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Effects of an exogenous enzyme preparation extracted from a mixed culture of *Aspergillus* spp. on lactational performance, metabolism, and digestibility in primiparous and multiparous cows

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ABSTRACT

The objective of this study was to investigate the effects of an exogenous enzyme preparation from *Aspergillus oryzae* and *Aspergillus niger* on lactational performance of dairy cows. Forty-eight Holstein cows (32 primiparous and 16 multiparous) averaging (± SD) 36.3 ± 8.7 kg/d milk yield and 141 ± 52 d in milk were enrolled in a 10-wk randomized complete block design experiment (total of 24 blocks) and assigned to 1 of 2 treatments: basal diet, no enzyme supplementation (CON) or the basal diet supplemented with 4.2 g/kg dry matter intake (DMI) of an exogenous enzyme preparation containing amylolytic and fibrolytic activities (ENZ). After a 2-wk covariate period, premixes with the enzyme preparation or control were top-dressed daily by mixing with approximately 500 g of total mixed ration. Production data were collected daily and averaged by week. Milk samples were collected every other week, and milk composition was averaged by week. Blood, fecal, and urine samples were collected over 2 consecutive days at 0, 4, 8, 12, and 36 h after feeding during the last week of the experiment. Compared with CON, cows fed ENZ tended to increase DMI and had increased milk concentrations of true protein, lactose, and other solids. Milk fat content tended to be higher in CON cows. A treatment × parity interaction was found for some of the production variables. Primiparous cows receiving ENZ had greater yields of milk, energy-corrected milk, milk true protein, and lactose compared with CON primiparous cows; these production variables did not differ between treatments for multiparous cows. Intake and total-tract digestibility of nutrients did not differ between treatments. Concentrations of blood glucose and total fatty acids were not affected by ENZ supplementation, but β-hydroxybutyrate concentration tended to be greater in ENZ cows. Overall, the exogenous enzyme preparation used in this study increased milk protein and lactose concentrations in all cows, and milk production in primiparous but not multiparous cows. The differential production response between primiparous and multiparous cows was likely a result of a greater increase in DMI with ENZ supplementation in the younger animals.

Key words: fibrolytic activity, milk production, parity, dairy cow

INTRODUCTION

The development and adoption of strategies to enhance the performance of dairy cows while reducing feeding costs (i.e., increasing efficiency) is ultimately the main goal of dairy nutrition professionals. The use of exogenous enzyme preparations has been evaluated as a strategy to improve forage utilization and, consequently, productive efficiency of ruminants. However, results from previous research with lactating cows are inconsistent and, when positive, they are generally small (e.g., standardized mean difference of 0.25 kg/cow per day of milk yield between enzyme and control treatments in a meta-analysis by Arriola et al., 2017).

Effects of exogenous enzymes can be categorized by their mode of action as preconsumptive (acting on the feed), ruminal, or postruminal (McAllister et al., 2001). Based on their enzymatic activities, exogenous enzymes can be defined as amylases, cellulases, β-glucanases, hemicellulases, xylanases, pectinases, and proteases (Beauchemin et al., 2003). *Aspergillus* spp. are fila-

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mentous fungi widely used to produce various enzymes (e.g., cellulases, pectinases, amylases) and metabolites (e.g., citric acid) that can be used in animal nutrition, but the benefits of their supplementation to the diet of dairy cows remain unclear. Studies have demonstrated that *Aspergillus oryzae* extracts increase the number of ruminal bacteria (Newbold et al., 1992a,b) and that synergism between *A. oryzae* and rumen microbes enhances the release of nutrients from plant cell walls in the rumen (Newbold, 1995). However, improvements in lactational performance of dairy cows fed diets supplemented with *A. oryzae* were either small or not evident in recent (Zilio et al., 2019; Vittorazzi et al., 2021) and older (Kellems et al., 1990; Sievert and Shaver, 1993; Higginbotham et al., 1994) studies. Supplementation of diets with *Aspergillus niger* extracts alone has not been investigated, and only 2 studies (Oh et al., 2019; Silvestre et al., 2022) have evaluated the effects of a combination of *A. niger* and *A. oryzae* on lactational performance of dairy cows. Oh et al. (2019) reported increased total VFA concentration and Silvestre et al. (2022) suggested increased postprandial blood glucose concentration in cows fed a reduced-starch diet supplemented with *A. oryzae* and *A. niger*. The effects of *A. oryzae* and *A. niger* on total-tract digestibility of nutrients and lactational performance of the cows were either small or not evident in the latter 2 studies.

