Amylase addition increases starch ruminal digestion in first-lactation cows fed high and low starch diets

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ABSTRACT

The objective of this study was to evaluate the effect of an exogenous amylase preparation on digestion of low- and high-starch diets in dairy cattle. Rumen and total-tract nutrient digestibility were measured in a 4 × 4 Latin square design with 28-d periods using 4 first-lactation cows cannulated at the rumen and duodenum. Corn silage-based diets had 20 or 30% starch, attained by changing the composition of concentrate, with or without addition of an exogenous amylase preparation. Effects of the enzyme additive were observed on ruminal digestibility but not at the total-tract level. Ruminal digestibility of starch increased from 75% in control to 81% with amylase supplementation. This difference in ruminal starch digestion was compensated postruminally, so that the total-tract digestibility of starch was almost complete and did not differ between treatments. The amylase supplement also increased the true ruminal digestibility of organic matter but did not affect microbial N flow to the duodenum. Amylase supplement reduced the proportion of acetate and butyrate and increased that of propionate, particularly in the high-starch diet, where it tended to increase the concentration of total volatile fatty acids in the rumen. Other effects were a higher amylase activity in the solid-associated microbial community and a tendency for lower numbers of protozoa. In contrast, we observed no changes in intake, production, dry matter and fiber (neutral detergent fiber and acid detergent fiber) digestibility, or ruminal digestion, and no or small changes on selected fibrolytic and amylolytic bacteria and on the microbial community in general. We conclude that the exogenous amylase improved starch digestion in the rumen in first-lactation cows with moderate intake and production levels.

Key words: amylase, ruminal digestion, starch, dairy cow

INTRODUCTION

Dairy cows of high genetic merit require an energy-dense diet to fulfill their production potential. This is why starchy cereals are so prevalent in the diets of high-producing dairy cows. However, cereals are also a human staple food, and compared with other feed components, they are expensive and their prices are expected to rise due to increasing costs of production and higher demand. Increases in cereal price also directly affect the profitability of dairy farms. For these reasons, the pressure is growing to reduce starch in dairy cow rations, making optimization of starch digestibility an important area of research if performance of milk production is to be maintained at a high level. In this context, the use of exogenous amylase as a feed additive for high-producing ruminants is of interest (McCarthy et al., 2013). Several studies have demonstrated that exogenous amylase preparations resistant to ruminal degradation are able to improve OM digestibility in dairy cows (Hristov et al., 2008; Klingerman et al., 2009; Gencoglu et al., 2010) or beef steers (DiLorenzo et al., 2011). This increased OM digestibility was associated, in some cases, with improved NDF digestibility, whereas no effect on starch digestibility (Gado et al., 2009; Klingerman et al., 2009; Gencoglu et al., 2010) or on true digestibility of OM in the rumen (Hristov et al., 2008) was found. Although there is abundant information on the use and mode of action of exogenous fibrolytic enzymes in ruminants (Beauchemin et al., 2003, 2004), the number of studies on exogenous amylase is still small and the exact mechanisms by which amylases might improve digestibility have not been fully explored. The objective of the present work was to evaluate the effect of an exogenous amylase preparation on ruminal and total-tract digestion of low- and high-starch diets in dairy cows.

MATERIALS AND METHODS

The experiment was conducted in respect of the national legislation on animal care (Certificate of Authori-

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sation to Experiment on Living Animals, no. 004495, Ministry of Agriculture, France). The experiment was approved by the Auvergne regional ethic committee for animal experimentation (approval number CE30-10).

Animals, Feeding, and Experimental Design

Four Holstein first-lactation cows in mid lactation, weighing 545 ± 25 kg (means $\pm$ SD) and at 82 ± 3 DIM at the beginning of the trial were used in a 4×4 Latin square design. Six weeks before the start of the experiment, cows were fitted with ruminal cannulas (external and internal diameter = 123 and 106 mm) made of polyamide-polyvinyl chloride, and T-shaped cannulas (external and internal diameter = 30 and 19 mm) made of plastisol with a gutter-type flange placed at the proximal duodenum, before the bile duct entrance. Cannulas were manufactured in the INRA workshop (St-Genès Champanelle, France). Surgery was performed under general anesthesia. Four dietary treatments were applied to the cows during 4 successive 4-wk periods. Treatments consisted of 30% (high starch) or 20% (low starch) starch in the ration with or without addition of amylase.

