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# Influence of fibrolytic enzymes mixture on performance, nutrient digestion, rumen fermentation and microbiota in Holstein bulls

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**ABSTRACT.** The aim of the study was to evaluate the influences of fibrolytic enzymes mixture (FEM) – a mixture of cellulase, xylanase, pectinase and laccase – on nutrient digestion and ruminal fermentation in bulls. Eight Holstein bulls fitted with rumen cannula were used in a replicated 4 × 4 Latin square design and allocated to four treatment groups: control, low-FEM (LFEM), medium-FEM (MFEM) and high-FEM (HFEM) with 0, 0.25, 0.50 and 0.75 g/kg FEM, respectively. Even though the addition of FEM had a positive linear impact on dry matter (DM) intake and average daily gain, it had no influence on feed conversion ratio. Supplemented FEM linearly promoted nutrients total-tract digestibility, reaching greater values at 0.50 and 0.75 g FEM/kg DM. Rumen pH value and the ratio of acetate to propionate were reduced linearly, the concentration of total volatile fatty acids (VFA) elevated linearly but the content of ammonia-N was not influenced with increasing dose of FEM. For corn silage rumen degradation, effective degradability (ED) of DM and organic matter were quadratically reduced, reaching greater values in MFEM, whereas ED of neutral detergent fibre was linearly elevated. Supplemented FEM linearly increased activities of cellulolytic enzymes, α-amylase and protease and populations of bacteria, fungi, protozoa, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Prevotella ruminicola*, *Butyrivibrio fibrisolvens* and *Ruminobacter amylophilus*, but had no impact on populations of methanogens and *Ruminococcus flavefaciens*. Supplemented FEM linearly increased urinary total purine derivative excretion. So, it was indicated that FEM enhanced nutrient digestibility and total VFA production, the optimum dose was 0.50 g/kg DM in bull diets.

## Introduction

Supplementation of fibrolytic enzymes (FE) into ruminant diets had been used as a nutritional strategy to improve performance via enhancing nutrient digestion (Beauchemin et al., 2004). It was previously demonstrated that supplementation with a mixture of xylanase and cellulase enhanced performance and nutrient digestibility in steers (Gómez-Vázquez et al., 2011) or cows (Yang et al., 2000). However, other researchers observed that average daily gain (ADG) and feed conversion ratio (FCR) were unchanged with supplementing xylanase and cellulase mixture (Krueger et al., 2008). The divergent responses should be related to the differences in activities and formulations of enzymes used in these studies. Eun et al. (2007) demonstrated that the optimum dose proportions and activities of supplied FE were critical for the magnitude of the improvement in forage utilization. The factors limiting the digestion of dietary fibre by rumen microbes were some plant cell wall

compounds, such as cellulose, hemicellulose, pectin and lignin, as well as the cross-linked matrix formed among these compounds (Wang and Mcallister, 2002). Results of *in vitro* studies suggested that the action of enzymes was substrate-specific (Colombatto et al., 2003), and that the FE combination to reach the highest reducing sugars release included xylanase, carboxymethyl cellulase, β-glucanase and ferulic acid esterase (Yang and Xie, 2010). Laccase is yielded by bacteria and fungi and can be used to degrade lignin. Yue et al. (2020) reported that supplementing laccase into bull diets enhanced daily gain and nutrient digestibility. Based on this research, the hypothesis was that a fibrolytic enzymes mixture (FEM) containing cellulase, xylanase, pectinase and laccase should have the potential to enhance performance and feed digestion in bulls.

The enhancement in feed digestion was usually attributed to a stimulatory impact of exogenous FE on nutrient digestibility in the rumen (Beauchemin et al., 2004). Studies *in vivo* observed that FE addition increased nutrient apparent digestibility in dairy cows (Gado et al., 2009) or steers (Krueger et al., 2008; Gómez-Vázquez et al., 2011), and that supplementation with a FE preparation increased total volatile fatty acids (VFA) concentration and total viable bacteria numbers in the rumen of cows (Nsereko et al., 2002). Giraldo et al. (2007) found that degradability of dry matter (DM) and fibre, production of VFA and acetate, microbial growth and cellulolytic enzymes activities increased with exogenous cellulase addition *in vitro*. The amount of microbial protein synthesis is usually measured by urinary total purine derivatives (PD) excretion (Yue et al., 2020). Application of enzymes containing primarily cellulase and xylanase activities increased rumen microbial protein synthesis in cows (Yang et al., 1999). These data suggested that the increased nutrient digestibility and microbial protein synthesis with exogenous FE addition might be associated with an enhancement in rumen microbial growth and enzymes activities. However, the *in vivo* studies investigating the influences of FE supplementation on enzymes activities and microbiota in the rumen are limited.

Therefore, this study was conducted to examine the influences of supplementing FEM into bull diets on growth performance, total-tract and rumen nutrient digestibility, rumen fermentation parameters, enzymes activities, microbiota and urinary total PD excretion.

