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Effects of alfalfa flavonoids on the production performance, immune system, and ruminal fermentation of dairy cows

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Objective: The objective of this study was to examine the effects of alfalfa flavonoids on the production performance, immunity, and ruminal fermentation of dairy cows.
Methods: The experiments employed four primiparous Holstein cows fitted with ruminal cannulas, and used a 4×4 Latin square design. Cattle were fed total mixed ration supplemented with 0 (control group, Con), 20, 60, or 100 mg of alfalfa flavonoids extract (AFE) per kg of dairy cow body weight (BW).
Results: The feed intake of the group receiving 60 mg/kg BW of AFE were significantly higher ($p<0.05$) than that of the group receiving 100 mg/kg BW. Milk yields and the fat, protein and lactose of milk were unaffected by AFE, while the total solids content of milk reduced ($p = 0.05$) linearly as AFE supplementation was increased. The somatic cell count of milk in group receiving 60 mg/kg BW of AFE was significantly lower ($p<0.05$) than that of the control group. Apparent total-tract digestibility of neutral detergent fiber and crude protein showed a tendency to increase ($0.05<p\leq0.10$) with ingestion of AFE. Methane dicarboxylic aldehyde concentration decreased ($p = 0.03$) linearly, whereas superoxide dismutase activity showed a tendency to increase ($p = 0.10$) quadratically, with increasing levels of AFE supplementation. The lymphocyte count and the proportion of lymphocytes decreased ($p = 0.03$) linearly, whereas the proportion of neutrophil granulocytes increased ($p = 0.01$) linearly with increasing levels of dietary AFE supplementation. The valeric acid/total volatile fatty acid (TVFA) ratio was increased ($p = 0.01$) linearly with increasing of the level of AFE supplementation, the other ruminal fermentation parameters were not affected by AFE supplementation. Relative levels of the rumen microbe *Ruminococcus flavefaciens* tended to decrease ($p = 0.09$) quadratically, whereas those of *Butyrivibrio fibrisolvens* showed a tendency to increase ($p = 0.07$) quadratically in response to AFE supplementation.
Conclusion: The results of this study demonstrate that AFE supplementation can alter composition of milk, and may also have an increase tendency of nutrient digestion by regulating populations of microbes in the rumen, improve antioxidant properties by increasing antioxidant enzyme activities, and affect immunity by altering the proportions of lymphocyte and neutrophil granulocytes in dairy cows. The addition of 60 mg/kg BW of AFE to the diet of dairy cows was shown to be beneficial in this study.

Keywords: Flavonoids; Alfalfa; Ruminal Fermentation; Nutrient Digestibility; Dairy Cow

INTRODUCTION

The antibiotics commonly used to supplement ruminant feed play a role in improving production performance; however, the unreasonable use of antibiotics in animal feedstuff can lead to drug resistance and drug residues in food products, threatening food safety and human health [1]. The use of some antibiotics has been forbidden worldwide. For this reason, it is necessary to evaluate the potential of natural antimicrobials, such as plant extracts, for modification of rumen microbial fermentation [2].

Flavonoids are a large group of naturally occurring polyphenolic compounds with a basic struc-

ture consisting of an A and C ring of benzo-1-pyran-4-quinone and a B ring. The majority of flavonoids generally remain conjugated with sugars as glycosides [3]. As plant secondary metabolites, flavonoids play important roles in plant growth, development, and blossom formation. Flavonoids can also promote the growth and development of animals, and improve the quality of animal products, due to their anti-microbial and anti-oxidative properties; therefore, flavonoids are widely used as feed additives in animal production. Oskoueian et al [4] found that the addition of the flavonoids naringin and quercetin to ruminate diets could suppress methane production without influencing rumen microbial fermentation, while the use of flavone, myricetin, and kaempferol decreased the total volatile fatty acid (TVFA) concentration of rumen microbes cultured *in vitro*. Moreover, flavonoid-rich plant extracts can decrease ruminal methane emission without adversely affecting ruminal fermentation characteristics *in vitro* [5]. In contrast, flavonoids from propolis extract had no effect on production performance of dairy cows, although there were positive effects on milk quality and protein metabolism [6]. Daidzein increases the production performance of cows under heat stress [7]. These published data illustrate that flavonoids can influence the production performance and ruminal metabolism of ruminant animals, and that different types of flavonoids from various sources have distinctive effects.

