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Effects of a combination of fibrolytic and amylolytic enzymes on ruminal enzyme activities, bacterial diversity, blood profile and milk production in dairy cows

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ABSTRACT

We hypothesised that adding a combination of fibrolytic and amylolytic enzymes to the diet of early-lactation dairy cows would improve rumen enzyme activity and bacterial diversity, promote energy metabolism, and benefit milk production in cows. Twenty multiparous early-lactation (90 ± 5 d) Holstein cows with similar body conditions were randomly allocated to control (**CON**, $n = 10$) and experimental (**EXP**, $n = 10$) groups in a completely randomised single-factor design. The CON was fed only a basal total mixed ration diet, and the diet of the EXP was supplemented with a combination of fibrolytic and amylolytic enzymes at 70 g/cow/d (cellulase 3 500 CU/g, xylanase 2 000 XU/g, β-glucanase 17 500 GU/g, and amylase 37 000 AU/g). The experiment lasted 28 days, with 21 days for adaptation and 7 days for sampling. Enzyme addition increased the activity levels of α-amylase and xylanase, and the ammonia-N concentration ($P < 0.05$) tended to increase the activity of β-glucanase ($P = 0.08$) in rumen fluid. However, there was no significant difference in the rumen bacterial richness and diversity, phylum (richness > 0.1%) or genus (richness > 1%) composition between the CON and EXP groups ($P > 0.05$). A tendency of difference was found between CON and EXP ($R = 0.22$, $P = 0.098$) in principal component analysis. Ten genera showed different abundances across the CON and EXP groups (linear discriminant analysis effect size, linear discriminant analysis > 2). EXP increased the ratio of albumin to globulin and the concentrations of total cholesterol and low-density lipoprotein cholesterol ($P < 0.05$) and tended to increase triglycerides ($P = 0.09$) in blood. Milk yield, 3.5% fat-corrected milk yield and energy-corrected milk yield increased with enzyme supplementation ($P < 0.05$). The production levels of milk fat and lactose increased, but the percentage of solids, not fat and protein, decreased in EXP ($P < 0.05$). Although the DM intake was not affected, the feed efficiency tended to increase ($P = 0.07$) in EXP. In conclusion, dietary supplementation with a mixture of fibrolytic and amylolytic enzymes on multiparous early-lactation dairy cows increased α-amylase and xylanase activity levels in rumen fluid, enhanced milk performance and tended to improve the feed efficiency in cows.

Implications

We aimed to detect the effects of a combination of fibrolytic and amylolytic enzymes on ruminal enzyme activities, bacterial diversity, blood profile and milk production in dairy cows. We found that dietary supplementation with enzymes increased α-amylase and xylanase activity levels in rumen fluid, enhanced milk performance and tended to improve feed efficiency in cows. These findings provide evidence in the field of combined supplementation of fibrolytic and amylolytic enzymes in dairy cows. Further research to determine metagenomics and metabolomics of rumen content, transcriptomics and nutrient absorption of rumen epithelium is needed.

Introduction

Supplementation with exogenous enzymes is a potential way to enhance animal production. In addition, the production cost of exogenous enzymes is becoming lower and has attracted the attention of scientists (Zilio et al., 2019). Previously, researchers have worried that the added exogenous enzymes would be suppressed by ruminal proteolysis (Chesson, 1994). In this century, a great

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number of dietary enzyme trials were carried out (Zilio et al., 2019) and certified that some exogenous enzymes have the potential to promote rumen fermentation (microorganisms and/or microbial enzymes) (McAllister et al., 2001) and enhance production performance in dairy cows (Adesogan et al., 2019; Zilio et al., 2019).

Cellulose and xylanase are the two main evaluated fibre-degrading enzymes (Zilio et al., 2019) in dairy cows. Inconsistent effects were found on nutrient utilisation (Kung et al., 2000; Yang et al., 2000; Elwakeel et al., 2007) and production performance (Murad and Azzaz, 2010; Dean et al., 2013; Kholif and Aziz, 2014). Recently, two meta-analyses (Arriola et al., 2017; Tirado-González et al., 2018) showed that supplementing diets with exogenous fibre-degrading enzymes has positive overall effects on lactation in dairy cows (Adesogan et al., 2019). Factors responsible for variations in responses to exogenous enzymes include the forage to concentrate ratio (Tirado-González et al., 2018), application method (to concentrate, forage, or total mixed ration (**TMR**)) (Adesogan, 2005; Arriola et al., 2017), type of enzyme and forage (Tirado-González et al., 2018; Yang et al., 2019), duration of experiment (Adesogan et al., 2014; Arriola et al., 2017), lactation stage (Schingoethe et al., 1999; Beauchemin et al., 2003), and production level (Refat et al., 2018) of dairy cows.

In addition to fibre-degrading enzymes, amylase has also attracted wide attention. Supplementing dairy cow diets with exogenous amylolytic enzymes promoted rumen fermentation (ruminal starch digestibility and propionate proportion) (Noziere et al., 2014), milk yield (Tricarico et al., 2005; Klingerman et al., 2009), and feed efficiency (Gencoglu et al., 2010; Andreazzi et al., 2018) without increasing the risk of acidosis. Nevertheless, inconsistent effects have been reported on ruminal fermentation (Andreazzi et al., 2018), milk yield (Gencoglu et al., 2010; Weiss et al., 2011), and feed efficiency (Weiss et al., 2011), indicating that responses to amylolytic enzymes are affected by the concentration (Noziere et al., 2014; Andreazzi et al., 2018) and type (Andreazzi et al., 2018) of dietary starch, the nature of the dietary fibre (Andreazzi et al., 2018), and the lactation stage (Bachmann et al., 2018) of dairy cows.