Effects of enzyme supplementation on lactational performance of primiparous versus multiparous cows have not been adequately investigated. Only a few studies have evaluated an enzyme × parity interaction on performance, digestibility, and metabolism. For instance, enzyme dose (Holtshausen et al., 2011) and forage-to-concentrate ratio (Arriola et al., 2011) did not interact with parity to affect the performance of dairy cows. Golder et al. (2019), in contrast, observed an enzyme × parity effect on milk protein concentration but no interactions for other production variables. Van Soest (1994) described a strong positive relationship between BW and the capacity of gastrointestinal tract in herbivores, which could be observed by limited meal size and reduced rate of feed intake in primiparous compared with multiparous cows (Bowman et al., 2003). Considering that the supplementation of exogenous enzymes in the diets would enhance rumen fermentation in dairy cows, primiparous (i.e., smaller animals with lower rumen capacity) would benefit to a greater extent than multiparous cows; however, the effects of exogenous enzyme supplementation of dairy diets on production variables in multiparous versus primiparous cows remains unclear.

The objective of the current study was to investigate the effects of an enzyme extract from *A. oryzae* and *A. niger* on performance, energy metabolism markers, total-tract digestibility of nutrients, and N use in lactating dairy cows. A second objective was to determine whether the response to enzyme supplementation is different between primiparous and multiparous cows. We hypothesized that enzyme preparation would improve total-tract digestibility of nutrients and, consequently, lactational performance in both multiparous and primiparous cows.

MATERIALS AND METHODS

All procedures involving animals used in this experiment were approved by The Pennsylvania State University's Institutional Animal Care and Use Committee.

Animals, Experimental Design, and Treatments

Forty-eight Holstein cows were enrolled in a randomized complete block design experiment. Cows were blocked according to parity (primiparous = 32; multiparous = 16), DIM, and milk yield (**MY**); average MY and DIM at the beginning of the study were (±SD) 36.3 ± 8.7 kg/d and 141 ± 52 d. Cows within block (2 cows/block) were randomly assigned to 1 of 2 treatments (24 cows/treatment): basal diet without enzyme supplementation (**CON**) or basal diet supplemented with an exogenous enzyme preparation from *A. oryzae* and *A. niger* (Cellulo-Gest, Purina Animal Nutrition; **ENZ**). According to the manufacturer, the enzymatic activities of the enzyme preparation were as follows: amylase, 1.2×10^6 modified Wohlgemuth units/kg (hydrolysis of interior α-1,4-glucosidic bonds of starch at 30°C); hemicellulase, 1.9×10^5 hemicellulase units/kg (hydrolysis of the interior glucosidic bonds of a defined locust bean gum at 40°C); cellulase, 1.1×10^4 cellulase units/kg (hydrolysis of interior β-1,4-glucosidic bonds of a defined carboxymethylcellulose at pH 4.5 and at 40°C); β-glucanase, 1.0×10^3 glucanase units/kg (hydrolysis of lichenin substrate at pH 6.5 and at 40°C); and pectinase, 1.0×10^3 pectinase units/kg (hydrolysis of interior α-1,4 glucosidic bonds of a defined polygalacturonate at pH 3.5 and at 40°C). The experiment had a 2-wk covariate period followed by 2 wk of adaptation and 6 wk of data and sample collections. Data collected during the covariate period were used to formulate a basal diet to meet or exceed NE_L and MP requirements (NRC, 2001) of a multiparous cow with BW of 680 kg, MY of 33 kg/d (with 3.80% fat and 3.20% true protein), and DMI of 23.4 kg/d. Premixes with the enzyme preparation or placebo were provided by the exogenous enzyme manufacturer and top-dressed daily by mixing with approximately 500 g of TMR. The premixes had similar composition (see Table 1) and the amount fed to animals in the ENZ

group corresponded to supplementation of 113.4 g/d of the enzyme preparation (corresponding to a dose of 4.2 g/kg of TMR DM and assuming DMI of 27 kg/cow per day). Cows were housed in a freestall barn at The Pennsylvania State University's Dairy Teaching and Research Center that was equipped with a Calan Broadbent Feeding System (American Calan Inc.) for individual monitoring of DMI. Feeding was ad libitum, targeting 5 to 10% refusals. A Rissler model 1050 TMR mixer (I. H. Rissler Mfg. LLC) was used to deliver the TMR once daily at approximately 0800 h. Cows had free access to drinking water.

