Postruminal digestion of starch infused into the abomasum of heifers with or without exogenous amylase administration

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ABSTRACT: The effect of an exogenous amylase on postruminal digestion of starch infused into the abomasum of cattle was studied. Four rumen-cannulated heifers were fed 5.5 kg DM/d of a diet without starch, and assigned randomly to a crossover design. The experiment consisted of 2 periods lasting 23 d each with 10 d for adaptation to the diet followed by 13 d of abomasal infusion and sample collection. During the first 3 d of each infusion phase, isotonic saline solution was infused (1 liter/h) for measurement of baseline values in feces, followed by daily infusions of 880 g DM corn starch (1 kg/10 liters of water) without or with the addition of 2% of amylase. Titanium dioxide (10 g/d) was ruminally administered for estimation of fecal excretion. Digestion of starch in small intestine was calculated as the difference between the amounts of infused starch, disappeared from hindgut and fecal excretion. The apparent disappearance of starch from the hindgut was estimated based on the increment of microbial nitrogen (N) excretion due to starch infusion (1 g microbial N/100 g fermented starch) compared to baseline values. The concentration of purine bases in feces was used to estimate excretion of microbial N. Microbial N excretion

increased with starch infusion ($P < 0.05$) but was not influenced by amylase ($P = 0.81$). Starch disappearance from the small intestine was not improved by amylase ($P = 0.78$) and averaged 85%. Amylase affected neither blood concentration of glucose ($P = 0.80$) nor of insulin ($P = 0.26$), but glucagon was lower without ($P < 0.0001$) than with amylase. The infusion of starch increased fecal excretion of total VFA (acetate, propionate, and butyrate) by 53% ($P < 0.05$), which indicates increased carbohydrate fermentation in the hindgut and incomplete digestion of starch in the small intestine. However, the excretion of total VFA was not affected by amylase ($P = 0.66$). Lactate excretion was higher at the second day of starch infusion ($P < 0.05$) without than with amylase, which suggests lower flow of starch from the small intestine to the hindgut due to a possible effect of amylase addition in animals not adapted to starch digestion. However, lactate excretion returned near to baseline values within 2 d, which was probably due to increase of lactate-utilizing bacteria and the adaptation of the microbial population in the hindgut. Further studies with higher starch levels and addition of amylase are recommended.

Key words: amylase, cattle, hindgut fermentation, lactate, starch, volatile fatty acid

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INTRODUCTION

Starch usually provides an important proportion of energy in diets for lactating cows and high-producing beef cattle. However, on average

only 11% to 90% of the postruminal starch flow disappear in the small intestine (Owens et al., 1986; Giuberti et al., 2014; Moharrery et al., 2014). Studies with diets containing high amount of grain (Karr et al., 1966) or with abomasal infusion of corn starch (Little et al., 1968; Kreikemeier et al., 1991; Matthé, 2001; Richards et al., 2002) have shown that digestion of starch in the small intestine is limited. Some aspects influencing the digestibility of starch in the small intestine are intrinsic feed factors like the structural formation (Giuberti et al., 2014) and the grain type. Feed processing methods (Owens et al., 1986), particle size, and interactions with other nonstarch components affect the extent of starch digestion as well (Giuberti et al., 2014). Also reduced capacity of glucose absorption, the time for starch hydrolysis, the access of enzymes to starch granules or time and surface exposure (Owens et al., 1986), and limited supply and activity of pancreatic amylase (Huntington, 1997) have been stated as possible factors limiting starch digestion. Thus, supply of exogenous amylase may be an option to improve starch digestibility in the small intestine. In line with this, the effect of amylase addition on starch digestion in diets of dairy cows has been evaluated (Gencoglu et al., 2010; Weiss et al., 2011; McCarthy et al., 2013; Nozière et al., 2014; Vargas-Rodriguez et al., 2014). However, amylase was added to the concentrate making it difficult to differentiate the site and extent of the effect of amylase. Therefore, the present study aimed to evaluate the effect of an exogenous amylase on postruminal digestion of starch infused into the abomasum of cattle not adapted to starch digestion. We hypothesized that small intestinal digestion of starch in unadapted cattle is limited by amylase activity, which might be improved by exogenous administration of amylase, and that this effect might be especially pronounced during adaptation to the starch digestion.

MATERIALS AND METHODS

The animal study reported herein was performed in accordance with the animal German Animal Welfare Act (Federal Republic of Germany, 2014) and approved by the Animal Welfare Commission of the Ministry of Energy, Agriculture, Environment and Rural Areas of the federal state of Schleswig-Holstein (V 244-7224.121-25).

Animals and Diets

Four adult heifers (2 German Black Pied and 2 Jersey × German Black Pied), with an average

BW of 565 ± 29 kg at the beginning of the experiment and fitted with rumen cannulas (#2C, 10 cm i.d.; Bar Diamond, Inc., Parma, ID) were used. The heifers were fed 5.5 kg/d of a diet targeted to contain no starch and consisted of 64.9% grass hay (primary growth of *Lolium perenne*-dominated sward), 33% dried beet pulp (J. August Plambeck GmbH, Brügge, Germany), 0.9% feed urea (Piarumin; Stickstoffwerke Piesteritz GmbH, Wittenberg, Germany) and 1.2% of a mineral and vitamin premix (Panto-Mineral; Hamburger Leistungsfutter GmbH, Hamburg, Germany) on DM basis in 2 equal meals at 0700 and 1500 h. The ingredients and chemical composition of the diet is shown in Table 1. The diet was formulated to meet requirements for maintenance according to the GfE (2001). Animals had free access to drinking water. The heifers were housed in a tie stall with individual cubicles and bedded with rubber mats.

