Running title: Starch digestion in horses

The effects of processing barley and maize on metabolic and digestive responses in Horses[1]

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[1] Felleskjøpet Fôrutvikling (Trondheim, Norway) financed the experiment. The authors gratefully acknowledge Jon Anders Næsset for assistance with feed production and Agnieszka Waliczek and Mette Henne for technical assistance during the animal trial.

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ABSTRACT

The competition for customers increases the search for new grain processing methods for equine feed, but the effect on starch digestibility and metabolic responses varies. Therefore, to evaluate the effect of the processing methods, toasting and micronizing, on starch digestion and the effect on metabolic responses, the mobile bag technique (MBT) and plasma glucose and insulin concentrations in the blood were used to estimate nutrient disappearance and metabolic responses pre-caecally. Further, caecal pH, ammonium nitrogen (N) and short chain fatty acid (SCFA) concentrations were used to estimate the metabolic response in the caecum. Four caecally cannulated horses (body weight [BW] 565 ± 35 kg) were used in a 4x4 Latin square design with

four periods of 8 d diet adaptation and 2 d of data collection. Diets were formulated using hay and processed grains: micronized barley (MB), toasted barley (TB), micronized maize (MM) and toasted maize (TM) and were balanced to provide 1 g starch/kg BW in the morning meal. On day 9 in each period, blood and caecal fluid samples were taken before the morning meal and hourly thereafter for 8 h. On day 10 in each period, 15 bags of either MB, TB, MM or TM (1x1x12 cm; 15 µm pore size; 1 g feed) were placed in the stomach, respectively. The dry matter (DM) disappearance was highest for the MM at all timepoints compared to the other feedstuffs (P<0.001). Maize and micronizing had the highest starch disappearance (P=0.048) compared to barley and toasting. No treatment effect was measured for any of the glucose and insulin parameters. No feed effect was measured for the insulin parameters. Plasma glucose peaked later (P=0.045) for maize than for barley, and TB had a larger area under the curve (AUC) for glucose than MB, MM and TM (P=0.015). The concentration of total SCFA increased after feeding (P<0.001), with a higher concentration for barley than for maize (P=0.044). No treatment or feed effects were measured for ammonium N or pH, but both were affected by time (P<0.001). In conclusion, toasting was not as efficient as micronizing to improve pre-caecal starch digestibility; therefore, the preferred processing method for both barley and maize is micronizing. Further, the amount of starch escaping enzymatical digestion in the small intestine was higher than expected.

Key words Glucose, insulin, mobile bag technique, pH, short chain fatty acid

ABBREVIATIONS

Acid detergent fibre	ADF
Association of Official Analytical Chemists	AOAC
Area under the curve	AUC
Body weight	BW
Crude fat	Cfat
Crude protein	CP
Degree of gelatinisation	DG
Dry matter	DM
Micronized barley	MB
Mobile bag technique	MBT
Micronized maize	MM
Near-infrared radiation	NIR
Neutral detergent fibre	NDF
Nitrogen	N
Short chain fatty acid	SCFA
Toasted barley	TB
Toasted maize	TM

INTRODUCTION

Introduction

The apparent total tract digestibility of starch in grains is found to be nearly 100% in horses (Jensen *et al.*, 2014), whereas larger variations (21.5-90.1%) are found for pre-caecal starch digestion (Meyer *et al.*, 1995). In horses the pre-caecal starch digestion depends on several factors, such as the type of grain and its characteristics, meal size and passage rate of digesta (Kienzle, 1994). Further, grain processing involving heat and moisture is associated with improving the availability of starch for enzymatic degradation, thereby increasing starch digestion in the small intestine (Svihus *et al.*, 2005). Using the mobile bag technique (MBT), Philippeau *et al.* (2014) found that pre-caecal starch digestion depended on processing, with the lowest digestion for untreated barley and the highest for ground barley, 55.1% and 97.4%, respectively. Enzymatic starch digestion in the small intestine is preferred, as starch fermentation in the hindgut is associated with a higher concentration of short chain fatty acids (SCFA) and lactate, decreased pH and microbial disturbance in equines (Willard et al., 1977; de Fombelle et al., 2003). Therefore, compound feeds and grains used for horses are often processed, and one of the most common processing methods is micronizing (Julliand *et al.,* 2006). It includes thermal heat processing with high temperatures (85-125°C) for a short time using near-infrared radiation (NIR) (Farrell *et al.,* 2015). Processing methods that include endosperm disruption and heat above 80°C in combination with moisture will restructure the starch granules, causing gelatinisation (Svihus *et al.*, 2005). Gelatinisation increases amylolytic degradation, because part of the crystalline structure is lost (Svihus *et al.*, 2005). Holm *et al.* (1988) found the degree of starch gelatinisation and digestion rate in rats to be positively correlated, assuming more starch to be digested and thereby change the metabolic responses, as more glucose will be absorbed in the small intestine. Vervuert *et al.* (2008) found

68 that thermal processing increased serum glucose and insulin responses when horses were fed
69 extruded barley compared to rolled or micronized barley, reflecting a higher digestibility of starch
70 in the small intestine with extrusion than with the other methods. However, from the literature, it
71 is unclear whether the degree of gelatinisation from processing is followed by higher glucose and
72 insulin responses (Vervuert et al., 2003; Vervuert et al., 2007; Vervuert et al., 2008). The
73 competition for customers increases the search for other processing methods so feed producers can
74 achieve a differential product. Toasting is one of the 'new' processing methods employed by some
75 equine feed companies. This method is often used in products for human consumption, such as
76 breakfast cereals, flour and wine (Fares and Menga, 2012; Chira and Teissedre, 2013), primarily
77 to enhance taste as a result of the Maillard reaction (Martins *et al*., 2001), and it includes
78 temperatures ranging from 90-240°C (Grala *et al.,* 1994; Mosenthin *et al.*, 2016). Hence, toasting
79 could potentially be as effective as micronizing for improving the small intestine's digestibility of
80 starch. Nonetheless, to our knowledge, no study has been conducted on toasting's effect on nutrient
81 digestibility in horses. Therefore, the objective of this experiment was to compare the effects of
82 micronizing and toasting on starch digestion of barley and maize. It is hypothesised that: 1) toasting
83 is as efficient as micronizing for improving the small intestine's digestibility of starch; 2) starch
84 digestibility in the small intestine is highly reflected in the blood glucose and insulin responses
85 after feeding, independent of processing method; 3) the amount of starch escaping digestion in the
86 small intestine is low; and 4) fluctuations in caecal pH and SCFA concentrations and proportions
87 after feeding are small, independent of processing method.
88
89 **MATERIALS AND METHODS**
90 ***Experimental design***

All housing, management and experimental procedures followed the laws and regulations for experimental animals in Norway (i.e. Regulations on the Use of Animals in Experiments, July 2015). The experiment was designed as a 4x4 Latin square experiment with four experimental periods. Each period consisted of 8 d of diet adaptation followed by 2 d of data collection. Blood and caecal samples for pH and SCFA analyses were collected on day 9, and digestibility in the small intestine was measured on day 10 in each period.