# Material and methods

## Holstein dairy bulls, experimental design and feeding

The feeding experiment was conducted according to the regulations established by the Animal Care and Use Committee of Shanxi Agriculture University. Eight Holstein bulls fitted with ruminal cannulas (418 ± 10.2 kg body weight (BW) and 11 ± 0.33 months old) were arranged into a 4 × 4 Latin square experimental design and each experimental period lasted 24 days: 14 days for adaptation and 10 days for data collection. Dietary supplementation level of FEM in four treatments was as follows: control – 0 g/kg FEM, low-FEM (LFEM group) – 0.25 g/kg FEM, medium-FEM (MFEM group) – 0.50 g/kg FEM and high-FEM (HFEM group) – 0.75 g/kg. The FEM contained 200 g/kg cellulase ($1.0 \times 10^5$ U/g), 300 g/kg xylanase ($5.0 \times 10^4$ U/g), 200 g/kg pectinase ($3.0 \times 10^{11}$ U/g) and 300 g/kg laccase ($1.0 \times 10^5$ U/g). All enzymes were purchased from Shanxi Dayu Biotechnology Co., Ltd., Ruicheng, China and determined activities at 39 °C and pH 6.0 according to the procedures reported by Agarwal et al. (2000). The formulation and addition level of FEM were determined based on the results of Yang and Xie (2010) and Yue et al. (2020). Before the trial, the FEM was mixed with the premix and then added into the bull concentrate, and mixed with roughage 3 h before the feeding. Bulls were housed individually and fed twice daily (07:00 and 190:0) a total mixed ration (TMR; Table 1) which was formulated according to the recommendations of NRC (2001). The DM content of TMR was 54.3%.

## Measurements and analytical methods

The individual bull BW at the beginning and at the end of each 24 days period was determined. From day 15 to day 24, DM intake (DMI) was measured, and feed and refusals were collected daily for each bull and then stored at –20 °C. From day 15 to day 24, daily total excretion of faeces of each bull was collected, measured (weight or volume) and sampled. Accurately 200 g wet faecal sample was blended with 50 ml tartaric acid solution (100 g/l), then stored at –20 °C. The collected feed, refusals and faeces samples were dried at 50 °C for 48 h, and ground in a feed mill to pass a 1-mm sieve for chemical analyses. Analytical DM content of oven-dried samples was analysed by drying at 135 °C for 3 h (AOAC International, 2006). Organic matter (OM) content was calculated as the difference

**Table 1**. Ingredient and chemical composition of basal diet

| Ingredients | Contents, g/kg dry matter |
|---|---|
| Corn silage | 400 |
| Lucerne hay | 70 |
| Oat hay | 30 |
| Maize, ground | 244 |
| Wheat bran | 35 |
| Soybean meal | 40 |
| Rapeseed meal | 30 |
| Cottonseed cake | 128 |
| Calcium carbonate | 10 |
| Salt | 5 |
| Calcium phosphate | 3 |
| Mineral and vitamin premix[1] | 5 |
| Chemical composition | |
| Dry matter | 543 |
| organic matter | 916 |
| crude protein | 155 |
| ether extract | 29 |
| neutral detergent fibre | 370 |
| acid detergent fibre | 236 |
| calcium | 8.4 |
| phosphorus | 4.3 |

[1] contained per kg premix: g: Fe 20, Cu 1.6, Mn 8, Zn 7.5; mg: I 120, Co 20; IU: vit. A 820 000, vit. D 300 000, vit. E 10 000

between DM and ash contents, with ash determined by combustion at 550 °C for 5 °C h. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were analysed according to the procedure of Van Soest et al. (1991) with heat stable alpha amylase and sodium sulphite used in the NDF procedure, and expressed inclusive of residual ash. The nitrogen content of samples was analysed according to the Kjeldahl method (AOAC International, 2006). The sample of urine was diluted 10 times using distilled water, composited by the animal. Uric acid and allantoin of urine were analysed based on the description of Yue et al. (2020).

Rumen degradation of diet was examined using the nylon bag technique on days 15 – 17. Diet samples were dried (65 °C and 48 h), milled to 2.5-mm, and weighted into nylon bags (8 cm × 12 cm; pore 47 ± 1.5 μm (Sino-french Beef Cattle Cooperation Centre of China Agricultural University, Beijing, China)) with 3 g of each. After incubating for 0, 4, 8, 12, 24, 36, 48 and 72 h, the duplicated sample bags and an empty bag were taken out from the rumen, washed with running cold water to clean, dried (65 °C for 48 h), weighted, and then DM, CP and NDF were determined (Van Soest et al., 1991; AOAC International, 2006). The degradation parameters of diet were calculated based on the model of McDonald (1981), and kinetics of nutrient disappearance *in situ* in the rumen was estimated using the nonlinear regression procedure of SAS (2002). The value of k, the articulate passage rate out of the rumen determined based on the procedure of Yang et al. (1997), was 0.049/h for diet.