Experimental Design and Procedure

The experiment consisted of 2 periods lasting 23 d each with a 10-d phase for adaptation to the diet containing no starch followed by a 13-d phase of abomasal infusion and sample collection. Between periods, a 7-wk period without treatment application was set to avoid carryover effects of adaptation to starch digestion. The animals were assigned randomly to a crossover design. Firstly, from day 1 to 3 of each infusion period isotonic saline solution (0.9% NaCl wt/vol; B. Braun Melsungen AG, Melsungen, Germany) was infused into the abomasum to obtain baseline values of fecal and blood

Table 1. Ingredients and chemical composition of the diet

Item	% of dietary DM
Ingredient	
Grass hay	64.9
Dried beet pulp	33.0
Feed urea	0.90
Mineral and vitamin mix ^a	1.20
Chemical composition ^b	
CA	6.60
CP	9.28
Ether extract	0.45
NDF	55.7
ADF	32.6
ADL	4.50

^aContained (per kilogram) 210 g calcium, 100 g sodium, 40 g magnesium, 30 g phosphorus, 1,000 mg copper, 8,000 mg zinc, 5,000 mg manganese, 60 mg iodine, 50 mg selenium, 40 mg cobalt, 5,000 mg vitamin E, 1,000,000 IU vitamin A, and 100,000 IU vitamin D3.

^bCalculated from chemical composition of individual ingredients.

variables. Treatments were daily abomasal infusion of native corn starch containing 95.7% starch, 0.4% of CP, and 0.2% of crude ash (CA) on DM basis (Maisita 21.000; Agrana AG, Vienna, Austria) with or without an exogenous amylase. The starch product was mixed at a rate of 1 kg starch per 10 liters of water and maintained in suspension by continuous stirring. Abomasal infusion began half an hour after morning feeding and lasted for 10 h/d from day 4 to 13 of each infusion period. The average effective total dosage of starch was 880 g/d. A peristaltic pump (rotarus Flow 50; Hirschmann Laborgeräte GmbH & Co KG, Eberstadt, Germany) was used for infusion. The α -amylase (EC.3.2.1.1; 13,000 U/mL, Distizym BA-N, 1,4- α -D-Glucan-Glucanohydrolase; Erbslöh Geisenheim AG, Geisenheim, Germany) solution (2% vol/vol in distilled water) was infused into the tube used for starch infusion at a rate of 50 mL/h using a syringe pump (540060-HP Single Syringe; TSE Systems GmbH, Bad Homburg, Germany; syringe used: Omnifyx Solo; B. Braun Melsungen AG, Melsungen, Germany). According to manufacturer, the amylase was produced from a selected strain of *Bacillus subtilis*, with an optimum activity at pH 5.8 to 6.0 and at 70 to 80 °C. The amylase activity of Distizym BA-N was determined using the bicinchoninic assay where Distizym BA-N is added to a 1% soluble corn starch solution (wt/vol) at 37 °C for 10 min at pH 7.5. The reducing sugars were determined at a wavelength of 560 nm and expressed as units (U) using a maltose standard, where 1 U of α -amylase activity is defined as 1 μ mol maltose formed per minute. According to the manufacturer, 1,560 U of Distizym BA-N/kg of grain starch is the recommended standard dosage in alcohol production for the liquefaction of starch-containing mashes. In our experiments we used a clearly higher dosage (147,727 U/kg starch) to compensate for any possible impairment of enzyme activity by low pH in the abomasum. The abomasal infusion was performed using a tool to insert lines into the abomasum described by Westreicher-Kristen and Susenbeth (2017). Succinctly, an insertion device with the infusion line was passed through the rumen cannula, then through the *sulcus omasi*, and finally through the omaso-abomasal orifice into the abomasum. The insertion device and the infusion lines remained in the abomasums for the entire phase of abomasal infusion of 13 d each and 24 h a day. Their position in the abomasums was controlled twice daily according to Westreicher-Kristen and Susenbeth (2017) to ensure that the suspension containing the starch and the amylase solution was always

completely infused into the abomasum. To infuse the solutions into abomasum, a thin Tygon tubing (2.06 mm i.d.; Hirschmann, Eberstadt, Germany) was passed through the whole infusion line from the peristaltic pump into the abomasum with the insertion device. Total fecal excretion was determined using titanium dioxide (TiO₂; Kronos 1171; Harold Scholz & Co. GmbH, Recklinghausen, Germany). For this, 5 g TiO₂ were weighed into gelatine capsules (HKG size 30 × 90 mm, 59 mL, limpid-transparent, type 36; Capsula GmbH, Ratingen, Germany) and ruminally administered to each heifer twice daily immediately after each feeding. The TiO₂ administration was initiated 5 d before the phase of abomasal infusion to reach the equilibrium of TiO₂ excretion after initial administration (Glindemann et al., 2009). For blood sampling, heifers were surgically fitted with an indwelling long-term catheter (Long Term Catheter Guidewire Style A1410, 14 ga × 20 cm; MILA International Inc., Florence, KY) in 1 jugular vein at the second day of each phase of abomasal infusion. For this, heifers were sedated with 0.7 mL Rompun 2% (Xylazine; Bayer AG, Leverkusen, Germany) and surgical intervention was carried out under aseptic conditions and local anesthesia with 4 mL of Isocain 2% (Procainhydrochlorid; Selectavet Dr. Otto Fischer GmbH, Weyarn-Holzolling, Germany). The catheters were held in place by suturing them onto the skin. To protect the catheters from blockage with coagulated blood, they were permanently infused with isotonic saline solution (0.9% NaCl wt/vol; B. Braun Melsungen AG, Melsungen, Germany) at a rate of approximately 1 mL/min during the whole phases of blood sampling.