The proportion of corn silage was the same for all diets (Table 1). Diets were isoenergetic on a net energy basis and isonitrogenous on CP and digestible protein basis, and not deficient in rumen-fermentable N (INRA, 2007). Differences in starch content were achieved by changes in formulation of the concentrate, with corn

grain in the high-starch diet being replaced by soybean hulls and citrus pulp in the low-starch diet. Cows were housed in individual stalls and fed *ad libitum* during adaptation wk 1 and 2, and were fed at 95% of *ad libitum* intake during adaptation wk 3 and during wk 4 when measurements were done. Rations were distributed as TMR twice a day. For practical reasons, cows were fed at 0900 h (60% of the daily ration) and 1700 h (40% of the daily ration) during wk 1 and 2, and at 0945 and 2145 h (2 equal meals) during wk 3 and 4. Fifteen minutes before each meal, each cow received 300 g of ground concentrate alone or supplemented with amylase [Ronozyne RumiStar 600 (CT), DSM Nutritional Products, Basel, Switzerland]. The enzyme dose (10 g/d) was calculated to be equivalent to a dosage of 300 kilo novo units (KNU)/kg of DM total ration, assuming an average daily feed intake of 20 kg of DM. Corn silage, hay, and additional concentrate without any enzyme supplementation were distributed after the ground concentrate had been entirely consumed. Water and mineral salt were freely available. Cows also received 200 g/d of a mineral and vitamin complement (Galaphos midi duo, CCPA, Janzé, France; 4.5% P, 20% Ca, Mg 4.5%, 5% Na). Milking times were 0630 and 1530 h.

Measurements and Sampling

Intake and Weight. Cows were weighed at the start of each experimental period. Water consumption

Table 1. Ingredients and chemical composition of the diets

Item	High starch (30%)		Low starch (20%)	
	Control	Amylase	Control	Amylase
Ingredient composition, % of DM				
Corn silage	59.0	59.0	58.5	58.9
Hay	7.9	8.0	8.1	7.9
Pelleted high starch concentrate ¹	33.1	33.0	—	—
Pelleted low starch concentrate ²	—	—	33.4	33.2
Amylase activity, ³ KNU/kg of DM		354		350
Chemical composition, g/kg of DM				
CP	159	158	161	160
NDF	313	314	349	349
ADF	156	156	185	184
Starch	299	299	197	199
Feed value, /kg of DM				
NE _L , MJ	6.97	6.97	6.76	6.76
Digestible protein, g of PDI ⁴	105	105	104	104

¹High starch concentrate: 44.4% corn grain, 6.3% citrus pulp, 16.9% tanned soybean meal, 30% soybean meal 48, 2.5% urea, on a DM basis.

²Low starch concentrate: 1.3% corn grain, 15.6% soybean hulls, 33.8% citrus pulp, 16.9% tanned soybean meal, 30% soybean meal 48, 2.5% urea, on a DM basis.

³Amylase activity calculated from the actual analyzed enzyme activity of the test product Ronozyne RumiStar 600 [CT; DSM Nutritional Products, Basel, Switzerland; declared amylase activity: 600 kilo novo units (KNU)/g; analyzed amylase activity: 669 KNU/g], intake of the enzyme (10 g/cow per day), and actual DMI.

⁴PDI = protein digestible in the intestine (INRA, 2007).

was measured once a week. Intake was recorded 4 d per week on wk 1 and 2, and daily on wk 3 and 4. The DM content (24 h in a 103°C forced-air dry oven) of feeds was measured once a week for concentrates and hay, and twice (wk 1, 2, 3) or thrice (wk 4) a week for silage. The DM and N intakes were corrected for losses of volatile compounds (ethanol, NH_3 , and acetic and lactic acids) from corn silage during drying (Dulphy et al., 1975). A 100-g sample of each ingredient of the diet was collected daily during wk 4 of each period, pooled per period, and stored at -20°C for silage or at ambient temperature for other ingredients until analysis of chemical composition. When refusals exceeded 1 kg, DM content was measured and refusals were stored at -20°C for subsequent analysis of chemical composition; otherwise, the chemical composition was assumed to be similar to the offer.

Total-Tract Digestibility, N Balance, Nutrient Flow, and Microbial Synthesis. Total-tract digestibility and N balance measurements were performed in wk 4 by total feces and urine collection during 6 d. A urine collection device fixed to the animal was used to separate urine from feces. The device was connected by a flexible pipe to a 30-L carboy containing 500 mL of 10% sulfuric acid. Fresh weight (feces and urines) and DM content (feces) were determined daily at 0900 h. Total daily fecal excretion of each cow was mixed and a 0.5% aliquot pooled over the 6-d collection period and stored at -20°C . Similarly, for urine, a 1% aliquot of the daily excretion of each cow was sampled, pooled, and stored at -20°C .

Duodenal digesta fluxes were determined using ytterbium(III) chloride (YbCl_3) as an external indigestible marker (Siddons et al., 1985). A solution of 1 g of Yb was continuously infused in the rumen at a rate of 1.2 L/d through the ruminal cannula using a peristaltic pump for 10 consecutive days, (i.e., 6 d before the first duodenal sampling) to ensure a steady state (Owens and Hanson, 1992). Twelve duodenal samples of 250 mL, representing 2-h intervals over a day, were collected over 3 d in wk 4. Samples were pooled per animal and per period and stored at -20°C .

