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Original Article

Effects of Flammulinavelutipes Stem Base on Microflora and Volatile Fatty Acids In Caecum of Growing Layers under Heat Stress Condition

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■Keywords

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ABSTRACT

The present study was undertaken to investigate the effects of *Flammulinavelutipes* stem base (FVS) on growth performance, microbial flora and volatile fatty acids of growing layers under heat stress condition. A total of 72 ISA Brown hens were randomly divided into six treatments: thermoneutral temperature control group (CON), heat stress control group (HS), heat stress antibiotic group (ANT) as positive control and heat stress FVS groups (20, 40 or 60 g/kg FVS). The experimental period had a duration of 28 d (days 84-112). On day 98, daily gain average was significantly higher (p<0.05) in the FVS groups than in the HS group. The number of bands in the FVS groups were higher (p<0.05) than in the HS group on day 98. The microbial similarity between the 60 g/kg FVS group and the HS group were the lowest on day 98. FVS group's specific bacteria were mainly *Coprococcus comes*, *[Clostridium] papyrosolvens*, *Butyricicoccuspullicaecorum*on day 98. Whereas on day 112, the FVS groups specific bacteria were mainly *Parabacteroides distasonis*, *Coprobacterfastidiosus*, *Elusimicrobiumminutum*. The content of acetic acid and butyric acid were higher (p<0.05) in 20 g/kg FVS group than in the CON group on day 112. In conclusion, FVS can lighten the adverse effect of heat stress by increasing the diversity of intestinal flora in growing layers.

INTRODUCTION

The condition of the gut system affects the nutrients utilization for organ development, tissue growth and immune system maturation in the host. Cecal microbial populations are the indication of gut health in animals (Mahfuz *et al*., 2017). The cecum is a complex ecosystem of microbial colonization in poultry. Volatile fatty acids (VFA) usually is produced through bacterial fermentation in the cecum that is necessary for the intestinal function and intestinal integrity (Meimandipour *et al*., 2010). In recent years, high-through put sequencing has been used by many researchers to investigate the gut microbial diversity in animals (Wang *et al*., 2017). Intestinal flora has an important influence on host health (Chang *et al*., 2016). The composition and activities of intestinal microflora can be altered by dietary patterns, such as feed additives (Maesschalck *et al*., 2015) and antibiotics (Kalter *et al*., 2010; Zou *et al*., 2016).

Since the last few decades, antibiotic shave been used in the poultry to promote growth performance. These antibiotics products include bambermycin, avilamycin, and the flavomycin (Butaye *et al.*, 2003). The product flavomycin (synonyms: moenomycin, flavophospholipol and bambermycin) is a glycolipid antibiotic produced by *Streptomyces* species including *S. bambergiensis*, *S. ghanaensis*, *S. geysirensis*, and *S. ederensis* (Huber *et al.*, 1965; Wallhausser *et al.*, 1965). In addition,

this antibiotic as been commonly used in poultry industry to prevent infectious diseases (Osweiler *et al*., 2010). However, the overuse of antibiotics may lead to antibiotic-resistant genes which spread extensively by promoting the selection of antibiotic-resistant bacteria in host animals (Zhou *et al*., 2016). In addition, the constant application of antibiotics in poultry feed with the purpose to improve production performance has led to human health hazards (Mahfuz *et al*., 2018). Therefore, it was urgent to find antibiotic substitutes, optimize the composition of intestinal flora, and promote the health of the birds.

Heat stress caused by extreme hot weather was a very common issue in poultry breeding. Heat stress can damage the intestinal micro ecological balance of poultry, may cause great economic losses by declining production performance, and even by the death of the host. At high ambient temperature (36±1°C, minimum temperature 31±1°C), it can lead to minor cracks in the duodenum, jejunum, ileum, and may lead to the rupture of the villi, and even severe absence of the villi in chicken (Quinteiro-Filho *et al*., 2010). Ramnath *et al.* (2008) reported that heat stress (40±1°C, non-heat stressed temperature 30±1°C) affected concentrations of certain oxidative stress markers. The study of Abidin (2013) showed that temperature ranging from 18°C to 26°C was comfortable for broilers.

*Flammulinavelutipes*s tem base (FVS) is known as waste material commonly found in the mushroom industry. FVS is an agricultural byproduct having nutritional and medicinal values and its availability is abundant but up to now its utilization is limited for animal production (Mahfuz *et al*., 2017). *Flammulinavelutipes* is a saprophytic fungus. The optimum temperature for its growth is 22-25°C. *Flammulinavelutipes* is widely distributed in nature, including China, Japan, Russia, Europe, North America and Australia (Yoshihama *et al*., 1994). *Flammulinavelutipes* contain dietary fiber, are low in calories, have a high content of protein consisting of all the essential amino acids, minerals and vitamins and are free of cholesterol (Karaman *et al*., 2010). It has been highly valued as a functional food for its good antioxidant, anti-inflammatory, immunomodulatory, anti-tumour, and cholesterol-lowering effects (Wu *et al*., 2014; Yan *et al*., 2014; Chen *et al*., 2015; Xia, 2015). Laying hens fed with FVS (3-5%) could reduce the pathogenic bacteria like *Salmonella spp.*, *E. coli* and *Clostridium spp.*, as well as increase the number of beneficial bacteria of *Lactobacillus spp.*, and *Bifidobacterium spp.*(Lee *et al*., 2012; Lee *et al*., 2014). However, there was no previous study on the effect of *Flammulinavelutipes*s tem base on animals submitted to heat stress.