Sampling and Sample Preparation

During each sampling phase, 5 samples (~200 g fresh matter each) of the grass hay and dried beet pulp were collected and pooled by feed, divided into 2 subsamples and ground to pass a 2-mm and a 1-mm sieve (ZM1; Retsch GmbH, Haan, Germany) for chemical analysis. Fecal grab samples (~500 g fresh matter) were collected during the 13-d sampling phase from the rectum (exception day 4 because of transition time between the phases of NaCl and starch infusion) 4 times a day at 0900, 1200, 1500, and 1800 h.

The applied sampling pattern was chosen to compensate for possible diurnal variation in the concentration and excretions of TiO₂ via feces (Glindemann et al., 2009). After each collection time, fecal samples were cooled down for ~15 min

at -20°C to quickly minimize microbial activity. Afterwards, samples were placed in a cooling room at 4°C and composited per animal at the end of each sampling day. From this, 6 subsamples ($4 \times 100\text{ g}$ and $2 \times 250\text{ g}$) were obtained and stored at -20°C for analyses. Blood samples were collected at day 3 (baseline), 4, 6, and 8 of each sampling phase. The first daily blood sample was taken directly after beginning of the infusion at 0730 h (time point zero), followed by blood sampling after 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, and 10 h. For each sampling, the first 2 mL of blood were discarded. Afterwards, blood samples ($\sim 9\text{ mL}$) were drawn into collection tubes containing potassium EDTA as anticoagulant to obtain plasma (Monovette K3 EDTA, $92 \times 16\text{ mm}$; Sarstedt AG & Co., Nümbrecht, Germany) and placed on ice immediately for $\sim 10\text{ min}$. Additionally, blood samples ($2 \times \sim 9\text{ mL}$) were drawn into collection tubes containing clotting activators to obtain serum (Serum Monovette, $92 \times 16\text{ mm}$; Sarstedt AG & Co., Nümbrecht, Germany) and maintained at room temperature for 60 min. Plasma and serum samples were obtained by centrifugation ($1,041 \times g$, at 20°C for 10 min) and samples were stored at -20°C until analysis.

Analytical Procedures

Diets. Feed samples (1-mm particle size) were analyzed in duplicate for concentrations of moisture, CA, CP, and ether extract according to the official analytical methods in Germany (VDLUFA, 2007; methods 3.1, 8.1, 4.1.1, and 5.1.1, respectively). Feed samples (2-mm particle size) were analyzed in triplicate for NDF, ADF, both expressed on ash-free basis, and ADL as described by Van Soest et al. (1991) using an ANKOM 200 Fiber Analyzer (ANKOM Technology, Macedon, NY). Heat-stable α -amylase was added during NDF extraction. Native corn starch used for abomasal infusion was analyzed for starch concentration based on polarimetric procedure (method 7.2.1; VDLUFA, 2007). **Feces.** At the end of the experiment, feces samples were defrosted at room temperature overnight and homogenized for the following described analyses. Samples were analyzed in duplicate for moisture, CA, and N according to VDLUFA (2007), and for concentration of TiO_2 as described by Brandt and Allam (1987). In brief, Kjeldahl digestion solution obtained by heating fecal sample for 6 h in 96% (vol/vol) sulfuric acid was filtered (MN 640 w, black ribbon No. 41, 150 mm i.d.; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and

added with 35% (vol/vol) hydrogen peroxide. The TiO_2 concentration was measured based on light absorbency in a spectrophotometer (Jenway 6300; Bibby Scientific Ltd, Stone, UK) at a wavelength of 405 nm. Concentration of starch was determined in lyophilized samples (0.2-mm particle size) based on glucose liberated after incubation with thermostable α -amylase (Thermamyl; Novozymes, Bagsvaerd, Denmark) at 95°C for 45 min and amyloglycosidase (Roche Diagnostics GmbH, Mannheim, Germany) at 60°C for 30 min as described by Brandt et al. (1987). Glucose concentration was measured based on light absorbency after incubation with glucose oxidase (GOD FS; DiaSys Diagnostic Systems GmbH, Holzheim, Germany) at 37°C for 10 min in a spectrophotometer (Jenway 6300; Bibby Scientific Ltd, Stone, UK) at a wavelength of 500 nm. Concentration of VFA (acetate, propionate, and butyrate) was analyzed in a gas chromatograph (ATI Unicam 610; Unicam Chromatography GmbH & Co. KG, Kassel, Germany) equipped with a flame ionization detector. Separation of VFA was performed at 103°C for 25 min along a 2 mm column (GP 10%, SP-1000, 1% phosphoric acid, 100/120 Chromosorb WAW; Supelco, Inc., Bellefonte, PA). The concentration of lactate was determined using a Lactate-UV Test Kit (D-Lactic acid/L-Lactic acid-UV Test; Boehringer Ingelheim Pharma GmbH & Co. KG, Mannheim, Germany). The concentration of purine bases (**PB**) was determined in lyophilized samples in duplicate by HPLC (Merck-Hitachi HPLC; Merck KGaA, Darmstadt, Germany) using a reversed-phase C18 Hypersil Gold column ($250 \times 4\text{ mm}$; Thermo Fisher Scientific GmbH, Dreieich, Germany) after sample hydrolysis in perchloric acid as described in detail by Dickhoefer et al. (2016).

Blood. Serum was analyzed for glucose concentration using the glucose hexokinase method (Glucose Hexokinase Fluid 5 + 1; mti-diagnostics GmbH, Idstein, Germany) and for insulin concentration by immunoradiometric assay (Insulin IRMA KIT; Immunotech, Inc., Beckman Coulter, CA). Glucagon was analyzed in plasma by radioimmunoassay (Glucagon RIA, MI13021; IBL International GmbH, Hamburg, Germany).