On days 23 and 24, rumen fluid (200 ml) was sampled at 0, 3, 6 and 9 h after bulls were fed in the morning. Samples of rumen fluid were determined for pH immediately (HK-1309 pH, Beijing Huakeyi technology Co. LTD, Beijing, China), and then filtered to pass 4 layers of cheesecloth. The ruminal fluid of 5 ml was acidified with 1 ml of 250 g/l metaphosphoric acid and another 5 ml was acidified with 1 ml of 20 g/l (w/v) $H_2SO_4$ for the determination of VFA and ammonia-N, respectively, and frozen at –20 °C. The ruminal fluid of 5 ml and 50 ml were stored at –80 °C for analysing microbial populations and enzymatic activities, respectively. The VFA was analysed by gas chromatography (GC-7800; Beijing Purui Analytical Instrument Co., Beijing, China) as described by Filípek and Dvořák (2009), and ammonia-N was analysed using UV-1100 spectrophotometer (Meipuda Instrument Co., LTD, Shanghai, China) as described by AOAC (2006). According to the methods described by Agarwal et al. (2000), total enzymatic activities of ruminal fluid were analysed.

Microbial DNA was isolated from homogenized rumen fluid (1.5 ml) as described by Yu and Morrison (2004). The integrity and purity of isolated DNA was assessed by agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, NanoDrop Technologies, Rockland, DE, USA). Primer pairs used for real-time PCR are summarized in Table 2. The standards were generated from the treatment pool set of microbial DNA by using regular PCR. The PCR product was purified using a PureLinkTM Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific Co., Ltd., Shanghai, China) and quantified by a spectrophotometer. The copy number concentration of each standard was examined based on the length and mass concentration of the PCR product. The serial 10-fold dilutions were used for the quantification of target DNA. Amplification and detection of real-time PCR were carried out in StepOne™system (Thermo Fisher Scientific Co., Ltd., Shanghai, China). The amplification samples were assayed in triplicate by using a Biotools QuantiMix EASY SYG KIT (B&M Labs, S. A., Madrid, Spain) in a 20 μl reaction mixture as described by Yue et al. (2020).

## Statistical analyses

Data were analysed using the mixed model procedure of SAS (Proc Mixed; SAS, 2002) with

**Table 2.** PCR primers for real time PCR assay

| Target species | Primer sequence (5') | GeneBank accession no. | Size, bp |
|---|---|---|---|
| Total bacteria | F: CGGCAACGAGCGCAACCC<br>R: CCATTGTAGCACGTGTGTAGCC | AY548787.1 | 147 |
| Total anaerobic fungi | F: GAGGAAGTAAAAGTCGTAACAAGGTTTC<br>R: CAAATTCACAAAGGGTAGGATGATT | GQ355327.1 | 120 |
| Total protozoa | F: GCTTTCGWTGGTAGTGTATT<br>R: CTTGCCCTCYAATCGTWCT | HM212038.1 | 234 |
| Total methanogens | F: TTCGGTGGATCDCARAGRGC<br>R: GBARGTCGWAWCCGTAGAATCC | GQ339873.1 | 160 |
| *Ruminococcus albus* | F: CCCTAAAAGCAGTCTTAGTTCG<br>R: CCTCCTTGCGGTTAGAACA | CP002403.1 | 176 |
| *Ruminococcus flavefaciens* | F: ATTGTCCCAGTTCAGATTGC<br>R: GGCGTCCTCATTGCTGTTAG | AB849343.1 | 173 |
| *Butyrivibrio fibrisolvens* | F: ACCGCATAAGCGCACGGA<br>R: CGGGTCCATCTTGTACCGATAAAT | HQ404372.1 | 65 |
| *Fibrobacter succinogenes* | F: GTTCGGAATTACTGGGCGTAAA<br>R: CGCCTGCCCCTGAACTATC | AB275512.1 | 121 |
| *Ruminobacter amylophilus* | F: CTGGGGAGCTGCCTGAATG<br>R: GCATCTGAATGCGACTGGTTG | MH708240.1 | 102 |
| *Prevotella ruminicola* | F: GAAAGTCGGATTAATGCTCTATGTTG<br>R: CATCCTATAGCGGTAAACCTTTGG | LT975683.1 | 74 |

a repeated 4 × 4 Latin square design to account for effects of a square, period within the square, bull within square and treatment. The treatment was considered as a fixed effect; square, a period within square, and bull within square were considered as random effects. Data for rumen fermentation parameters, enzymes activities and number of populations of microbiota were summarized by sampling time and analysed using the same mixed model procedure but with time included as a repeated measure. Linear and quadratic orthogonal contrasts were tested using the CONTRAST statement of SAS with coefficients estimated based on the FEM application rates. Significant differences were determined with a threshold of $P < 0.05$, while trends that suggested possible significance were identified at $0.05 < P < 0.10$.