This study examined the effect of FVS on growth performance, caecal microflora and VFA concentration in ISA Brown growing layers under heat stress condition.

MATERIALS AND METHODS

Test materials, experimental condition and feeding management

Experimental chickens (ISA Brown) were purchased from Changchun Octavia Farms and FVS was collected from the local domestic mushroom farm in Changchun City, Jilin, China.

The experiment was carried out at the Animal Unit, College of Chinese Medicine Materials, Jilin Agricultural University, and all the procedures were approved by the animal care and use committee of Jilin Agricultural University. A total of 72 hens, aged 84d derived from ISA Brown strain were divided into 6 groups, with 3 replications having 4 chickens each. The birds were housed into a wire cage (100cm, 60cm, 50cm, length, width, height) and an average homogeneous not significant body weight (1192±15.32 g; Table 2) was considered for each replication. Dietary treatment included thermoneutral temperature control group (CON, basal diet, 28±1°C), heat stress control group (HS, basal diet, 38±1°C), heat stress antibiotic group (ANT, basal diet supplemented with 5 mg/kg flavomycin, 38±1°C) as positive control and heat stress FVS group (basal diet supplemented with 20, 40, and 60 g/kg FVS, 38±1°C). Heat stress was not constant throughout the experimental period. During the experimental period, room temperature was maintained at 38±1°C from 8:00-18:00, after the heat stress time, the room temperature was 28±1°C, until the next morning. The incandescent lamps with room heater were used to maintain the heat stress and the spray method was used to control the relative humidity at 50%-60%. A wet and dry bulb thermometer were used to record the temperature and humidity throughout the experimental period. The trial lasted for 28 days from day 84 to day 112. All procedures were applied for heat stress, only. Feed and water were provided *ad libitum* throughout the whole period. Mushroom and antibiotics were mixed with the growing layers diet formulated according to NRC (1994) specification in Feed Mill (Jilin Hanghong Animal Husbandry Co. Ltd, China). The analyzed nutritional composition of the experimental diet and FVS are presented in Table 1.

Table 1 – Experimental diet with nutritional composition[1]

Item	CON	HS	ANT	20 g/kgFVS	40 g/kgFVS	60g/kgFVS
Ingredient						
Corn	673.50	674.00	674.50	670.00	645.00	615.00
Soybean meal (CP 47%)	256.50	256.00	255.00	240.00	245.00	255.00
FVS				20.00	40.00	60.00
Lysine	2.00	2.00	2.00	2.00	2.00	2.00
Methionine	2.50	2.50	2.50	2.50	2.50	2.50
Dicalcium	30.00	30.00	30.00	30.00	30.00	30.00
Limestone	31.00	31.00	31.00	31.00	31.00	31.00
Common salt	2.50	2.50	2.50	2.50	2.50	2.50
Vit-mineral premix[2]	2.00	2.00	2.00	2.00	2.00	2.00
Antibiotics			0.50			
Total	1000	1000	1000	1000	1000	1000
Chemical analysis						
DM[3](g/kg)	914.70	913.40	914.40	913.60	915.50	914.50
CP(g/kg)	166.40	167.80	166.90	168.70	168.60	167.60
Ca(g/kg)	11.20	11.10	11.10	11.20	11.20	11.10
P(g/kg)	5.40	5.50	5.60	5.60	5.50	5.70
EE(g/kg)	28.30	28.30	28.60	28.68	28.59	28.30
CF(g/kg)	25.50	25.40	25.60	27.70	32.10	37.30
Calculated analysis(g/kg)						
ME(g/kg)	11.55	11.54	11.57	11.60	11.59	11.63
Lysine(g/kg)	10.00	9.90	10.00	9.98	10.01	10.10
Methionine(g/kg)	4.80	4.80	5.10	4.90	4.80	5.00
Cystine(g/kg)	2.80	2.82	2.90	2.88	2.81	2.81

[1]CON=thermoneutral temperature control group (basal diet, 28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40or60 g/kg FVS, 38±1°C).

[2]Provided per kg of the complete diet: retinyl acetate, 4500 IU; cholecalciferol, 1200 IU; DL-α-tocopheryl acetate, 2500 IU; thiamin, 5000 mg; riboflavin,20000 mg; phylloquinone,10000 mg; niacin, 45000 mg; pantothenic acid,35000 mg; biotin, 1500 mg; folic acid, 3000 mg; cyanocobalamin, 40 mg; zinc, 45 mg; manganese 50 mg; iron, 30 mg; copper, 4 mg; cobalt, 100 µg; iodine, 1 mg; selenium, 100 µg.

