.00
Vitamin-mineral Premix[4]	4.00
Total	100.00
Calculated Nutrient levels	
DE,(Mcal/Kg)	3.00
CP, %	21.00
Lys, %	1.22
Met+Cys, %	0.98
Ca, %	1.00
AP, %	0.47

[1]Nutrient level of the diets was based on NRC (1994).
[2]Crude protein content is 62.5%, and ME is 2.79 Mcal/kg.
[3]Metabolizable energy is 8.8 Mcal/kg.
[4]Supplied per kilogram of diet: vitamin A (retinyl acetate), 1,500 IU; cholecalciferol, 200 IU; vitamin E (DL-α-tocopheryl acetate), 10 IU; riboflavin, 3.5 mg; pantothenic acid, 10 mg; niacin, 30 mg; cobalamin, 10 μg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine, 1.5 mg; pyridoxine, 3.0 mg; Fe, 80 mg; Zn, 40 mg; Mn, 60 mg; I, 0.18 mg; Cu, 8 mg; Se, 0.15 mg.

The genomic DNA was isolated with the PowerFecal® Fecal DNA Kit (Mo Bio Laboratories, Inc, US). The 16S rDNA V3 region was amplified with primer 341F-GC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and 519R (5'-ATT ACC GCG GCT GCT GG-3'). The PCR-DGGE analysis was performed using the Bio-Rad DCode Universal Detection System (BioRad, Hercules, CA). Dendrograms and sequencing of DGGE Bands were followed by Sun *et al.* (2013). The aimed DNA fragments were sequenced using the primer M13F (CGC CAG GGT TTT CCC AGT CAC GAC) and M13R (AGC GGA TAA CAA TTT CAC ACA GGA) by Sangon Biotech Company, Ltd. (Shanghai, China). The aimed sequences were compared directly with non-redundant nucleotides in the GenBank database using Basic Local Alignment Search Tool (BLAST). The VFAs of cecal content, Shannon index were statistically analyzed with One-way ANOVA SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). The group differences were determined by the Duncan's test, and differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Adhesion ability: The adhesion ability of *B. amylolique faciens* to Caco-2 cells was shown in Fig.1 and Fig.S1. Result showed that the counts of *B. amyloliquefaciens* attached to Caco-2 cells was increased by the hatch time, which nearly 700 BAF/ 100 cells at 2h. Similarly, trials have indicated the variation of Bacillus strain in adhesion for decades (Batista *et al.*, 2014).

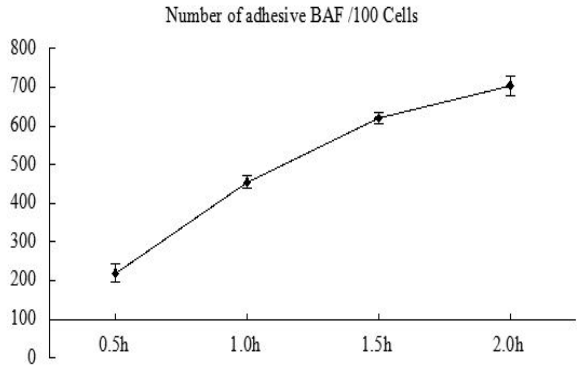

Fig 1: The number of BAF attached to Caco-2 cells.
Number of adhesive BAF /100 Cells means the co-culture of Baf and Caco-2 cells after 0.5 h, 1.0 h, 1.5 h, 2 h by gram staining.

Cytokine production: Data showed that the Baf and Anti birds had higher (P<0.05) concentration of mucosal S-IgA than Cont birds on d 7 and 21 (Table 2). The serum concentration of mucosal S-IgA and IL-6 was significantly increased by the supplementation of *B. amyloliquefaciens* compared with control birds on d 7, 21, and 42. The *B. amyloliquefaciens*-supplemented birds had higher (P<0.05) concentration of TNF-α than control birds on d 7. Moreover, the birds supplemented with antibiotic had higher concentration of IL-6 than control birds on d 7, 21, and 42. Molnár *et al.* (2011) find that the supplementation of *B. subtilis* modulated the immunological responses by increasing the immunological tissues, such as lympho-histiocytic infiltration and solitary lymphoid follicles, in the ileal mucosa of broiler chickens. Similarly, a study also found that the application of *Bacillus subtilis* induced the secretion of IL-6 and TNF-α in the ileum of broilers (Rajput *et al.*, 2013). Lee *et al.* (2015) found that the bacillus-based direct-fed microbials enhanced the innate immune responses by inducing the transcriptional expression of 37 genes related with the inflammatory response in the intestine of broilers. However, more studies are needed, given that the immunomodulation of *B. amyloliquefaciens* in broiler chickens is still unclear.