Alfalfa (*Medicago sativa* L.) is one of the most widely cultivated perennial forage legumes, and has traits of high biomass production, good nutritional quality, and adaptability. Flavonoids are a major class of alfalfa secondary metabolites. Gao [8] found that the total flavonoid content in 73.3% of 45 alfalfa varieties cultivated in China was 0.6% to 0.9%. Alfalfa is currently widely used in ruminant feed and some of its benefits may be attributable to its flavonoid content. The previous studies found that alfalfa flavonoids extraction could reduce fat deposits in muscle of Chongren chickens and improve the growth and reproductive performance, immunity of mice in China; however, there have been few reports of the effects of alfalfa extracts rich in flavonoids on dairy cows. Therefore, the aim of this study was to evaluate the effects of alfalfa flavonoids extract (AFE) on the production performance, immune responses, and ruminal fermentation of dairy cows.

MATERIALS AND METHODS

Preparation of alfalfa flavonoids extract

The AFE (purity, 50%) was produced by Shaanxi Green Bio-engineering Co. Ltd (Xi'an, China). Fresh alfalfa was harvested during the full-blossom period and dried at 65°C for 2 days. The dried alfalfa was extracted using water at 100°C for 2 h, and the process repeated three times. Extracted solutions were collected and concentrated at 78°C and 0.02 mpa. The resulting concentrated solution was spray-dried and filtered through an 80 to 100-mesh screen. The AFE used in this study consisted of water-soluble polysaccharides (15.3%), crude protein (10.2%), ash (15.5%), and unknown compounds (8.8%).

Animals, diet, and feeding

Four primiparous Holstein cows fitted with ruminal fistulas were chosen from Xinghuo Dairy Farm No. 2, Shanghai, for use in this experiment. The cows were in milk for 79±6 d and their average body weight (BW) was 500±25 kg. The experimental design was a 4×4 Latin square, consisting of four experimental periods of 24 days each, with 10 days for adaptation. Experiments were divided into four groups, where animals were fed with total mixed ration (TMR) (Table 1) supplemented with 0 (control group, Con), 20, 60, or 100 mg AFE per kg of BW. Cows were fed the similar weight of TMR in the morning every day. At first, AFE was mixed evenly into a portion of TMR and fed to the dairy cows. Then, the remaining TMR was given to the dairy cows when the TMR with AFE was consumed. Cows were housed in tie stalls and provided with diet and drinking water *ad libitum* during the experiment. Cows were managed according to the Feeding Standard of Dairy Cattle in China (NY-T 34-2004) guidelines. The cows were fed three times daily at 06:30, 13:30, and 19:30, and milked three times daily at 10:00, 16:00, and 22:00. The milk yield of cows was recorded during the experimental period.

Sample collection and chemical analyses

Milk samples were collected three times per day from each cow in each experimental period; 20 mL, 15 mL, and 15 mL samples were collected in the morning, afternoon, and night, respectively.

Table 1. Ingredient and chemical composition of basal diet

Items	Content (g/kg)
Ingredient	
Concentrate[1)]	333.5
Cottonseed	37.9
Beet pulp, pellet	32.8
Premix[2)]	1.8
Corn silage	453.2
Oat grass	140.8
Chemical composition[3)]	
NEL (Mcal/kg)	1.7
Crude protein	175.0
Ether extracts	25.0
Neutral detergent fiber	311.0
Acid detergent fiber	151.0
Calcium	6.6
Phosphorus	2.8

NEL, net energy for lactation.
[1)] The concentrate was from Shanghai Bright Holstan Co., Ltd. It contained 158.3 mg/kg corn, 35.3 mg/kg soybean meal, 52.9 mg/kg soybean hull, 54.9 mg/kg dried distillers grains with solubles, 16.7 mg/kg brewer's grain, 2.2 mg/kg sodium chloride, 3.5 mg/kg calcium carbonate, 4.4 mg/kg calcium hydrophosphate, 3.3 mg/kg sodium bicarbonate.
[2)] The premix per kilogram diet contained 3,000 IU vitamin A, 31,400 IU vitamin D, 30 IU vitamin E, 100 mg iron, 10 mg copper, 35 mg zinc, 20 mg manganese, 0.3 mg iodine, 0.1 mg selenium, 0.08 mg cobalt.
[3)] NEL was calculated and others were measured.