Calculations and Statistical Analysis

Fecal DM excretion was calculated as TiO_2 administration (g/d) divided by the TiO_2 concentration (g/kg DM) in the feces, assuming a fecal recovery of TiO_2 of 100% (Glindemann et al., 2009). Fecal excretion of OM, total N, microbial N,

starch, VFA, and lactate was calculated based on their concentrations in feces (g/kg DM) and fecal DM excretion. The OM concentration in feces was calculated as $1000 - \text{CA}$ (g/kg DM). The apparent disappearance of starch from the small intestine was calculated as the difference between amounts of infused starch and disappeared from the hindgut and excreted with the feces. The apparent disappearance of starch by fermentation from the hindgut was estimated based on the increment of microbial N excretion due to starch infusion compared to baseline values. The concentration of microbial N in feces was calculated based on PB excretion using a PB-N:microbial N ratio of 0.116 according to [Chen \(1989\)](#). The baseline values of microbial N excretion were obtained from the 3-d phase of NaCl infusion. In line with this, it was assumed that 1 g microbial N originates from 100 g of fermented starch according to [Ørskov et al. \(1970\)](#). They measured the microbial N synthesis when starch was infused through cannula into the caecum of sheep. The apparent disappearance of starch from total intestine tract was calculated as the difference between infusion and excretion amount of starch. For baseline, apparent total tract digestibility of DM (ATTD_{DM}) and OM (ATTD_{OM}) was calculated as the quotient of the total amount that apparently disappeared (intake – fecal excretion) and the amount of feed intake. For the starch infusion phase, the amount of starch infused was additionally considered as intake.

All data were statistically analyzed by the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). Differences between baseline and starch infusion in fecal excretion of DM, OM, total and microbial N, VFA, and lactate were calculated prior to statistical analysis by subtracting the baseline values (average of 3-d NaCl infusion) from values with starch infusion. The LSM and SEM of the differences between baseline and starch infusion were calculated and compared using the LSMEANS procedure with repeated measurements. Period, d, treatment, and $d \times$ treatment interactions were included as fixed effects and animal as a random factor. An autoregressive covariance structure for fecal variables was chosen because of equally spaced sampling times. Fecal excretion of total and microbial N, lactate, and propionate on a day-to-day basis were analyzed using the same model. Differences between baseline and starch infusion in blood variables were calculated prior to statistical analysis by subtracting the baseline values (day 3 with NaCl infusion) from values with starch infusion for each time point. The LSM and SEM of hourly concentrations of blood

variables and of the differences between baseline and starch infusion were calculated and compared using the LSMEANS procedure with repeated measurements. Period, d, treatment, time, and $d \times$ treatment and time $\times$ treatment interactions were included as fixed effects and animal as a random factor. An unstructured covariance structure for blood was chosen because of unequally spaced sampling times. Treatment effects were considered significantly different at $P < 0.05$ and tendencies with P -values between 0.05 and 0.10.

RESULTS

Fecal Excretion and ATTD

The effects of starch infusion with or without amylase on fecal excretion of DM, OM, N, lactate, and VFA as well as ATTD are presented in [Table 2](#). Baseline values of total and microbial N excretion averaged 34.6 and 8.8 g/d, respectively, and were increased with starch infusion ($P < 0.05$). The increments of total and microbial N with starch infusion compared to baseline were not influenced by amylase administration ($P = 0.46$ and 0.81 , respectively) and averaged 7.1 and 1.1 g/d, respectively. Changes of total and microbial N excretion on a day-to-day basis are shown in [Fig. 1A](#) and [B](#), respectively, and were not affected by amylase administration ($P = 0.53$ and 0.49 , respectively). Total VFA, acetate, and butyrate excretions increased with starch infusion ($P < 0.05$, for all; [Table 2](#)), whereas propionate excretion was unaffected ($P = 0.65$). Molar proportion of butyrate increased and of acetate and propionate decreased through starch infusion ($P < 0.05$), but they were unaffected by amylase treatment ($P = 0.73$, 0.86 , 0.74 , respectively). On a day-to-day basis, propionate excretion was higher at the second day of starch infusion with than without amylase ($P < 0.05$; [Fig. 2A](#)). Afterwards, propionate excretion decreased to similar values of propionate excretion as without amylase. Baseline values of lactate excretion averaged 0.02 g/d and increased numerically when starch was infused to 7.9 g/d (average) but without any influence without ($P = 0.13$) or with amylase ($P = 0.30$). Lactate excretion was not affected by amylase treatment ($P = 0.72$; [Table 2](#)). On a day-to-day basis, lactate excretion was higher at the second day of starch infusion without than with amylase (72.8 vs. 43.3 g/d, respectively; $P < 0.05$) ([Fig. 2B](#)). Furthermore, lactate excretion was drastically reduced without differences between treatments to 11.4 and 0.62 g/d in the third and fourth day of starch infusion, respectively. Lactate

Table 2. Fecal excretion of DM, OM, N, lactate, and VFA in heifers infused abomasally with NaCl (basal) or 880 g/d corn starch with or without amylase administration