# Results

## Performance, total-tract nutrient digestibility and rumen fermentation

The performance, nutrient digestion and ruminal fermentation parameters were summarized in Table 3. Dry matter intake elevated linearly ($P = 0.001$) with incrementing the application level of FEM and was greater ($P < 0.05$) for bulls in MFEM and HFEM groups in comparison with those in control. The BW of bulls at the start and end of the trial were similar among the four groups. Increasing addition level of FEM linearly increased ($P = 0.043$) ADG but did not affect FCR in bulls. Greater ($P < 0.05$) ADG was observed in MFEM and HFEM than in LFEM and control animals. Nutrient digestibility responded linearly ($P < 0.05$) to the elevated FEM addition. In comparison with the control, bulls receiving 0.50 and 0.75 g/kg DM FEM had greater ($P < 0.05$) digestibility of DM, OM, CP, ADF and NDF. Ruminal pH, total VFA, acetate to propionate ratio and percentages of acetate, propionate and isovalerate responded linearly ($P < 0.05$) but ammonia-N and proportions of butyrate, valerate and isobutyrate were unchanged with elevating the dose of FEM. Ruminal pH, the molar proportion of acetate, and the ratio of acetate to propionate were lower ($P < 0.05$), while total VFA concentration and molar percentage of propionate were greater ($P < 0.05$) for bulls consuming diets with FEM than for those in control one. The percentage of isovalerate was greater ($P < 0.05$) for bulls in MFEM and HFEM groups in comparison with those in LFEM and control.

## Ruminal degradation

For DM, the soluble fraction (a) elevated linearly ($P < 0.05$), ED increased quadratically ($P < 0.05$), but the slowly degradable fraction (b) and degradation rate (c) were unchanged with increasing dose of FEM (Table 4). The soluble fraction (a) and ED of DM were greater ($P < 0.05$) for MFEM than for control group. For NDF, the soluble fraction (a) and degradation rate (c) were not affected, but the slowly degradable fraction (b) and ED linearly elevated ($P < 0.05$) with increasing FEM inclusion level.

**Table 3.** Effects of fibrolytic enzymes mixture (FEM) supplementation on dry matter intake (DMI), average daily gain (ADG), feed conversion ratio (FCR), nutrient digestibility and ruminal fermentation in Holstein bulls

| Item | Treatments[1] | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | control | LFEM | MFEM | HFEM | | treatment | linear | quadratic |
| DMI, kg/day | 10.1$^{b}$ | 10.7$^{ab}$ | 11.7$^{a}$ | 11.9$^{a}$ | 0.23 | 0.005 | 0.001 | 0.62 |
| Body weight, kg | | | | | | | | |
| beginning of the trial | 544 | 543 | 541 | 542 | 3.15 | 0.98 | 0.84 | 0.78 |
| end of the trial | 572 | 573 | 577 | 579 | 3.09 | 0.93 | 0.54 | 0.86 |
| ADG, kg/day | 1.39$^{b}$ | 1.49$^{b}$ | 1.82$^{a}$ | 1.83$^{a}$ | 0.14 | 0.031 | 0.043 | 0.12 |
| FCR, kg/kg | 7.26 | 7.16 | 6.79 | 6.86 | 0.12 | 0.46 | 0.16 | 0.72 |
| Digestibility, % | | | | | | | | |
| dry matter | 66.9$^{b}$ | 68.4$^{ab}$ | 70.9$^{a}$ | 71.8$^{a}$ | 1.86 | 0.045 | 0.027 | 0.48 |
| organic matter | 67.6$^{b}$ | 69.9$^{ab}$ | 71.6$^{a}$ | 72.1$^{a}$ | 1.79 | 0.047 | 0.034 | 0.54 |
| crude protein | 65.1$^{b}$ | 67.2$^{ab}$ | 69.4$^{a}$ | 70.3$^{a}$ | 1.64 | 0.027 | 0.001 | 0.55 |
| neutral detergent fibre | 46.4$^{b}$ | 49.5$^{ab}$ | 53.6$^{a}$ | 54.9$^{a}$ | 1.01 | 0.022 | 0.001 | 0.46 |
| acid detergent fibre | 44.9$^{b}$ | 47.0$^{ab}$ | 51.0$^{a}$ | 51.9$^{a}$ | 1.19 | 0.014 | 0.001 | 0.38 |
| Ruminal fermentation | | | | | | | | |
| pH | 6.62$^{a}$ | 6.35$^{b}$ | 6.27$^{b}$ | 6.22$^{b}$ | 0.047 | 0.002 | 0.001 | 0.081 |
| Total VFA, mmol/l | 121$^{b}$ | 129$^{a}$ | 132$^{a}$ | 134$^{a}$ | 1.49 | 0.001 | 0.001 | 0.085 |
| % | | | | | | | | |
| acetate (A) | 68.3$^{a}$ | 66.5$^{b}$ | 66.2$^{b}$ | 65.3$^{b}$ | 0.32 | 0.001 | 0.001 | 0.14 |
| propionate (P) | 19.2$^{b}$ | 20.6$^{a}$ | 20.7$^{a}$ | 20.6$^{a}$ | 0.28 | 0.043 | 0.024 | 0.065 |
| butyrate | 9.44 | 9.68 | 9.43 | 10.3 | 0.15 | 0.12 | 0.073 | 0.26 |
| valerate | 1.09 | 1.24 | 1.39 | 1.52 | 0.096 | 0.46 | 0.12 | 0.96 |
| isobutyrate | 0.73 | 0.80 | 0.81 | 0.80 | 0.037 | 0.89 | 0.55 | 0.64 |
| isovalerate | 1.17$^{b}$ | 1.21$^{b}$ | 1.47$^{a}$ | 1.45$^{a}$ | 0.068 | 0.028 | 0.047 | 0.79 |
| A:P | 3.56$^{a}$ | 3.23$^{b}$ | 3.19$^{b}$ | 3.17$^{b}$ | 0.051 | 0.006 | 0.002 | 0.084 |
| Ammonia N, mg/100 ml | 8.80 | 8.35 | 8.25 | 8.25 | 0.13 | 0.47 | 0.18 | 0.43 |