Samples were mixed evenly with the preservative, 2-bromo-2-nitro-1,3-propanediol, and sent to the testing center at Shanghai Bright Holstan Co. Ltd. (Shanghai, China) for measurement of milk components, including concentrations of protein, fat, and lactose, and somatic cells counts.

The method of measurement of apparent total-tract digestibility was described in detail by Benchaar et al [9]. Samples of TMR were collected daily and stored at –20°C, before being combined and dried in an oven (DHG 9240A, Shanghai Jing Hong Laboratory Instrument Co. Ltd., Shanghai, China), crushed using a pulverizer (FW80, Tianjin Taisite Instrument Co. Ltd., Tianjin, China), and analyzed for dry matter (DM), crude protein (CP), ether extracts (EE), neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents. Feces were collected each day from each group, mixed uniformly, and a representative sample (200 g) collected and stored at –20°C. Samples of feces were dried and ground using a pulverizer for subsequent analysis of DM, EE, CP, NDF, ADF, and ash content. For both TMR and fecal samples, DM was detected using AOAC method 930.15; EE was measured using AOAC method 920.85; CP was tested using the method described by Kjeldahl, with an azotometer (Scino KT260, FOSS, Hillerod, Denmark); NDF and ADF were measured using the methods described by Van Soest and a Fiber Analyzer (2000i, Ankom, New York, UAS); and ash content was measured according to AOAC method 938.08.

Blood samples were collected from the caudal vein of cattle using a vacuum blood collection tube coated with ethylenediaminetetraacetic acid on day 18, 2 h after feed intake, and sent to the laboratory at the Shanghai Animal Research Center for blood cell count analysis using an automatic hematology analyzer (LH750, Beckman Coulter Inc., Fullerton, CA, USA). Blood samples were processed by leaving them at room temperature for 30 min, and then centrifuging for 15 min at 1,500×*g* in a 5810R centrifuge (Eppendorf, Hamburg, Germany). Serum samples were then stored at –20°C, pending analysis, for which they were sent to the Beijing Sino-uk Institute of Biological Technology, where immunoglobulin A (IgA), IgG, and IgM concentrations, and anti-oxidative indices, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities, and methane dicarboxylic aldehyde (MDA) levels, were determined.

Ruminal fluid (250 mL) was collected 2 h after feed intake from day 20 to day 22. Samples of ruminal fluid were collected from different locations within the rumens of each cow, and passed through four layers of gauze. The pH of ruminal fluid samples was measured using a pH meter (PHS-25, Shanghai INESA Scientific Instrument Co. Ltd., Shanghai, China), then 10 mL aliquots were added to 0.1 mL of 6 mol/L HCl, and the acidified fluid stored at –20°C prior to determination of ammonia nitrogen concentration according to the method of Weatherburn [10]. In addition, ruminal fluid was collected in cryogenic vials, frozen in liquid nitrogen for 10 min, and then stored at –80°C pending further analysis.

Ruminal fluid samples were thawed in ultrapure water and centrifuged for 10 min at 15,000×*g*, then supernatants (1.5 mL) were removed to centrifuge tubes containing 25% metaphosphoric acid, and stored at –20°C for analysis of TVFA, using a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) as described by Guo et al [11]. The yield of crude microbial proteins was determined according to the method described by Hall and Herejk [12].

Lactic acid concentration was measured by p-hydroxybiphenyl colorimetry. Samples of ruminal fluid were first thawed in ultrapure water, centrifuged at 1,300×*g* for 10 min, supernatants (1 mL) added to 3 mL of 10% (w/v) trichloroacetic acid, and mixed thoroughly. The mixed fluid was allowed to stand for 30 min at room temperature and then centrifuged at 1,700×*g* for 10 min. The supernatant was then mixed thoroughly with 50 mg of calcium hydroxide and 0.8 mL of 20% (w/v) copper sulfate, boiled in water for 3 min, then cooled with water and centrifuged at 1,000×*g* for 5 min. Supernatants were transferred to glass tubes and mixed slowly with 6 mL of 98% (w/v) sulfuric acid. The mixed fluid was boiled in water for 5 min and cooled with water, then added to 125 μL of 1.5% (w/v) p-hydroxydiphenyl. Finally, the samples were allowed to stand for 15 min, boiled in water for 5 min, and then cooled with water. The optical density of samples was determined at a wavelength of 565 nm using a Multiskan Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, USA).