	Starch ^b		Starch + amylase ^b		Effect of amylase	
	Basal ^a	Δ	Basal ^a	Δ	SEM ^c	<i>P</i>
DM, kg/d	1.56	+0.16*	1.54	+0.20*	0.02	0.24
OM, kg/d	1.33	+0.15*	1.33	+0.18*	0.02	0.26
Total N, g/d	34.6	+7.38*	34.5	+6.80*	0.97	0.46
Microbial N ^d , g/d	8.81	+1.08*	8.91	+1.17*	0.43	0.81
Lactate, g/d	0.02	+9.39	0.02	+6.35	6.02	0.72
Total VFA ^e , g/d	17.9	+11.1*	20.0	+9.11*	3.22	0.66
Acetate, g/d	13.5	+6.69*	15.0	+6.37*	2.43	0.93
Propionate, g/d	2.81	-0.25	3.09	-0.63	0.53	0.60
Butyrate, g/d	1.56	+4.98*	1.82	+3.73*	1.28	0.37
VFA, % of total VFA						
Acetate	74.9	-4.42*	75.3	-4.20*	1.98	0.86
Propionate	16.1	-7.38*	15.6	-7.13*	0.96	0.74
Butyrate	8.99	+11.8*	9.08	+11.3*	2.27	0.73
ATTD _{DM} ^f , %	71.4	+1.36*	71.8	+0.79*	0.33	0.24
ATTD _{OM} ^f , %	73.8	+1.27*	73.8	+0.82*	0.43	0.25

^aData presented for baseline represent LSM of 3 d with NaCl infusion within each treatment ($n = 4$).

^bValues represent LSM of the difference between baseline and starch infusion without or with amylase administration ($n = 4$).

^cSEM of the LSM of Δ (difference between baseline and starch infusion).

^dCalculated as 0.116 g purine bases N/g microbial N according to [Chen \(1989\)](#).

^eSum of acetate, propionate, and butyrate.

*Significant difference between baseline and starch infusion within each treatment ($P < 0.05$).

excretion further decreased numerically from 0.34 to 0.09 g/d from day 5 to 10 of starch infusion, however did not numerically return to the baseline values (0.02 g/d). The baseline values of ATTD_{DM} and ATTD_{OM} averaged 71.6% and 73.8%, respectively. These values increased with starch infusion ($P < 0.05$), but the increments were not affected by amylase supplementation ($P = 0.24$ and 0.25 , respectively; [Table 2](#)).

Apparent Disappearance of Starch from Intestinal Tract

Excretion and apparent disappearance of infused starch with or without amylase are shown in [Table 3](#). Fecal excretion of starch was very small and averaged 4.4 and 5.6 g/d with and without amylase, respectively; though the difference was not significant ($P = 0.64$). Consequently, the apparent disappearance of starch from the total intestinal tract averaged 99%. The starch disappearance from the small intestine averaged 85% and was not improved by the addition of amylase ($P = 0.78$). From the starch reaching the hindgut, 96% apparently disappeared.

Blood Glucose, Insulin, and Glucagon Concentration

Blood glucose, insulin, and glucagon concentrations in heifers infused abomasally with starch

without and with amylase treatment are shown in [Table 4](#). Compared to baseline, glucose concentration was unaffected by starch infusion ($P = 0.27$), whereas insulin concentration increased ($P < 0.05$). Additional administration of amylase had no effect on glucose ($P = 0.80$) and insulin ($P = 0.26$) concentrations. Glucagon concentration did not differ from baseline values when starch plus amylase were infused ($P = 0.45$), but decreased with starch infusion without amylase administration ($P < 0.05$). Consequently, amylase administration affected blood glucagon concentration ($P < 0.0001$). Diurnal variations of blood glucose, insulin, and glucagon concentrations during abomasal starch infusion are illustrated in [Fig. 3](#). During the first phase of starch infusion (time points 0 to 1.5 h) concentrations of glucose and insulin increased. Thereafter (time points 1.5 to 6 h) they remained relatively constant (plateau phase). Concentrations of glucagon remained higher (time points 1.5 to 6 h) when starch was infused with than without amylase ($P < 0.05$; [Fig. 3C](#)).

DISCUSSION

During the present trial, neither feed intake nor heifers' behavior was influenced by the presence of the infusion device in the abomasum. For the present study, we intended to infuse starch at a level

Figure 1. Daily fecal excretion of total N (A) and microbial N (B) of heifers abomasally infused with 0.9% NaCl (1 to 3 d) or 880 g/d corn starch (4 to 13 d) without (open circle) or with (closed circle) amylase administration. Values are LSM $\pm$ SEM ($n = 4$). Graph A: day $P < 0.0001$, treatment $P = 0.53$, day $\times$ treatment interactions $P = 0.79$. Graph B: day $P = 0.01$, treatment $P = 0.49$, day $\times$ treatment interactions $P = 0.01$.

that would not develop severe diarrhea based on results reported by Kreikemeier et al. (1991). In our study, feces had a soft consistence only during the first 2 d after beginning of starch infusion. During the afternoon of the first day of starch infusion (~8 h after initiation of infusion), animals started to excrete feces with a softer consistence and lactic acid smell. Excreted feces during the second and third day of starch infusion were even softer and with a more intensive acidic smell. However, these effects were reduced during the following days. In general, cases of diarrhea were not observed and animals remained healthy during the infusion periods. It has to be noted that the results of the present study are valid only for the amount and rate of infusion of 880 g starch/d. However, similar infusion rates and even higher amounts of starch into the abomasum are reported in the literature, which indirectly supports the present approach (Oldick et al., 1997; Knowlton et al., 1998; Reynolds et al., 2001; Gressley and Armentano, 2007).

Figure 2. Daily fecal excretion of propionate (A) and lactate (B) of heifers abomasally infused with 0.9% NaCl (1 to 3 d) or 880 g/d corn starch (4 to 13 d) without (open circle) or with (closed circle) amylase administration. Values are LSM $\pm$ SEM ($n = 4$). *Indicate significant differences between treatments ($P < 0.05$); otherwise $P \geq 0.05$. Graph A: day $P = 0.27$, treatment $P = 0.87$, day $\times$ treatment interactions $P = 0.31$. Graph B: day $P < 0.0001$, treatment $P = 0.64$, day $\times$ treatment interactions $P = 0.93$.