Animals

Four healthy caecum-cannulated Norwegian cold-blooded trotter geldings (age 14-26 yr) with an initial body weight (BW ± SEM) of 565 ± 35 kg were used in the experiment. Horses were followed routinely with veterinarian check-ups including vaccinations, dental examinations and teeth floating. All horses were housed in individual stalls (3x3 m) with rubber mats and wood shavings as bedding material. In the adaptation period, horses were allowed access to a gravel paddock for 3-4 h/d. In the collection periods, one outdoor visit for 1 h was allowed daily after sampling had ended.

Diets

Treatments were arranged as 2x2 factorial, with two processing methods: micronizing and toasting. Two feeds were used: barley and maize. The chemical composition of the feedstuffs is shown in Table 2. Four experimental diets were formulated using hay and processed grains (same batches): micronized barley (MB), toasted barley (TB), micronized maize (MM) and toasted maize (TM) (Table 3). The micronizing and toasting processes are described below. All concentrate was fed once a day at 0600 h. Seven days prior to the first adaptation period, a mix of the four diets

was fed to gradually increase starch intake from 0 to 1 g/kg BW per day. Thereafter, all diets were balanced to provide 1 g starch/kg BW, and the amount of hay was adjusted to a total DM intake of 3 g/kg BW in the meal at 0600 h. The horses were fed a total of 15.7 ± 0.03 g DM/kg BW per day, which was divided into three meals fed at 0600 h, 1400 h and 2000 h (Table 3). A commercial supplement of vitamins and minerals (Champion Multitiskud, Felleskjøpet Forutvikling, Trondheim, Norway) and sodium chloride (80 and 25 g/d, respectively) was included with the morning meal. Water was available in the individual stalls' automatic water troughs, and from buckets in the gravel paddock.

Table 1.

Table 2.

Table 3.

Processing

Micronizing and toasting of barley and maize occurred at Felleskjøpet Agri (Skansen, Norway). Approximately 14.5 h prior to the micronizing treatment, the raw maize was preconditioned with water to raise the moisture content to 15.5%. The barley did not receive any preconditioning with water, as it had a moisture content of 11.2%. The barley and maize were then micronized for approximately 45 sec at 90-105°C using an infrared micronizer with a heat output of 525 kW (M600/72/HRS, Micronizing Company UK Ltd, Suffolk, United Kingdom) (Table 1). After micronizing, the heated barley and maize were run through a roller (0.15 mm, TECOM AB, X, Sweden) to produce a flaked product and then cooled down (custom-made cooler, Felleskjøpet Agri, Skansen, Norway). Prior to the toasting treatment (approximately 15 h and 12.5 h for maize

and barley, respectively), the raw grains were preconditioned with water to raise the moisture content to 20.6 and 22.6% (maize and barley, respectively). Thereafter, the grains were toasted for 30 min at 150°C (ECOTOAST 600, Agrel GmbH agrar Entwicklungs labor, Germany). After toasting, the heated barley and maize were run through a roller (0.35 mm and 1 mm for barley and maize, respectively (Strukturvalse T80, Vestjysk Smede, Denmark) to produce a flaked product and then cooled down.

143 ***Data collection***

144 ***Feedstuffs***

145 Samples of all feedstuffs were collected regularly during the four data collection periods and stored
146 in sealed plastic bags for later analysis.

147

148 ***Mobile bag technique***

149 The mobile bag technique was used to estimate the small intestinal starch digestibility. Bags
150 (1x1x12 cm) were made from precision-woven open mesh fabric with a porosity of 15 µ (Sefar
151 Nitex, 03-15/10, Sefar AG, Heiden, Switzerland). The bags were prepared by cutting a piece of
152 mesh (large enough for the heat sealing) and folding it in the middle. The mesh was then heat
153 sealed at one end and one side, and then turned inside out to avoid sharp edges. A steel washer (1
154 cm external diameter, weight 0.3 g) was sealed into the end of each bag, allowing for capture with
155 a magnet in the caecum. Lastly, the bags were marked with a permanent marker for identification.
156 The weights of the bags when empty and when filled with individual feed (1 g/bag) were recorded.
157 All feeds were milled to pass a 1.5 mm screen. The bags (15 bags/horse per period) were soaked
158 in cold tap water before they were placed in the stomach with a nasogastric tube flushed with
159 approximately 1.5 L of tap water. Bags were administered after feeding half of the morning meal,
160 and before feeding hay. The rest of the morning meal and the hay were fed afterwards. A string
161 (40 cm long) with a double-sided magnet (approximately 2 cm in diameter) was introduced into
162 the caecum through the cannula to retrieve the bags upon arrival. The bags were removed from the
163 magnet at hourly intervals for 8 h after feeding. Bags not harvested in the caecum were collected
164 in the faeces throughout the following days. The capture time of each bag was noted as soon as
165 the bags were collected and, thereafter, hand-rinsed in cold tap water and stored at -20°C. At the

end of the experiment, all bags were thawed at room temperature, washed in cold water for 35 min (Woolprogram, Avantixx 7 Varioperfect, Bosch, Gerlingen-Schillerhöhe, Germany) and then dried at 45°C for 48 h. The bags were left at room temperature (approximately 25°C) for equilibration for 24 h prior to weighing. Control bags (4 bags per feedstuff) were soaked for 1 h before washing and drying as described above to determine their nutrient loss. To obtain enough residue for chemical analyses, the collected bags of each feedstuff were pooled to a specific collection time (0-3, 4-6 and 7-9 h), regardless of which horse they came from. All bags found in the faeces were pooled for each feedstuff.

Blood samples

Blood samples were collected by jugular vein puncture into 10 ml heparinized tubes (BD Vacutainer, Becton, Dickinson and Company, USA) before the morning meal (time: 0) and hourly thereafter (time: 1-8 h). The blood samples were centrifuged (Heraeus labofuge 300, Thermo Fisher Scientific, Waltham, USA) immediately after sampling at 3000×g for 10 min and plasma was harvested and stored at -20°C for later analysis of insulin and glucose concentrations.