Sample Collection

Diet and Feed Ingredients. Weights of feed offered and orts were recorded daily, and daily feed intake was measured during the entire experiment. Samples of the concentrates were collected weekly, and samples of the forages, TMR, and orts were collected twice weekly and stored at −20°C. Feed samples were later thawed overnight at room temperature, dried for 72 h at 55°C in a forced-air oven, and ground in a Wiley mill (Thomas Scientific) through a 1-mm sieve. Thereafter, forage, TMR, and orts samples were composited, on an equal DM basis, by experimental week and concentrates for the entire experiment. The DM content of the weekly TMR and orts composites samples was used to calculate DMI of the cows. Feed ingredients were submitted to Cumberland Valley Analytical Services (CVAS, Waynesboro, PA) for wet chemistry analysis of CP (method 990.03; AOAC International, 2000), amylase-treated NDF (Van Soest et al., 1991), ether extract (method 2003.05; AOAC International, 2006), ADF (method 973.18; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), minerals (Ca and P; method 985.01; AOAC International, 2000), and estimated NFC (NRC, 2001). Starch concentration was analyzed in corn silage samples according to Hall (2009). Table values for other feed ingredients analyzed in previous experiments were used for calculating dietary starch concentration. The nutrient composition of the diet (i.e., CP, NDF, ADF, ether extract, starch, ash, Ca, and P) was reconstituted from the analyzed chemical composition of the individual feed ingredients and their inclusion rate in the TMR. Balances of RDP, RUP, NE_L, and MP were estimated based on NRC (2001) using average DMI, MY, milk composition, and BW of the cows.

Milk Production, Milk Composition, and BW. Cows were milked twice daily at 0600 and 1800 h, and milk production was recorded at each milking (Afimilk system; Kibbutz Afikim). Milk samples were collected every other week in 4 consecutive milkings (p.m. and a.m.) into 50-mL tubes containing bromo-2-nitropropane-1,3-diol and submitted to Dairy One Cooperative Inc. (Ithaca, NY). Milk samples were analyzed for fat, true protein, lactose, other solids, TS, SCC, and MUN by infrared spectroscopy (MilkScan 4000, Foss Elec-

Table 1. Ingredient and chemical composition of the basal diet fed to dairy cows during the experiment

Item	Diet
Ingredient, % of DM or as indicated	
Corn silage[1]	43.0
Alfalfa haylage[2]	13.2
Hay-straw mixture[3]	4.0
Whole roasted soybeans	7.5
Whole cottonseed	4.0
Corn grain, ground	8.0
Canola meal	9.5
SoyPlus[4]	4.0
Molasses[5]	4.5
Mineral and vitamin premix[6]	1.8
Treatment premix with ENZ[7] or placebo[8]	0.5
Composition, % of DM	
CP[9]	16.5
RDP[10]	11.1
RUP[10]	5.4
NDF[9]	31.7
ADF[9]	21.6
Starch[11]	24.4
Ether extract[9]	5.0
NFC[10]	44.0
NE_L,[10] Mcal/kg DM	1.64
NE_L balance,[10] Mcal/d	3.60
MP balance,[10] g/d	31.0
Ca[9]	0.80
P[9]	0.40

[1]Corn silage was 44.0% DM and contained (% of DM) 7.4 CP and 36.6 NDF.

[2]Alfalfa haylage was 33.0% DM and contained (% of DM) 20.4 CP and 41.5 NDF.

[3]Hay-straw mixture was 95.4% DM and contained (% of DM) 4.6 CP and 79.5 NDF.

[4]Dairy bypass feed ingredient (Landus Cooperative).

[5]Liquid molasses was from Westway Feed Products.

[6]The premix (Cargill Animal Nutrition, Cargill Inc.) contained (%, as-is basis): trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg; vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

[7]ENZ premix contained 8.8% exogenous enzyme extract from *Aspergillus oryzae* and *Aspergillus niger* (Cellulo-Gest, Purina Animal Nutrition), 56.1% corn gluten feed, and 35.1% limestone.

[8]Placebo (control) premix contained 60% corn gluten feed and 40% limestone.

[9]Values calculated using the nutrient analysis of the feed ingredients (Cumberland Valley Analytical Services Inc.) and their inclusion in the diets.

[10]Estimated based on NRC (2001) using actual DMI, MY, milk composition, and BW of the cows throughout the experiment.

[11]Calculated from starch concentration in corn silage samples analyzed according to Hall (2009), and table values for other feed ingredients analyzed in previous experiments.

tric). Milk composition data were weighted for the corresponding a.m. and p.m. MY; and milk fat, milk true protein, and lactose yields were calculated from the averages of MY and weighted milk composition during each sampling wk. Energy-corrected milk yield was calculated as follows: ECM, kg/d = kg of milk × [(38.3 × % milk fat × 10 + 24.2 × % milk true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140] (Sjaunja et al., 1990). Body weight was recorded using an AfiFarm 3.04E scale system (SAE Afikim) twice daily when cows exited the milking parlor. Average BW was calculated for each cow in each experimental week. Body weight change was calculated as follows: BW change, kg/d = (average BW during experimental wk 8 – average BW during covariate wk 2) ÷ days on study.