[3]DM=dry matter; CP=crude protein; Ca=calcium; P=phosphorus; EE=ether extract; CF=crude fiber; ME=metabolisable energy.

Sample collection

Mushroom stem was harvested from a domestic mushroom farm at Changchun city, and sun dried properly. Then the sub sample was grinded and was prepared (0.01mm) for proximate component analysis. Feed sample and FVS were analyzed (n=6) following the method of AOAC (2000). Dry matter, ether extract, crude fiber, and total ash were analyzed according to the procedures of AOAC (2000). Nitrogen was determined using an FP528 nitrogen determinator (LECO Corporation, Joseph, MI, USA).The analyzed results were presented in Table 1. Analyzed compositions of *Flammulinavelutipes* mushroom stem were dry matter=88.50±0.80g/kg, crude protein=13.55±0.42g/kg, crude fiber=21.05±0.11g/kg, ether extract=2.3±0.014g/kg, Ash=11.4±0.085g/kg, calcium=4.0±0.1 g/kg and phosphorus=6.2±0.28g/kg. A total of 36 birds (two from each replicate, n=6) were slaughtered on day 98 and day 112 to collect cecum and samples were kept in a freezer(-80°C) for further analysis.

Growth performance

Body weight and feed intake per pen were recorded from day 84 to day 98 and day98 to 112 and used to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Analysis of the cecal digesta microbial flora

Cecal chime were taken to extract total bacterial DNA by using AxyPrep genomic DNA mini kit following the instructions of the manufacture Co. The 16SrDNA V3 region of the total bacterial DNA was amplified by PCR. JY-TD331A PCR-DGGE (denaturing gradient gel electrophoresis) and Vilber gel scanning imaging system were used for DGGE test. The common and specific bands in the DGGE map were recovered by gel cutting and amplified where DGGE electrophoresis was carried out according to the above PCR method to confirm the correctness of the retrieved target fragments. DNA was amplified and purified by universal primers (without GC clamps). The PCR product of the target template was connected by

a carrier linked conversion kit (pMD18-TVector). The competent cell of *E. coli* DH5 was transferred into the junction product coated agar plates that contained amplicillin. Cloning sequencing was completed by Shanghai Biological Engineering Co. ltd. Chromas software was used to analyze and sort out the sequence and then compared the sequence of close relatives with the GenBank database.

Determination of VFA content in the cecal digesta

The experimental conditions were as follows; the chromatographic column was DB-FFAP capillary column (30m×250 μm×0.25μm); the inlet temperature of 220°C; FID detector temperature of 250°C ; column temperature program: initial temperature of 65°C, and then to 20°C/min to 190°C. Split ratio 25: 1; gas flow rate; N_2 25 mL/min; hydrogen H_2 40 mL/min; air 400 mL/min. Agilent gas chromatograph 7890A was used to detect the concentration of acetic acid, propionic acid and butyric acid in the cecal digesta.

Statistical analysis

The experimental data were processed by Microsoft Excel, and then subjected to one-way analysis of variance using SPSS software. Multiple comparisons were performed using Tukey-kramer's test. Cluster analysis was performed using Quantity one. $p<0.05$ indicates significant difference.

RESULTS

Effect of FVS on growth performance

The effects of FVS on growth performance growing layers were presented in Table 2. On day 98, the ADG in the FVS groups were higher ($p<0.05$) than that of the HS group. On day112, the ADG was higher ($p<0.05$) in the 60 g/kg FVS group than in the HS group. The ADG in the FVS groups had no significant difference with the CON group and ANT group in the whole period of the test. On day 98, the ADFI was higher ($p<0.05$) both in the 20 g/kg FVS group and 40 g/kg FVS group than in the HS group. On day 112, the ADFI was lower ($p<0.05$) in the FVS groups than in the CON group. There was no significant difference in FCR among groups throughout the test period.

Table 2 – Effect of *Flammulinavelutipes*stembase on growth performance of growing layers[1,2]

Item	CON	HS	ANT	20 g/kg FVS	40 g/kg FVS	60 g/kg FVS	SEM	*p*-value
Day 98								
ADG(g/d per bird)	9.18^{b}	7.06^{a}	8.73^{b}	8.23^{b}	8.42^{b}	8.67^{b}	0.259	0.002
ADFI (g/d per bird)	33.25^{cd}	31.58^{b}	34.48^{e}	34.07^{de}	32.92^{c}	30.08^{a}	0.525	0.001
FCR %	3.45	4.72	3.95	4.14	3.91	3.47	0.209	0.106
Day 112								
ADG (g/d per bird)	11.87^{ab}	10.87^{a}	12.1^{ab}	12.03^{ab}	12.36^{ab}	12.55^{b}	0.248	0.037
ADFI (g/d per bird)	62.37^{c}	60.91^{abc}	61.97^{bc}	59.79^{ab}	59.04^{a}	59.18^{a}	0.562	0.021
FCR %	5.27	5.61	5.12	4.97	4.79	4.72	0.159	0.199
IBW	1187	1182	1210	1183	1178	1212	15.32	0.984

[1]CON=thermoneutral temperature control group (basal diet, 28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40 or 60 g/kg FVS, 38±1°C).