For calculation of bacterial N flow and protein synthesis, rumen content samples were taken in each period on the first day of wk 4, before and 2.5 h after the morning feeding. The solid and liquid phases were separated by filtration through a polyester monofilament fabric (250- μm mesh aperture). Solid-adherent bacteria (SAB) in the retentate and liquid-associated bacteria (LAB) in the filtrate were isolated by washing, stomaching, and differential centrifugation, as described in Bauchart et al. (1990). Pre- and postfeeding bacterial samples were pooled and stored at -20°C .

Rumen Fermentation Traits. Rumen contents were sampled before and at 1, 2.5, 5, and 8 h after the morning feeding on 2 consecutive days. Samples were maintained on a magnetic stirrer for pH determination using a digital pH meter (CG837, Ag/AgCl electrode, Schott Geräte, Hofheim, Germany) and then immediately strained through a 250- μm -pore polyester monofilament fabric. The rumen pH was also measured continuously using immersed probes (Sentinel Rumen Monitoring system, Kahne Ltd., Auckland, New Zealand). For VFA, 0.8 mL of rumen filtrate was transferred to a microcentrifuge tube containing 0.5 mL of a 0.5 N HCl solution containing 2% (wt/vol) metaphosphoric acid and 0.4% (wt/vol) crotonic acid, kept at 4°C for at least 2 h, and stored at -20°C until analysis. For ammonia, 1 mL of rumen filtrate was transferred to a microcentrifuge tube containing 0.1 mL of 5% orthophosphoric acid and stored at -20°C until analysis. Viscosity was measured on the rumen filtrate 5 h after feeding using a micro Ubbelohde viscometer and a ViscoClock (Schott Geräte).

Rumen Microbial Traits. For protozoa, strained rumen fluid samples collected 2.5 h after the morning feeding were mixed with a methyl green-formalin solution in a 1:1 ratio and stored at room temperature in the dark until used for counting. Samples were diluted in methyl green-formalin solution if necessary and enumeration was done using a Neubauer counting chamber. The activities of carboxymethyl cellulase (CMCase), xylanase, and amylase from SAB were determined before and 2.5 h after the morning feeding according to the procedure described by Martin et al. (1993). Total genomic DNA was extracted from rumen samples collected before and 2.5 h after feeding using the method described by Yu and Morrison (2004) and quantified by spectrophotometry using a NanoQuant Plate on an Infinity spectrophotometer (Tecan, Männedorf, Switzerland). The cellulolytic bacteria *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*; the starch-degrading lactate-producing *Streptococcus bovis*; the *Prevotella* spp.; and total bacteria were quantified by quantitative PCR (qPCR) using primers targeting the 16S rRNA gene, as reported before (Edwards et al., 2007; Stevenson and Weimer, 2007; Lettat et al., 2012). Denaturing gradient gel electrophoresis PCR targeting the bacterial V3 variable region of the 16S rRNA gene was performed as described before (Sadet et al., 2007).

Milk Production and Composition. Cows were milked in their stalls twice daily at 0630 and 1530 h. Milk yield was recorded by an automatic infrared flowmeter (DeLaval, Elancourt, France). Aliquots (100 mL) of morning and evening milkings were taken 2 d (wk 1,

2 and 3) or 3 d (wk 4) per week for determination of milk composition.

Chemical Analysis

Milk fat, protein, and lactose concentrations were determined by infrared spectrophotometry (Combi-Foss 5000, Foss Electric, Hillerød, Denmark). Milk urea was determined by the dimethylaminobenzaldehyde colorimetric method (Potts, 1967). Milk fat, protein, lactose, and urea yields were calculated from morning and evening milk yields and composition. Diet ingredients, refusals, duodenal contents, and feces were analyzed for DM (103°C for 24 h), ash (550°C for 6 h), and N by Kjeldahl method (AOAC, 1990). Neutral detergent fiber using α -amylase and ADF (Van Soest et al., 1991) were analyzed on samples dried at 60°C for 48 h and ground through a 1-mm screen. Starch was analyzed by spectrophotometry after enzymatic analysis on fresh feeds and refusals and on lyophilized duodenal and fecal samples (Faisant et al., 1995). Ytterbium concentration was determined in infusion solutions, feces, and duodenal contents by atomic absorption spectrophotometry (model 2380 spectrophotometer, Perkin-Elmer, Bois d'Arcy, France) at a wavelength of 398.8 nm with an acetylene/N₂O flame after extraction of the marker from lyophilized samples (Hart and Polan, 1984). Purine and pyrimidine bases were measured in LAB, SAB, and duodenal contents, as described by Fanchone et al. (2013). Briefly, lyophilized samples were hydrolyzed by perchloric acid (70%) at 100°C and neutralized by addition of sodium hydroxide (0.6 N). Purine and pyrimidine bases were then quantified using HPLC (Waters, Saint Quentin en Yvelines, France. Nitrogen (Dumas method) and ash (550°C for 6 h) were measured in lyophilized LAB and SAB samples. For VFA and ammonia determination, samples from 2 consecutive days were pooled per animal for each period and sampling time. The VFA content of the rumen liquid was determined by GLC using crotonic acid as an internal standard (Morgavi et al., 2003). Ammonia content of rumen liquid and of duodenal samples was determined by colorimetry using the phenol-hypochlorite method (Weatherburn, 1967) on a spectrophotometer (Infinity M200, Tecan Austria GmbH, Grödig, Austria). α -Amylase concentration in samples of the test product Ronozyme RumiStar 600 (CT) was measured according to Jung and Vogel (2008). Samples were extracted with 0.1 M sodium phosphate buffer pH 5.0 at room temperature. The centrifuged extract was appropriately diluted with 0.1 M sodium phosphate buffer, pH 5.0, and incubated in the presence of a dyed starch (Red Starch, Megazyme International Ireland, Bray, Ireland) at 40°C for 1 h. The reaction was stopped with ethanol (95%),