Total DNA extraction and real-time polymerase chain reaction

Ruminal fluid was thawed in ultrapure water for 20 min, 300 μL aliquots transferred to centrifuge tubes, and total DNA extracted from ruminal microbes using a genome extraction kit (Tiangen Biotechnology Co. Ltd., Beijing, China), according to the manufacturer's instructions. The concentration and purity of total DNA were measured using a NanoDrop ND-1000 ultramicrospectrophotometer (Thermo Fisher, USA).

Primer sequences are presented in Table 2 and oligonucleotides were synthesized by a subsidiary of Invitrogen (Shanghai, China). Real-time polymerase chain reaction (PCR) assays were performed on a LightCycler 96 system, using its Sequence Detection Software (Roche, Basel, Switzerland). Reactions were performed in triplicate in reaction volumes of 20 μL in optical reaction plates (Roche, Switzerland) sealed with optical adhesive film. Reactions were performed using the LightCycler 96 system, and contained Master Mix (10 μL), 50 to 100 ng genomic DNA (5 μL), PCR grade water (3 μL), forward primer (1 μL), and reverse primer (1 μL). Real-time PCR was carried out according to the manufacturer's instructions, with the following cycles: pre-incubation at 95°C for 600 s, and 45 cycles of melting at 95°C for 10 s, annealing at 60°C/50°C (*Ciliate protozoa*) for 60 s, and extension at 72°C for 10 s.

Table 2. Primer used for the real-time polymerase chain reaction

Gene name	Primer sequence 5'-3'	Amplicon size (bp)
General bacteria	F:CGGCAACGAGCGCAACCC R:CCATTGTAGCACGTGTGTAGCC	130 [13]
Ruminococcus flavefaciens	F:CGAACGGAGATAATTTGAGTTTACTTAGG R:CGGTCTCTGTATGTTATGAGGTATTACC	132 [13]
Butyrivibrio fibrisolvens	F:ACCGCATAAGCGCACGGA R:CGGGTCCATCTTGTACCGATAAAT	65 [14]
Prevotella ruminicola	F:GCGAAAGTCGGATTAATGCTCTATG R:CCCATCCTATAGCGGTAAACCTTTG	78 [14]
Lactobacillus spp.	F:AGCAGTAGGGAATCTTCCA R:ATTCCACCGCTACACATG	345 [15]
Streptococcus bovis	F:TTCCTAGAGATAGGAAGTTTCTTCGG R:ATGATGGCAACTAACAATAGGGGT	127 [14]
General fungi	F:GAGGAAGTAAAAGTCGTAACAAGGTTTC R:CAAATTCACAAAGGGTAGGATGATT	120 [13]
Ruminobacter amylophilus	F:CTGGGGAGCTGCCTGAAT R:CATCTGAATGCGACTGGTTG	100 [14]
Ciliate protozoa	F:GCTTTCGWTGGTAGTGTATT R:CTTGCCCTCYAATCGTWCT	234 [16]

Statistical analyses

The gene 'general bacteria' was used as a reference gene. Relative gene expression of microbes was calculated using the $2^{-\Delta\Delta Ct}$ method as follows:

$$\text{Relative quantification} = 2^{-[(Ct\ \text{target gene} - Ct\ \text{reference gene})\ \text{treatment group} - (Ct\ \text{target gene} - Ct\ \text{reference gene})\ \text{control group}]}$$

Data were managed using Microsoft Excel 2007 and analyzed using the PROC general linear model procedure in SAS version 9.2 (Inst. Inc., Cary, NC, USA). The mathematical model used for the analysis was:

$$Y_{ijk} = \mu + P_i + C_j + T_k + E_{ijk}$$

Where, Y_{ijk} = observed variables, μ = overall mean, P_i = effect of period i (range = 1 to 4), C_j = effect of animal j (range = 1 to 4), T_k = effect of treatment k (range = 1 to 4), and E_{ijk} = random error. A Duncan multiple comparison test was used to compare treatment means. Less than 0.05 p value was taken to indicate significance and $0.05<p\leq0.10$ was taken as an indication of tendency.