Short chain fatty acid, ammonium nitrogen and pH

Caecal fluid was collected before the morning meal (time: 0) and thereafter hourly (time: 1-8 h). A collection tube and a pH electrode (Sentix 41, WTW, Weilheim, Germany) attached to a data logger (ProfiLine 340i, WTW, Weilheim, Germany) were placed in the caecum according to Jensen *et al.* (2016) approximately 30 min before first collection (time: 0). Caecal fluid was sampled (~100 ml) with a 400 ml syringe connected to the tube placed in the caecum. The pH was measured immediately as caecal fluid samples were taken and in-situ in the caecum every minute

189 throughout the 8 h time frame with the pH electrode. From this, two subsamples of each 9.5 ml
190 caecal fluid were mixed with 0.5 ml of formic acid and stored at 3°C for later analysis of SCFA
191 and ammonium nitrogen (N) concentrations.
192
193 ***Chemical analyses***
194 Feed samples from each period were analysed in duplicate for DM, starch and crude protein (CP)
195 (Table 2). Samples were milled to pass a 1 mm screen (Cutting mill SM 200, Retsch GmbH, Haan,
196 Germany). For starch, feed samples were milled to pass a 0.5 mm screen before analysis. Dry
197 matter content was measured by drying to a constant weight (24 h at 105 ± 2°C) and samples were
198 incinerated at 550°C for 16 h for ash determination. Starch was measured according to the
199 Association of Official Analytical Chemists (AOAC, method 996.11.) by using heat-stable α-
200 amylase, and water-soluble carbohydrates (WSC) were determined by the method described in
201 Randby *et al.* (2010). Nitrogen was determined according to the Dumas method (Elementar
202 Analysensysteme GmbH, Hanau, Germany), and CP was calculated as N x 6.25. Crude fat (CFat)
203 was analysed according to the accelerated solvent extractor method (Dionex ASE 350, Thermo
204 Fisher Scientific, Waltham, USA). Neutral detergent fibre and ADF were analysed using the filter
205 bag technique described by ANKOM (2017a and 2017b). Residues from the mobile bags were
206 analysed for starch and N as described above. Plasma glucose was analysed by the hexokinase-
207 method according to Tietz *et al.* (1995), and insulin was analysed using the ELISA test (Mercodia
208 AB, Uppsala, Sweden). Caecal fluid was analysed for the concentration of SCFA (times: 0, 1, 3,
209 5 and 7 h) and ammonium N (times: 0 and 3 h). The concentrations of SCFA were determined by
210 gas chromatography (Trace 1300 GC, Thermo Fisher Scientific, Waltham, USA), and ammonium
211 N was measured according to AOAC (method 2001.11), besides the first digestion step. The degree

of gelatinisation (DG) was evaluated using the differential scanning calorimetry (DSC) method. The DSC method relies on the enthalpy measurement of non-processed and processed samples, and the difference between the two represents the extent of gelatinisation with a greater difference indicating greater gelatinization. A DM feed sample weighing approximately 30 mg (ground through a 0.5 mm screen) was weighed in a stainless-steel pan, and deionized water (1:2, feed/water, wt/wt, total weight 90 mg) was added. Thermal scans were conducted using a differential scanning calorimeter (DSC 823, Mettler Toledo, Stockholm, Sweden). The measurement was performed by heating the pan in the DSC from 10 to 120°C at a heating rate of 10°C/min. The onset, peak, and conclusion gelatinisation temperatures and the enthalpy of gelatinisation (ΔH) were then determined. The DG is calculated as DG (%) = $[(\Delta H_0 – \Delta H_1)/\Delta H_0] \times 100$, in which ΔH_0 is the gelatinisation enthalpy of starch (J/g starch) in a non-processed sample and ΔH_1 is the gelatinisation enthalpy of starch in a processed sample (J/g starch). A 100% DG equates to completely processed starch, whereas 0% equates to unprocessed starch and negative values indicate lower DG in the processed sample than the non-processed sample. All measurements were performed in duplicate.

Statistical analyses

All statistical analyses were performed in Rstudio (version 1.1.456, Rstudio Inc., Boston, USA). Analysis of variance was done on the chemical composition of the feedstuffs with a model comprising nutrient as response and feed and treatment as predictors. The dry matter, starch and CP disappearance were subjected to ANOVA, with the nutrient disappearance as response and feed, treatment and time (DM) or time interval (starch and CP) and their interactions as predictors. Mean concentrations, peak concentration, time to peak and number of peaks were calculated for

plasma glucose and insulin. Calculations of area under the curve (AUC) above baseline (without considering area beneath) were performed for glucose and insulin in GraphPad Prism (version 8.0.1, GraphPad Software, San Diego, USA), and ANOVA were performed in a model compromising either mean concentration, peak concentration, time to peak or number of peaks and AUC as response, with feed, treatment and their interactions (if present) as predictors. Analyses of SCFA, ammonium N concentrations and pH were performed using mixed models for repeated measurements. The model comprised the fixed effect of feed (barley or maize), treatment (micronizing or toasting), time (after feeding), interaction (feed x treatment) and the random effect of horse. Significant differences of least-square means were analysed by Tukey's Honest Significant Difference test (Rstudio, version 1.1.456, Rstudio Inc., Boston, USA). All results are presented as least-square means with SEM as a measure of variance. Effects are considered significantly different if $P < 0.05$ and a tendency if $P < 0.10$.

RESULTS

All horses remained healthy and in good condition throughout the experiment. Residues from the previous evening meal were collected for two horses on the day of sampling (one horse in period 3: 1.6 kg DM and two horses in period 3: 0.7 and 1 kg DM, respectively). The residue was offered to the horses and eaten after sampling had ended.

Chemical composition of the feedstuffs

The chemical composition of the feedstuffs is shown in Table 2. Hay has the highest numerical DM content compared to maize and barley. An effect of treatment ($P < 0.001$) was measured for DM, with micronizing having the highest content for both maize and barley. Barley had the highest content of CP ($P < 0.001$) compared to maize. Toasting had the highest ($P = 0.003$) WSC content for both barley and maize. The starch content was highest in maize compared to barley ($P < 0.001$), whereas hay had the lowest numerical content. Crude fat was highest in maize compared to barley ($P < 0.001$). Neutral detergent fibre and ADF were highest in barley compared to maize ($P < 0.001$). The degree of gelatinisation was highest for MM compared to the other diets (Table 2). However, DG for processed barley was negative, indicating that processed barley had a lower DG than whole barley. The negative DG for barley was interpreted as zero DG for barley. The dry matter intake for each meal and daily nutrient intake is shown in Table 3. The size of the grain meal within each diet varied to ensure similar starch intake.