which precipitates substrate molecules of high molecular weight. The precipitated high-molecular-weight starch fragments were removed by centrifugation, and absorbance in the supernatant was measured at 510 nm. The α -amylase in the assay solution was quantified by using an α -amylase standard curve and the activity was expressed as KNU per kilogram. One KNU is defined as the amount of enzyme that releases, in a 2-step reaction containing α -amylase and excess amount of α -glucosidase, 6 μ mol of *p*-nitrophenol/min from 1.86 mM 4,6-ethylidene-G7-*p*-nitrophenyl-maltoheptaoside at pH 7.0 and 37°C.

Calculations and Statistical Analysis

Duodenal DM flows were calculated as the ratio between daily amount of Yb excreted in feces and Yb content of duodenal samples. Daily amount of DM and Yb excreted in feces were corrected for the estimated amount of undigestible DM removed by sampling, assuming 0.4 and 0.5 g of undigestible DM/g of DM at the ruminal and duodenal levels, respectively. Microbial protein synthesis was calculated using a reference bacterial sample consisting either in LAB, or in a mix (MB) of LAB and SAB at a 44:56 ratio; that is, a 25:75 ratio in the rumen, with an outflow rate of 0.12 and 0.05 h⁻¹, respectively (Doreau and Ottou, 1996). Ruminal protein balance was calculated as the difference between N intake and duodenal NAN multiplied by 6.25, and expressed as grams of CP per gram of DMI. The 4% FCM was calculated as milk yield $\times$ (0.4 + 0.015 $\times$ milk fat concentration), with milk yield and milk fat concentration being expressed in kilograms per day and grams per kilogram, respectively.

Data were analyzed by ANOVA using the Mixed procedure of SAS software, version 9 (SAS Institute Inc., Cary, NC), with mean per animal and treatment as experimental unit. The model included cow as a random effect, and period, starch (high vs. low), exogenous amylase (without vs. with), time, and interactions starch $\times$ amylase, starch $\times$ time, amylase $\times$ time, and starch $\times$ amylase $\times$ time as fixed effects. For rumen kinetics, the REPEATED statement within the Mixed procedure of SAS was used. Significance was declared at $P < 0.05$. Probability values between 0.05 and 0.10 were considered as trends.

RESULTS

BW, DMI, Nitrogen Partition, and Milk Yield and Composition

The BW of animals increased during the trial from 547 to 572 kg between periods 1 and 4 ($P = 0.04$). No

Table 2. Effects of starch level and amylase addition on BW, DMI, milk yield and composition, and N partition¹

Item	High starch (30%)		Low starch (20%)		SEM	<i>P</i> -value		
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase
BW, kg	560	568	568	566	6	0.65	0.68	0.43
DMI, kg/d	18.4	18.9	19.2	19.1	0.45	0.33	0.64	0.59
Milk yield, kg/d	24.7	25.3	24.0	23.7	0.6	0.12	0.82	0.47
4% FCM yield, kg/d	24.6	24.4	25.5	26.0	1.1	0.29	0.89	0.78
Milk fat, g/kg	39.8	37.9	44.4	46.8	2.7	0.04	0.93	0.46
Milk protein, g/kg	33.3	33.6	32.4	32.8	0.3	0.03	0.27	0.83
Milk lactose, g/kg	51.9	51.3	50.9	50.4	0.4	0.05	0.23	0.83
Milk urea, g/kg	0.330	0.302	0.361	0.339	0.017	0.09	0.19	0.88
SCC, 10 ³ cells/mL	138	134	133	257	47	0.26	0.25	0.22
N intake, g/d	467	477	494	489	12	0.14	0.86	0.56
N feces, g/d	153	152	159	151	6	0.76	0.50	0.57
N urine, g/d	172	180	176	187	8	0.51	0.29	0.87
N milk, g/d	135	139	128	128	4	0.05	0.62	0.58

¹Means of 4 first-lactation cows per treatment.

effect of treatment was observed on BW, DMI, milk yield, N intake, or N excretion in urine and feces (Table 2). The high-starch diet increased milk protein and lactose contents as well as N secretion in milk but it decreased milk fat and urea concentrations compared with the low-starch diet. No effect of exogenous amylase and no starch × amylase interactions were observed for these traits.