Excretion of Total and Microbial N

It is well known that fecal N excretion is associated with DM intake (6 g/kg; Van Soest, 1994). Using this figure, total N would equal 32.7 g/d for baseline, which is quite similar to our measured value (34.5 g/d of total N). As expected, N excretion was increased with starch infusion when compared to baseline values. Simultaneously, excretion of microbial N was increased, which indicates an enhanced fermentation of carbohydrates and in turn a higher microbial mass in the hindgut. Fecal microbial N was estimated from PB excretion using a PB-N:microbial N ratio of 0.116. This ratio was determined by Chen (1989) in mixed (liquid- and solid-associated) ruminal microbes. This could be a matter of concern because this ratio could be different in ruminal and fecal microbes. Moreover, previous studies demonstrated that the PB-N:N ratio of

Table 3. Fecal excretion and apparent disappearance of starch from different segments of the intestinal tract of heifers infused abomasally with 880 g/d corn starch without or with amylase treatment

	Treatment ^a		SEM	P
	Starch	Starch + amylase		
Fecal excretion, g/d	5.63	4.38	1.86	0.64
Apparent disappearance, g/d				
Small intestine	752	742	35.6	0.78
Hindgut ^b	122	133	34.7	0.75
Total tract	874	875	1.86	0.65
Apparent disappearance, %				
Small intestine ^c	85.5	84.4	4.05	0.78
Hindgut ^c	97.2	95.4	1.38	0.33
Total tract	99.4	99.5	0.21	0.64

^aData presented are LSM of phase of starch infusion without or with amylase treatment ($n = 4$). Previous to starch and starch + amylase treatments, a 3-d phase of NaCl infusion was applied for baseline measurements.

^bCalculated as 1 g microbial N/100 g starch fermented (Ørskov et al., 1970). Microbial N calculated based on purine bases excretion using a PB-N: microbial N ratio of 0.116 according to Chen (1989).

^cBased on the amount reaching each respective segment of the intestine.

ruminal microbes markedly vary depending on the microbial composition with values ranging from 0.03 in liquid-associated to 0.14 in solid-associated microbes (Dickhoefer et al., 2016). However, as far as we know, values of PB-N:N ratio for fecal microbial matter of ruminants are missing. It must be considered that the factor used may have led to an over- or underestimation of microbial N in baseline values, but the latter probably affected all estimations at the same degree, therefore not affecting differences between treatments. In the present study around 16% of the additional N excretion due to starch infusion was of microbial origin. The CP content of the infused starch was very low (~0.4% in DM) and its contribution to CP appearing in the hindgut can be neglected. Consequently, 84%

of the increased N excretion by starch infusion can be considered as endogenous N. This represents around 6.8 g endogenous N/kg infused corn starch, which is far higher than values for fecal endogenous losses of 2.2 and 1.9 g N/kg DM intake given by GfE (2001) and NRC (2001). The main reason for this difference is that the latter values are related to intake and the greatest proportion of the feed does not reach the abomasum (e.g., fermentation and absorption in the rumen). In line with this and assuming a disappearance of feed DM by two-third in the rumen, both values are rather well in agreement.

Excretion of VFA and Lactate

The VFA, which are not absorbed and metabolized by colonic epithelial cells or transferred into the blood, are excreted via feces. Therefore, the increased fecal excretion of total VFA when starch was infused into the abomasum indicates enhanced carbohydrate fermentation in the hindgut. In this study, total VFA excretion increased by 53% with starch infusion. Similarly, Siciliano-Jones and Murphy (1989) reported an increase of 80% of total VFA concentrations in the cecum of steers when diets were changed from high forage to high grain proportions. Moreover, changes of the molar proportions of VFA in feces may provide information about which carbohydrate source was primarily fermented. The baseline values of the molar proportion of acetate, propionate, and butyrate averaged 75%, 16%, and 9%, respectively. With starch infusions, they were changed to 71%, 9%, and 20%, respectively. Similarly, Ørskov et al. (1970) found reduced proportion of acetate in both colon and cecum when starch was infused into the terminal ileum of sheep, and a 3-fold incremented proportion of butyrate in the colon, cecum, and feces. Consequently, the increased proportion of

Table 4. Concentration of glucose and insulin in serum, and glucagon in plasma of heifers infused abomasally with 880 g/d corn starch without or with amylase treatment

	Starch ^b		Starch + amylase ^b		SEM ^c	Effect of amylase P
	Basal ^a	Δ	Basal ^a	Δ		
Glucose, mmol/L	4.02	-0.24	4.19	-0.28	0.22	0.80
Insulin, ng/mL	1.16	+0.46*	1.22	+0.60*	0.14	0.26
Glucagon, ng/mL	152	-19.0*	143	+6.94	9.10	<0.0001

^aData presented for baseline represent LSM obtained at day 3 of NaCl infusion from 1.5 to 6 h after beginning of infusion within each treatment ($n = 4$).

^bValues represent LSM of the difference between baseline and starch infusion with or without amylase treatment from 1.5 to 6 h after beginning of infusion ($n = 4$).

^cSEM of the LSM of Δ (difference between baseline and starch infusion).

*Significant difference between baseline and starch infusion within each treatment ($P < 0.05$).