Theoretically, exogenous fibrolytic and amylolytic enzymes are expected to have synergistic effects when dietarily supplemented together with dairy cows (Tricarico et al., 2008; Bajaj and Mahajan, 2019). The addition of α-amylase is hypothesised to promote fibre digestion because more starch hydrolysis products are provided to rumen microbes, and some products, such as maltodextrin, are substrates for both amylolytic and fibrolytic bacteria (Tricarico et al., 2008). In addition, α-amylase can produce more oligosaccharides from amylose and amylopectin, which may affect rumen fermentation (Tricarico et al., 2008). Moreover, the supplemented fibrolytic enzymes can digest more cellulose and hemicellulose in the cell wall and release more sugars and starch, therefore promoting amylolytic microbes (Bajaj and Mahajan, 2019). However, to the best of our knowledge, only two experiments have studied the effects of fibrolytic and amylolytic enzymes in combination (Hristov et al., 2008; Zilio et al., 2019). Moreover, they reported no effects on nutrient intake and digestibility, ruminal fermentation, or production performance. This may have been caused by the unsuitable enzyme delivery method (intraruminal) (Hristov et al., 2000), the limited number of animals (only four cows) for the experiment of Hristov et al., 2008, the inappropriate time and diet portion of enzyme supplementation (once a week into the concentrate during its preparation) (Beauchemin et al., 2003), and the lactation stage of cows (Schingoethe et al., 1999; Beauchemin et al., 2003; Hristov et al., 2008; Zilio et al., 2019) for the trial of Zilio et al., 2019. In the current study, 20 early-lactation cows were used, and the enzymes were supplemented into the TMR during its production twice daily. The enzyme activities and bacterial diversity of the rumen were not measured in two previous studies (Hristov et al., 2008; Zilio et al., 2019) but were determined in the current study, which could help us better understand the effects and mechanism of enzyme supplementation.

In this context, we hypothesised that adding a mixture of fibrolytic (cellulase, xylanase, and β-glucanase) and amylolytic (amylase) enzymes into the diet of early-lactation multiparous dairy cows would improve rumen enzyme activity and bacterial diversity, promote energy metabolism, and benefit milk production of cows.

Material and methods

Experimental design, animals, and diets

The experiment was carried out in the Modern Farm (Baoji, China) and the Laboratory of Animal Nutrition at Northwest A&F University (Yangling, Shaanxi, China). All animal research procedures were approved by the Northwest A&F University Animal Care and Use Committee (protocol number: NWAFAC1211; Yangling, Shaanxi, China). Twenty multiparous (average parity 3.5) early-lactation (90 ± 5 days in milk) Holstein Friesian dairy cows of similar body condition score were randomly assigned to control (**CON**, $n = 10$) and experimental (**EXP**, $n = 10$) groups as a completely randomised single-factor design. All cows were fed a TMR (Table 1), and the experimental group was supplemented with a complex enzyme preparation at 70 g/cow/d, with enzyme preparation added to the TMR during its production twice daily. The TMR (Table 1) was prepared twice per day before feeding by Stationary Mixer Feeders (Trioliet *Solomix 3*). The mixture of fibrolytic and amylolytic enzymes (Guangdong VTR Bio-Tech Co., ltd.) contained fibrolytic enzymes (cellulase 3 500 CU/g, xylanase 2 000 XU/g, and β-glucanase 17 500 GU/g) and amylolytic enzyme (amylase

Table 1
Ingredients of the TMR diet and the nutrient composition for dairy cows.

Items	Value
Ingredients (% of DM)	
Corn silage	48.3
Alfalfa hay	8.04
Steam flaked corn	13.7
Corn	5.36
Soybean meal	6.70
Soybean hull	2.68
Cottonseed meal	4.29
Corn bran	6.70
Cottonseed	2.68
Vitamin-mineral mix[1]	1.09
Rumen-protected fat[2]	0.40
Optigen[3]	0.13
Calculated nutrient values	
DM (%)	57.1
ADF (% of DM)	15.9
NDF (% of DM)	24.9
CP (% of DM)	18.9
EE (% of DM)	4.80
Starch (% of DM)	23.8
Ca (% of DM)	0.52
P (% of DM)	0.37

Abbreviations: TMR = total mixed ration; EE = ethanol extract.
[1] Each kilogram contained 400 mg Cu, 2 400 mg Fe, 4 000 mg Zn, 2 000 mg Mn, 40 mg I, 30 mg Se, 50 mg Co, 40 mg vitamin B1, 1 mg vitamin B12, 1 200 mg nicotinic acid, 700 mg pantothenic acid, 45 mg vitamin K3, 300 KIU vitamin A, 100 KIU vitamin D3, and 6 500 IU vitamin E.
[2] Megalac protected fat, a calcium salt of palm fatty acid distillate, produced by Yihai Kerry Arawana Holdings Co., ltd. (Shanghai, China).
[3] A product of Alltech Inc. (Nicholasville, Kentucky, USA), which provides a controlled release of non-protein nitrogen to the rumen over time.