Volatile fatty acids levels: On d 21, the Baf birds had higher level of acetic acid than control birds (Table 3). Compared with the control and antibiotics birds, the Baf birds had higher (P<0.05) concentration of methylacetic on d 21 and 42. On d 42, both the *B. amyloliquefacien*- and antibiotic-supplemented birds had greater (P<0.05) acetic acid levels than the control birds. No significant differences were found

Table 2: Effects of *B. amyloliquefaciens* on the concentration of mucosal S-IgA, TNF-α and IL-6 in broilers[1.]

Item	Age	Treatment			SEM	P-val
		Cont	Anti	Baf		
S-IgA(ng/L)	D 7	1146^{c}	1590^{a}	1303^{b}	48.81	0.001
	D 21	1122^{b}	1455^{a}	1433^{a}	42.17	0.001
	D 42	1170^{b}	1278^{b}	1478^{a}	38.10	0.001
TNF-α(ng/L)	D 7	49.99^{b}	62.65^{a}	50.54^{b}	1.416	0.001
	D 21	53.07	51.39	49.84	1.000	0.309
	D 42	53.13^{a}	40.84^{b}	42.41^{b}	1.726	0.001
IL-6(ng/L)	D 7	39.00^{c}	61.62^{a}	55.54^{b}	1.082	0.001
	D 21	32.28^{b}	51.39^{a}	48.85^{a}	2.143	0.001
	D 42	35.44^{c}	40.84^{b}	45.87^{a}	2.497	0.001

$^{a-c}$ Means in the same row with different superscript letters differ significantly (P<0.05).

[1] Each mean represents 6 birds. Cont = birds were fed a basal diet; Anti = birds were fed a basal diet supplemented with 20 mg colistin sulfate/Kg; Baf = birds were fed a basal diet supplemented with 10^{9} cfu *B. amyloliquefaciens*/Kg.

Table 3: Effects of *B. amyloliquefaciens* on the concentration of major volatile fatty acids of cecal contents in broilers[1]

Age	Groups	VFAs					
		Acetic acid	Methy-lacetic	Isobutyric acid	Butyrate	Isovaleric acid	Valeric acid
D 7	Cont	83.86	3.829	0.487	7.229	0.549	0.454
	Anti	90.34	3.868	0.385	6.728	0.372	0.482
	Baf	88.23	4.876	0.591	10.334	0.662	0.504
	SEM	3.208	0.316	0.063	0.713	0.074	0.034
	P-val	0.757	0.351	0.463	0.052	0.308	0.643
D 21	Cont	103.6	7.377^{b}	0.821	17.73	0.879	1.487
	Anti	109.1	6.257^{b}	0.668	21.12	0.583	1.605
	Baf	141.7	11.01^{a}	0.672	24.53	0.600	1.613
	SEM	10.36	0.855	0.057	1.617	0.088	0.101
	P-val	0.302	0.026	0.521	0.251	0.315	0.883
D 42	Cont	90.67^{b}	7.439^{c}	1.029	13.14	1.210^{b}	1.491
	Anti	122.9^{a}	9.078^{b}	0.526	19.93	2.160^{a}	2.301
	Baf	140.0^{a}	15.13^{a}	0.889	19.59	0.950^{b}	1.812
	SEM	8.168	1.367	0.118	1.413	0.192	0.185
	P-val	0.010	0.019	0.213	0.059	0.001	0.211

$^{a-c}$ Means in the same volume with different superscript letters differ significantly (P<0.05).