Feed Digestion and Ruminal Microbial Synthesis

The high-starch diet had a negative effect on total-tract digestibility of NDF and ADF but increased that of starch (Table 3). In contrast, no effects of starch level were observed on total-tract apparent DM and OM digestibility; ruminal digestibility of OM, NDF, ADF, and starch; duodenal microbial and nonmicrobial N flows; or efficiency of microbial protein synthesis. Amylase supplementation increased ruminal digestibility of starch by 6.5%. Amylase supplementation also increased the true ruminal digestibility of OM by an average of 4%, irrespective of the microbial population (LAB or MB) used in calculations or the starch level of the diet. However, the lower ruminal digestibility of OM and starch in the absence of exogenous amylase addition was compensated postruminally, as no differences were observed on total-tract digestibility of OM and starch. Also, we observed no effect of amylase supplementation on DM total-tract digestibility, ruminal and total-tract digestibility of NDF and ADF, duodenal N flows, or efficiency of microbial synthesis.

Ruminal Fermentations

Mean ruminal pH and N-NH₃ did not differ among treatments, averaging 6.30 and 261 mg/L, respectively

(Table 4). Similar conclusions were obtained with continuous measurement of pH by probes (not shown). A starch × enzyme interaction ($P = 0.04$) revealed that minimal pH was slightly higher with the low-starch control diet (5.52) than with other treatments (on average 5.42); the maximal pH was slightly higher ($P = 0.05$) with high-starch than with low-starch diets (7.02 vs 6.90, respectively). Time under pH 5.8 was low and similar among treatments, averaging 9 min/d (data not shown). High-starch diets induced a shift in the proportion of VFA with lower acetate and a relative higher propionate, isovalerate, valerate, and caproate (Table 4). These differences were mainly observed between diets supplemented with amylase. Amylase supplementation tended to increase total ruminal VFA concentration in high-starch diets. No interaction between sampling time and experimental factors (starch level or amylase addition) was observed on rumen fermentation measurements, except a trend for isobutyrate (starch × amylase × time, $P = 0.05$). Amylase supplementation also marginally reduced ($P = 0.04$) the viscosity of rumen fluid from 1.23 to 1.15 cSt (centistokes, where 1 cSt = 1 mm²/s) independently of the diet (data not shown).

Rumen Microbial Populations and Enzymatic Activities

The concentration of total bacteria and that of selected bacterial populations, as estimated by qPCR of 16S rRNA gene copies, did not show any major change among treatments from samples taken before feeding (data not shown). However, some effects were observed after feeding; the high-starch diet slightly increased the concentration of total bacteria, and amylase supplementation slightly decreased the concentration of *F.*

Table 3. Effects of starch level and amylase addition on OM, NDF, ADF, starch and N digestion, and microbial synthesis¹

Item	High starch (30%)		Low starch (20%)		SEM	P-value		
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase
Apparent total-tract digestibility, %								
DM	70.1	70.3	70.5	69.62	0.7	0.86	0.62	0.48
OM	71.4	71.5	72.1	70.8	0.7	0.99	0.44	0.41
NDF	50.7	50.0	56.0	52.9	1.4	0.03	0.23	0.42
ADF	48.4	46.0	52.7	49.3	1.9	0.10	0.18	0.81
Starch	98.4	99.2	97.6	97.6	0.3	0.004	0.22	0.16
True ruminal digestibility, %								
OM, using LAB ²	57.0	60.6	56.2	61.1	1.1	0.92	0.03	0.68
OM, using MB ²	64.6	67.3	62.6	67.9	1.1	0.52	0.01	0.27
Apparent ruminal digestibility, %								
NDF	44.6	47.5	48.8	49.9	3.2	0.33	0.55	0.79
ADF	40.6	41.6	46.1	45.7	3.5	0.21	0.93	0.85
Starch	73.9	81.1	76.2	82.0	2.3	0.52	0.03	0.76
Duodenal N flow, g/d								
Total N	497	500	530	491	21	0.59	0.41	0.35
NH ₃ -N	3.01	3.23	3.30	3.08	0.21	0.76	0.98	0.33
NAN	494	497	527	488	21	0.59	0.41	0.35
Microbial N								
using LAB	251	283	266	270	13	0.91	0.21	0.31
using MB	361	378	365	375	19	0.98	0.51	0.86
Nonammonia-nonmicrobial N								
using LAB	244	214	261	218	20	0.63	0.13	0.75
using MB	133	119	162	113	19	0.58	0.16	0.41
Efficiency of microbial synthesis, g of N/kg of OM truly digested in the rumen								
using LAB	27.2	27.7	27.7	26.3	1.5	0.76	0.76	0.56
using MB	34.0	32.9	33.8	32.5	2.0	0.90	0.56	0.96

¹Means of 4 first-lactation cows per treatment.²Using liquid associated bacteria (LAB) or mixed bacteria (MB) as microbial reference sample.