37 000 AU/g). The activities of xylanase, cellulase and β-glucanase were determined using the dinitrosalicylic acid method. Cellulose, β-glucan and birchwood xylan (each at 10 g/l in 50 mM sodium phosphate buffer, pH 7.0) were used as substrates to react with the rumen fluid supernatant, and the OD was read at 540 nm. The reaction time of xylanase (Khanna, 1993) was 15 min, and those of cellulase and β-glucanase (Zhang et al., 2009) were both 30 min. Cellulase units (**CUs**), xylanase units (**XUs**), and β-glucanase units (**GUs**) are defined as μmol of reducing sugars released per minute. The activity of amylase (Visvanathan et al., 2016) was measured by a kit (Jiancheng Bioengineering Institute, Nanjing, China). This kit determines the amylase activity by measuring soluble starch dextrinised at 60 °C, pH 6.0, with iodine solution, using 10 mg/ml soluble starch in 126 mmol/l phosphate buffer at pH 6.0. Amylase units (**AUs**) are defined as mg of soluble starch dextrinised per hour. The trial lasted 28 days, including 21 d for adaptation followed by 7 d for sampling. Cows were fed twice daily (0600 and 1400 h) with at least 5% refusals in the feed trough, had free access to water and were milked three times per day (0000–0100, 0700–0800, and 1400–1500 h).

Sample collection and laboratory analyses

Ruminal enzymes and microbes

At 0900 h (3 h after the 0600 h morning feeding) on the fourth day of the sampling period, 400 ml of rumen liquid was collected per cow (n = 3 cows/group) by oral intubation. The pH was detected immediately by a pH metre. Two hundred millilitres of rumen liquid was filtered by four layers of gauze and then divided into 10 ml centrifuge tubes. These tubes were transported on ice in an insulated container to the laboratory of Northwest A&F University within approximately 30 min to determine rumen enzyme activity and rumen bacterial diversity. Some of the rumen fluid samples were centrifuged at 1 000*g* at 4 °C for 10 min. The supernatant was used for analyses of the activities of amylase, xylanase, cellulase and β-glucanase as described above. The ammonia nitrogen concentration of rumen fluid was determined on a spectrophotometer using the phenol-hypochlorite method (Weatherburn, 1967). Total genomic DNA was extracted from rumen samples using hexadecyl trimethyl ammonium bromide, and the concentration and purity were assessed using agarose gel (1%) electrophoresis (Quast et al., 2012). The V3-V4 variable region of bacterial 16S rRNA was amplified by PCR using the specific primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-CCGTCAATT CCTTTGAGTTT-3′). The PCRs were conducted using the following program: 2 min of predenaturation at 95 °C; 30 s for denaturation at 95 °C, 30 s for annealing at 55 °C, 30 s for extension at 72 °C, cycle 30 times; and a final extension at 72 °C for 5 min. The PCRs were performed in a 50 μl mixture containing 0.25 μl of Tap enzyme (5 U/μl), 5.0 μl of 10 × buffer, 1.0 μl of dNTPs (10 mM/l), 1.25 μl of each primer (10 μM/l), 40.25 μl of ddH_2O, and 1.0 μl of template DNA (50 ng/μl). The resulting PCR products were first gel purified using a 2% agarose gel and an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) and quantified using QuantiFluor-ST (Promega, Madison, WI) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA) according to standard protocols by Majorbio Bio-Pharm Technology Co. ltd. (Shanghai, China). Raw FASTQ files were demultiplexed, quality-filtered using Trimmomatic (https://www.usadellab.org/cms/index.php?-page=trimmomatic), and merged using FLASH (https://ccb.jhu.edu/software/FLASH/) with the following criteria. (1) The reads were truncated at any site receiving an average quality score < 20 over a 50-bp sliding window. (2) The merged reads had identical barcodes and no more than two nucleotide mismatches in the primer regions, and reads containing ambiguous bases were removed. (3) The reads whose overlaps were longer than 10 bp were merged according to their overlap sequence. Operational taxonomic units were clustered with a 97% similarity cut-off using UPARSE (version 7.1, https://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME (https://www.drive5.com/uchime/). The taxonomy of each operational taxonomic unit was assigned by classifying its representative sequence using the RDP Classifier algorithm (https://rdp.cme.msu.edu/) against the Silva128 16S rRNA database (SSU123; https://www.arb-silva.de/) using a confidence threshold of 70%.

Blood profile

At 0900 h (3 h after the 0600 h morning feeding) on the fifth day of the sampling period, a 5 ml blood sample was collected per cow (n = 10 cows/group) from the tail vein with a procoagulant vacuum blood collection tube. Then, the tubes were centrifuged (4 °C, 3 500 r/min, 5 min) to separate the serum. Serum samples were frozen at −40 °C. The plasma concentrations of total cholesterol and insulin were determined using a kit (Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of plasma glucose, blood urea nitrogen (**BUN**), total protein, globulin, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol (**LDL-c**) were measured by an automatic blood biochemical analyser (Jinan Hanfang Medical Instrument Co., ltd).