Nutrient disappearance

The DM loss from the control bags was 7.3 ± 1.4%, 9.9 ± 1.9%, 6.5 ± 1.6% and 9.6 ± 1.0% for MM, TM, MB and TB, respectively. The effects of feed, treatment, time and their interactions on DM, starch and CP pre-caecal disappearance are shown in Fig. 1. There was an effect of the interaction, feed x treatment x time ($P < 0.001$), and the DM disappearance from the mobile bags increased over time; it was at all times highest for the MM compared to the other feedstuffs. Starch disappearance increased with later time intervals, and an interaction between feed x treatment ($P = 0.048$) was measured with maize and micronizing having the highest disappearances compared to barley and toasting. Disappearance of CP increased over time ($P = 0.041$), regardless of feed or treatment.

Metabolic response in plasma

The effects of feed, treatment and their interaction on plasma glucose and insulin measurements are shown in Table 4. Treatment did not affect any of the measured variables for plasma glucose and insulin. Feed had no effect on the measured variables for plasma insulin. There was no effect of feed on peak and the number of peaks for plasma glucose. However, plasma glucose peaked later ($P = 0.045$) for maize than for barley. Regarding AUC, an interaction between feed and treatment was found for glucose ($P = 0.015$), with a larger AUC for toasted barley than for micronized barley and micronized or toasted maize.

Digestive response in the caecum

The effects of feed, treatment, time and their interactions on SCFA concentrations and molar proportions are shown in Fig. 2. The concentration of total SCFA increased after feeding ($P <$

290 0.001), with a higher concentration for barley than for maize (P = 0.044) (Fig. 2a). Generally, the
291 molar proportion of acetate was the greatest, followed by propionate and then butyrate for all diets
292 at all time points. However, the molar proportion of acetate (P = 0.004) first increased and then
293 decreased with time (Fig. 2b), whereas the opposite was found for propionate (P = 0.006) (Fig.
294 2c). Firstly, the proportion of butyrate (P = 0.086) tended to increase and thereafter decrease with
295 time (Fig. 2d), whereas iso-butyrate (P < 0.001) (Fig. 2e) and iso-valerate (P < 0.001) (Fig. 2g)
296 decreased after feeding. Further, butyrate tended to be higher (P = 0.058) for micronizing than for
297 toasting (Fig. 2d). An interaction between feed and time (P < 0.001) was present for valerate, as
298 the proportion after feeding increased for barley; however, maize remained the same (Fig. 2f). The
299 (C2+C4)/C3 ratio (P = 0.055) tended to first increase and then decrease after feeding, reflecting
300 the changes in molar proportions of acetate, propionate and butyrate over time (Fig. 2h). No effects
301 of feed, treatment or their interaction were found on ammonium N. But mean concentrations of
302 ammonium N decreased over time (P < 0.001), with MM from 57.5 to 23.2 mg/L, MB from 65.7
303 to 22.3 mg/L, TM from 65.9 to 17.2 mg/L and TB from 65.8 to 19.5 mg/L. The pH decreased after
304 feeding, reaching a minimum pH after 195, 173, 180 and 150 min for MM, MB, TM and TB,
305 respectively (Fig. 3). The pH then fluctuated before increasing again. Feed, treatment and their
306 interaction had no effect on caecal pH.

307 **TABLE 4.**

DISCUSSION

Starch digestion has been previously investigated in horses using different direct and indirect methodologies. Small intestinal cannulated horses (Meyer *et al.,* 1993), slaughter experiments (de Fombelle *et al.,* 2003) and the MBT (Philippeau *et al.,* 2014) have been used as more direct methods for quantifying starch digestion in different segments of the gastrointestinal tract of horses. Blood glucose and insulin responses (Healy *et al.,* 1995; Vervuert *et al.,* 2004: Vervuert *et al.,* 2007; Jensen *et al.,* 2016) and changes in fermentation parameters in the caecum (McLean *et al.,* 2000) of horses have been used as a proxy to evaluate the degree of starch digestion in the small intestine and caecum, respectively. However, the results have been inconclusive. To the authors' knowledge, this is the first study to include both metabolic responses in blood and the digestive responses in caecum in combination with results from the MBT. The results presented here show the complexity of evaluating starch digestion in horses by only including one of the above-mentioned methodologies.

Pre-caecal disappearances of starch and protein

It is assumed that nutrients lost from mobile bags harvested in the caecum are digested in the small intestine. In the present study, the pre-caecal disappearance of starch and protein varied from 55-81% and 82-95%, respectively. This is in accordance with previous studies using the MBT (Hymøller *et al.,* 2012; Philippeau *et al.,* 2014). Protein digestion was relatively high and not affected by processing, while high starch digestibility was expected due to the maize and barley being processed. However, some variation was measured in the starch disappearance. In the present study, average starch intake was 565 g/d, and according to MBT, starch measurements of approximately 107, 164, 122 and 254 g/d escaped digestion in the small intestine for MM, MB,

TM and TB diets, respectively. Since the apparent total tract digestibility of starch in grains is found to be nearly 100% (Jensen *et al.*, 2014), it is expected that the undigested starch was fermented mainly in the hindgut. Some starch might be fermented by gastric microbiota present in the saccus caecus in the non-glandular region of the stomach (Coenen *et al*., 2006; Varloud *et al*., 2007). However, to what extend starch is fermented in the stomach still needs to be quantified. The site of starch digestion in the gastrointestinal tract of the horse (pre-caecal or hindgut) is expected to influence the metabolic responses, as discussed below.

Metabolic response in plasma

In the present study, it was hypothesised that starch digestion in the small intestine was reflected in the blood glucose and insulin responses after feeding, independent of the processing method. This was the case, as both plasma glucose and insulin increased after feeding. This was also measured in earlier studies (Vervuert *et al.,* 2003; Vervuert *et al.,* 2004; Vervuert *et al*., 2009). In the present study, MM had a higher pre-caecal DM and starch disappearance from mobile bags compared to the other diets, but no differences were found between feeds or treatments for either plasma glucose or insulin. Similar findings for whole versus thermal processed barley on starch disappearance and glucose and insulin responses were measured by Philippeau *et al.* (2014). This contradicts the theory that increased starch digestibility should increase the glucose concentration in the blood and further increase the insulin response (Palumbo *et al.,* 2013). Yet, it is unclear to what degree the disappeared starch from MM was enzymatically digested or possibly degraded by microbes, as they are present along the entire gastrointestinal tract including the stomach (de Fombelle *et al.,* 2003).

