Effects of fibrolytic and amylolytic compound enzyme preparation on rumen fermentation, serum parameters, and production performance in primiparous early-lactation dairy cows

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SUPPLEMENTARY FILE

Materials and Methods

Animals, diets, and experimental design

The activities of xylanase, cellulase and β-glucanase were determined using dinitrosalicylic acid method. Cellulose, β-glucan and birchwood xylan (each at 10 g/L in a 50 mM sodium phosphate buffer, pH 7.0) were used as substrates to react with the rumen fluid supernatant respectively and OD was read at 540 nm. The reaction time of xylanase (Khanna, S. 1993) was 15 min, and that of cellulase and β-glucanase (Zhang et al. 2009) was 30 min. Cellulase units (CU), Xylanase units (XU), β-glucanase units (GU) are defined as μmol of reducing sugars released per minute. The activity of amylase (Visvanathan et al. 2016) was measured by kit (Jiancheng Bioengineering Institute, Nanjing, China). This kit is equal to the determination of amylase activity by measuring soluble starch dextrinized at 60℃, pH 6.0 with iodine solution, using 10 mg/ml soluble starch in 126 mmol/L phosphate buffer with pH 6.0. Amylase units (AU) is defined as mg of soluble starch dextrinized per hour.

Sampling procedure and laboratory analyses

From the first to third day of the experimental period, the TMR feed samples were collected homogeneously when cows were fed at 0600 h. From the second day of the experimental period,

the residues of previous day were removed and weighed daily before 0600 h to calculate the dry matter intake (DMI). The chemical composition and particle size distribution of feed samples were determined on samples collected on the first to third day of experimental period.

Some collected feed samples were dried at 65 °C for 48h, then were ground to pass a 1-mm screen for further chemical analysis. The contents of dry matter (DM) (method 930.5), crude protein (CP) (method 990.03), ethanol extract (EE) (method 920.39), and ash (method 942.05) were determined using the AOAC Official Method (Lee, 1995). The method of Van Soest et al. (1991) was used to determine the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents. The non-fibrous carbohydrates (NFC) (g/kg DM) was calculated as 1000 - CP (g/kg DM) - EE (g/kg DM) - NDF (g/kg DM) - ash (g/kg DM) (Zhang et al., 2020). Other fresh feed samples were used to measure diet particle size by using the Penn State Particle Separator with three sieves (19, 8, and 1.18 mm) and a bottom pan (Kononoff and Heinrichs, 2003). Physical effectiveness factors (pef) were calculated as the total proportion of DM retained on the 19 and 8 mm (pef8.0) (Lammers et al., 1996) or the 19, 8, and 1.18 mm (pef1.18) sieves (Kononoff and Heinrichs, 2003). The physically effective NDF (peNDF8.0, peNDF1.18) were calculated by multiplying the diet NDF content (% of DM) and pef8.0 and pef1.18, respectively (Cao et al., 2021).

At 0900 h (three hours after the 0600 h morning feeding) on the fourth day of experimental period, 400 ml rumen liquid was collected per cow by oral intubation. The pH was recorded immediately by pH meter. Two hundred ml rumen liquid was filtered by four layers gauze, then was divided into 10 ml centrifuge tubes. These tubes were transported on ice in an insulated container to the lab of Northwest A&F University within about 30 min to determine the volatile fatty acid (acetate to valerate, isobutyrate, and isovalerate) contents by gas chromatography, following the procedure described by Li et al. (2014).

At 0800 h (two hours after the 0600 h morning feeding) on the fifth day of experimental period,

five-ml blood samples were collected per cow from the tail vein with procoagulant vacuum blood collection tube (sodium heparin tube, Shandong Tianai Medical Instrument Co., Ltd). Then tubes were centrifuged (4 °C, 3500 g/min, 15 min) to separate serum. Serum samples were frozen transported into Yangling Demonstration Zone Hospital (Yangling, Shaanxi, China) to detect physiological and biochemical indexes.

During 1400-1500 h (midday milking) on the first to third day of experimental period, the suction cups were manually took off after they attached teats for 2 minutes and 200 ml milk sample was manually collected per cow from 4 teats. The regular chemical indices and somatic cell count (SCC) of milk sample were analysed by milk composition analyser (UL40AC-8, Hangzhou Ultrasun Technologies Co., Ltd) at the farm lab. Milk production was electronically recorded every day. The yield of 4% fat corrected milk (FCM; kg/d) was calculated as actual milk yield (kg/d) × (0.4 + 15 × milk fat content (%)) (NRC, 2001). The energy corrected milk yield (ECM; kg/d) was calculated as actual milk yield (kg/d) × (0.3246 + 12.86 × Milk fat content (%) + 7.04 × Milk protein content (%)) (NRC, 2001). Feed efficiency was calculated as FCM yield (kg/d) / DMI (kg/d).

Statistical analysis

The study was performed under a completely randomized single-factor design. Feed intake and milk production were recorded daily. The chemical composition and particle size distribution of feed samples, as well as the physical and chemical indices and SCC of milk samples were measured for three consecutive days (the first to third day of experimental period); the daily averages of these data were calculated and used for further statistical analysis. Due to the limitation of labour, the rumen pH and volatile acid profile, and the serum parameters were sampled for one day. For statistical analyses, SPSS software (Version 22.0, SPSS Inc., Chicago, USA) was used to determine the differences of all measures between control and experimental

77 groups, with supplementation of compound enzyme preparation as the fixed factor and the cow
78 as a random factor.