Dry matter intake, milk composition, and milk yield

From the beginning of the adaptation period to the end of the sampling period, the refusals were weighed, and the dry matter intake (**DMI**) of cows was calculated at 0600 daily. The daily averages of these data were calculated and used for further statistical analysis. During 1400–1500 h (midday milking) on the first to third days of the sampling period, milk was collected manually from each teat after 2 min of milking (100 ml/cow, n = 10 cows/group) from four teats. The regular physical (density, freezing point depression, and acidity) and chemical parameters (milk fat, protein, lactose, total solids, and solids not fat) of the milk samples were analysed by a milk composition analyser (UL40AC-8, Hangzhou Ultrasun Technologies Co., ltd) at the farm laboratory. Milk yield was electronically recorded every day. The yield of 3.5% fat-corrected milk (kg/d) was calculated as actual milk yield (kg/d) × 0.4324 + milk fat yield (kg/d) × 16.216 (Tyrrell and Reid, 1965). The energy-corrected milk yield (kg/d) was calculated as actual milk yield (kg/d) × 0.327 + milk fat yield (kg/d) × 12.95 + milk protein yield (kg/d) × 7.20 (Tyrrell and Reid, 1965). Feed efficiency was calculated as energy-corrected milk yield (kg/d)/DMI (kg/d).

Statistical analysis

All data were detected for normality and outliers by using the Shapiro–Wilk and Grubbs's tests, respectively. SPSS statistical software (version 22.0, SPSS Inc., Chicago, USA) was used to determine the differences in all measures between the control and experimental groups, with supplementation of a combination of fibrolytic and amylolytic enzymes as the fixed factor and the cow as a random factor.

$$Y_{ij} = \mu + \text{treatment}_i + \text{cow}_j + \varepsilon_{ij}$$

where Y_{ij} = the kth observation of the jth cow in the ith treatment, μ = the overall mean, treatment_i = the fixed effect of the ith treatment (i = 0–1), cow_j = the random effect of the jth cow (j = 1–10), and ε_{ij} = the residual error associated with the jth cow in the ith treatment. All results are expressed as the mean and SEM (Tables

2–7). Significance was declared at $P \leq 0.05$, and trends were defined at $0.05 < P \leq 0.10$.

Alpha diversity (Chao, ACE, Sobs, Shannon, Simpson, Coverage, Shannoneven, and Simpsoneven) was measured, and CON and EXP were compared by Student's *t*-test. Differences in rumen bacterial relative abundances at the phylum (>0.1%) and genus (>1%) levels between CON and EXP samples were analysed by a two-tailed Student's *t*-test with false discovery rate multiple check calibration at a 0.95 confidence interval. Principal coordinates analysis of morisita-horn dissimilarity (Wolff et al., 2019) with the default 999 permutations, which was calculated from the operational taxonomic unit sequence count table, was used to visualise the difference in the bacterial community between CON and EXP by the ANOSIM statistical test. The strict version of linear discriminant analysis effect size with absolute abundance was used to determine significant genera differing between CON and EXP (linear discriminant analysis > 2).

Table 2
Effects of the combination of fibrolytic and amylolytic enzymes on the enzyme activity levels of rumen fluid in dairy cows, $n = 3$ cows/group.

Item	CON	EXP	*SEM*	*P* value
pH	6.35	6.49	0.059	0.256
α-Amylase (AU/ml)	0.31[b]	0.35[a]	0.005	0.001
Cellulase (CU/ml)	10.3	10.5	0.133	0.527
β-Glucanase (GU/ml)	37.3	38.4	0.322	0.081
Xylanase (XU/ml)	20.8[b]	23.2[a]	0.436	0.004
Ammonia nitrogen (mg/l)	235[b]	257[a]	3.48	0.001

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes.
[a,b] Different superscripts within a row indicate a significant difference ($P < 0.05$).

Table 3
Effects of the combination of fibrolytic and amylolytic enzymes on rumen bacterial diversity in dairy cows, $n = 3$ cows/group.

Item	CON	EXP	*SEM*	*P* value
Chao	1 270.60	1 268.20	37.938	0.979
ACE	1 254.70	1 249.50	34.546	0.950
Sobs	1 012.00	1 003.00	41.499	0.927
Shannon	4.00	4.24	0.286	0.724
Simpson	0.10	0.10	0.028	0.962
Coverage	0.99	0.99	0.001	0.458
Shannoneven	0.58	0.61	0.039	0.710
Simpsoneven	0.01	0.03	0.008	0.376

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes.

Table 4
Effects of the combination of fibrolytic and amylolytic enzymes on phyla of rumen bacteria with a relative richness content greater than 0.1% in dairy cows, $n = 3$ cows/group.

Phylum	CON	EXP	*SEM*	*P* value[1]
Bacteroidetes	81.30	82.33	1.743	0.804
Firmicutes	15.41	15.60	1.472	0.957
Proteobacteria	2.33	0.79	0.599	0.232
Tenericutes	0.47	0.47	0.040	0.964
Fibrobacteres	0.16	0.26	0.070	0.529
Spirochaetae	0.15	0.38	0.129	0.432
Cyanobacteria	0.15	0.12	0.022	0.579

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes.
[1] The difference was analysed by a two-tailed Student's *t*-test with false discovery rate (FDR) multiple check calibration at a 0.95 confidence interval.

Table 5
Effects of the combination of fibrolytic and amylolytic enzymes on the genera of rumen bacteria with a relative richness content greater than 1% in dairy cows, $n = 3$ cows/group.