succinogenes (Table 5). Amylase supplementation also affected the proportion of *Prevotella* spp. relative to total bacteria that decreased in the low-starch diet and increased in the high-starch diet. The numbers of total rumen protozoa, total small entodiniomorphs,

total large entodiniomorphs, and *Dasytricha* were not affected by the level of starch or by amylase supplementation. In contrast, the numbers of *Isotricha* were lower in high-starch diets and tended to be lower in amylase-supplemented diets. Similarly to qPCR data

Table 4. Effects of starch level, amylase addition, and sampling time¹ on rumen pH, N-NH₃ concentration, and VFA concentrations and molar proportions²

Item	High starch (30%)		Low starch (20%)		SEM	P-value			
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase	Time
Mean pH	6.32	6.26	6.30	6.31	0.04	0.64	0.54	0.31	<0.0001
N-NH ₃ , mg/L	257	280	270	235	26	0.55	0.82	0.27	0.03
Total VFA, mM	114.2	122.8	120.9	120.0	2.8	0.49	0.17	0.09	<0.0001
VFA molar proportions, mol/100 mol									
Acetate	64.7	60.6	64.9	65.3	0.6	<0.0001	0.002	<0.0001	<0.0001
Propionate	16.3	21.7	17.5	17.3	0.7	0.03	0.001	<0.0001	0.24
Isobutyrate ³	0.61	0.64	0.64	0.59	0.05	0.92	0.82	0.44	<0.0001
Butyrate	15.2	13.4	14.6	14.1	0.55	0.95	0.05	0.26	<0.0001
Isovalerate	1.37	0.77	0.85	0.69	0.08	<0.0001	<0.0001	0.005	<0.0001
Valerate	1.36	2.09	1.13	1.39	0.10	<0.0001	<0.0001	0.02	<0.0001
Caproate	0.51	0.80	0.45	0.58	0.05	0.009	<0.0001	0.15	<0.0001
Acetate:propionate	4.04	3.05	3.77	3.83	0.11	0.03	<0.0001	<0.0001	0.001

¹Before and 1, 2.5, 5, and 8 h after the morning feeding.²Means of 4 first-lactation cows × 5 sampling times per treatment.³Starch × amylase × time interaction: *P* = 0.052.

Table 5. Effects of starch level and amylase addition on selected bacteria and protozoa 2.5 h after feeding¹

Item	High starch (30%)		Low starch (20%)		SEM	P-value		
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase
Bacteria, Log ₁₀ 16S rRNA copy number/g of DM rumen content								
Total bacteria	10.8	10.7	10.7	10.6	0.041	0.04	0.17	0.87
<i>Prevotella</i> spp.	10.3	10.2	10.2	10.1	0.057	0.15	0.23	0.44
% in total bacteria	30.2	32.6	34.0	31.2	1.662	0.24	0.84	0.03
<i>Fibrobacter succinogenes</i>	9.26	9.13	9.23	9.07	0.034	0.25	0.002	0.71
% in total bacteria	2.98	2.48	3.48	2.91	0.335	0.22	0.16	0.92
<i>Ruminococcus albus</i>	7.77	7.77	7.69	7.70	0.155	0.25	0.89	0.96
% in total bacteria	0.11	0.12	0.13	0.14	0.037	0.30	0.51	0.98
<i>Ruminococcus flavefaciens</i>	8.37	8.33	8.14	8.20	0.089	0.08	0.92	0.58
% in total bacteria	0.41	0.42	0.28	0.38	0.083	0.33	0.51	0.61
<i>Streptococcus bovis</i>	6.87	6.73	6.73	6.75	0.103	0.46	0.47	0.33
% in total bacteria	0.02	0.01	0.01	0.01	0.004	0.95	0.61	0.21
Protozoa, Log ₁₀ cells/mL of rumen fluid								
Total protozoa	5.60	5.33	5.55	5.48	0.158	0.67	0.16	0.40
Small entodiniomorphs	5.58	5.30	5.50	5.40	0.182	0.92	0.16	0.48
Large entodiniomorphs	3.98	3.98	3.98	4.20	0.353	0.72	0.72	0.72
<i>Isotricha</i>	3.58	3.05	3.75	3.65	0.145	0.03	0.06	0.16
<i>Dasytricha</i>	2.53	2.42	2.45	2.45	0.221	0.91	0.80	0.80

¹Means of 4 first-lactation cows per treatment

on bacterial populations, the bacterial community as evaluated by denaturing gradient gel electrophoresis analysis revealed no clear effect of treatments (data not shown).

Starch level as well as amylase supplementation did not affect xylanase and CMCase activities of SAB (Table 6). In contrast, total amylase activity increased in the high-starch diet and was higher with diets supplemented with amylase. The effect of amylase supplementation was more marked after feeding in the high-starch diet and before feeding in the low-starch diet (starch × amylase × time interaction, $P = 0.02$).

Fecal Fermentations

The main observed effect on fecal fermentations (Table 7) was a reduction in total VFA concentration in high-starch diets supplemented with amylase, whereas an opposite effect was observed with the low-starch diet (starch × amylase interaction). The proportion of butyrate was slightly increased in high-starch diets.