RESULTS

Effects of AFE on production performance and apparent total-tract digestibility of nutrients in dairy cows

As shown in Table 3, the feed intake of the group receiving 60 mg/kg BW AFE was significantly higher ($p<0.05$) than that animals receiving 100 mg/kg BW AFE. The fat and protein content of milk showed decrease, and the lactose content of milk showed increase with ingestion of AFE, but each group had no significant difference. The total solids content of milk reduced ($p = 0.05$) linearly in response to increasing AFE supplementation. The somatic cell counts of milk exhibited a tendency to decrease ($p = 0.10$) quadratically with increasing AFE supplementation, and the somatic cell counts of milk was significantly lower ($p<0.05$) in the group receiving 60 mg/kg BW AFE than in the control group. Apparent total-tract digestibility of CP and NDF showed a tendency to increase ($0.05<p\leq0.10$) with increasing AFE supplementation; however, AFE had no effect on apparent total-tract digestibility of DM, ADF, or EE (Table 4).

Effects of AFE on anti-oxidants, immunoglobulin concentrations, and whole blood cell counts in dairy cows

As shown in Table 5, SOD activity showed a tendency to increase ($p = 0.10$) quadratically with increasing AFE supplementation; however, MDA concentration decreased ($p = 0.03$) linearly in response to AFE supplementation. In addition, MDA concentration in control group was significantly higher ($p<0.05$) than that animals receiving 60 mg/kg BW and 100 mg/kg BW AFE. GSH-Px activity levels increased with increasing AFE supplementation; however, the observed difference was not significant. Concentrations of IgG, IgM, and IgA were not affected by AFE (Table 6). As shown in Table 7, lymphocyte counts and propor-

Table 3. Effects of AFE on production performance of dairy cows

Items	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
Feed intake (kg/d)	29.49[ab]	29.77[ab]	30.81[a]	28.57[b]	0.61	0.55	0.07
Milk yield	28.76	30.99	34.31	30.76	2.54	0.43	0.28
Composition of milk (g/kg)							
Fat	34.67	32.98	32.81	32.65	1.93	0.49	0.70
Protein	30.43	28.85	27.00	27.56	1.97	0.26	0.60
Lactose	48.80	49.91	50.12	49.55	0.62	0.40	0.20
Milk total solid	123.23	120.38	118.56	118.44	1.65	0.05	0.43
Somatic cells count ($\times 10^3$/mL)	28.17[a]	23.92[ab]	21.08[b]	23.42[ab]	1.17	0.12	0.10

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.
[ab] In the same row, values with different letters mean significant differences ($p>0.05$), and the same or no letter mean no significant differences ($p<0.05$).

Table 4. Effects of AFE on apparent total-tract digestibility (%) of nutrients of dairy cows

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
Dry matter	74.43	75.62	76.90	78.19	1.82	0.17	0.98
Ether extracts	70.26	75.75	76.81	75.51	2.42	0.17	0.21
Crude protein	81.26	81.05	82.57	84.82	1.21	0.07	0.35
Acid detergent fiber	40.61	45.45	47.22	53.94	6.94	0.23	0.89
Neutral detergent fiber	41.87	47.03	47.89	55.22	4.74	0.10	0.82

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.

Table 5. Effects of AFE on anti-oxidative resistance of dairy cows

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
Superoxide dismutase (U/mL)	42.25	46.37	49.62	47.64	2.49	0.12	0.10
Glutathione peroxidase (U/mL)	454.49	535.73	455.44	535.93	25.04	0.19	0.99
Catalase (U/mL)	59.62	60.96	60.14	58.56	2.04	0.67	0.50
Methane dicarboxylic aldehyde (nmol/mL)	1.72[a]	1.37[ab]	1.25[b]	1.24[b]	0.14	0.03	0.23

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.
[ab] Means with the same letter in the row differ at $p < 0.05$.

tions decreased (p = 0.03) linearly with increasing doses of AFE, whereas the proportion of neutrophil granulocytes increased (p = 0.01) linearly. In addition, lymphocyte counts and proportions in control group was significantly higher (p<0.05) than that animals receiving 100 mg/kg BW AFE, and the proportion of neutrophil granulocytes showed the opposite result. The neutrophil granulocyte count for the groups receiving 20 to 100 mg/kg BW AFE supplementation tended to increase (p = 0.09) compared with controls.

Effects of AFE on ruminal fermentation parameters and relative expression of microbial genes in dairy cows

The valeric acid/TVFA ratio was increased (p = 0.01) linearly with increasing of the level of AFE supplementation. The ratio

Table 6. Effects of AFE on immunoglobulin concentration (g/L) of dairy cows

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
Immunoglobulin G	11.42	11.00	12.09	9.41	0.98	0.29	0.27
Immunoglobulin M	2.70	2.65	2.45	2.55	0.17	0.40	0.68
Immunoglobulin A	0.73	0.75	0.75	0.68	0.07	0.57	0.52

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.