Fecal and Urine Sampling. Fecal samples were collected from each cow by rectal stimulation to estimate apparent total-tract digestibility of nutrients. Spot fecal samples (approximately 500 g/cow) were collected over 2 consecutive days, at 0, 4, 8, 12 and 36 h after feeding (i.e., 0800 and 1200 h on d 1 and 1600 and 2000 h on d 2) during the last experimental week. Fecal samples were oven-dried at 55°C for 72 h and ground using a Wiley mill (Thomas Scientific) through a 1-mm sieve. Ground samples were composited by cow and week and were analyzed for CP (N × 6.25) using the Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.), and NDF and ADF using an Ankom 200 fiber analyzer (Ankom Technology Corp.). Total-tract digestibility of nutrients was estimated using indigestible NDF as an internal marker according to Huhtanen et al. (1994), with the sample bag modification proposed by Lee et al. (2012). Urine samples (approximately 300 mL/cow) were collected at the same time points as for fecal samples and added to 2 M H_2SO_4 in a ratio of 60 mL of acid per 1,000 mL of urine to reach a pH <3.0. Acidified samples were diluted 1:10 with distilled water and stored at −20°C for further analyses. Urine samples were composited on an equal volume basis per cow and analyzed for urea N (**UUN**; Urea nitrogen kit 580; Stanbio Laboratory Inc.) and creatinine (Creatinine kit 420; Stanbio Laboratory Inc.). Composite urine samples were freeze-dried (VirTis Ultra 35L; SP Scientific) and analyzed for N using a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc.). Daily urinary volume was estimated based on urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW (unpublished total urine collection data from Hristov et al., 2011). Estimated daily urine output was used to calculate daily excretions of urine N and UUN. Total excreta N was calculated as the sum of excreted urine and fecal N. Unaccounted N was calculated as follows: Unaccounted N, g/d = [N intake, g/d – (total excreta N, g/d + milk N g/d)]. Milk N secretion was determined as follow: Milk N, g/d = (milk true protein yield, g/d ÷ 6.38) + MUN, g/d.

Blood Sampling. Blood samples were collected at the same time points as fecal samples by coccygeal venipuncture using a 20-gauge × 2.54-mm needle into 9-mL Vacutainer tubes containing EDTA (Becton, Dickinson and Co.). Samples were placed on ice until being centrifuged at 1,500 × g at 4°C for 15 min for plasma collection. Plasma samples were frozen at −20°C and composited by cow on an equal volume basis. Composite samples were analyzed for total fatty acids (HR Series NEFA-HR, Wako Diagnostics), BHB (Autokit 3-HB Microliter Procedure; Wako Diagnostics), and glucose (Idexx Laboratories Inc.).

Statistical Analysis

Statistical analysis of the data was performed using SAS (release 9.4, SAS Institute Inc.). Data were tested for normality using the UNIVARIATE procedure and processed for outlier identification based on an absolute studentized residual value >3 using PROC REG. Log-transformed data were analyzed when the W statistic of the Shapiro-Wilk test was <0.05 (e.g., SCC data). Statistical analysis was completed using the MIXED procedure. Production data (DMI, MY, milk composition, milk components yield, feed efficiency, ECM, ECM feed efficiency, and BW) were averaged per experimental week, and the averaged data were analyzed as repeated measures using the first-order autoregressive [AR(1)] covariance structure. The statistical model included the fixed effects of treatment, week, parity, treatment × week, and treatment × parity interactions, and a covariate measurement. Block and block × treatment were random effects. The statistical model for nutrient intake, total-tract nutrient digestibility, blood metabolites, BW change, and N utilization contained the fixed effects of treatment, parity, and treatment × parity interaction. Statistical differences were considered at $P \leq 0.05$, and tendency was declared at $0.05 < P \leq 0.10$. Data are presented as covariate-adjusted (production data) least squares means.

RESULTS AND DISCUSSION

Supplementation of ENZ in the diet of mid-lactation cows tended to increase ($P = 0.08$) DMI by 0.9 kg/d compared with CON and increased ($P < 0.01$) MY by 2.3 kg/d in primiparous but not multiparous cows (i.e., treatment × parity interaction; Table 2). Feed efficiency did not differ between treatments for primiparous cows but was reduced ($P = 0.01$) by ENZ compared with CON in multiparous cows. Milk fat content tended to

Table 2. Production variables in dairy cows fed a diet supplemented with an exogenous enzyme preparation from *Aspergillus oryzae* and *Aspergillus niger*