DISCUSSION

This 4 × 4 Latin square trial was designed to obtain robust information on digestive traits, rather than animal production performance. Therefore, only digestive aspects, with a particular attention to the effects of amylase additive, are discussed.

The main observed effects of exogenous amylase were on ruminal digestion of starch, which increased by 6.5% on average. In contrast, Hristov et al. (2008) reported

no effect of exogenous amylase on ruminal starch digestibility in dairy cows fed a hay-based diet containing 21% starch. Although differences in responses between studies could be explained by differences in enzyme preparation or diet composition, the present work showed, for the first time to our knowledge, the ability of an exogenous amylase preparation to improve ruminal starch digestion in vivo. This finding was in line with the concomitant increase in true ruminal OM digestibility, whereas ruminal NDF digestibility was not affected. In agreement with the increased ruminal starch digestibility, the amylase activity isolated from SAB increased in amylase-supplemented diets. Given that rumen contents are washed before enzyme extraction, only microbial enzymes are expected to be isolated, with no contamination from the exogenous amylase activity, although adsorption (or attachment) of the exogenous enzyme to small particles cannot be excluded. The increase in microbial amylase activity and ruminal starch digestibility observed in this trial was not associated with changes in the bacterial community or changes in the amylolytic *Strep. bovis*. Many bacteria are able to utilize starch as an energy source and changes might have been too subtle to be picked up by the techniques used. The results on protozoa were contrasting, as exogenous amylase tended to decrease the number of small entodiniomorphs and holotrichs, which are the groups involved in degradation of starch and sugars.

Despite the improved ruminal starch digestion with amylase supplementation, total-tract starch digestion was not modified, reflecting the ability of the intes-

Table 6. Effects of starch level, amylase addition, and sampling time on xylanase, carboxymethyl cellulase (CMCase), and amylase activities of solid-associated microorganisms in the rumen¹

Item ²	High starch (30%)		Low starch (20%)		SEM	P-value			
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase	Time
Xylanase specific activity ³									
Before meal	1.96	1.13	1.24	1.96					
2.5 h after meal	1.09	1.08	2.70	1.14	0.50	0.22	0.25	0.99	0.84
Xylanase total activity									
Before meal	20.06	12.15	13.35	19.60					
2.5 h after meal	8.64	5.18	12.89	8.78	4.80	0.54	0.51	0.33	0.04
CMCase specific activity ⁴									
Before meal	1.68	1.43	2.61	1.96					
2.5 h after meal	2.05	1.60	0.74	0.98	0.69	0.81	0.58	0.88	0.25
CMCase total activity									
Before meal	19.61	13.92	13.32	18.57					
2.5 h after meal	11.03	11.34	10.12	9.72	5.00	0.77	0.97	0.48	0.12
Amylase specific activity									
Before meal	5.81	4.59	5.47	4.10					
2.5 h after meal	9.48	8.34	5.78	3.86	2.15	0.16	0.37	0.88	0.23
Amylase total activity ⁵									
Before meal	39.82	41.49	23.74	37.69					
2.5 h after meal	27.99	56.08	26.71	26.28	5.76	0.006	0.02	0.33	0.73

¹Means of 4 first-lactation cows per sampling time per treatment.²Specific activity measured in μmol of reducing sugar/mg of protein per hour; total activity measured in μmol of reducing sugar/g of DM per hour.³Starch × amylase × time interaction: $P = 0.041$.⁴Starch × time interaction: $P = 0.099$.⁵Starch × amylase × time interaction: $P = 0.022$.

time to digest starch escaping ruminal digestion, which can be up to 3 g/d per kg of BW (Huntington, 1997; Offner and Sauvant, 2004; Nozière et al., 2010). Previous studies reported a numerical increase in total-tract digestibility of starch with amylase-supplemented diets in dairy cows (Hristov et al., 2008; Klingerman et al., 2009; Weiss et al., 2011) or in beef steers (DiLorenzo et al., 2011), but it never exceeded 1.7% and was not always observed (McCarthy et al., 2013). It is worth noting that starch was highly degradable in the present work, with an average digestibility of 78 and 98% at the ruminal and total-tract levels, respectively. These

values are in accordance with the expected values estimated from INRA (2007) feed tables.