[2]data represented the mean value of 12 birds per treatment. a, b, c, d, e-means in the same row with different letters are significantly different at $p<0.05$

SEM-pooled standard error of the means.

Effect of FVS on the microbial diversity

The number of microbial flora bands was shown in Table 3. On day98, the band number was higher ($p<0.05$) in the FVS groups than in the HS group, and

Table 3 – Effect of *Flammulinavelutipes* stem base on microbial flora bands number(Fig. 1) in caecum of growing layers[1,2]

Item	CON	HS	ANT	20 g/kg FVS	40 g/kg FVS	60 g/kg FVS	SEM	*p*-value
Day 98								
	47.33^{bc}	40.33^{a}	45.33^{b}	49^{bc}	51.33^{c}	44.67^{b}	1.299	0.001
Day 112								
	38.0	38.0	38.0	42.67	36.33	42.0	1.330	0.311

[1]CON=thermoneutral temperature control group (basal diet, 28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40 or 60 g/kg FVS, 38±1°C).

[2]data represented the mean value of 12 birds per treatment. a, b, c-means in the same row with different letters are significantly different at $p<0.05$

SEM-pooled standard error of the means.

there was no significant difference in the number of bands both in the FVS groups with the CON group. The number of the bands in the 20 g/kg FVS groups and 60 g/kg had no significant difference with the ANT group. On day 112, there was no significant difference among the groups.

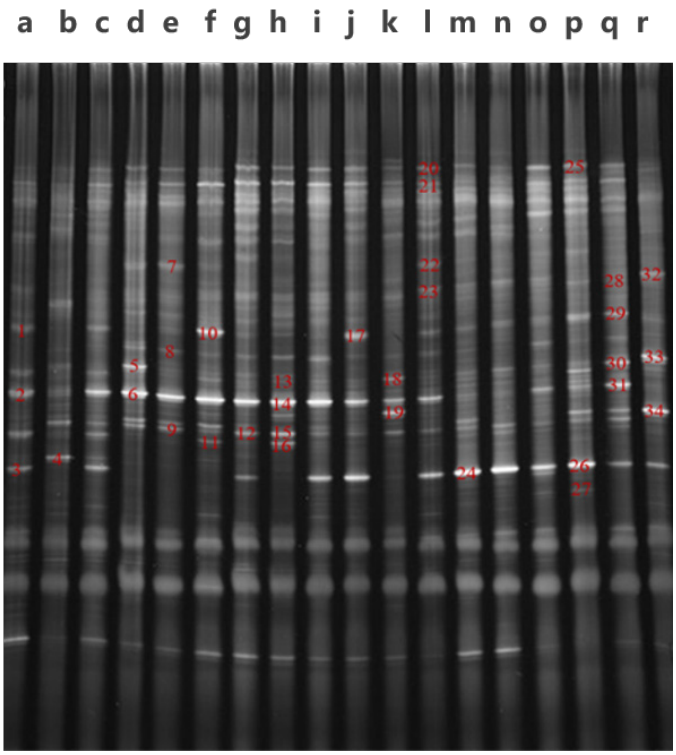
The results of sequence alignment of bands in DGGE maps (Fig. 1) are shown in Table 4. Under heat stress on day 98, the specific bacteria in the CON group were *Ruminococcuslactaris*, *Alistipes senegalensis* (1,3). The specific bacteria in the HS group were *Helicobacter pullorum*, *Stomatobaculumlongum* (5, 7). The specific bacteria in the FVS groups were *Selenomonasruminantium* strain, *Coprococcus comes*, *Intestinimonasbutyriciproducens* strain, *Merdimonasfaecis*strain, *[Clostridium] papyrosolvens*, *Butyricicoccuspullicaecorum*, *Terasakiellapusilla* (16, 18, 22, 23, 28, 29, 32, 33, 34). All the groups contain

Table 4 – Sequencing results of microbial florabands in DGGE map (Fig. 1)[1]