353 The AUC is often used as a parameter to describe both overall plasma glucose and insulin
354 responses after feeding. However, contradicting results are found for grain processing on AUC.
355 Vervuert *et al.* (2003) and Vervuert *et al.* (2004) did not measure any effect of processing oats or
356 maize (untreated vs. thermal processing) on glucose or insulin AUC, respectively. Yet, Vervuert
357 *et al.* (2008) measured a larger glucose AUC for extruded compared to rolled and micronized
358 barley, along with a larger insulin AUC for extruded and micronized barley compared to rolled
359 barley. In the present study, an interaction between feed x treatment was found for AUC, with TB
360 having a higher AUC for glucose compared to MB, MM and TM. Toasted barley peaked twice
361 during the sampling time, whereas MB, MM and TM only peaked once. The time for peaks to
362 occur and the number of peaks could indicate differences in gastric contractions and thereby,
363 gastric emptying. Lorenzo-Figueras *et al.* (2005) describes gastric emptying as a combination of
364 relaxation of the proximal portion of the stomach, suppression of antral motility and stimulation
365 of the pyloric contractions, all working together at once. The composition of the meal combined
366 with volume, physical structure, energy density and osmolarity can affect the rate of gastric
367 emptying (Meyer *et al.,* 1986). Slower gastric emptying is measured with a starch-rich meal (1.25
368 g starch/kg BW) compared to a meal low in starch (0.66 g starch/kg BW) (Metayer *et al.*, 2014).
369 However, in the present study, all meals were similar in starch content. Yet, plasma glucose peaked
370 later for maize than for barley. In general, meals containing maize were smaller in volume
371 compared to those containing barley, as the starch content was higher in maize than barley;
372 thereby, less was required to obtain 1 g starch/kg BW/meal. This contradicts smaller meals
373 resulting in faster gastric emptying compared to larger meals (Metayer *et al.,* 2014). On the other
374 hand, the difference in meal size is small in the present study, and the effect on gastric emptying
375 may have been limited. Another approach could be physical structure, osmolarity or even the ratio

between amylose and amylopectin in the grains. In general, maize has a higher swelling- and water-binding capacity than barley (Brøkner *et al.,* 2012). This suggests a higher ratio of amylopectin to amylose, as it is easier to solubilize (Cowieson *et al*., 2018). Furthermore, Hymøller *et al.* (2012) measured a longer average pre-caecal passage time of mobile bags containing soaked maize compared to soaked barley (7.99 and 6.82 h, respectively), supporting the theory of why plasma glucose peaked later for maize than for barley. Maize and barley contain approximately similar ratios between amylose and amylopectin (approximately 25 and 75%, respectively) (Svihus *et al.,* 2005; Cowieson *et al.,* 2018), but it cannot be excluded that maize had a higher amylopectin ratio, as it was not measured in the present study.

Digestive response in the caecum

In general, plasma glucose and insulin concentrations are parameters of pre-caecal digestion, whereas the caecal SCFA concentration together with pH gives an indication of fermentation in the hindgut of the horse. Further, the time to reach maximum SCFA concentration and minimum pH in caecum can indicate the passage rate of the feed from the stomach to the caecum and the fermentability of the escaped starch. In the present study, SCFA concentrations increased relatively fast after feeding (approximately 1-2 h), and maximum SCFA concentrations were measured approximately 3 h after feeding. Jensen *et al.* (2016) measured both an increase in SCFA concentration and a corresponding pH drop approximately 3 h after feeding horses a pelleted barley meal (2 g starch/kg BW). In the present study, barley had a higher total SCFA concentration compared to maize, with TB having the highest SCFA concentration, and furthermore, a lower pre-caecal starch disappearance up to 6 h after administration, reflecting starch being fermented in the caecum. The proportions of acetate and propionate also indicate fermentation of starch. McLean *et al.* (2000) measured higher lactate and total SCFA with both higher acetate and

propionate concentrations and lower caecal pH 4-8 h after feeding rolled barley compared to micronized and extruded barley, indicating that less starch reached the caecum when using these processing techniques compared to rolling. Similar results are measured for propionate, lactate and pH by increasing rolled barley in the ration, thereby increasing daily starch intake (Julliand *et al*., 2001). Starch intake was approximately 2 g/kg BW/meal in the studies by Julliand *et al.* (2001), McLean *et al.* (2000) and Jensen *et al.* (2016), and the minimum pH varied from 6.26-6.40, which is lower than the minimum pH in the present study. When feeding either starch at approximately 2 g/kg BW/meal or hay-only diets, caecal pH varied from 6.26-6.40 and 6.50-6.74, respectively (McLean *et al.* 2000; Julliand *et al.* 2001; Jensen *et al.* 2016). In this study, the decrease in caecal pH was in between the above studies. Altogether, this indicates that processed starch meals fed at a level of 1 g/kg BW can to some extent escape the enzymatic digestion in the small intestine, thereby interfering with the microbiota, concentrations and ratios of SCFA and pH.

In this study, it is possible that the processing methods that included thermal heat increased the pre-caecal starch digestibility as a result of an increased DG. When comparing the DG in the present study, no gelatinisation occurred for either of the two barley diets. Whereas, for maize, micronizing had a larger impact on DG compared to toasting. Vervuert *et al.* (2004) also measured an increased DG when maize was micronized compared to untreated maize. In general, maize has a higher gelatinisation enthalpy, meaning lower temperatures and moisture content are required to gelatinise maize starch compared to barley starch (Tan *et al.,* 2008). However, both Vervuert *et al.* (2007) and Philippeau *et al.* (2014) measured the effect of processing barley on DG. From these two studies, ground barley had a DG varying from 15-18%, indicating a possibility of a lower DG for TB and MB in the present study. Yet, Rosenfeld and Austbø (2009) did not measure an effect of micronizing grains on pre-caecal starch disappearance as in the present study. An in vitro study

423 demonstrated lower starch digestibility of peas when toasted compared to being extruded and
424 expanded (Masoero *et al.,* 2005). This is also confirmed in pigs, where a lower ileal starch
425 digestibility of toasted peas compared to dried was measured (Canibe and Bach Knudsen, 1997).
426 However, it can be difficult to compare results across studies, as the processing conditions
427 (moisture content, duration, temperature and pressure) vary.
428