Table 7. Effects of AFE on blood cells count of dairy cows

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
White blood cells (10^9/L)	13.52	13.54	13.70	13.41	0.23	0.83	0.57
Neutrophil granulocyte (10^9/L)	4.17	4.42	4.90	4.87	0.90	0.09	0.64
Lymphocyte (10^9/L)	8.78[a]	8.56[ab]	8.29[ab]	8.09[b]	0.14	0.03	0.20
Monocyte (10^9/L)	0.23	0.27	0.25	0.24	0.05	0.80	0.80
Eosinophilic granulocyte (10^9/L)	0.30	0.23	0.21	0.16	0.07	0.36	0.43
Basophilic granulocyte (10^9/L)	0.058	0.055	0.048	0.050	0.008	0.40	0.75
Neutrophil granulocyte (%)	33.05[c]	34.37[b]	37.42[ab]	38.50[a]	0.91	0.01	0.23
Lymphocyte (%)	62.05[a]	60.95[ab]	58.25[ab]	57.82[b]	1.08	0.03	0.51
Monocyte (%)	2.02	2.40	2.32	1.75	0.29	0.25	0.74
Eosinophilic granulocyte (%)	2.37	1.80	1.62	1.52	0.30	0.28	0.30
Basophilic granulocyte (%)	0.50	0.48	0.38	0.40	0.09	0.35	0.79

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.
[ab] Means with the same letter in the row differ at $p < 0.05$.

of valeric acid/TVFA in both the control group and group receiving 20 mg/kg BW AFE was significantly lower ($p<0.05$) than that of group receiving 100 mg/kg BW AFE. However, the other ruminal fermentation parameters were unaffected by AFE (Table 8). It was observed a trend for a quadratic response in the relative expression levels of *Ruminococcus flavefaciens* (*R. flavefaciens*) (Table 9). The relative expression levels of *R. flavefaciens* was decreased firstly and then increased with increasing doses of AFE supplementation. The relative expression levels of *Butyrivibrio fibrisolvens* (*B. Fibrisolvens*) showed a tendency to increase ($p = 0.07$) quadratically with increasing AFE supplementation. The relative expression levels of other microbial genes were unaffected by AFE supplementation.

DISCUSSION

Effects of flavonoid supplementation on the production performance of dairy cows

Gessner et al [17] and Winkler et al [18] reported that a diet containing grape marc meal extract or green tea and curcuma extracts, which are rich in flavonoids, could increase the milk yield of dairy cows, although the authors reported no effects of these substances on feed intake. In contrast, it was not observed significant differences in the feed intake and milk yield when cows fed a diet supplemented with AFE in the present study, which agrees with previous studies that used a propolis extract rich in flavonoids in the diets of dairy cows [6].These results demonstrate that, although flavonoids may improve the production performance of dairy cows, flavonoids from various sources exhibit different effects. Cant et al [19] showed that milk fat and protein content were decreased with increased lactose synthesis and secretion of water into milk. The results of our study indicate that AFE may increase lactose synthesis and then promote secretion of milk. The total solids content of milk reduced with increasing AFE supplementation. It showed that AFE reduced the total solids content of milk in relation to decreasing the contents of milk fat and protein. In addition, we found that feed intake and milk yield was decreased in animals fed 100 mg/kg BW AFE. The results showed high dose of AFE may have negative effect of production performance in dairy cows. Therefore, our results differ somewhat from those of previous reports, which may reflect the fact that different materials contain different types of flavonoids, which have distinct effects on the production performance of dairy cows. Somatic cells in milk comprise approximately 98% to 99% white