No effect of amylase supplementation was observed on ruminal and total-tract digestibility of NDF and ADF, which is in good agreement with Hristov et al. (2008) and Weiss et al. (2011). In contrast, several studies in dairy cows (Gado et al., 2009; Klingerman et al., 2009; Gencoglu et al., 2010) and beef steers (DiLorenzo et al., 2011) reported a numerical increase in NDF total digestibility. However, in all these studies, digestibility was not directly measured from total feces collection but was calculated from partial collection of feces or marker

Table 7. Effects of starch level and amylase addition on fecal pH and VFA concentration and proportion¹

Item	High starch (30%)		Low starch (20%)		SEM	P-value		
	Control	Amylase	Control	Amylase		Starch	Amylase	Starch × Amylase
pH	6.63	6.68	6.95	6.62	0.139	0.24	0.21	0.09
DM, %	12.6	13.6	13.2	12.9	0.32	0.76	0.32	0.05
Total VFA, mM	71.5	56.9	57.0	64.3	4.34	0.43	0.42	0.02
VFA molar proportions, mol/100 mol								
Acetate	77.4	77.9	78.6	78.5	0.49	0.09	0.67	0.51
Propionate	14.4	14.4	14.5	14.3	0.46	0.92	0.67	0.72
Butyrate	6.6	6.0	5.2	5.4	0.40	0.003	0.55	0.20
Isobutyrate + isovalerate	0.9	0.9	0.9	1.0	0.10	0.52	0.45	0.64
Valerate + caproate	0.7	0.7	0.7	0.8	0.03	0.03	0.03	0.23

¹Means of 4 first-lactation cows per treatment

dilution. The ruminal digestion of NDF accounted for, on average, 91% of their total digestion. This is in good agreement with published data (Nozière et al., 2010). The difference in NDF digestibility between the high-starch and low-starch diets may be explained by the high digestibility of NDF in the concentrate fed in the low-starch diet and by a lower digestibility of forage NDF in the high-starch diet. The former is likely the main explanation because other traits such as pH, fibrolytic activity of SAB, and numbers of fibrolytic microbes all indicate that the rumen fibrolytic function was not affected by the high-starch diet. Under these conditions, which were favorable for fiber degradation, amylase supplementation could not have had any additional positive effect on this trait.

The ruminal metabolism of nitrogen; that is, the duodenal flows of microbial and nonmicrobial N fractions and N-NH₃ ruminal concentrations, was not affected by treatments. Although amylase supplementation increased the true digestibility of OM in the rumen, this positive effect did not translate into a higher microbial protein synthesis in the present study. In contrast, Gado et al. (2009), with a low N diet (10.3% of CP) reported increased microbial N duodenal flow with a commercial exogenous enzyme mixture but this flow was estimated from urinary excretion of allantoin and uric acid. Hristov et al. (2008), using ¹⁵N as a microbial marker, reported no effect of exogenous amylase on microbial N duodenal flows, which is in good agreement with the present results. As already reported (Doreau and Ottou, 1996; Fanchone et al., 2013), the microbial flow to the duodenum was higher when using MB as microbial reference compared with LAB. However, the LAB method led to a more realistic quantification of dietary N degradation in the rumen (on average 59 vs. 80% of N intake with LAB and MB, respectively, assuming 2 g of endogenous N/kg of DMI) compared with the value of 62% calculated from INRA (2007) feed tables.

Total bacterial numbers measured by qPCR were also in agreement with the measures on microbial protein flow to the small intestine. The N-NH₃ concentration in the rumen was high, on average 260 mg of N-NH₃/L, whereas rumen N balance did not differ from zero. This may be due to both the high level of dietary rumen-degradable N, and the high level of N recycling via saliva favored by a high level of dietary NDF (>30% DM) mainly provided by forage. The high NDF level may also have stimulated saliva production, helping to stabilize mean rumen pH in the high-starch diet.

The main effect of amylase supplementation on VFA was observed with the high-starch diet. Amylase supplementation tended to increase total concentration and induced a shift in the molar proportions of propionate

and valerate that increased at the expense of acetate and butyrate. It may be expected that the glucose released from starch hydrolysis by the exogenous amylase was predominantly fermented via the odd-chain VFA pathways. This may contribute to regulate the production of ATP required for microbial growth, because these odd-VFA pathways produce less ATP per mole of fermented carbon (Sauvant and Van Milgen, 1995). Nevertheless, changes were rather moderate, which is in line with DeFraen et al. (2005) and Hristov et al. (2008), who reported no change in VFA profiles following amylase supplementation. In the present work, the pattern of major VFA was only slightly modified by starch level. This is in line with the fact that the digested NDF:digested OM ratio was only slightly modified between high-starch and low-starch diets (from 0.23 to 0.28, respectively). This ratio has been shown to be the main explanatory variable of VFA molar proportions in the rumen (Nozière et al., 2010), with an expected minor change in molar proportions of acetate, propionate, and butyrate (-1.3 ± 1.5 , $+0.9 \pm 1.5$, and $+0.34 \pm 0.9$ mol/mol, respectively) when this ratio increased from 0.23 to 0.28.

CONCLUSIONS

The exogenous amylase used in this study improved starch digestion in the rumen of first-lactation cows, whereas the previously reported effect on NDF digestion was not confirmed. The first-lactation cows used in this trial had moderate intakes and production levels and, based on the numerous indicators of rumen function measured, ruminal fermentation was well balanced, as reflected by the good feed digestibility. Further research on high-producing animals is necessary to assess the usefulness of this exogenous amylase to improve starch digestion when ruminal conditions are less favorable.

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