429 *Methodical and practical recommendations*

430 In summary, the results presented here show the complexity of evaluating starch digestion in
431 horses. Future studies should include detailed information regarding processing (duration,
432 temperature, moisture content, pressure and machinery), diet characteristics (composition and DG)
433 and feeding management (g/kg BW/meal, number of meals and feeding order of hay and
434 concentrate), as well as information regarding techniques used to study starch digestion. This
435 would provide a better basis for comparing and interpreting results.
436 From a practical point, the results presented in this study indicate that processing affected the DG
437 in maize more than in barley. Furthermore, compared to toasting, the preferred processing
438 technique for improving the starch digestion based on the disappearance of starch from the mobile
439 bags is micronizing. The metabolic responses in plasma and digestive responses in the caecum
440 revealed more of a change over time than an effect of processing and type of grain on the measured
441 variables. However, the SCFA concentration was highest in the TB compared to the MB, TM and
442 MM, supporting the lower digestibility of starch in the small intestine from the TB. The effect of
443 the changes measured in the caecum in this study on hindgut health can be questioned. Whereas,
444 the energy value of starch is lower when fermented to SCFA than with enzymatical digestion in
445 the small intestine with absorption of glucose. The results from this study revealed that when

446 feeding only 1 g processed starch/kg BW/meal, starch escapes the enzymatic digestion in the small
447 intestine, and there is still a lack in our knowledge regarding diet effects on gastric emptying and
448 passage rate through the small intestine for improving enzymatical starch digestion.
449

450 ## CONCLUSION

451 In the present study, it was hypothesised that toasting was as efficient as micronizing to improve
452 starch digestibility. However, this was not the case when evaluating the small intestinal
453 digestibility of starch. Therefore, to increase the pre-caecal starch digestibility, the preferred
454 processing method is micronizing for both barley and maize when measured by the MBT. Further,
455 starch digestibility for both barley and maize was highly reflected in the metabolic responses of
456 plasma glucose and insulin after feeding, but no effect of processing method was measured.
457 Fluctuations in both caecal pH and SCFA concentrations after feeding were significant, and the
458 starch escaping the enzymatical digestion in the small intestine was higher than expected.
459

460 **Conflict of interest**

461 The authors have no declaration of interest associated with this publication.

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600 **Figure legends**

601

602 Figure 1. Dry matter, starch and crude protein (CP) pre-caecal disappearance for each of the four
603 diets (micronized maize = MM, micronized barley = MB, toasted maize = TM and toasted barley
604 = TB) for each hour or time interval (1 = 0-3 h, 2 = 4-6 h and 3 = 7-9 h), respectively. Differences
605 given for feed (F), treatment (T) and time/time interval (Ti) and interactions.

606

607 Figure 2. Concentration of SCFA (mmol/L) and molar proportions (%) measured hourly (mean ±
608 SEM) in caecal fluid after feeding the four diets (MM = micronized maize, TM = toasted maize,
609 MB = micronized barley and TB = toasted barley). Differences given for feed (F), treatment (T)
610 and time (Ti) and interactions.

611

612 Figure 3. pH fluctuations in caecum measured in 30-min intervals for the average of the four diets
613 after feeding (MM = micronized maize, TM = toasted maize, MB = micronized barley and TB =
614 toasted barley). Differences given for feed (F), treatment (T) and time (Ti).

615

616 **Tables**

617

618 **Table 1**. Processing conditions for barley and maize

	Toasting				Micronizing			
	Temp.[1]	Duration (min)	Heat source	Roller (mm)	Temp.	Duration (sec)	Heat source	Roller (mm)
Barley	150	30	Steam	0.35	90-105	45	NIR[2]	0.15
Maize	150	30	Steam	1.00	90-105	45	NIR	0.15

619 [1] Temp. = temperature in ˚C

620 [2] NIR = near-infrared radiation

621 **Table 2**. Dry matter (g/kg), chemical composition (g/kg DM) and degree of gelatinisation (DG,

622 %) of hay, micronized or toasted maize and barley (mean ± SEM).

	Hay	Maize		Barley		*P*-value[2]	
Nutrient[1]		Micronized	Toasted	Micronized	Toasted	F	T
DM	898 ± 1.46	874 ± 2.47^{a}	840 ± 4.27^{b}	881 ± 1.27^{A}	830 ± 3.03^{B}	0.338	<0.001
Ash	78.2 ± 0.85	14.2 ± 0.31	13.8 ± 0.65	19.8 ± 0.12	20.4 ± 0.30	<0.001	0.862
CP	147 ± 5.59	86.3 ± 2.42^{a}	84.2 ± 1.77^{b}	120 ± 2.10^{B}	126 ± 0.71^{A}	<0.001	0.302
CFat	18.6 ± 1.59	43.4 ± 3.25^{a}	36.0 ± 1.10^{b}	14.3 ± 0.70	15.6 ± 0.57	<0.001	0.058
Starch	28.9 ± 0.80	721 ± 7.89	719 ± 9.69	601 ± 5.00	577 ± 7.88	<0.001	0.145
WSC	84.9 ± 2.18	27.7 ± 0.88^{b}	35.4 ± 1.55^{a}	32.6 ± 0.50	38.5 ± 0.60	0.557	0.003
NDF	616 ± 6.62	95.8 ± 4.61^{b}	119 ± 1.30^{a}	224 ± 2.46	227 ± 7.32	<0.001	0.051
ADF	341 ± 4.92	46.7 ± 0.89	47.8 ± 1.10	78.6 ± 0.56	77.1 ± 1.94	<0.001	0.859
DG		56.8 ± 1.49	39.1 ± 3.10	-12.7 ± 12.0	-34.3 ± 1.53	<0.001	0.021

623 [1] CP = crude protein, Cfat = crude fat, WSC = water soluble carbohydrates, NDF = neutral

624 detergent fibre, ADF = acid detergent fibre, DG = degree of gelatinisation.

625 [2] The effect of feedstuff (F) and treatment (T).

626 [a, b or A, B] Values within a row for each feedstuff are different if superscript differs ($P < 0.05$).

Table 3. Dry matter intake (kg DM) and daily nutrient intake (g DM/kg BW) for the four diets (mean ± SEM).