Stripe number	Sequence length (bp)	The closest name of the spawn in the GenBank database (login number)	Similarity %
1	171	*Ruminococcuslactaris* ATCC 29176 Scfld_02_7(NZDS990170.1)	99
2,15,20,25,31	170	*[Clostridium] saccharolytic*umstrainAn168contig_53 (NZNFKU01000053.1)	100
3	190	*Alistipessenegalensis* JC50 (NZCAHI01000040.1)	97
4,13,24,26,35,41,51,55,68	191	*Bacteroides uniformis* ATCC 8492 Scfld_3.0.1_32(NZ_DS362249.1)	99
5	171	*Helicobacter pullor*um MIT 98-5489 supercont2.11 (NZDS990451.1)	100
6,10,11,14,17,30	191	*Alistipestimonensis* JC136 strain DSM 25383 (NZFNRI01000015.1)	97
7,62	172	*Stomatobaculumlongu*mstrain ACC2 supercont1.5 (NZJH590865.1)	87
8,9	172	*Hathewayaproteolytica* DSM 3090(NZ_FRAD01000002.1)	89
12,27	191	*Alistipesinops*strain 627 contig00044(NZ_JRGF01000044.1)	97
16	197	*Selenomonasruminantium*strain WCT3(NZFNCM01000034.1)	93
18	170	*Coprococcus comes* ATCC 27758 Scfld1(NZGG662006.1)	99
19	173	*[Clostridium] termitidis* CT1112 Ct_contig00106(NZAORV01000077.1)	93
21	172	*Acholeplasmaequifetale* ATCC 29724 T434DRAFT_scaffold00026.26_C(NZJH XL01000026.1)	79
22	174	*Intestinimonasbutyriciproducens*strain AF211(NZCP011307.1)	94
23,28	171	*Merdimonasfaecis*strainBR31_L001_R1_001_paired_contig_46 (NZMIEH01000046.1)	100
29	172	*[Clostridium] papyrosolvens* DSM 2782 ctg56 (NZACXX02000011.1)	90
32	191	*Butyricicoccuspullicaecorum* 1.2 acBRa-supercont1.4 (NZKB976106.1)	99
33,34	171	*Terasakiellapusilla* DSM 6293 Q397DRAFT_scaffold00068.68_C(NZJHYO01000068.1)	89
36	190	*Olivibactersitiensis* DSM 17696 A37SDRAFT_scaffold_13.14_C(NZ_ATZA01000014.1)	83
37	191	*Leeuwenhoekiella sp.* MAR_2009_132 P164DRAFT_scf7180000000008_ quiver.2_C(NZJPOL01000002.1)	90
38,48	174	*Intestinimonas sp*. GD2 genomeassemblyIntestinimonasmassiliensis, scaffold00006 (NZLN869528.1)	91
39,50,53,56,61,65	191	*Arenibacteralgicola*strain TG409 U735DRAFT_scf7180000000011_quiver.3_C (NZJPOO01000003.1)	90
40	192	*Bacteroides coprophilus* DSM 18228=JCM 13818 strain DSM 18228 Scfld2 (NZEQ973630.1)	100
42	191	*Prevotelladentasini* JCM 15908 (NZBAKG01000039.1)	94
43	170	*Alistipesfinegoldi*i DSM 17242, complete genomeSequence ID:(NC018011.1)	99
44,46,52	190	*Bacteroides stercoris* ATCC 43183 Scfld_02_15 (NZ_DS499676.1)	94
45	197	*Desulfitobacterium dichloroeliminans* LMG P-21439 (NC019903.1)	89
47	191	*Parabacteroides johnsonii* CL02T12C29 supercont1.5 (NZJH976469.1)	93
49	172	*Ruminococcusbicirculans* chromosome II (NZHF545617.1)	88
54	191	*Odoribactersplanchnicus* DSM 20712 (NC015160.1)	92
57	173	*Oscillibacter sp.* KLE 1745 Scaffold82 (NZKI271778.1)	97
58	191	*Parabacteroides distasonis* ATCC 8503 (NC009615.1)	95
59	191	*Bacteroides coprocola* DSM 17136 Scfld_02_75 (NZDS981502.1)	95
60	191	*Rikenellamicrofusus* DSM 15922 RikmiDRAFT_RMD.2 (NZ_KE386488.1)	97
63	171	*Tyzzerellanexilis* DSM 1787 Scfld665 (NZDS995667)	99
64	191	*Coprobacterfastidiosus* NSB1 scaffoldcontig19 (NZKI440788.1)	92
66	191	*Bacteroides ovatus* strain ATCC 8483 (NZCP012938.1)	92
67	174	*Elusimicrobiumminutum* Pei191 (NC010644.1)	92

[1]DGGE=denaturing gradient gel electrophoresis.

bacteria *Bacteroides uniformis* (4, 13, 24, 26) except HS group. The ANT group and the FVS groups contain the bacteria *Alistipesinops* strain, *[Clostridium] termitidis* (12, 19, 27).



(a) Day 98

Figure 1 – Cecal digesta PCR-DGGE map in growing layers[1,2,3]

[1]CON=thermoneutraltemperature control group (basal diet,28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40 or 60 g/kg FVS, 38±1°C).