Genus	CON	EXP	*SEM*	*P* value[1]
Prevotella_7	48.16	36.61	8.973	0.580
Prevotella_1	23.12	30.35	4.692	0.504
unclassified_f__Prevotellaceae	2.92	3.54	0.439	0.538
Succinivibrionaceae_UCG-001	2.28	0.68	0.608	0.219
Ruminococcaceae_UCG-014	2.07	1.68	0.186	0.348
Oribacterium	1.89	1.02	0.468	0.413
Prevotellaceae_YAB2003_group	1.43	1.21	0.229	0.680
Roseburia	1.40	0.88	0.215	0.261
norank_f__Bacteroidales_S24-7_group	1.18	3.18	0.897	0.315
Prevotellaceae_UCG-001	0.92	1.13	0.130	0.475
norank_f__Bacteroidales_BS11_gut_group	0.86	2.46	0.857	0.411
Lachnospiraceae_NK3A20_group	0.83	0.91	0.152	0.830
Ruminococcaceae_NK4A214_group	0.78	1.93	0.567	0.366
Rikenellaceae_RC9_gut_group	0.70	1.37	0.365	0.413
Succiniclasticum	0.53	1.36	0.380	0.326

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes.
[1] The difference was analysed by a two-tailed Student's *t*-test with false discovery rate (FDR) multiple check calibration at a 0.95 confidence interval.

Table 6
Effects of the combination of fibrolytic and amylolytic enzymes on blood indices in dairy cows, $n = 10$ cows/group.

Item	CON	EXP	*SEM*	*P* value
Insulin (μIU/ml)	25.85	21.63	3.148	0.518
Total protein (g/l)	51.22	50.66	2.266	0.906
Albumin (g/l)	24.02	27.04	0.994	0.132
Globulin (g/l)	27.20	23.62	1.576	0.267
Albumin: Globulin	0.93[b]	1.17[a]	0.052	0.016
Blood urea nitrogen (mmol/l)	4.34	4.73	0.223	0.393
Glucose (mmol/l)	2.69	2.67	0.125	0.924
Total cholesterol (mmol/l)	3.90[b]	5.16[a]	0.268	0.014
Triglyceride (mmol/l)	0.07	0.09	0.006	0.099
High-density lipoprotein cholesterol (mmol/l)	2.13	2.40	0.101	0.183
Low-density lipoprotein cholesterol (mmol/l)	0.62[b]	0.83[a]	0.044	0.015

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes.
[a,b] Different superscripts within a row indicate a significant difference ($P < 0.05$).

Results

Ruminal enzyme activities

The addition of enzyme preparation increased the activity levels of α-amylase and xylanase and the ammonia-N concentration in rumen fluid ($P < 0.05$, Table 2). In addition, the activity of β-glucanase tended to be increased ($P = 0.081$). No effect was found on pH or the activity of cellulase ($P > 0.05$, Table 2).

Ruminal bacterial diversity

After sequencing, a total sequence number of 342 926 was obtained, with an average of 57 154 reads per sample. The average length of the reads was approximately 370 bp. Sequences were

Table 7
Effects of the combination of fibrolytic and amylolytic enzymes on production performance in dairy cows, $n = 10$ cows/group.

Item	CON	EXP	*SEM*	*P* value
DMI (kg/d)	25.8	26.8	0.154	0.13
Milk yield (kg/d)				
Actual milk yield	42.3[b]	45.0[a]	0.334	0.001
3.5% FCM[1]	45.2[b]	48.3[a]	0.358	<0.001
ECM[2]	46.2[b]	50.8[a]	0.579	0.007
Fat	1.63[b]	1.87[a]	0.030	0.026
Protein	1.56	1.63	0.014	0.199
Lactose	1.95[b]	2.19[a]	0.030	0.039
Feed efficiency[3]	1.82	1.96	0.022	0.070
Milk composition (%)				
Fat	3.80	3.82	0.392	0.972
Protein	3.63[a]	3.32[b]	0.085	0.001
Lactose	4.55	4.46	0.106	0.474
Solids not fat	9.33[a]	8.91[b]	0.171	0.035
Density (g/cm^3)	1 033.90	1 032.57	0.792	0.153
Freezing point depression (℃)	0.53	0.51	0.010	0.103
Acidity (°T)	6.82[a]	6.28[b]	0.221	0.036

Abbreviations: CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes; DMI = dry matter intake; 3.5% FCM = 3.5% fat-corrected milk; ECM = energy-corrected milk.
[1] 3.5% Fat-corrected milk yield = actual milk yield (kg/d) × 0.4324 + milk fat yield (kg/d) × 16.216.
[2] Energy-corrected milk yield = actual milk yield (kg/d) × 0.327 + milk fat yield (kg/d) × 12.95 + milk protein yield (kg/d) × 7.20.
[3] Feed efficiency = energy-corrected milk yield (kg/d)/DM intake (kg/d).
[a,b] Different superscripts within a row indicate a significant difference ($P < 0.05$).

clustered into 1 488 operational taxonomic units. Overall, a total of 14 phyla, 24 classes, 37 orders, 63 families, and 188 genera from bacteria were identified, while 420 operational taxonomic units were identified to the species level. As the sequencing reads increased, the number of operational taxonomic units gradually plateaued in all samples, indicating that the amount of sequencing sampling reads was sufficient to reach plateau levels (Supplementary Figure S1). As shown in Table 3, there was no significant difference in the bacterial richness and diversity between the CON and EXP groups ($P > 0.05$).