[1] control – 0 g/kg FEM; LFEM – 0.25 g/kg FEM, MFEM – 0.50 g/kg FEM; HFEM – 0.75 g/kg FEM; SEM – standard error of the mean; VFA – volatile fatty acid; $^{abc}$ means with different superscripts in each row are significantly different at $P < 0.05$

**Table 4.** Effects of fibrolytic enzymes mixture (FEM) supplementation on *in situ* ruminal digestion kinetics and effective degradability (ED) of diet

| Item | Treatments[1] | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | control | LFEM | MFEM | HFEM | | treatment | linear | quadratic |
| DM | | | | | | | | |
| a[2] | 0.262$^{b}$ | 0.296$^{ab}$ | 0.315$^{a}$ | 0.310$^{ab}$ | 0.007 | 0.047 | 0.032 | 0.106 |
| b | 0.589 | 0.599 | 0.618 | 0.595 | 0.009 | 0.384 | 0.356 | 0.192 |
| C, $h^{-1}$ | 0.036 | 0.035 | 0.035 | 0.033 | 0.001 | 0.591 | 0.378 | 0.331 |
| L, h | 1.34 | 1.29 | 1.23 | 1.20 | 0.134 | 0.456 | 0.064 | 0.529 |
| ED | 0.511$^{b}$ | 0.546$^{ab}$ | 0.572$^{a}$ | 0.549$^{ab}$ | 0.011 | 0.043 | 0.069 | 0.033 |
| NDF | | | | | | | | |
| a[2] | 0.191 | 0.194 | 0.197 | 0.204 | 0.005 | 0.879 | 0.234 | 0.865 |
| b | 0.653$^{b}$ | 0.689$^{ab}$ | 0.776$^{a}$ | 0.752$^{ab}$ | 0.019 | 0.015 | 0.008 | 0.31 |
| C, $h^{-1}$ | 0.028 | 0.028 | 0.026 | 0.025 | 0.001 | 0.536 | 0.197 | 0.78 |
| L, h | 2.38 | 2.35 | 2.26 | 2.24 | 0.184 | 0.503 | 0.069 | 0.569 |
| ED | 0.428$^{b}$ | 0.445$^{ab}$ | 0.466$^{a}$ | 0.458$^{a}$ | 0.009 | 0.031 | 0.047 | 0.358 |
| CP | | | | | | | | |
| a[2] | 0.273$^{c}$ | 0.334$^{b}$ | 0.375$^{a}$ | 0.344$^{ab}$ | 0.014 | 0.047 | 0.021 | 0.031 |
| b | 0.505$^{b}$ | 0.537$^{ab}$ | 0.594$^{a}$ | 0.585$^{a}$ | 0.008 | 0.040 | 0.031 | 0.021 |
| C, $h^{-1}$ | 0.051 | 0.050 | 0.053 | 0.052 | 0.001 | 0.332 | 0.565 | 0.285 |
| L, h | 0.13 | 0.11 | 0.10 | 0.09 | 0.009 | 0.476 | 0.054 | 0.528 |
| ED | 0.531$^{c}$ | 0.605$^{b}$ | 0.684$^{a}$ | 0.645$^{a}$ | 0.009 | 0.041 | 0.017 | 0.042 |

[1] control – 0 g/kg FEM; LFEM – 0.25 g/kg FEM, MFEM – 0.50 g/kg FEM; HFEM – 0.75 g/kg FEM; [2] parameters were calculated from the fitted equation: $y = a + b(1 - e^{-c(t-L)})$ for $t > L$, where: y – percentage of dry matter (DM) disappearance from the nylon bag at time t, a – soluble fraction, b – slowly degradable fraction, c – fraction rate constant at which b is degraded, L – lag time (h), and t – time of incubation (h); ED – effective degradability calculated using equation: $a + bc/(c + k)$, where $k = 0.049\ h^{-1}$ for diet, respectively; NDF – neutral detergent fibre; CP – crude protein; SEM – standard error of the mean; $^{abc}$ means with different superscripts in each row are significantly different at $P < 0.05$

In comparison with the control group, the slowly degradable fraction (b) of NDF was higher ($P$ = 0.015) in MFEM and ED of NDF was higher ($P$ = 0.047) in MFEM and HFEM groups. For CP, the soluble fraction, potentially degradable fraction and ED of CP increased quadratically ($P$ < 0.05) with increasing FEM supplementation. The ED of CP was the highest for MFEM and HFEM groups, the lowest for the control, and the intermediate for LFEM.