[1] Each mean represents 6 birds. Cont = birds were fed a basal diet; Anti = birds were fed a basal diet supplemented with 20 mg colistin sulfate/Kg; Baf = birds were fed a basal diet supplemented with 10^{9} cfu *B. amyloliquefaciens*/Kg.

on the concentrations of isobutyric acid, or valeric acid within the three treatments through the whole trial.

Thanh *et al*. (2009) indicated that the maize soybean-based diet combined with metabolites produced from *Lactobacillus plantarum* increased the concentrations of fecal VFAs especially acetic acid and butyrate in broilers. Svihus *et al*. (2013) found that the ceca produces major VFAs in largely conservative molar proportions of acetic acid > butyric acid > propionic acid, which is supported data. Additionally, our data about the PCR-DGGE profiles suggested that the *Bacteroides sp*. was only detected in the birds fed with *Bacillus amyloliquefaciens* on d 42, which may also provide one reason.

DGGE Profile of cecal contents: The within-group-similarity index in cecal contents of the Baf- supplemented birds were 83%, 83%, and 88%, respectively, on d 7, 21, and 42 (Fig 2-4). The control birds showed 78%, 74%, and 89% similarity within the group on d 7, 21, and 42, respectively. While the antibiotics-supplemented group showed 82%, 75%, and 89% similarity, respectively. After pooling the repeated samples together, we ran the PCR-DGGE (Fig 5). Our data suggested that the supplementation of *B. amyloliquefaciens* and antibiotics changed the composition of cecal microflora. Furthermore, the supplementation of *B. amyloliquefaciens* increased the

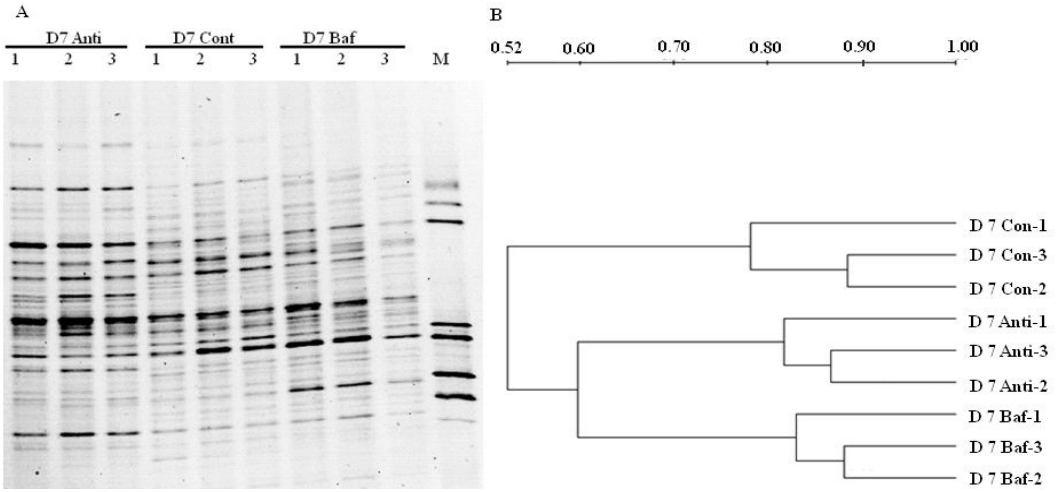

Fig 2: (A) PCR-DGGE of the 16S rRNA V3 region amplified from the DNA of cecal microflora in broilers on d 7. (B) Dendrogram relatedness.

The numbers (1, 2, 3) indicate the replicates in one group (N =3, pooled of 6 birds).

Sample D 7 Cont, Anti and Baf were taken from the cecal content of control, antibiotic and *B. amyloliquefaciens*- supplemented birds on d 7, respectively.