At the phylum level, the rumen bacteria mainly included *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Table 4 and Supplementary Figure S2). There was no significant difference between CON and EXP in each phylum of bacteria with a relative richness content greater than 0.1%. The rumen bacterial composition at the genus level is shown in Table 5 and Supplementary Figure S3. *Prevotella_7* and *Prevotella_1* are the dominant genera, of which *Prevotella_7* accounts for the largest proportion. However, no significant difference was found between CON and EXP in each genus of bacteria with a relative richness content greater than 1%. To display the different abundances in the bacterial communities between CON and EXP, a principal coordinates analysis of morisita-horn dissimilarity (Wolff et al., 2019) with the default 999 permutations, which was calculated from the operational taxonomic unit sequence count table, was performed (Fig. 1). A tendency was found between CON and EXP ($R = 0.22$, $P = 0.098$). Linear discriminant analysis effect size was conducted, and bacteria with linear discriminant analysis scores greater than 2 were speculated to have different abundances across the CON and EXP (Fig. 2). Ten genera were found. Within them, five genera were more abundant in the CON, including *unclassified_f_Veillonellaceae*, *norank_f_Veillonellaceae*, *Syntrophococcus*, *Selenomonas*, and *Lachnospiraceae_UCG-001*. The other five genera were more abundant in the EXP, including *Ruminococcaceae_UCG-001*, *Hydrogenoanaerobacterium*, *Sphaerochaeta*, *Veillonellaceae_UCG-001*, and *[Eubacterium]_nodatum_group*.

Blood profile

As shown in Table 6, supplementation with the enzyme preparation increased the ratio of albumin to globulin (**A/G**), total cholesterol and low-density lipoprotein cholesterol ($P < 0.05$). Triglycerides tended to be increased in the EXP ($P = 0.09$). Apart

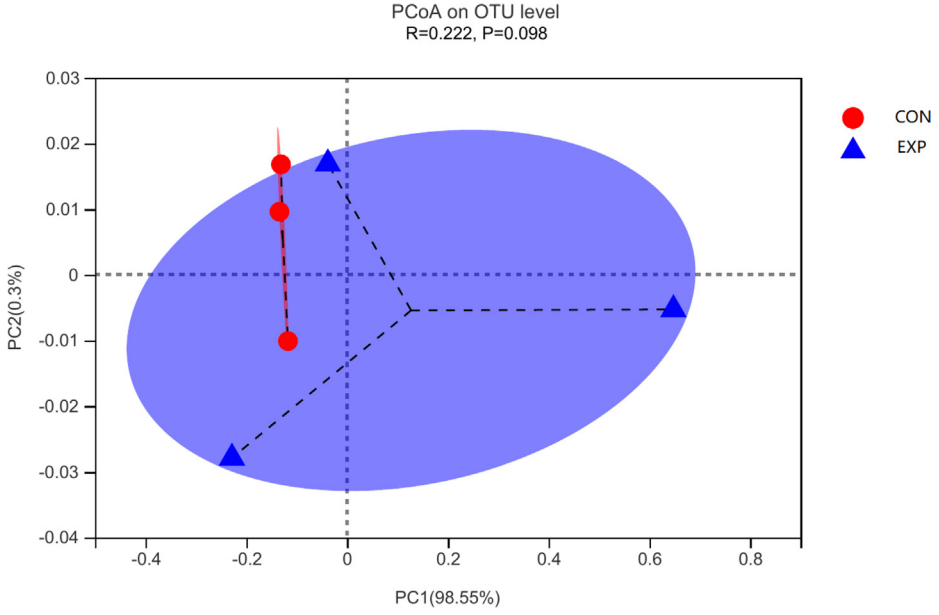

Fig. 1. Principal component analysis (PCoA) of the dairy cow's rumen bacterial community between CON and EXP at the operational taxonomic unit (OUT) level based on Morisita-horn dissimilarity with the default 999 permutations and the ANOSIM statistical test. CON = control group, cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes, $n = 3$ cows/group.