## Rumen enzymtic activities and microbiota

Increasing level of FEM addition linearly elevated ($P$ < 0.05) activities of cellulolytic enzymes, α-amylase and protease (Table 5). In comparison with the control, activities of carboxymethylcellulase in MFEM, cellobiase and laccase in HFEM and xylanase, pectinase, α-amylase and protease in MFEM and HFEM groups were higher ($P$ < 0.05). Populations of total bacteria, fungi, protozoa, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Butyrivibrio fibrisolvens* and *Ruminobacter amylophilus* responded linearly ($P$ < 0.05) but populations of total methanogens and *Ruminococcus flavefaciens* were unaltered with increasing addition level of FEM. Populations of total bacteria, *B. fibrisolvens* and *P. ruminicola* were greater ($P$ < 0.05) in MFEM and HFEM than in LFEM and control animals. Total fungi population was the greatest in MFEM, intermediate for LFEM and HFEM and lowest for control. Populations of total protozoa and *F. succinogenes* were greater ($P$ < 0.05) in MFEM and HFEM groups in comparison with control one. The population of *R. albus* was higher ($P$ = 0.003) for bulls in HFEM groups in comparison with those in control and LFEM groups. Higher ($P$ = 0.001) population of *Rb. amylophilus* was observed in FEM treatments than in control one.

**Table 5.** Effects of fibrolytic enzymes mixture (FEM) supplementation on ruminal microbial enzyme activity and microflora in Holstein bulls

| Item | Treatments[1] | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | control | LFEM | MFEM | HFEM | | treatment | linear | quadratic |
| Microbial enzyme activity | | | | | | | | |
| carboxymethyl-cellulase, μmol glucose/min/ml | 0.36$^{b}$ | 0.41$^{ab}$ | 0.46$^{a}$ | 0.44$^{ab}$ | 0.014 | 0.046 | 0.024 | 0.15 |
| cellobiase, μmol glucose/min/ml | 0.55$^{b}$ | 0.61$^{ab}$ | 0.65$^{ab}$ | 0.68$^{a}$ | 0.016 | 0.013 | 0.002 | 0.61 |
| xylanase, μmol xylose/min/ml | 1.16$^{b}$ | 1.25$^{ab}$ | 1.35$^{a}$ | 1.37$^{a}$ | 0.036 | 0.013 | 0.027 | 0.65 |
| pectinase, μmol D-galactouronic acid/min/ml | 0.77$^{b}$ | 0.85$^{ab}$ | 0.89$^{a}$ | 0.88$^{a}$ | 0.018 | 0.045 | 0.015 | 0.16 |
| laccase, U/l | 6.07$^{b}$ | 6.21$^{ab}$ | 6.25$^{ab}$ | 6.49$^{a}$ | 0.11 | 0.046 | 0.042 | 0.83 |
| α-amylase, μmol glucose/min/ml | 0.13$^{b}$ | 0.15$^{ab}$ | 0.17$^{a}$ | 0.17$^{a}$ | 0.005 | 0.037 | 0.026 | 0.89 |
| protease, μg hydrolysed protein/min/ml | 0.88$^{b}$ | 0.92$^{ab}$ | 1.06$^{a}$ | 1.04$^{a}$ | 0.025 | 0.047 | 0.041 | 0.58 |
| Microbiota, copies/ml | | | | | | | | |
| total bacteria, ×10$^{11}$ | 1.66$^{b}$ | 1.77$^{b}$ | 2.12$^{a}$ | 1.97$^{a}$ | 0.072 | 0.011 | 0.043 | 0.34 |
| total anaerobic fungi, ×10$^{8}$ | 3.52$^{c}$ | 4.44$^{b}$ | 5.64$^{a}$ | 4.86$^{b}$ | 0.25 | 0.001 | 0.001 | 0.051 |
| total protozoa, ×10$^{8}$ | 5.38$^{b}$ | 5.86$^{ab}$ | 6.22$^{a}$ | 6.40$^{a}$ | 0.13 | 0.013 | 0.002 | 0.44 |
| total methanogens, ×10$^{9}$ | 6.20 | 6.32 | 6.69 | 6.24 | 0.17 | 0.77 | 0.77 | 0.45 |
| *Ruminococcus albus,* ×10$^{8}$ | 1.23$^{b}$ | 1.31$^{b}$ | 1.60$^{ab}$ | 2.08$^{a}$ | 0.10 | 0.003 | 0.001 | 0.16 |
| *Ruminococcus flavefaciens,* ×10$^{9}$ | 1.19 | 1.24 | 1.44 | 1.40 | 0.062 | 0.45 | 0.16 | 0.72 |
| *Fibrobacter succinogenes,* ×10$^{10}$ | 1.87$^{b}$ | 2.37$^{ab}$ | 2.83$^{a}$ | 3.01$^{a}$ | 0.14 | 0.009 | 0.001 | 0.49 |
| *Butyrivibrio fibrisolvens,* ×10$^{9}$ | 5.73$^{b}$ | 6.34$^{b}$ | 7.93$^{a}$ | 8.50$^{a}$ | 0.33 | 0.001 | 0.001 | 0.95 |
| *Prevotella ruminicola,* ×10$^{10}$ | 4.11$^{b}$ | 4.52$^{b}$ | 5.11$^{a}$ | 4.99$^{a}$ | 0.12 | 0.001 | 0.001 | 0.078 |
| *Ruminobacter amylophilus,* ×10$^{8}$ | 5.45$^{b}$ | 6.88$^{a}$ | 7.16$^{a}$ | 7.13$^{a}$ | 0.19 | 0.001 | 0.001 | 0.13 |