Item	Diet[1]		SEM[2]	*P*-value[3] Treatment
	CON	ENZ		
DMI, kg/d	23.7	24.6	0.41	0.08
Primiparous	23.4	24.7	0.55	0.26
Multiparous	24.0	24.5	0.82	0.67
Milk yield, kg/d	32.1	32.9	0.45	0.22
Primiparous	29.5	31.8	0.52	<0.01
Multiparous	37.7	36.8	0.74	0.42
Feed efficiency,[4] kg/kg	1.38	1.36	0.022	0.43
Primiparous	1.35	1.40	0.029	0.26
Multiparous	1.43	1.32	0.024	0.01
Milk fat, %	4.20	4.00	0.080	0.08
Milk fat yield, kg/d	1.36	1.29	0.036	0.18
Primiparous	1.27	1.31	0.038	0.45
Multiparous	1.52	1.34	0.068	0.09
ECM, kg/d	32.8	32.4	0.66	0.67
Primiparous	30.4	32.4	0.63	0.03
Multiparous	37.3	34.5	1.35	0.18
ECM ÷ DMI, kg/kg	1.36	1.34	0.028	0.55
Primiparous	1.37	1.43	0.03	0.13
Multiparous	1.37	1.25	0.05	0.12
Milk true protein, %	3.12	3.21	0.025	0.01
Milk true protein yield, kg/d	1.00	1.06	0.020	0.02
Primiparous	0.92	1.04	0.024	<0.001
Multiparous	1.16	1.14	0.028	0.61
Milk lactose, %	4.91	4.96	0.012	0.01
Milk lactose yield, kg/d	1.58	1.64	0.025	0.06
Primiparous	1.46	1.61	0.031	<0.01
Multiparous	1.82	1.79	0.032	0.54
Other solids, %	5.83	5.89	0.012	<0.01
TS, %	13.2	13.1	0.08	0.22
MUN, mg/dL	10.8	10.9	0.23	0.95
SCC[5] ($\times 10^3$ cells/mL)	1.71 (66.0)	1.55 (43.5)	0.046 (7.21)	0.02
BW, kg	642	647	2.5	0.21
BW change,[6] kg/d	0.65	0.73	0.056	0.33

[1]CON = control; ENZ = CON supplemented with an enzyme extract preparation from *Aspergillus oryzae* and *Aspergillus niger* mixed culture at 4.2 g/kg of DMI.

[2]Largest SEM published in table; n = 220 for DMI, milk yield, and BW; n = 44 for BW change; n = 128 to 132 for all other variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: milk yield, milk fat yield, ECM, ECM ÷ DMI, milk true protein yield, MUN and BW change, $P \leq 0.03$. For all other variables, $P \geq 0.07$. Treatment × parity interaction: milk yield, feed efficiency, milk fat yield, ECM yield, milk true protein yield, milk lactose yield, $P \leq 0.03$. ECM ÷ DMI, $P = 0.07$. For all other variables, $P > 0.10$.

[4]Feed efficiency (kg/kg) = kg of milk ÷ kg of DMI.

[5]Statistical analysis was performed on log-transformed data. Actual data ($\times 10^3$ cells/mL) are given in parentheses.

[6]BW change: (average BW during experimental wk 8 – average BW during covariate wk 2) ÷ days on study.

be lower ($P = 0.08$) for ENZ compared with CON, and milk fat yield tended to be lower ($P = 0.09$) in multiparous cows fed the ENZ diet. A treatment × parity interaction was also observed for ECM yield; there was no difference in ECM yield for multiparous cows, whereas primiparous cows receiving the ENZ diet had greater ($P = 0.03$) ECM yield than those receiving the CON diet. Concentrations of milk true protein and lactose were greater ($P = 0.01$) for ENZ than CON for both primi- and multiparous cows. Primiparous cows on the ENZ diet had greater ($P < 0.01$) yields of milk true protein and lactose compared with CON, but no differences were observed for multiparous cows. Supplementation of the diet with ENZ resulted in increased ($P < 0.01$) other solids content in milk and decreased ($P = 0.02$) milk SCC. There were no differences between treatments for milk TS content, MUN, BW, or BW change.

The main hypothesis of our study was that an exogenous enzyme preparation containing fibrolytic and amylolytic activities would improve ruminal and, possibly, total-tract digestibility of dietary nutrients, which in turn would improve lactational performance of dairy cows. Although total-tract nutrient digestibility was

not affected, primiparous—but not multiparous cows—responded positively to the enzyme treatment.