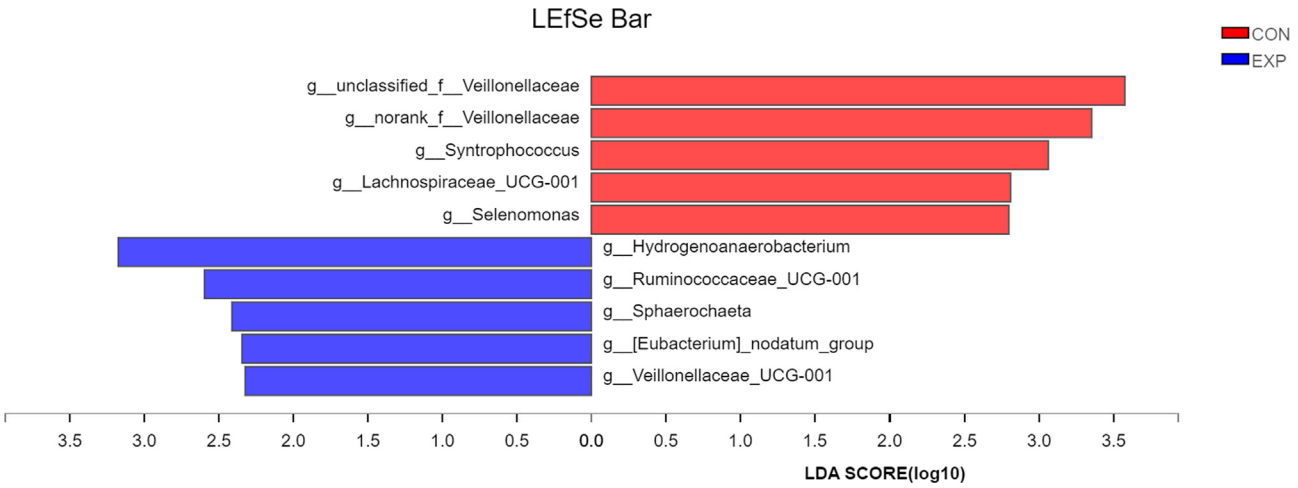

Fig. 2. Linear discriminant analysis effect size (LEfSe) with absolute abundance to determine significant rumen microbe genus differences between CON and EXP (histogram of linear discriminant analysis (LDA) scores greater than 2 could be speculated to have a different abundance). CON = control group, dairy cows were fed the basal total mixed ration (TMR) diet without supplementation of the combination of fibrolytic and amylolytic enzymes; EXP = experimental group, cows were fed the basal TMR diet with supplementation of the combination of fibrolytic and amylolytic enzymes, $n = 3$ cows/group.

from these differences, no effects were found on other parameters ($P > 0.05$).

Production performance

Table 7 shows that although the addition of enzyme preparation had no significant effect on DMI ($P > 0.05$), it increased milk yield, 3.5% fat-corrected milk and energy-corrected milk by 2.76, 3.10 and 4.60 kg/d, respectively ($P < 0.05$), and tended ($P = 0.070$) to increase feed efficiency. Enzyme preparation also had some effect on milk composition. It increased the yields of milk fat and lactose ($P < 0.05$) and reduced the milk protein percentage, solids not fat and acidity ($P < 0.05$).

Discussion

Ruminal enzyme activities

Due to a technical problem, volatile fatty acid concentrations were not measured. Enzyme supplementation promoted the activity levels of α-amylase and xylanase and the ammonia-N concentration in rumen fluid ($P < 0.05$). In addition, the activity of β-glucanase tended to be increased ($P = 0.081$). These results agree with the statement of Hristov et al. (1999a and 1999b) that some exogenous polysaccharide-degrading enzymes are partially resistant to rumen degradation and therefore have the potential to enhance rumen degradation, improving the productivity of dairy cows. In addition, the rumen pH was not affected in this research. This result is consistent with previous studies supplemented with fibrolytic (Cotta, 1993) and amylolytic enzymes (Tricarico et al., 2005; Tricarico et al., 2008) and their combination (Hristov et al., 2008; Zilio et al., 2019), indicating that the cows were healthy and that no acidosis occurred.

Ruminal bacterial diversity

Enzyme preparations had no effect on rumen bacterial richness and diversity or phylum and genus composition. However, a tendency was found between CON and EXP ($R = 0.22$, $P = 0.098$) in principal coordinates analysis, and ten genera showed different abundances across the CON and EXP groups through linear discriminant analysis effect size. *In vitro*, Tricarico et al., 2005 reported increased acetate and butyrate and decreased propionate molar proportions in steers, lactating dairy cows, and ruminal-simulating continuous cultures with the dietary addition of α-amylase. These authors (Tricarico et al., 2008) later reported that bacteria (*Streptococcus bovis S1* and *Butyrivibrio fibrisolvens 49*) that could grow quickly on starch did not benefit from α-amylase addition, but bacteria (*Butyrivibrio fibrisolvens D1*, *Selenomonas ruminantium GA192*, and *Megasphaera elsdenii T81*) that could not grow or could only grow slowly on starch grew rapidly with α-amylase supplementation. Our data supported their hypothesis of a cross-feeding mechanism: the addition of α-amylase produces maltodextrins (oligosaccharides from amylose and amylopectin) that provide substrate and a competitive advantage to non-amylolytic bacteria that produce acetate and butyrate (Tricarico et al., 2008). Moreover, considering the result of Russell (1985) that cellodextrins produced by cellulolytic bacteria could be utilised by non-cellulolytic species and the result of Cotta (1993) that xylooligosaccharides from xylan hydrolysis could be utilised by non-xylanolytic species, Tricarico et al. (2008) hypothesised that the oligosaccharide cross-feeding mechanism may also be the case for fibrolytic exogenous enzymes. *In vivo*, Chung et al., 2012 added cellulose-degrading enzymes into the diets of dairy cows and found that the population density of *Ruminobacter amylophilus* was increased and that of *Fibrobacter succinogenes* tended to be increased by the high-enzyme treatment (1 ml of enzymes/kg). However, *Streptococcus bovis* tended to be decreased by the low-enzyme treatment (0.5 ml of enzymes/kg). Amylase has no or little effect on the bacteria and the microbial communities that decompose fibre and starch (Noziere et al., 2014). This indicated that exogenous enzymes had little effect on rumen bacterial diversity. Further research is needed to explore the changes in rumen fungi and ciliates after adding exogenous enzymes.

Blood profile

Enzyme preparation increased cholesterol, low-density lipoprotein cholesterol and A/G and tended to increase triglycerides but did not affect insulin, total protein, BUN, albumin, globulin, glucose, or high-density lipoprotein cholesterol. The increased cholesterol and low-density lipoprotein cholesterol levels and the tendency for increased triglyceride levels indicate that the mobilisation of body fat may have increased in EXP (Puppel and

Kuczyńska, 2016). Recently, the A/G before dry-off was likely a rapid and useful parameter to predict the innate immune states and adaptation conditions in dairy cows, with high A/G cows showing less systemic inflammatory responses and higher milk yield than low A/G cows (Cattaneo et al., 2021). Therefore, the increased A/G in the present study may indicate a better health condition with less inflammation in the EXP.