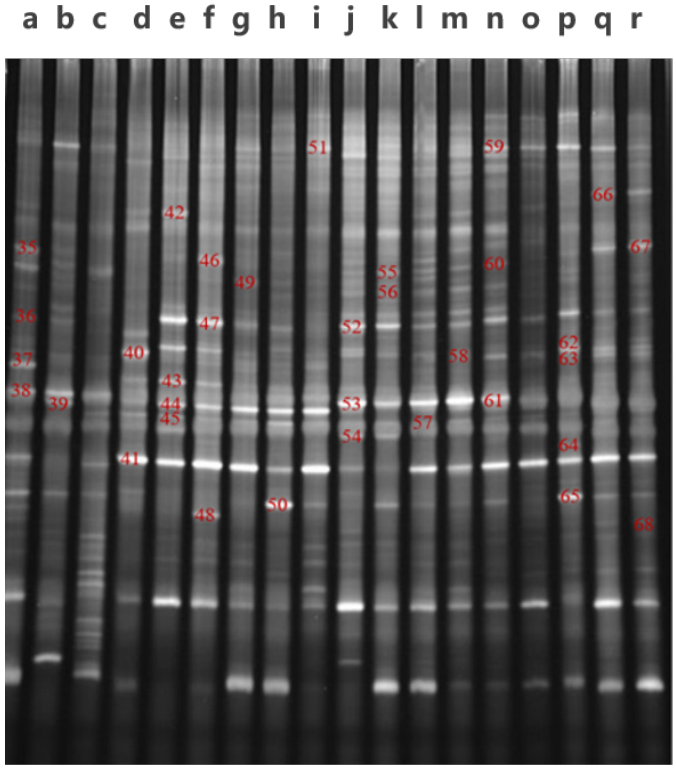
[2]The number presented inside the figure is the stripe number.

[3]a-c=CON group, d-f=HS group, g-i=ANT group, j-l=20 g/kgFVS group, m-o=40 g/kg FVS group, p-r=60 g/kg FVS group.

Under heat stress on day 112, the specific bacteria in the CON group were *Olivibactersitiensis* and *Leeuwenhoekiella sp.* (36, 37). The specific bacteria in the HS group were *Prevotelladentasini* and *Alistipesfinegoldii* (42, 43). The specific bacteria in the FVS groups were *Odoribactersplanchnicus*, *Parabacteroides distasonis*, *Rikenellamicrofusus*, *Tyzzerellanexilis*, *Coprobacterfastidiosus*, *Bacteroides ovatus* strain and *Elusimicrobiumminutum* (54, 58, 60, 63, 64, 66, 67). All the groups contain bacteria *Arenibacteralgicola* strain and *Bacteroides coprocola* (39, 50, 53, 56, 59, 61, 65) except the HS group. All the groups contain bacteria of *Bacteroides stercoris* (44, 46, 52) except the CON group. The ANT group and the FVS groups contain the bands of *Ruminococcusbicirculans* and *Oscillibacter sp.* (49, 57).

Effect of FVS on the microbial similarity

As shown in Fig. 2 (a), on day 98, the highest similarity between the 20 g/kg FVS group and the ANT group was 70.47%. The lowest similarity between the 60 g/kg FVS group and the HS group was 55.13%. Among the heat stress groups, the highest similarity was in the 40 g/kg FVS group with the CON group. As shown in Fig. 2 (b), on day 112, the highest similarity between the 20 g/kg FVS group and the ANT group was 62.58%. Among the heat stress groups, the ANT group had the highest results, similar to the CON group and followed by the 60 g/kg FVS group.



(b) Day 112

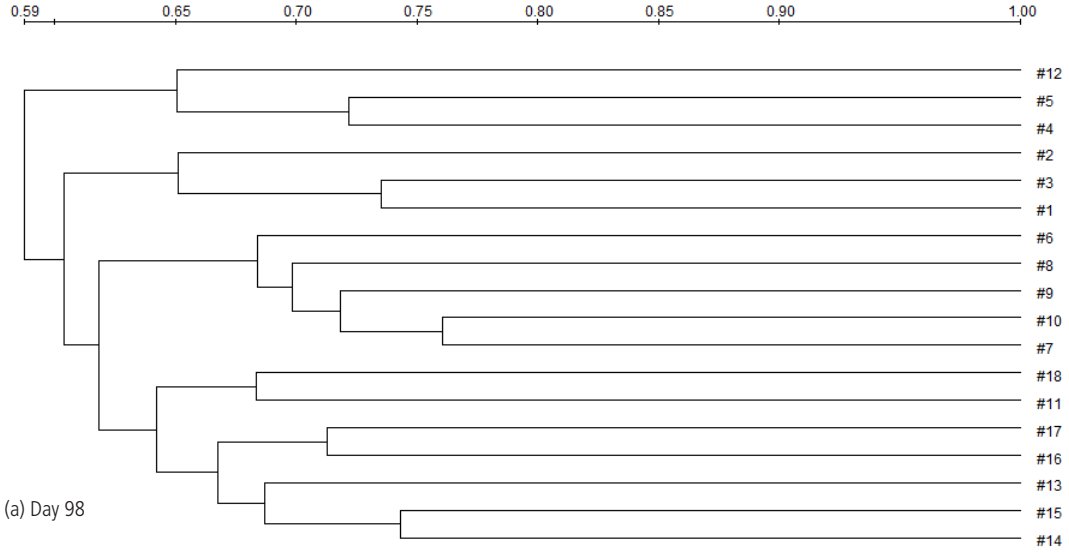
Figure 2 – Cecal digesta PCR-DGGE band clustering analysis in growing layers[1,2]

[1]CON=thermoneutral temperature control group (basal diet,28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40 or 60 g/kg FVS, 38±1 °C).