[1] control – 0 g/kg FEM; LFEM – 0.25 g/kg FEM, MFEM – 0.50 g/kg FEM; HFEM – 0.75 g/kg FEM; SEM – standard error of the mean; $^{abc}$ means with different superscripts in each row are significantly different at $P$ < 0.05

**Table 6.** Effects of fibrolytic enzymes mixture (FEM) supplementation on urinary excretion of purine derivative (PD) in dairy bulls

| Item | Treatments[1] | | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | control | LFEM | MFEM | HFEM | | teatment | linear | quadratic |
| Allantoin, mmol/day | 79.9$^{c}$ | 91.8$^{b}$ | 102$^{a}$ | 104$^{a}$ | 2.68 | 0.001 | 0.001 | 0.053 |
| Uric acid, mmol/day | 5.69 | 5.77 | 5.81 | 5.80 | 0.021 | 0.21 | 0.066 | 0.29 |
| Total PD, mmol/day | 86.6$^{c}$ | 99.6$^{b}$ | 111$^{a}$ | 114$^{a}$ | 2.95 | 0.001 | 0.001 | 0.051 |

[1] control – 0 g/kg FEM; LFEM – 0.25 g/kg FEM, MFEM – 0.50 g/kg FEM; HFEM – 0.75 g/kg FEM; SEM – standard error of the mean; $^{abc}$ means with different superscripts in each row are significantly different at $P$ < 0.05

### Urinary purine derivative

As shown in Table 6, the excretion of total PD and allantoin had a linear ($P < 0.05$) response and were the greatest in MFEM and HFEM, followed by LFEM and then control animals. Application of FEM had no impact on excretion of uric acid.

## Discussion

The increment in DMI was probably due to the increase in the slowly degradable fraction and ED of corn silage NDF which caused rumen fill to be relieved. Studies indicated that DMI was controlled by the rumen fill extend and fibre degradation rate (Allen, 2000), and that the increased DMI with supplementing exogenous enzymes mixture in cow diets was a result of the enhancement in nutrient digestibility (Gado et al., 2009). The increase in ADG with FEM addition should be attributed to the increment in DMI and nutrient digestibility which resulted in an enhanced intake of digestible energy. The increased DMI and ADG are not reflected in greater final BW, it might be the large differences in body weight between individuals. The FCR calculated via DMI divided by ADG was unchanged in the present study. Likewise, Gómez-Vázquez et al. (2011) observed that DMI and ADG were increased by supplementing a xylanase and cellulase preparation in the concentrate for steers. However, others reported that total DMI increased but ADG and feed efficiency were unchanged in steers fed hays treated with a mixture of cellulase and xylanase (Krueger et al., 2008). Yang et al. (2000) found that the increase in cow performance was greater when FE was supplemented in the concentrate than in the TMR. Eun et al. (2007) reported that the improved magnitude of forage utilization depended on the formulations and activities of supplied FE. Hence, the inconsistent responses of growth performance to exogenous FE supplementation were associated with the differences in application methods, activities and formulations of enzymes in these studies (Beauchemin et al., 2004).

The increase in apparent digestibilities of DM, OM, CP, NDF and ADF was in line with the increment in rumen VFA concentration and dietary degradability of DM, CP and NDF. The increased ruminal degradability of DM, CP and NDF indicated that supplementation of FEM have positive effects on ruminal nutrient degradation (Giraldo et al., 2007; Eun et al., 2007). In addition, nutrient digestibility in the intestine might also be stimulated by FEM inclusion. Previous studies indicated that a portion of dietary supplemented FE could bypass the rumen, causing an increase in FE activities in the intestinal digesta (Hristov et al., 2000). Beauchemin et al. (1999) observed that fibre digestion in the rumen and small intestine was promoted by the addition of enzymes containing primarily cellulase and xylanase activities in cows. Similarly, in studies on cows (Gado et al., 2009) or steers (Krueger et al., 2008; Gómez-Vázquez et al., 2011) positive responses of nutrient apparent digestibility to FE addition were observed.