Production performance

As hypothesised, the enzyme preparation increased the production of milk, 3.5% fat-corrected milk and energy-corrected milk. The enzymes used in this study were cellulose-degrading enzymes (xylanase, cellulase, β-glucanase) and amylase. Exogenous fibrolytic enzymes have been shown to improve ruminant performance. Golder et al., 2019 found that exogenous fibrolytic enzymes increased dairy cow milk production by 0.7 kg/d. Through meta-analysis, Arriola et al., 2017 reported that the application of exogenous fibrolytic enzymes increased milk yield (0.83 kg/d) and 3.5% energy-corrected milk (0.55 kg/d) and found a moderate level of heterogeneity in milk production. Exogenous amylase can improve rumen starch digestibility (Noziere et al., 2014). Andreazzi et al., 2018 found that adding amylase to a high-starch diet can improve milk production in cows. In the current study, the combination of cellulose-degrading enzymes and amylase increased milk yield by 2.7 kg/d. Previous experiments (Peters et al., 2015; Arriola et al., 2017; Golder et al., 2019) found that exogenous fibrolytic enzymes had no effect on the DMI of cows. Some studies (Gencoglu et al., 2010; Andreazzi et al., 2018) suggested that amylase decreased the DMI of cows. The mixture of fibrolytic and amylolytic enzymes had some effects on milk composition, including increased milk fat and lactose production and reduced milk protein percentage. Some studies (Gencoglu et al., 2010; Ferraretto et al., 2011; Weiss et al., 2011) found that amylase had no effect on milk fat, protein and lactose concentration and yields, and Andreazzi et al., 2018 only found an increased milk lactose yield. Thus, amylase has little effect on milk composition. Golder et al., 2019 found that milk fat percentage was not significantly increased by exogenous fibrolytic enzymes, but milk fat yield was significantly increased by 0.040 kg/d. The yield of milk protein increased by 0.010 kg/d despite the milk protein percentage being reduced by 0.020%. Exogenous fibrolytic enzymes can also increase the yields of milk protein (0.03 kg/d) and lactose (0.05 kg/d) (Arriola et al., 2017). In the current study, the numerically improved DMI (CON 25.6, EXP 26.8, $P = 0.13$, Table 7) and the probably increased ruminal mono- and oligosaccharides caused by the enhanced ruminal enzyme activities may contribute to the improvement of milk yield. Increased milk yield could be attributable to increased xylanase and amylase activity levels in the rumen, increased digestibility and increased supplies of volatile fatty acid and microbial protein.

No effect was found on milk yield or feed efficiency in the two previous studies using a combination of fibrolytic and amylolytic enzymes (Hristov et al., 2008; Zilio et al., 2019). One explanation for the unexpected effect in the study of Hristov et al., 2008 was an insufficient dose of enzyme, which was 10 g/cow/d (Hristov et al., 2000). In the current study, a dose of 70 g/cow/d was used. Another explanation for the study of Hristov et al., 2008 was the delivery method of direct supplementation into the rumen, which may have resulted in a lack of the pre-ingestive effects of enzymes on feed and the decreased homogeneity of feed and enzymes (Beauchemin et al., 2003). The results of the study of Zilio et al., 2019 could probably be attributed to the inappropriate time and diet portion of enzyme supplementation (Adesogan, 2005). The enzymes were added once a week into the concentrate during its preparation. The once-a-week time interval might be too long, and the enzyme activity might be decreased over such a period (Adesogan, 2005). The addition of exogenous enzymes into concentrates showed positive effects on production performance in a study using a high concentrate to forage ratio (62:38) diet but had no effect in studies using diets with lower concentrate to forage ratios (45:55–40:60) (Adesogan, 2005). Therefore, a suitable diet component for enzyme supplementation should consider the concentrate to forage ratio (Adesogan, 2005). The concentrate to forage ratio of Zilio et al., 2019 was approximately 52:48, which is closer to the range of 45:55–40:60. Therefore, the addition of enzymes into concentrate might be unsuitable in that study.

Conclusion

The dietary supplementation of a combination of fibrolytic (cellulase, xylanase, and β-glucanase) and amylolytic (amylase) enzymes on multiparous early-lactation dairy cows increased α-amylase and xylanase activity levels in rumen fluid, enhanced milk performance and tended to improve the feed efficiency in cows.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100595.

Ethics approval

All animal research procedures were approved by the Northwest A&F University Animal Care and Use Committee (protocol number: NWAFAC1211; Yangling, Shaanxi, China).

Data and model availability statement

The data/models were not deposited in an official repository. All data and models generated and/or used through the current study are available from the corresponding author on reasonable demand.

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Author contributions

Yangchun Cao and Junhu Yao conceived the study. Zhaokun Liu, Yong Li, Congcong Zhao, and Zhejia Liu performed the work. The animal experiment was carried out by Yong Li, Lamei Wang, Xiaoyong Li, and Zhejia Liu. The chemical and data analysis was finished by Zhaokun Liu and Congcong Zhao. The manuscript was written by Zhaokun Liu, Yong Li, Wilbert Pellikaan, and Yangchun Cao and checked by all co-authors.

Declaration of interest

The authors declare that they have no conflict of interest.

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