[2]#1-#3=CON group, #4-#6=HS group, #7-#9=ANT group, #10-#12=20 g/kg FVS group, #13-#15=40 g/kg FVS group, #16-#18=60 g/kg FVS group.

Effects of FVS on VFA content

As shown in Table 5, on day 98, the content of VFA in the FVS groups was lower(p<0.05) than in the HS group. The content of acetic acid and butyric acid in the 20 g/kg FVS group and the 60 g/kg FVS group had no significant differences with the CON group on day 98. There were no significant differences in the content of propionic acid in the FVS groups with the CON group on day 98. On day



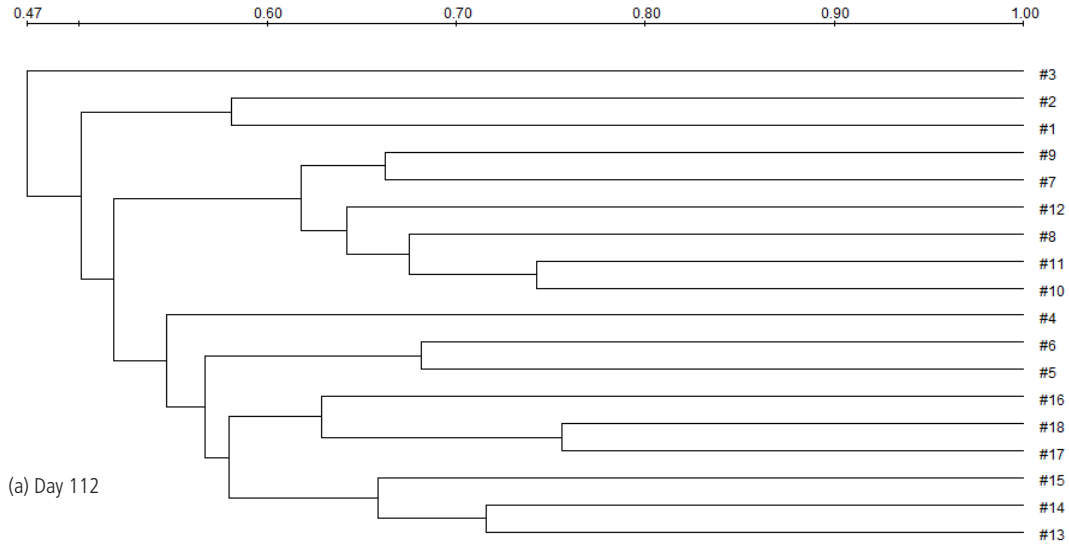


Table 5 – Effects of *Flammulinavelutipes* stem base on volatile fatty acids content in caecum of growing layers[1,2]

Item	CON	HS	ANT	20 g/kg FVS	40 g/kg FVS	60 g/kg FVS	SEM	*p*-value
Day 98								
Acetic acid	30.06^{a}	85.95^{c}	53.95^{b}	44.57^{ab}	53.45^{b}	28.52^{a}	7.027	0.001
Propionic acid	15.57^{ab}	38.06^{c}	20.59^{b}	16.59^{ab}	19.11^{b}	10.81^{a}	3.047	0.001
Butyric acid	11.78^{a}	43.46^{d}	23.77^{c}	16.29^{ab}	19.14^{bc}	11.1^{a}	3.846	0.001
Day 112								
Acetic acid	30.38^{a}	48.37^{ab}	63.78^{b}	55.5^{b}	32.18^{a}	29.56^{a}	5.265	0.002
Propionic acid	19.26^{b}	19.64^{b}	20.8^{bc}	24.41^{c}	13.6^{a}	14.85^{a}	1.377	0.001
Butyric acid	9.18^{a}	12.37^{ab}	21.95^{c}	15.19^{b}	9.14^{a}	10.82^{a}	1.605	0.001

[1]CON=thermoneutral temperature control group (basal diet, 28±1°C), HS=heat stress control group (basal diet, 38±1°C), ANT=antibiotic group (basal diet supplemented with 5 mg/kg flavomycin, 38±1°C), FVS=FVS group (basal diet supplemented with 20, 40 or 60 g/kg FVS, 38±1°C).

[2]data represented the mean value of 12 birds per treatment. a, b, c-means in the same row with different letters are significantly different at $p<0.05$

SEM-pooled standard error of the means.

112, the content of acetic acid and butyric acid in the 40 g/kg FVS group and the 60 g/kg FVS group had no significant difference with the CON group and the HS group respectively. The content of VFA in the 20 g/kg FVS group was higher ($p<0.05$) than in the CON group.