	Micronized		Toasted	
	Maize (n=4)	Barley (n=4)	Maize (n=4)	Barley (n=4)
Morning (0600 h)				
Hay	1.10 ± 0.03	0.91 ± 0.03	1.13 ± 0.04	0.95 ± 0.03
Supplement	0.88 ± 0.03	1.05 ± 0.03	0.90 ± 0.03	1.10 ± 0.03
Lunch (1400 h)				
Hay	3.95 ± 0.12	3.95 ± 0.12	3.95 ± 0.12	3.95 ± 0.12
Evening (2000 h)				
Hay	3.95 ± 0.12	3.95 ± 0.12	3.95 ± 0.12	3.95 ± 0.12
Daily nutrient intake[1]				
DM	15.6 ± 0.02	15.6 ± 0.02	15.7 ± 0.03	15.7 ± 0.03
Ash	1.13 ± 0.01	1.12 ± 0.01	1.14 ± 0.01	1.13 ± 0.01
CP	2.21 ± 0.08	2.25 ± 0.08	2.22 ± 0.08	2.27 ± 0.08
Cfat	0.32 ± 0.02	0.29 ± 0.02	0.31 ± 0.02	0.29 ± 0.02
Starch	1.39 ± 0.02	1.39 ± 0.02	1.37 ± 0.02	1.34 ± 0.02
WSC	1.25 ± 0.03	1.25 ± 0.03	1.27 ± 0.03	1.26 ± 0.03
NDF	8.91 ± 0.09	8.97 ± 0.09	8.97 ± 0.09	9.01 ± 0.09
ADF	4.92 ± 0.08	4.89 ± 0.08	4.94 ± 0.08	4.91 ± 0.08

[1] CP = crude protein, Cfat = crude fat, WSC = water soluble carbohydrates, NDF = neutral detergent fibre, ADF = acid detergent fibre.

[a, b] Values within a row are different if superscript differs ($P < 0.05$).

645 **Table 4**. Mean ± SEM peak (ng/L), time to peak (h) and area under the curve (AUC, ng x h/L) for

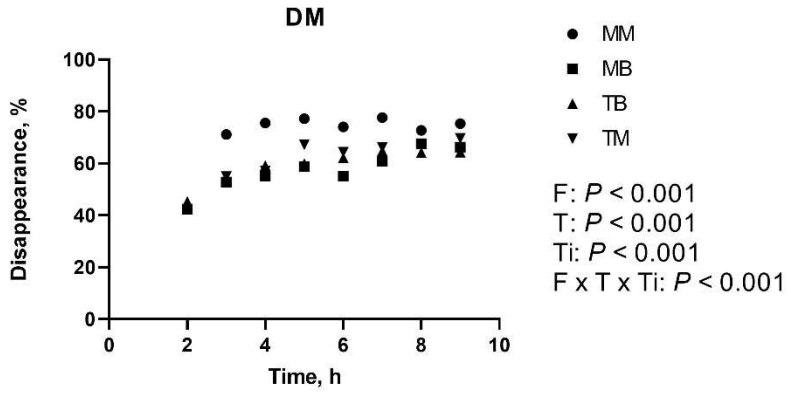
646 glucose (G) and insulin (I) with different diets.

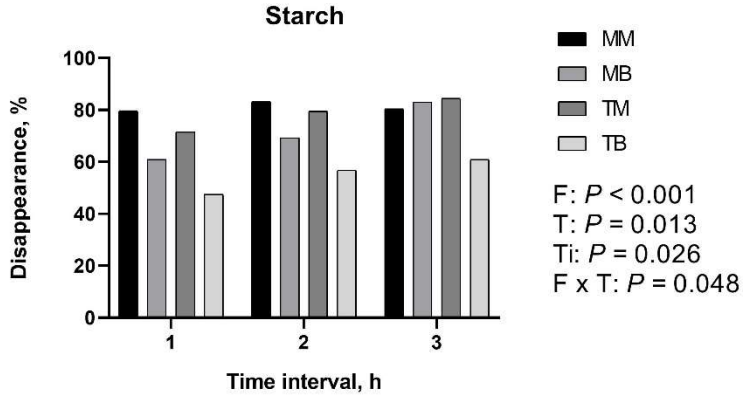
Feed		Barley		Maize		*P*-value[1]		
Treatment		Micronized	Toasted	Micronized	Toasted	F	T	FxT
Peak	G	5.88 ± 0.13	5.85 ± 0.18	5.85 ± 0.19	5.78 ± 0.23	0.794	0.794	0.794
	I	386 ± 56.8	354 ± 26.5	460 ± 64.7	394 ± 65.0	0.325	0.397	0.765
No. peaks	G	1.75 ± 0.48	1.25 ± 0.25	1.25 ± 0.25	1.50 ± 0.29	0.712	0.712	0.279
	I	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00			
Peak time	G	1.00 ± 0.00^{b}	1.25 ± 0.25^{b}	1.50 ± 0.29^{a}	2.00 ± 0.41^{a}	0.045	0.205	0.663
	I	1.25 ± 0.25	1.25 ± 0.25	1.00 ± 0.00	1.25 ± 0.25	0.574	0.574	0.574
AUC	G	2.32 ± 0.28^{ab}	3.48 ± 0.44^{a}	2.89 ± 0.57^{ab}	1.75 ± 0.25^{b}	0.177	0.983	0.015
	I	1373 ± 156	1433 ± 74.9	1444 ± 119	1220 ± 112	0.562	0.502	0.256

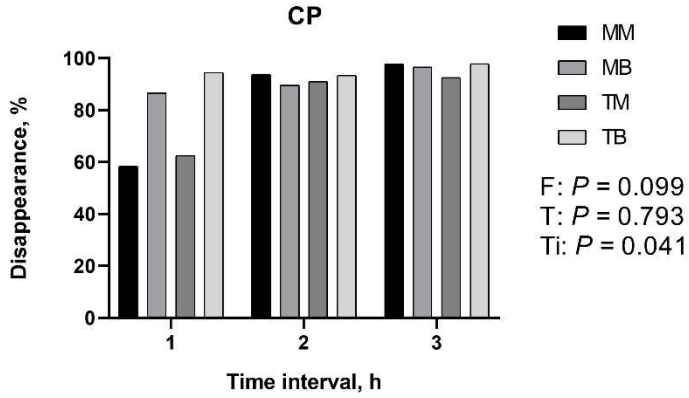
647 [1] The effect of feedstuff (F), treatment (T) and their interaction (F×T).