77 $Y_{ij} = \mu + \text{treatment}_i + \text{cow}_j + \varepsilon_{ij}$

78 where Y_{ij} = the kth observation of the jth cow in the ith treatment, μ = the overall mean,
79 treatment_i = the fixed effect of the ith treatment (i = 0 to 1), cow_j = the random effect of the
80 jth cow (j = 1 to 10), ε_{ij} = the residual error associated with the jth cow in the ith treatment. All
81 results were expressed as the mean and SEM (Table 1). Significance was declared at $P \leq 0.05$

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125 **Supplementary Table S1** Ingredients of the basic TMR diet.

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Ingredient	Content (g/kg)
Corn silage	482.6
Alfalfa hay	80.4
Steam flaked corn	136.7
Corn bran	67.0
Corn	53.6
Soybean meal	67.0
Soybean hull	26.8
Cottonseed meal	42.9
Cottonseed	26.8
Vitamin-mineral premix[1]	10.9
Rumen-protected fat[2]	4.0
Optigen[3]	1.3

127 [1]Each kilogram contained: 400 mg Cu, 2400 mg Fe, 4000 mg Zn, 2000 mg Mn, 40 mg I, 30 mg Se, 50 mg Co, 40 mg vitamin B1, 1 mg vitamin B12, 1200 mg nicotinic acid, 700 mg pantothenic acid, 45 mg vitamin K3, 300 KIU vitamin A, 100 KIU vitamin D3, and 6500 IU vitamin E.

128 [2] Megalac protected fat, a calcium salt of palm fatty acid distillate, produced by Yihai Kerry Arawana Holdings Co., Ltd. (Shanghai, China).

129 [3] A product of Alltech Inc. (Nicholasville, Kentucky, USA), which provides a controlled release of nonprotein nitrogen to the rumen over time.

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Supplementary Table S2

Effects of compound enzyme preparation on serum parameters in dairy cows (n = 10 cows/group).

Item	CON[1]	EXP[2]	SEM	*P*-value
ALP activity(U/L)	80.38[b]	110.43[a]	4.829	<0.01
Total protein (g/L)	67.70	68.43	0.893	0.66
Albumin (g/L)	31.53[b]	33.01[a]	0.319	0.01
Globulin (g/L)	36.08	35.41	0.812	0.70
Albumin: Globulin	0.88	0.94	0.023	0.09
Urea (mmol/L)	5.14	4.80	0.133	0.21
Glucose (mmol/L)	3.82	3.80	0.035	0.85
Total cholesterol (mmol/L)	5.10	4.66	0.234	0.37
Triglycerides (mmol/L)	0.12	0.11	0.003	0.21
High density cholesterol (mmol/L)	2.94	2.86	0.072	0.57
Low density cholesterol (mmol/L)	0.68	0.60	0.043	0.40

[1]Control (CON) group, without supplementation of compound enzyme preparation.
[2]Experimental (EXP) group, with supplementation of compound enzyme preparation.
[a,b]Different superscripts within a row indicate a significant difference (P<0.05). SEM, standard error of the mean; ALP, alkaline phosphatase.

Supplementary Table S3

Effects of compound enzyme preparation on chemical composition and particle size distribution of diet, and nutrient intake in dairy cows (n = 3, feed samples were collected on from the 1st to 3rd day of the experimental period, nutrient intakes were calculated by the feed residues collected on the same three days).

Item	CON[1]	EXP[2]	SEM	*P*-value
Diet				
DM (g/kg)	606.2	604.2	2.26	0.71
CP (g/kg DM)	209.8	197.6	5.92	0.36
EE (g/kg DM)	48.5	36.1	4.49	0.19
NDF (g/kg DM)	396.7	331.6	21.54	0.14
ADF (g/kg DM)	218.8	165.5	15.10	0.06
ash (g/kg DM)	67.0	73.9	2.15	0.11
NFC (g/kg DM)	278.0[b]	360.7[a]	22.36	0.04
$pef_{8.0}$	0.514	0.498	0.01	0.64
$pef_{1.18}$	0.867	0.856	0.01	0.48
$peNDF_{8.0}$ (g/kg DM)	204.9	164.6	13.62	0.15
$peNDF_{1.18}$ (g/kg DM)	344.3	283.6	19.84	0.13
Intake (kg/d)				
DM	19.87[b]	21.62[a]	0.168	<0.01
CP	4.19	4.23	0.128	0.91
EE	0.97	0.78	0.092	0.36
NDF	7.91	7.11	0.353	0.31
ADF	4.36	3.55	0.253	0.11
NFC	5.56[b]	7.74[a]	0.573	0.03
$peNDF_{8.0}$	4.08	3.53	0.229	0.28
$peNDF_{1.18}$	6.86	6.08	0.328	0.28

[1]Control (CON) group, without supplementation of compound enzyme preparation.
[2]Experimental (EXP) group, with supplementation of compound enzyme preparation.
[a,b]Different superscripts within a row indicate a significant difference ($P<0.05$). SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ethanol extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFC, non-fibrous carbohydrates = 100 - CP - EE - NDF - ash; $pef_{8.0}$ = DM retained on the 19, and 8 mm sieves/total DM; $pef_{1.18}$ = DM retained on the 19, 8, and 1.18 mm sieves/total DM; $peNDF_{8.0}$ = NDF × $pef_{8.0}$; $peNDF_{1.18}$ = NDF × $pef_{1.18}$.