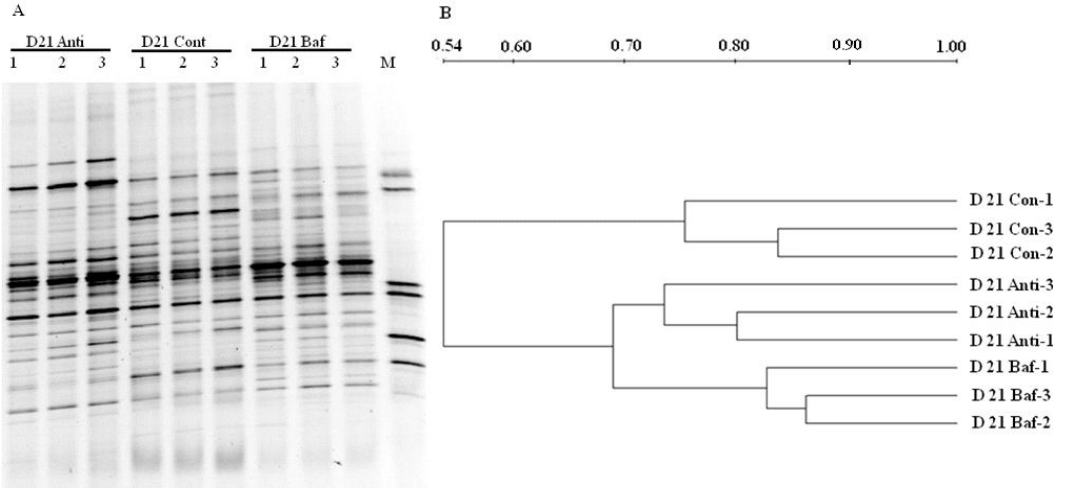

Fig 3: (A) PCR-DGGE of the 16S rRNA V3 region amplified from the DNA of cecal microflora in broilers on d 21. (B) Dendrogram relatedness.

The numbers (1, 2, 3) indicate the replicates in one group (N = 3, pooled of 6 birds).

Sample D 21 Cont, Anti and Baf were taken from the cecal content of control, antibiotic and *B. amyloliquefaciens*- supplemented birds on d 21, respectively.

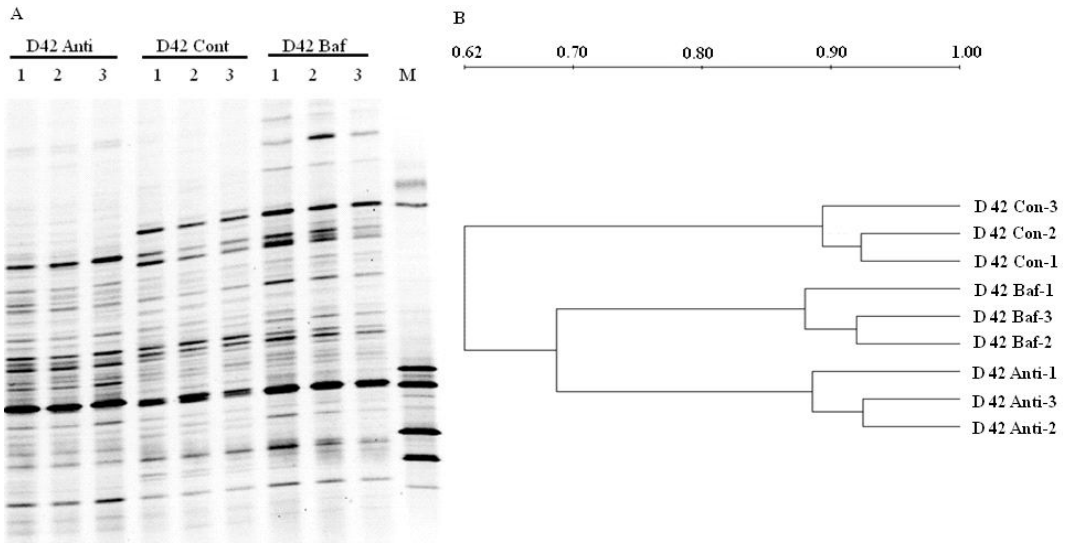

Fig 4: (A) PCR-DGGE of the 16S rRNA V3 region amplified from the DNA of cecal microflora in broilers on d 42. (B) Dendrogram relatedness

The numbers (1, 2, 3) indicate the replicates in one group (N = 3, pooled of 6 birds).