Table 8. Effects of AFE on ruminal fermentation parameters of dairy cows

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
pH	5.67	5.75	5.62	5.71	0.07	0.92	0.95
Ammonia nitrogen (mg/dL)	13.63	12.85	13.69	13.46	0.84	0.93	0.75
Total volatile fatty acid (mmol/L)	174.25	156.18	164.94	154.54	12.54	0.40	0.77
Acetic acid: TVFA	0.60	0.60	0.60	0.59	0.01	0.66	0.45
Propionic acid: TVFA	0.24	0.23	0.23	0.23	0.01	0.74	0.74
Butyric acid: TVFA	0.12	0.12	0.12	0.12	0.003	0.64	0.56
Valeric acid: TVFA	0.016[b]	0.016[b]	0.018[ab]	0.021[a]	0.001	0.01	0.35
Acetic acid: propionic acid	2.52	2.66	2.59	2.58	0.13	0.85	0.56
Isobutyric acid: TVFA	0.012	0.011	0.012	0.012	0.002	0.86	0.74
Isovaleric acid: TVFA	0.014	0.017	0.015	0.017	0.001	0.44	0.55
Lactic acid (μg/mg)	0.022	0.023	0.021	0.018	0.002	0.24	0.35
Microbial crude proteins (mg/mL)	4.92	4.85	4.86	5.74	0.70	0.42	0.42

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean; TVFA, total volatile fatty acid.
[ab] Means with the same letter in the row differ at $p<0.05$.

Table 9. Effects of AFE on relative expression of microbial gene

Item	AFE				SEM	p-values	
	Con	20 mg/kg BW	60 mg/kg BW	100 mg/kg BW		Linear	Quadratic
Streptococcus bovis	1.00	0.77	0.89	0.83	0.09	0.36	0.38
Lactobacillus spp.	1.00	0.78	0.89	0.85	0.15	0.63	0.56
Ruminobacter amylophilus	1.00	0.61	0.81	0.86	0.10	0.27	0.26
Ruminococcus flavefaciens	1.00	0.59	0.83	0.92	0.10	1.00	0.09
Butyrivibrio fibrisolvens	1.00	1.65	2.04	1.10	0.15	0.68	0.07
Prevotella ruminicola	1.00	0.95	1.21	1.06	0.13	0.49	0.72
General fungi	1.00	1.35	1.79	1.40	0.18	0.36	0.36
Ciliate protozoa	1.01	1.04	1.21	1.13	0.17	0.51	0.74

AFE, alfalfa flavonoids extract; BW, body weight; SEM, standard error of the mean.

blood cells and 1% to 2% mammary gland epithelial cells. Cows suffering from mastitis can exhibit increased milk somatic cell counts, hence, the results of the present study, demonstrating alterations in lymphocyte proportions, may indicate that AFE can reduce the incidence of mastitis in dairy cows.

Effects of flavonoids on the digestion and metabolism of dairy cows

Aguiar et al [20] found that the different level of propolis total flavonoids ingested by dairy cows did not affect DM, organic matter (OM), CP, and NDF compared with control diet. Furthermore, the digestibility of DM, CP, and NDF were significantly higher in a group of cows receiving 2.81 mg/kg DM propolis total flavonoids than those of animals receiving 1.22 mg/kg of the same substance. In addition, Paula et al [21] found the digestibility of CP had a linear tendency with increasing the level of propolis flavonoids when propolis total flavonoids were dosed in the rumen of water buffaloes. Our results were similar with the results of Paula et al [21]. The results showed that AFE may have a tendency of increasing the digestion of dietary protein and fiber. However, mulberry leaf flavonoid supplementation is reported to have no effect on the digestibility of DM, OM, NDF, and ADF [22]. Together, these results indicate that flavonoids can affect the digestibility and utilization of nutrients; however, flavonoids from different plants, and at different concentrations, produce different effects.