The decrease in rumen pH with FEM addition should be caused by the elevation in rumen total VFA concentration, but the pH values (6.35, 6.27 and 6.22 for LFEM, MFEM and HFEM group, respectively) were still optimal for microbial metabolism and feed digestion (Dijkstra et al., 2012). Acetate molar proportion was decreased by FEM addition, but acetate concentration was elevated due to the increase of total VFA content and was 82.6, 85.8, 87.4 and 87.5 mM for control, LFEM, MFEM and HFEM group, respectively. The changes of individual VFA in molar proportions and concentrations in the ruminal fluid can reflect the changes of individual VFA productions and nutrient degradation (Dijkstra et al., 2012). Hence, the increase in the concentration of acetate was in agreement with the positive responses of the slowly degradable fraction and ED of diet NDF, implying a stimulatory influence of FEM supplementation on fibre digestion in the rumen. Likewise, others reported that supplementing a FE preparation in cow diets increased ruminal total VFA concentration, and that the addition of cellulase or FE containing mainly endoglucanase and xylanase activities elevated concentrations of total VFA and acetate as well as degradability of DM and NDF *in vitro* (Giraldo et al., 2007; Eun et al., 2007). The enhancement in fibre digestion was attributed to the elevation in activities of cellulolytic enzymes and populations of microbes with FEM supplementation. In addition, the synergistic effects between dietary supplemented FE and ruminal microbial enzymes on feed degradation are also attributed to the increase in nutrient digestion (Morgavi et al., 2004; Wang et al., 2012). The FEM-fermented diet nutrients were then fermented by ruminal microbes. The plant cell walls are hydrolysed by cellulolytic enzymes to monosaccharides which then convert to acetate in the action of microbial intracellular enzymes (Wang and Mcallister, 2002). Studies indicated that exogenous FE addition reduced the thickness of plant cell wall, causing enhanced colonization and penetration of ruminal microbes and enzymes

to the surface of feed particles (Morgavi et al., 2004; Wang et al., 2012). The increase in activities of carboxymethyl-cellulase, cellobiase, xylanase, pectinase and laccase with FEM supplementation, is not only related to dietary supplemented FEM, but also to the increase in populations of total bacteria, fungi, protozoa, *F. succinogenes*, *R. albus* and *B. fibrisolvens.* The *R. albus*, *F. succinogenes* and *B. fibrisolvens* are main fibrolytic bacteria (Wang and Mcallister, 2002). All of the cellulolytic enzyme activities can be detected in protozoa and the activities of cellulases and xylanases produced by fungi are the highest in the rumen (Wang and Mcallister, 2002). Furthermore, the observed elevation in populations of microbes was likely due to that FEM addition promoted the release of feed reducing sugars which would provide available carbohydrates to encourage microbial growth (Beauchemin et al., 2004). In the *in vitro* study of Wang et al. (2012) increased release of reducing sugars from barley straw with the addition of FE in the buffer without ruminal fluid was observed. Similarly, other authors showed that rumen total viable bacteria numbers increased with the addition of a FE preparation into cow diets (Nsereko et al., 2002), and that ruminal cellulolytic enzymatic activities increased with exogenous cellulase addition *in vitro* (Giraldo et al., 2007). The increase in α-amylase activity with FEM addition was due to the increment in the populations of *B. fibrisolvens*, *P. ruminicola* and *Rb. amylophilus*, which are responsible for the degradation of starch to propionate (Wang and Mcallister, 2002). Thus, dietary FEM supplementation elevated propionate molar percentage and it was in accordance with the increment in both amylolytic bacteria population and α-amylase activity. The changes in propionate molar percentage resulted in the decreased acetate to propionate ratio and altered the rumen fermentation mode to higher propionate production.

Rumen isoacids are products of branched-chain amino acids degradation. The increment in the molar percentage of isovalerate was in accordance with the increase in the activities of *B. fibrisolvens*, *P. ruminicola* and *Rb. Amylophilus* populations as well as protease, suggesting that application of FEM might promote feed protein degradation in the rumen. Similarly, dietary supplementation of FE elevated ruminal population of *Rb. amylophilus* in cows (Chung et al., 2012). *In vitro* study demonstrated that isovalerate production increased with cellulases supplementation in Rusitec fermenters (Giraldo et al., 2007). Moreover, Yang et al. (1999) observed increased ruminal digestibility of CP with the addition of FE to cow diet. Furthermore, feed protein is degraded to ammonia-N which then is incorporated into microbial protein in the rumen. The increased ruminal CP degradation following FEM supplementation is due to the increased protease activity and population of proteolytic bacteria. The amount of microbial protein synthesis is usually measured by urinary total PD excretion (Yue et al., 2020). Therefore, the limited response of ammonia-N was inconsistent with the increased ruminal CP degradation following FEM supplementation. The conflicting results could be explained by the observed increment in urinary excretion of total PD which suggested that the conversion of ruminal ammonia-N to microbial protein was promoted by FEM addition. Likewise, Yang et al. (1999) observed that rumen microbial protein synthesis was increased by the application of enzymes containing primarily cellulase and xylanase activities in cows.

## Conclusions

Supplementation with fibrolytic enzymes mixture (FEM) promoted an increase of microbes populations and enzymes activities in the rumen, leading to an enhancement in feed intake, nutrient digestion and daily gain in bulls. The increased ruminal neutral detergent fibre degradability suggested the stimulatory effect of FEM addition on fibre digestion. The proper supplementation dose of FEM added into bull diets was 0.50 g/kg dry matter.

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## Conflict of interest

The Authors declare that there is no conflict of interest.

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