Sample D 42 Cont, Anti and Baf were taken from the cecal content of control, antibiotic and *B. amyloliquefaciens*- supplemented birds on d 42, respectively.

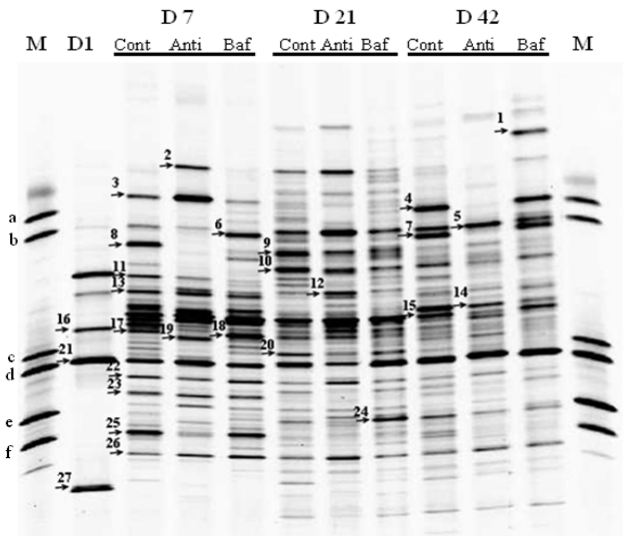

Fig 5: PCR-DGGE of the 16S rRNA V3 region amplified from the DNA of cecal microflora in broilers on d 7, 21 and 42

Marker: a represents *Staphylococcus aureus*, b represents *Clostridium perfringens*, c represents *Lactobacillus*, d represents *Escherichia coli*, e represents *Bacillus subtilis*, f represents *Salmonella*.

The numbers (1, 2, 3) indicate the replicates in one group (N = 3, pooled of 6 birds).

Sample of Cont, Anti and Baf were taken from the cecal content of control, antibiotic and *B. amyloliquefaciens*- supplemented birds on d 7, 21 and 42, separately

Shannon index of cecal microflora compared with control and antibiotics-supplemented birds, while no significant increase was found (Table 4). The Baf birds had greater number of bands ($P<0.05$) than control birds on d 21, and greater number of bands ($P<0.05$) than antibiotics birds through the experimental period. The intestinal microbiota and its metabolic activities play an important impact on the utilization of feed, the integrity of digestive tract and the general development of animals (Luo *et al.*, 2013). Data suggested that the supplementation of B. amyloliquefaciens and antibiotics changed the composition of cecal microflora in broilers, with a significant increase in the diversity of microflora in the cecal content of Baf-fed birds.

Detailed information about the cecal microflora of birds fed with differential diets revealed that bands 6 and 18 belonged to *Clostridiales bacterium* and *Clostridium innocuum* in the cecal content of Baf-supplemented birds, band 2 related to *Lactobacillus johnsonii* found in the antibiotic-supplemented birds, and band 8 related to *Enterococcus cecorum* detected in the control birds on d 7 (Fig 5, and Table 5). The predominant bacteria found amongst the three differentially-treated birds were *Enterococcus cecorum, Lactobacillus salivarius, Ruminococcus spp.*, and *Ruminococcus sp*. on d 7 (band 13, 14, 15, and 21). On d 21, band 24, related to *Clostridium spiroforme* was only detected in the cecal content of Baf-fed birds. The predominant bacteria detected in antibiotic birds (band 12, 22, 26) were related to *Eubacterium desmolans, Catonella sp*. and *Clostridium ramosum*. The predominant bacteria shared amongst the three treatments were related to *Ruminococcus sp.*, and *Clostridium ramosum*

Table 4: Effects of B. amyloliquefaciens supplementation on the diversity Of cecal microflora in broilers[1.]

Item	Age	Treatment			SEM	P-val
		Cont	Anti	Baf		
Shannonindex	D 7	2.200	2.103	2.405	0.064	0.196
	D 21	2.420	2.431	2.750	0.073	0.075
	D 42	2.220	2.247	2.260	0.044	0.947
Numbers of Band	D 7	20.7[a]	18.3[b]	23.0[a]	0.707	0.001
	D 21	22.7[b]	22.3[b]	27.3[a]	0.857	0.001
	D 42	22.7[ab]	21.7[b]	24.3[a]	0.484	0.045

[a, b] Means in the same row with different superscript letters differ significantly ($P<0.05$).