648 [a, b] Values within a row are different if superscript differs ($P < 0.05$)**.**

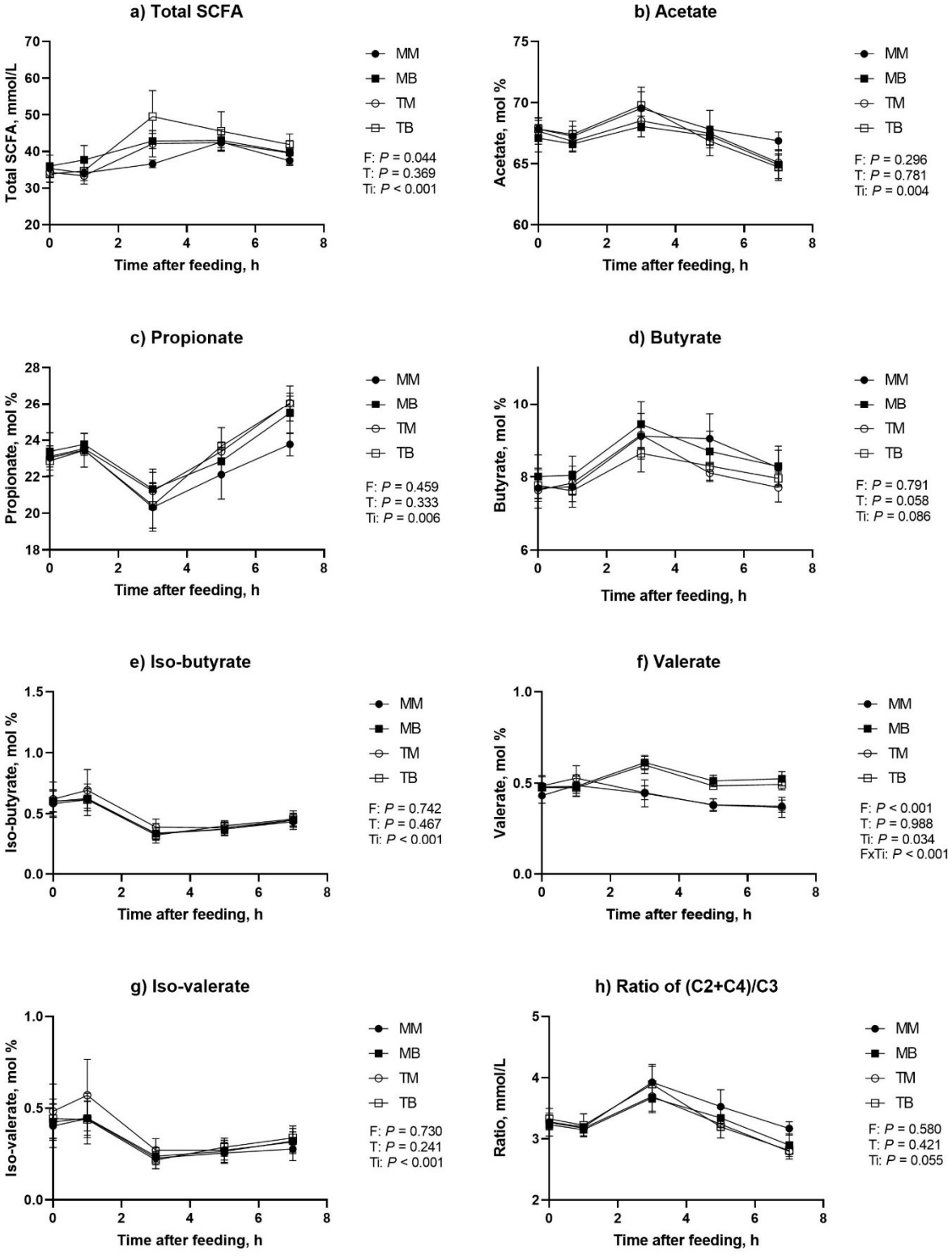
649


DM
Disappearance, %
Time, h
MM
MB
TB
TM
F: P < 0.001
T: P < 0.001
Ti: P < 0.001
F x T x Ti: P < 0.001


Starch
Disappearance, %
Time interval, h
MM
MB
TM
TB
F: P < 0.001
T: P = 0.013
Ti: P = 0.026
F x T: P = 0.048


CP
Disappearance, %
Time interval, h
MM
MB
TM
TB
F: P = 0.099
T: P = 0.793
Ti: P = 0.041

650


a) Total SCFA
Total SCFA, mmol/L
Time after feeding, h
MM
MB
TM
TB
F: P = 0.044
T: P = 0.369
Ti: P < 0.001
b) Acetate
Acetate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P = 0.296
T: P = 0.781
Ti: P = 0.004
c) Propionate
Propionate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P = 0.459
T: P = 0.333
Ti: P = 0.006
d) Butyrate
Butyrate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P = 0.791
T: P = 0.058
Ti: P = 0.086
e) Iso-butyrate
Iso-butyrate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P = 0.742
T: P = 0.467
Ti: P < 0.001
f) Valerate
Valerate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P < 0.001
T: P = 0.988
Ti: P = 0.034
FxTi: P < 0.001
g) Iso-valerate
Iso-valerate, mol %
Time after feeding, h
MM
MB
TM
TB
F: P = 0.730
T: P = 0.241
Ti: P < 0.001
h) Ratio of (C2+C4)/C3
Ratio, mmol/L
Time after feeding, h
MM
MB
TM
TB
F: P = 0.580
T: P = 0.421
Ti: P = 0.055

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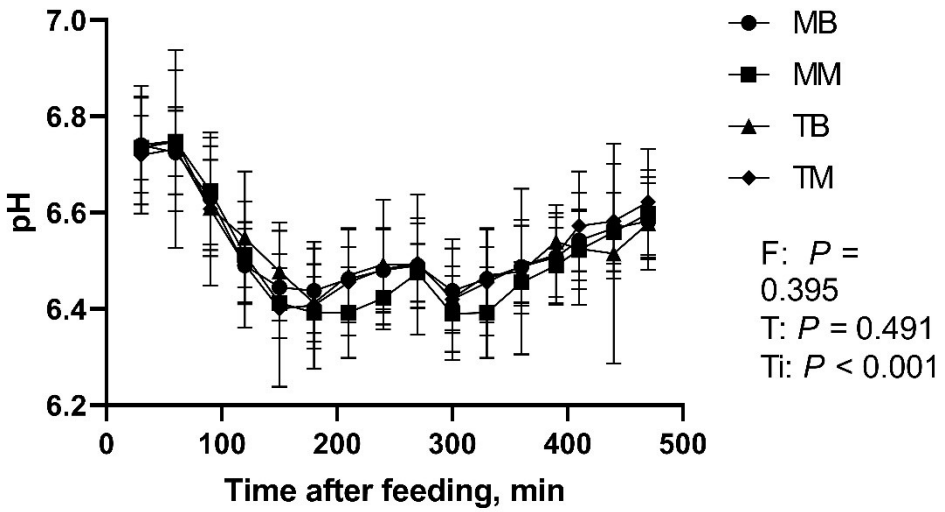
7.0
6.8
6.6
6.4
6.2
pH
0
100
200
300
400
500
Time after feeding, min
MB
MM
TB
TM
F: P = 0.395
T: P = 0.491
Ti: P < 0.001

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