Figure 3. Hourly concentrations of glucose (A) and insulin (B) in serum, and glucagon in plasma (C) of heifers ($n = 4$) infused abomasally with 880 g/d corn starch without (open circle) or with (closed circle) amylase treatment. Hourly concentrations represents LSM $\pm$ SEM based on blood samples collected at day 4, 6, and 8 from starch infusion phase (without baseline phase with 0.9% NaCl infusion, day 1 to 3). *Indicate significant differences between treatments ($P < 0.05$); otherwise $P \geq 0.05$. Graph A: day $P < 0.0001$, time $P < 0.0001$, treatment $P = 0.05$, time $\times$ treatment interactions $P = 0.98$, day $\times$ treatment interactions $P = 0.50$. Graph B: day $P < 0.0001$, time $P < 0.001$, treatment $P = 0.55$, time $\times$ treatment interactions $P = 0.03$, day $\times$ treatment interactions $P = 0.80$. Graph C: day $P = 0.57$, time $P < 0.001$, treatment $P < 0.0001$, time $\times$ treatment interactions $P = 0.09$, day $\times$ treatment interactions $P = 0.09$.

butyrate in feces indicates starch fermentation in the hindgut. The latter is supported by the observation in our study that lactate excretion increased drastically at the second day of starch infusion. According to Zhang and Cheryan (1991), starch is an ideal substrate for production of lactic acid by fermentation. Furthermore, the gradual reduction

of lactate excretion suggests an adaptation of the microbiota in the hindgut. Earlier studies have shown that the main fermentation product of lactate-utilizing bacteria, isolated from human feces, is butyrate (Duncan et al., 2004). Accordingly, the reduction of lactate excretion in the present study was accompanied by an increase of butyrate excretion (data not shown). Moreover, it is important to remark that the increase of lactate excretion at the second day of starch infusion was lower with than without amylase, probably due to lower availability of substrate for starch-fermenting bacteria, which support the hypothesis that amylase improved the digestion of starch in the small intestine in the nonadapted stage. However, the fact that lactate excretion during the whole phase of starch infusion never reached numerically the baseline values suggests that starch was never completely digested in the small intestine and that animal adaptation was not fully reached. It can be alternatively hypothesized that high starch flow to the hindgut occurred during many days, but the adaptation of microbiota in the hindgut—especially of lactate-utilizing bacteria—masked possible effects of amylase addition on starch digestion in the intestine by utilizing lactate in advanced stages of the starch infusion phase. Therefore, changes in composition of microbial community in the hindgut may have arisen without changes in total microbial N mass, and consequently, expected effects based on microbial N excretion could not have been observed. Another interesting observation is the results of DM content of feces. The baseline value of DM concentration of feces averaged 16.4%, remained unchanged with amylase administration with 16.3% at the second day of starch infusion, but tended to be lower without amylase administration (14.8%, $P = 0.07$; data not shown). Watery feces might indicate hindgut acidosis (Gressley et al., 2011) without amylase administration. An increment of propionate is normally expected by fermentation of soluble carbohydrates (e.g., starch). Because reduction of lactate excretion indicated that starch digestion was improved with amylase, a reduction of propionate excretion with amylase administration could be expected as well. Nevertheless, propionate excretion was higher with than without amylase administration during the second day of starch infusion. The latter suggests that glucose from hydrolyzed disaccharides and trisaccharides in the small intestine was probably not absorbed due to limitation of glucose absorption. Consequently, these highly soluble carbohydrates flew to the hindgut and were

fermented to propionate. Finally, it is important to remark that results of propionate and lactate excretion and DM content of feces at the second day of starch infusion match with each other, which suggests that amylase addition improves the digestion of starch in the small intestine of not adapted animals.

Apparent Disappearance of Starch

In the present study around 85% of infused starch apparently disappeared from the small intestine, which is within the range of 11% to 90% for cattle (Owens et al., 1986; Giuberti et al., 2014; Moharrery et al., 2014). From total starch that flows into the hindgut, 8% to 95% can be fermented by microbes (Moharrery et al., 2014). In the present study, the apparent disappearance of starch from the hindgut was high (average 96%). This was probably due to starch source used and the slow passage rate through the hindgut, which is characteristic in animals with low feed intake or at maintenance level as in our study. Moreover, the amount of starch that reached the hindgut was low (average 128 g/d), which can be related to the starch source used in the trial. Ørskov et al. (1970) reported in a study with sheep that starch infused into the cecum only in amounts higher than 138 g/d largely appeared in the feces. Thus, they showed that the hindgut has a substantial capacity for starch fermentation. In accordance, the apparent disappearance of starch from the total tract in our trial was nearly complete (>99%) and agrees with results with abomasal infusion of purified corn starch (Reynolds et al., 2001) and partially hydrolyzed starch (Knowlton et al., 1998). In the present study, the increase of microbial N excretion compared to the baseline was used as a proxy to calculate the amount of starch entering the hindgut. However, since microbial N was estimated from PB excretion, the calculated quantity of starch depends on the accuracy of the assumed values for the microbial yield per g starch fermented and the proportion of PB-N to total microbial N. As mentioned before, excretions of the different VFA indicate that besides starch probably mainly soluble sugars were fermented in the hindgut. The Cornell Net Carbohydrate and Protein System assumes that microbes that ferment soluble sugars produce around 18% more microbial CP than starch-fermenting microbes (Khezri et al., 2009). In line with this, an assumption of higher efficiency of microbial CP synthesis in the hindgut would result in a smaller amount of carbohydrates reaching the hindgut.