Several studies have investigated the efficacy of supplementing lactating cow diets with different enzyme preparations; however, responses have been inconsistent, and effects relatively small. Arriola et al. (2017) conducted a meta-analysis of 15 studies to evaluate the effects of adding exogenous fibrolytic enzymes in the diet and to determine which factors would affect treatment responses in dairy cows. These authors concluded that supplementation of enzyme preparations resulted in a slight increase in milk production and milk true protein and lactose yields, with no differences in DMI. To the best of our knowledge, the current study is the first to report a significant interaction between enzyme supplementation and parity on lactational performance of dairy cows (other than the milk true protein concentration effect reported by Golder et al., 2019). An earlier study that evaluated the effects of fibrolytic enzymes and parity on feeding behavior, salivation, and ruminal pH demonstrated that primiparous cows consume feed more slowly and tend to ingest smaller amounts of feed during a feeding event than multiparous cows (Bowman et al., 2003; production data were not reported in that study). Van Soest (1994) described a strong positive relationship between BW and the capacity of the gastrointestinal tract in herbivores, which supports the fact that DMI of primiparous cows, compared with multiparous cows, is affected more by rumen fill mechanisms (Dado and Allen, 1994). Bowman et al. (2003) did not report any interactions between enzyme supplementation and parity on feeding, chewing, or ruminating behavior in lactating dairy cows. However, the treatment × parity effects observed in our study demonstrate that primiparous cows responded positively to exogenous enzyme supplementation. The differential production response to ENZ compared with CON between parities in the current study was likely a result of a greater increase in DMI for primiparous (+1.3 kg/d) versus multiparous cows (+0.48 kg/d). This numerical increase in DMI was not reflected in increased milk production in multiparous ENZ cows, which might have influenced the lower feed efficiency observed in these animals.

A positive synergistic effect of *A. oryzae* and *A. niger* on nutrient digestibility, VFA production, and bacterial diversity was demonstrated in vitro when forage and TMR samples were incubated with 60 mg of enzyme preparation/g substrate (Kong et al., 2021). The effects of supplementing dairy cows with an enzyme preparation extracted from *A. oryzae* and *A. niger* were previously investigated in a study that also evaluated the supplementation of a *Saccharomyces cerevisiae*-based direct-fed microbial (Oh et al., 2019). Although digestibility of nutrients, DMI, and feed efficiency were not affected by the additives (Oh et al., 2019), direct-fed microbial and ENZ supplementation resulted in greater (41.8 kg/d) and intermediate (41.0 kg/d) milk production, respectively, compared with cows in the control group (39.8 kg/d). Moreover, cows fed the additives had increased concentration of total VFA in ruminal fluid, suggesting enhanced fermentation of dietary nutrients (Oh et al., 2019). Even though we did not measure ruminal fermentation variables in the current study, the increased performance of primiparous cows fed ENZ and the increased milk protein and lactose contents in the ENZ group (regardless of parity) may be indicative of enhanced ruminal fermentation that resulted in increased total VFA production (Arriola et al., 2011) and microbial protein synthesis. With ENZ supplementation increasing the availability of nutrients for rumen fermentation in the current study (as previously discussed), more gluconeogenic precursor (i.e., propionate) would be available to the cow. It is well known that propionate represents 60 to 74% of the substrate uptake for glucose synthesis in the liver of dairy cows and that the mammary gland is one of the tissues that have an obligatory demand for glucose (Aschenbach et al., 2010). Similarly, it is reasonable to assume that milk protein synthesis was enhanced because more AA were available for absorption in the small intestine as a consequence of increased microbial protein synthesis in the rumen (NRC, 2001). Because primiparous cows typically have lower DMI and nutrient supply (e.g., AA and glucose) to the mammary gland than multiparous cows, the enhanced lactational performance of primiparous cows in the current experiment can be associated with the greater DMI increase and availability of nutrients as a result of ENZ supplementation compared with CON.

Although ENZ did not affect nutrient intake or total-tract digestibility of dietary nutrients (Table 3), it is reasonable to hypothesize that ENZ may have increased the availability of nutrients for rumen fermentation (Kong et al., 2021) and thus, the supply of nutrients postruminally in primiparous cows (i.e., cows that are more sensitive to physical regulation of intake). Digestibility of nutrients is a competitive process of digestion and passage of the digesta through the gastrointestinal tract. Consequently, evaluation of in vivo digestibility of nutrients is subjected to fermentation and the retention time of digesta in the rumen, digestion and absorption in the small intestine, and fermentation in the large intestine (Oba and Allen, 1999). Thus, an increased DMI in ENZ cows could reflect, based on Allen (1996), a positive effect of ENZ on fiber degradability and a consequent reduction in ruminal retention time and increased digesta passage rate. Hence, we did

Table 3. Intake and total-tract digestibility of nutrients in dairy cows fed a diet supplemented with an exogenous enzyme preparation from *Aspergillus oryzae* and *Aspergillus niger*

Item	Diet[1]		SEM[2]	*P*-value[3] Treatment
	CON	ENZ		
Intake,[4,5] kg/d				
DM	24.5	25.3	0.58	0.37
OM	23.3	24.0	0.55	
CP	4.0	4.1	0.09	
NDF	7.8	8.0	0.18	
ADF	5.3	5.5	0.12	
Digestibility, %				
DM	73.8	74.1	0.48	0.68
OM	75.6	75.7	0.46	0.88
CP	75.2	75.4	0.88	0.89
NDF	55.1	54.3	0.61	0.34
ADF	52.0	52.9	0.88	0.48

[1]CON = control; ENZ = CON supplemented with an enzyme extract preparation from *Aspergillus oryzae* and *Aspergillus niger* mixed culture at 4.2 g/kg of DMI.