[1] Each mean represents 6 birds. Cont = birds were fed a basal diet; Anti = birds were fed a basal diet supplemented with 20 mg colistin sulfate/Kg; Baf = birds were fed a basal diet supplemented with 10^9 cfu *B. amyloliquefaciens*/Kg.

Table 5: Closet relative bacteria of the DGGE bands of broilers' cecum.

Band No.[2]	Length (bp)	Closest Relative (accession No.)[3]	Similarity (%)
1	189	Bacteroides sp. (JX519850.1)	100
2	194	Lactobacillus johnsonii (CP006811.1)	100
3	194	Clostridium spiroforme (NR_119030.1)	100
4	189	Alistipes sp. (KJ572413.1)	100
5	170	uncultured bacterium (KC805819.1)	100
6	194	Clostridiales bacterium (HQ452860.1)	100
7	189	Alistipes finegoldii (CP003274.1)	100
8	195	Enterococcus cecorum (NR_114779.2)	100
9	194	Lactobacillus amylovorus (KM112091.1)	100
10	171	Clostridiales bacterium (HM099644.1)	97
11	169	Ruminococcus sp. (AJ315979.1)	100
12	170	Eubacterium desmolans (NR_044644.2)	98
13	195	Enterococcus cecorum (HG316108.1)	99
14	194	Lactobacillus salivarius (KM361626.1)	100
15	169	Ruminococcus sp. (AJ315979.1)	100
16	169	Clostridiales bacterium (KF931641.1)	100
17	170	Clostridiales bacterium (EU728715.1)	97
18	194	Clostridium innocuum (AB971793.1)	100
19	194	Escherichia coli O157:H7 (CP010304.1)	99
20	169	Gemmiger formicilis (NR_104846.1)	98
21	169	Ruminococcus sp. (JN680878.1)	100
22	169	Catonella sp. (KM462124.1)	99
23	195	Acholeplasmatales bacterium (JN713486.1)	96
24	194	Clostridium spiroforme (NR_119030.1)	96
25	194	Clostridiales bacterium (HQ452860.1)	98
26	195	Clostridium ramosum strain (GU723322.1)	100
27	194	Clostridium ramosum (GU723321.1)	100

[1]Cecal content samples were obtained from the broilers on d 7, 21 and 42.

[2]Band numbers correspond to those in Figures 4.

[3]The bacterial relatives were the closest matches of named organisms located in GenBank by using BLAST.

(band 15, 21 and 26). On d 42, band 1 related to *Bacteroides sp*. was only found in the cecal of Baf-fed birds. The predominant bacteria shared with three treatments were related to uncultured bacterium, *Ruminococcus sp.* (band 5, 21). The predominant bacteria shared with Baf and control birds were related to *Alistipes sp.* and *Ruminococcus sp.* (band 4, 15). Other previous studies are in accordance with our data, which showed strains of *Fusobacterium*, *Bacteroides*, *Ruminococcus*, *Bifidobacterium*, *Sporomusa*, and *Enterobacteriaceae* dominate only in the ceca of broilers (Bjerrum *et al.*, 2006). The present study also found that *Clostridiales bacterium, Clostridium innocuum* and *Bacteroides spp.* were only detected in the cecum of Baf-supplemented birds, which we speculated resulted from *B. amyloliquefaciens* treatment. Similarly, studies have found that the supplementation of *B. amyloliquefaciens* increased the population of *Lactobacillus* compared with control groups (An *et al.*, 2008; Lei *et al*., 2015). Based on the above analysis, the dietary *B. amyloliquefaciens* and colistine sulfate indeed changed the composition of cecal microbial community in the broilers, in which the former increased the microbial diversity.

CONCLUSION

The supplementation of *B. amyloliquefaciens* had a good adhesion ability to epithelial cells, enhanced the mucosal immunity, increased the diversity microflora community, and the cecal concentration of major volatile fatty acids in broilers.

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