Blood Glucose, Insulin, and Glucagon Concentration

In the present study peripheral blood glucose concentration was not affected by starch infusion or amylase administration, which is in agreement with results of other findings (Larsen et al., 1956; Huber et al., 1961; Little et al., 1968; Huntington and Reynolds, 1986; Knowlton et al., 1998). This might be explained by an enhanced uptake of glucose in peripheral tissues or inhibition of liver gluconeogenesis due to increasing pancreatic insulin secretion. The observed increase in serum insulin due to starch infusion is in agreement with observations from Knowlton et al. (1998). In vitro studies with hepatocytes from cows indicated that hepatic gluconeogenesis from propionate is insulin-independent (Donkin and Armentano, 1995). Another explanation for the missing effect of infused starch on blood glucose concentration could be an enhanced utilization of glucose by gut tissues. Wahle et al. (1971) demonstrated that appreciable amounts of glucose are consumed by small intestinal tissue of sheep resulting in lactate as one of the end products of this glycolytic process. Kreikemeier et al. (1991) demonstrated that only 38% of starch disappearing from the small intestine could be accounted as net glucose absorption when starch was infused into the abomasum of Holstein steers. Similar observations were made by Huntington and Reynolds (1986). However, changes in insulin and glucagon concentrations with starch infusion indicated that glucose was absorbed in rates sufficient to affect the secretion of these hormones. On the other hand, concentrations of insulin and glucagon might have changed independently of blood glucose concentration. For example, an elevated insulin secretion was observed in earlier experiments with intravenous infusions of butyrate and propionate in sheep (Manns and Boda, 1967; Horino et al., 1968). Therefore, insulin secretion might have increased due to higher concentrations of these substances, which result from starch fermentation in the hindgut as discussed earlier. However, Huntington and Reynolds (1986) did not detect an effect of abomasal starch infusion on plasma concentration of propionate and butyrate. This might be due to the fact that the liver removes propionate and butyrate at rates nearly equal to their net absorption so that blood concentrations are normally quite low (Reynolds, 1995). In our study, blood glucose concentration seemed to be mainly influenced by feeding and not by starch infusion as glucose concentration decreased within the last 2 h before the second feeding and increased afterwards

although infusion was constant. The low concentration of glucose 8 h after beginning of infusion might be due to a reduced availability of propionate due to fasting, which would lead to a reduced gluconeogenesis. Finally, although the differences in glucagon concentrations found in the present study are difficult to explain, they might indicate some unknown effects of amylase administration.

Limiting Factors of Starch Disappearance from Small Intestine

A review of possible limitations of starch disappearance from small intestine in ruminants has been published by Owens et al. (1986): the time for starch hydrolysis, the access of enzymes to starch granules, the capacity for glucose absorption, and the activity of hydrolyzing enzymes involved. In the small intestine of ruminants, pancreatic amylase hydrolyzes amylose and amylopectin resulting in limit dextrins and oligosaccharides of 2 or 3 glucose units (Huntington, 1997). Disaccharidases located at the brush border membrane (e.g., maltase and isomaltase) are required for complete hydrolysis to glucose (Harmon, 1992). Karr et al. (1966) suggested the pancreatic amylase secretion to be the limiting factor of starch digestion in the small intestine, whereas Kreikemeier and Harmon (1995) stated a limitation due to the glucosidase activity at the brush border membrane. Huntington (1997) suggested that starch disappearance in the small intestine of not adapted ruminants is probably limited by the capacity for absorption of end products from starch digestion rather than by starch hydrolysis itself, whereas starch hydrolysis in adapted ruminants seems to be limited by amylase activity. However, our results (i.e., higher lactate excretion without amylase at the second day of starch infusion) demonstrated that the activity of amylase is also a limiting factor in cattle not adapted to starch digestion. Contrary, Remillard et al. (1990) found no effects of abomasal-infused amylase on intestinal digestibility of starch and suggested that amylase was not the first limiting factor in digestion of starch. However, animals were adapted to starch digestion through a preceding period of feeding. Missing effects of amylase administration in later days of starch infusion might be explained by reduced secretion of endogenous amylase due to amylase infusion. Swanson et al. (2002) observed a decreased secretion of pancreatic amylase when starch hydrolysate was infused into the abomasum of beef steers at rates of 20 to 40 g/h. Therefore, an indirect effect of amylase infusion on amylase

secretion due to increased amounts of hydrolysed starch in the lumen of the small intestine might exist. On the other hand, missing effects of amylase administration in later days of starch infusion might be explained by an adaptation due to an increased secretion of endogenous amylase. An adaptation of amylase activity in the small intestine was demonstrated in earlier studies as dietary components changed from forage to grain (Russell et al., 1981; Kreikemeier et al., 1990). In line with this, adaptation is also based on glucose uptake by induction of the sodium-dependent glucose cotransporter (SGLT1) in the small intestine as reviewed by Shirazi-Beechey (1995). In contrast, adaptation due to an increased secretion of brush border glucosidases seems to be limited (Harmon, 1992). Therefore, the missing effect of exogenous amylase in the last days of starch infusion in the present study might also be explained by an insufficient activity of these enzymes.

In conclusion, increased fecal excretion of microbial N and VFA indicates increased carbohydrate fermentation in the hindgut, and therefore limited digestion of starch in the small intestine. Lower lactate excretion during the beginning of starch infusion with amylase administration indicates an effect of amylase in not adapted animals, and suggests that addition of exogenous amylase might have the potential to improve intestinal digestibility of starch in not adapted animals. Missing effects of amylase administration after adaptation might be due to a sufficient activity of endogenous amylase at the infused level of starch of this trial or due to a rapid adaptation of hindgut microbes. Therefore, further studies are required to clarify whether the activity of endogenous amylase might be a limiting factor at higher levels of starch infusion. Intestinal digestibility of starch reported in this study is within the range of those values reported in the literature, which indirectly indicates a relative reliability of the present approach of calculating starch digestibility.

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