[2]Largest SEM published in table; n = 42 for all variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P < 0.05$ for all variables. Treatment × parity interaction: $P > 0.10$ for all variables.

[4]Intake during digestibility data collection period.

[5]*P*-values for intake of dietary nutrients are the same as for DMI.

not observe differences in total-tract digestibility of nutrients between ENZ and CON in the current study, because digestibility is inversely related to passage rate. Vittorazzi et al. (2021) found a positive linear response in total-tract fiber digestibility with increasing doses of a blend of xylanase and β-glucanase supplemented to the diet of mid-lactation cows producing 27.3 to 28.1 kg of milk/d and consuming 22.3 to 23.1 kg of feed DM/d. Arriola et al. (2017) reported that digestibility of dietary DM and NDF also tended to be slightly increased in cows fed exogenous enzymes; however, increasing the rate of enzyme application did not affect the performance of the cows (Vittorazzi et al., 2021). Golder et al. (2019) evaluated the use of fibrolytic enzymes (i.e., fermentation product of *Trichoderma reesei* with xylanase and cellulase activities) in the field using large commercial dairy herds and reported increased overall yields of milk (0.7 kg/d), fat (40 g/d), and milk true protein (10 g/d), and a numerical increase in DMI (0.2 kg/d). It is important to note that the responses to the enzyme treatment differed among farms in the Golder et al. (2019) study, which corroborates with the low to moderate heterogeneity levels and inconsistency in effect size reported in the meta-analysis of Arriola et al. (2017).

We observed no effects of treatment on N intake, N excretion in urine and feces, or N secretion in milk (Table 4). However, treatment × parity interactions for urine N, UUN, and total N excretions were observed. Compared with CON, excretion of urine N and UUN were increased ($P < 0.10$) by ENZ in primiparous but not multiparous cows. Excretion of urine N and UUN as a percentage of N intake, on the other hand, were not affected by treatment and there was no treatment × parity interaction on these variables. Total excreta N losses were greater ($P = 0.02$) for ENZ than for CON primiparous cows (413 vs. 353 g/d, respectively), but not for multiparous cows. Consequently, total excreta N as percentage of N intake was greater ($P = 0.03$) in ENZ than in CON primiparous cows (69.1 vs. 60.1%, respectively). Overall, treatment had no effect on N utilization in the current study, as evidenced by similar MUN concentration and N excretion and secretion variables. It is important to note, however, that MUN differences in cows fed the same diet can be caused by variation in the transport and recycling of urea in the gastrointestinal tract (Souza et al., 2021). Because primiparous cows fed the ENZ diet had increased milk protein yield, the greater total excreta N in this group was not expected. Wickersham et al. (2009) demonstrated that N recycling is increased in cattle fed a lower protein diet, which could be true for primiparous CON cows, assuming they had lower rumen fermentation rate and ammonia concentration than primiparous cows fed ENZ.

Concentrations of blood glucose and total fatty acids did not differ between CON and ENZ, but cows fed the ENZ diet tended to have greater ($P = 0.08$) blood BHB concentrations than CON cows (Table 5). Because plasma glucose concentration did not differ between treatments in the current study, it is plausible to infer that supplementation with ENZ resulted in greater utilization of glucose by the mammary gland (e.g., increased milk lactose content for ENZ compared with CON). Dietary factors such as starch and NDF concentrations (Silvestre et al., 2022) and the type of forage (Yang et al., 2019) can also influence responses in cows fed diets supplemented with enzyme preparations. For instance, Silvestre et al. (2022) hypothesized that supplementing a reduced-starch diet (i.e., a 26% reduction in dietary starch concentration by substitution of ground corn with wheat straw) with the same enzyme preparation as that used in the current study would compensate the deficit of energy compared with a normal-starch diet. Although the enzyme supplementation in the reduced-starch diet increased postprandial blood glucose concentration, cows were not able to maintain the same DMI and milk production levels as cows fed a normal-starch diet, even though feed efficiency was not affected by treatments (Silvestre et al., 2022).