The rumen is a classical host–microbe symbiotic system. The digestion of nutrient substances, such as plant materials, and their subsequent conversion to supply the energy requirements of the host ruminant are accomplished in the rumen. Seradj et al [23] found that flavonoids (Bioflavex) led to an increase in the levels of *Streptococcus bovis* (*S. bovis*) and *Megasphaera elsdenii* in the rumen of dairy cows, while those of hydrogenotrophic methanogenic archaea and *Methanosarcina* spp. were reduced. In addition, populations of the ciliates, *Ruminobacter albus* and *R. flavefaciens* were decreased, and the diversity of *Fibrobacter succinogenes* (*F. succinogenes*) was increased in response to flavonoid-rich plant extracts [5]. Flavonoids have an anti-microbial function, which may affect ruminal microorganisms either positively or negatively. Ruminal microbes have important roles in the degradation of nutrient substances. Flavonoids could, therefore, regulate the microbial flora of the rumen, with consequent effects on the digestion and metabolism of nutrient substances. Cellulose can be degraded by cellulolytic bacteria, such as *F. succinogenes*, *R. flavefaciens*, and *B. fibrisolvens*. Protein can be decomposed by proteolytic bacteria including *Ruminobacter Amylophilus*, *B. fibrisolvens*, *Prevotella* spp., *S. bovis*, fungi and protozoa. The growth of *F. succinogenes* needs valeric acid and isobutyric acid [24]. The valeric acid/TVFA ratio was increased linearly with increasing of the level of AFE supplementation in this study. The result showed AFE might promote the growth of *F. Succinogenes*. The results of this study indicated that the populations of *B. fibrisolvens* had a tendency to increase with increasing levels of AFE and the populations of *R. flavefaciens* showed a tendency to increase with supplementing 20 to 100 mg/kg BW AFE. The results indicate that AFE supplementation may increase degradation of cellulose and protein by elevating the populations of decomposing bacteria of cellulose and protein. Kim et al [5] reported that flavonoid-rich plant extracts do not affect ruminal fermentation characteristics. In addition, fermentation parameters are not affected by naringin and quercetin [4], consistent with the results of this study. In conclusion, flavonoids extracted from alfalfa may improve the digestion of fodder through regulating the population of ruminal microorganisms.

Effects of flavonoids on antioxidant capacity and immunity

Due to their strong oxidative characteristics, free radicals can damage the tissues and cells of organisms. The protective functions of flavonoids in biological systems are ascribed to their antioxidant capabilities [3]. Su et al [25] found that AFE could improve the antioxidant capacity of mammary epithelial cells by increasing the activities of CAT and GSH-Px, and decreasing the concentration of MAD. In the present study we found that MAD concentrations decreased, and SOD activity had a tendency to increase, in response to AFE, whereas CAT and GSH-Px activities were unaffected, similar with the findings of previous reports. Hence evidence indicates that flavonoids extracted from alfalfa can improve antioxidant capacity by increasing the activities of antioxidant enzymes, thereby protecting tissues and cells against damage mediated by free radicals.

Immunoglobulins are a type of globulin protein which either act as antibodies or have similar chemical structures to those of antibodies. Immunoglobulins function in antigen-specific binding and play important roles in regulating immune responses. Flavonoids from soybean can increase the concentrations of IgA, and IgG in both the serum and milk of dairy cows, whereas flavonoids extracted from propolis decrease the IgG and IgM concentrations in the serum of Holstein calves [26,27]. Flavonoids have been reported to affect humoral immune responses by regulating the concentrations of IgG and IgM. Satomi et al [28] found that levels of IgG, IgA, and IgM in mice were not affected by apigenin. Our results are, therefore, consistent with the previous report in mice and indicate that AFE can not influence humoral immune responses of dairy cows.

Neutrophil granulocytes have important roles in innate immunity, including killing pathogenic microorganisms and secreting antimicrobial substances during infections. We found that the proportion of neutrophil granulocytes ($p = 0.01$) among blood cells was positively correlated with the level of AFE supplementation, indicating that AFE may improve the ability of animals to withstand infection. Daidzein functions in both immunosuppression and immunostimulation. Low doses of daidzein can promote lymphocyte proliferation, whereas high doses have the

opposite effect [29]. In this study we found that lymphocyte counts and proportions reduced with increasing doses of AFE, consistent with the findings of Liu et al [29]. In addition, other studies have reported that flavonoids, such as baicalin and myricetin, may inhibit lymphocyte activation and proliferation [30], indicating that these flavonoids act as *in vitro* anti-inflammatory factors inhibiting lymphocyte activity; therefore, AFE is potentially an immunosuppressant.

CONCLUSION

In our study, we found that AFE supplementation exceeding 60 mg/kg BW AFE may decrease feed intake of dairy cows and milk total solid. AFE had a tendency of increasing CP and NDF apparent digestibility. Moreover, AFE could increase the valeric acid/TVFA ratio and had a tendency of increasing population of *B. Fibrisolvens*. The results showed AFE may enhance the digestion of CP and NDF by regulating populations of ruminal microbes. AFE supplementation had a tendency of increasing SOD activity and neutrophil granulocyte counts, whereas AFE supplementation decreased somatic cells count, MDA concentration and lymphocyte counts. These results indicate that AFE may prevent mastitis of dairy cows by increasing antioxidant capacity and improving non-specific immunity. In conclusion, the optimal dose of AFE supplementation is 60 mg/kg BW in this study.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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