DISCUSSION

Effect of FVS on growth performance

Heat stress is a common problem, specially in most of the tropical countries. Heat stress is a hazard to commercial poultry production in most areas of China, especially in the summer season. This study highlighted that FVS could alleviate the effect of heat stress on the growth performance in laying hens. This may be related to FVS rich in crude fiber speeding up the intestinal peristalsis of the chicken under heat stress and promoting digestion and absorption. He *et al*. (2014) reported that heat stress could reduce the egg weight, growth performance, digestive enzyme activities, beneficial bacteria and increase harmful bacteria in the cecum of chicken. Similarly, this study found that found that heat stress can lead to the decline of growth performance of growing layers. The study of Ai (2008) showed that the early heat acclimatization to poultry could effectively alleviate the decline in production performance at the later stage of growth. On day 112, there were no significant differences in the ADG, ADFI and FCR between the HS group and CON group, which may be the result of heat stress adaptation in laying hens. Garriga *et al*. (2006) showed that stress can cause intestinal villus injury on the intestinal mucosa associated with poor nutrient absorption. This may be the reason for the decrease of ADG in the HS group.

Effect of FVS on the microbial diversity

This study found that FVS could alleviate the effect of heat stress in the intestinal flora of growing layers, increase the diversity of intestinal flora, mainly beneficial bacteria and reduce the colonization of intestinal harmful bacteria. These findings were similar to Zeng *et al*. (2016), who reported that the diversity of cecal microflora in broilers was significantly higher in FVS supplemented diets than in the control. Some past studies found that FVS could increase the beneficial bacteria and could decrease the harmful bacteria (Lee *et al*., 2012; Lee *et al*., 2014). In this study, the FVS groups did not contain the pathogenic species *Helicobacter pullorum* (Steinbrueckner *et al*., 1997) and *Alistipesfinegoldii* (Dziarski *et al*.,2016) which were found in the HS group only. This indicated that FVS can optimize the composition of the intestinal flora and inhibit harmful bacteria. On day 98, *Bacteroides uniformis* was found in the CON group, ANT group and FVS groups. However, *Bacteroides uniformis* was not found in the HS group. *Bacteroides uniformis* has an important role in the breakdown of complex polysaccharides, starch and cellulose to simple ingredients (Lan *et al*., 2006), and then promote digestion and absorption of the intestinal tract.

Effect of FVS on the microbial similarity

This study found that FVS could alleviate the effect of heat stress on the similarity of intestinal flora, and the effect was similar to that of antibiotics. This study showed that the highest similarity in results were between the 20 g/kg FVS group and the ANT group. This ensured that the role of the 20 g/kg FVS group and the ANT group were the most similar to the composition of the intestinal flora. On day 98, the lowest similarity was found between the 60 g/kg FVS group and the HS group. The 40 g/kg FVS group was the highest similarity among the heat stress groups with the CON group. These indicated that feeding FVS may alleviate the effect of heat stress on the composition of the intestinal flora. On day 112, among the heat stress groups, the ANT group was the highest similar to the CON group and followed by the 60 g/kg FVS group. This showed that feeding high doses of FVS could alleviate the effect of heat stress on the composition of the intestinal flora. These results were similar to the study by Guo *et al*. (2004), who reported that the cecal viscosity and microbial populations were significantly improved by feeding mushroom extracts.

Effects of FVS on VFA content

VFA and other organic acids (such as lactic acid and succinic acid) are the key metabolites of carbohydrate fermentation in the large intestine. Previous studies have shown that acetate and propionate have a good therapeutic effect on colitis (Tedelind *et al*., 2007). Van Der Wielen *et al*. (2000) stated that VFA are responsible for the reduction of *Enterobacteriaceae* in the ceca of broiler chickens during growth. Among the VFA, butyric acid stands out as a preferred energy source for enterocytes and takes part in cellular differentiation and proliferation within the intestinal mucosa (Rinttilä *et al.*, 2013). In addition, the butyrogenic effect of different prebiotics in the broiler cecum has been previously reported (Rehman *et al*., 2008). This study found that FVS could alleviate the effect of heat stress on the content of VFA in the intestine. This may be due to the fact that the FVS groups contained specific bacterial flora that were different from the other groups and resulted in changes in the VFA content. The concentration of VFA was related to the composition of feed, number and type of anaerobic bacteria (Rehman *et al.*, 2007).

The FVS groups contained a lot of beneficial bacteria that could increase the VFA concentration directly and indirectly. *Coprococcus comes* produces butyric acid primarily. *Intestinimonasbutyriciproducens* strain is mainly responsible for the production of acetic acid and butyric acid. *Alistipesinops* strain mainly produces acetic acid and succinic acid. *Merdimonasfaecis* strain can ferment glucose to produce acetic acid. *[Clostridium] Papyrosolvens* mainly produces volatile fatty acids, acetic acid, propionic acid, and butyric acid. *Butyricicoccuspullicaecorum* belongs to the genus *Clostridium* and has the potential of probiotics whose metabolites are acetic acid and butyric acid.

CONCLUSIONS

FVS could alleviate the effect of heat stress on growth performance, intestinal flora and VFA in growing layers. In conclusion, this study provides a new way to find environmentally friendly alternatives to antibiotics, by the utilization of edible and medicinal fungi wastes, which has great significance for alleviating heat stress in poultry production. In order to achieve better performance and sound gut health, FVS may be considered an alternative potential feed supplement for growing layers under heat stress condition.

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