In the current study, ENZ did not affect blood concentration of total fatty acids but tended to slightly increase BHB concentration. Ruminal butyrate concen-

Table 4. Nitrogen utilization in dairy cows fed a diet supplemented with an exogenous enzyme preparation from *Aspergillus oryzae* and *Aspergillus niger*

Item	Diet[1]		SEM[2]	*P*-value[3] Treatment
	CON	ENZ		
N intake,[4] g/d	640	660	14.6	0.33
N excretion or secretion, g/d				
Urine N	256	266	14.8	0.64
Primiparous	217	264	15.8	0.05
Multiparous	296	269	27.9	0.50
UUN[5]	136	133	8.8	0.78
Primiparous	111	135	9.9	0.10
Multiparous	161	130	15.7	0.18
Fecal N	160	163	5.7	0.72
Total excreta N	419	433	16.1	0.52
Primiparous	353	413	17.0	0.02
Multiparous	484	454	30.7	0.49
Milk N	162	171	7.0	0.39
Unaccounted N[6]	58	56	17.9	0.93
As % of N intake				
Urine N	40.1	40.1	2.33	0.85
UUN[5]	21.2	19.5	1.21	0.33
Fecal N	24.1	24.6	0.63	0.54
Total excreta N	63.9	66.1	2.16	0.47
Primiparous	60.1	69.2	2.82	0.03
Multiparous	67.7	63.0	3.17	0.31
Milk N	25.4	26.0	0.92	0.62
Unaccounted N[6]	9.0	7.8	2.79	0.77

[1]CON = control; ENZ = CON supplemented with an enzyme extract preparation from *Aspergillus oryzae* and *Aspergillus niger* mixed culture at 4.2 g/kg of DMI.

[2]Largest SEM published in table; n = 41 to 44 (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect, $P \leq 0.05$ for all variables except UUN (g/d), unaccounted N (g/d), urine N (%), UUN (%), total excreta N (%), milk N (%), and unaccounted N (%). Treatment × parity interaction: UUN (g/d), total excreta N (g/d), total excreta N (%; see text for discussion), $P \leq 0.05$. Urine N (g/d), $P = 0.08$. For all other variables, $P > 0.10$.

[4]Intake during digestibility and urine data collection period.

[5]UUN = urinary urea nitrogen.

[6]Unaccounted N = N intake – (urinary N + fecal N + milk N).

tration was increased in cows fed a diet supplemented with amylolytic enzymes in the study of Zilio et al. (2019), and in vivo studies have demonstrated that most of the butyrate absorbed by the ruminal epithelium is converted to BHB and some acetoacetate before release in portal circulation (Pennington and Pfander, 1957; Weigand et al., 1975; DeFrain et al., 2004). Moreover, acetoacetate is subsequently removed from circulation and converted to BHB by the liver (Heitmann et al., 1986, 1987), and BHB synthesis appears to be stimulated by propionate production in isolated ruminal epithelial cells (Baldwin and Jesse, 1996). Thus, the tendency for increased plasma BHB concentration in ENZ cows appears to support the hypothesis for greater VFA concentration and butyrate production in the rumen of ENZ cows. In addition, the lack of differences between treatments in markers of energy metabolism (i.e., glucose and total fatty acids) and BW change, and considering the stage of lactation of the cows (i.e., mid-lactation), suggests that the tendency for greater concentrations of BHB was not caused by low energy balance in ENZ cows.

Diet composition, level and type of forage in the diet (Refat et al., 2018; Yang et al., 2019), feed ingredients

Table 5. Blood plasma concentrations of energy metabolites in dairy cows fed a diet supplemented with an exogenous enzyme extract from *Aspergillus oryzae* and *Aspergillus niger*

Item	Diet[1]		SEM[2]	*P*-value[3] Treatment
	CON	ENZ		
Glucose, mg/dL	49.5	50.1	2.39	0.87
Total fatty acids, μM	126	135	7.3	0.38
BHB, μM	498	578	31.3	0.08

[1]CON = control; ENZ = CON supplemented with an enzyme extract preparation from *Aspergillus oryzae* and *Aspergillus niger* mixed culture at 4.2 g/kg of DMI.

[2]Largest SEM published in table; n = 44 for BHB; n = 41 for total fatty acids; n = 43 for glucose (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment. Parity effect: $P > 0.08$ for all variables. Treatment × parity interaction: $P > 0.10$ for all variables.

presentation (Weiss et al., 2011), stage of lactation (Beauchemin et al., 2003), and interaction with dietary nutrients (Tirado-González et al., 2018; Silvestre et al., 2022) have all been shown to affect responses to exogenous enzyme supplementation. In addition, parity should be considered as a factor when using exogenous enzyme additives in the diet of dairy cows, as reported in the current study.

CONCLUSIONS

Supplementing the diet of mid-lactation dairy cows with an exogenous enzyme preparation from *A. oryzae* and *A. niger* increased DMI, milk true protein, and lactose concentrations. Total-tract digestibility of nutrients, energy metabolism, and N utilization were not affected by enzyme treatment. Supplementation of the exogenous enzyme preparation was more beneficial to the lactational performance of primiparous cows than multiparous cows. The observed increased yields of milk, ECM, milk true protein, and lactose in primiparous cows are likely a result of enhanced ruminal fermentation and feed intake in animals that may be more responsive to increased nutrient availability due to their lower DMI.

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