MINISTERIUM
FÜR EIN
LEBENSWERTES
ÖSTERREICH

HBLFA RAUMBERG - GUMPENSTEIN
LANDWIRTSCHAFT

Abschlussbericht optGERSTE

Wissenschaftliche Tätigkeit Nr. 2394

Ernährungsphysiologische Bewertung einer chemisch-thermischen Verarbeitungstechnik von Futtergerste

Nutritional evaluation of a chemical-heat processing technology of barley grain in the diets of early lactation dairy cows

Projektleitung:
Univ.-Doz. Dr. Leonhard Gruber, HBLFA Raumberg-Gumpenstein

Projektmitarbeiter:
Dipl. ECBHM Dr. Johann Gasteiner, HBLFA Raumberg-Gumpenstein
Ing. Anton Schauer, HBLFA Raumberg-Gumpenstein

Projektpartner:
Univ.-Prof. Dr.sc.agr. Qendrim Zebeli,
Veterinärmedizinische Universität Wien

Projektlaufzeit:
2012 – 2017

raumberg-gumpenstein.at

INHALTSVERZEICHNIS

ARCHIVES OF ANIMAL NUTRITION, 2016
http://dx.doi.org/10.1080/1745039X.2016.1253226

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Long-term influence of feeding barley treated with lactic acid and heat on performance and energy balance in dairy cows

Leonhard Gruber[a*], Annabella Khol-Parisini[b*], Elke Humer[b], Sherief M. Abdel-Raheem[c] and Qendrim Zebeli[b]

[a]Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Irdning, Austria; [b]Department for Farm Animals and Veterinary Public Health, Institute of Animal Nutrition and Functional Plant Compounds, Vetmeduni Vienna, Vienna, Austria; [c]Department of Animal Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

ABSTRACT

The study evaluated the long-term influence of feeding ground barley treated with lactic acid (LA) alone or with LA and heat on performance, energy and protein balance in dairy cows. Thirty cows were fed three diets differing in the treatment of barley grain, either unprocessed ground barley (Control), ground barley steeped in 1% LA at room temperature (LA-treated barley) or ground barley steeped in 1% LA with an additional heating at 55°C (LAH-treated barley). Cows were studied from week 3 to 17 post-partum. Dry matter intake (DMI), milk yield and composition and body weight (BW) were measured daily. Estimated energy and protein balances were calculated and blood samples were collected three times during the experiment and analysed for common metabolites of energy and lipid metabolism. Digestibility of different treated barley and other dietary ingredients was investigated *in vivo* using four wethers. The treatment of barley with LA and LAH increased the digestibility of organic matter (OM) by approximately 5% and the content of metabolisable energy by 0.5–0.6 MJ/kg DM. Data showed no effect of feeding diets containing LA- or LAH-treated barley at 39% of DM on overall DMI, BW, BW change, milk production and composition and on the blood variables studied. Diet influenced the estimated balances of net energy of lactation ($p < 0.01$) and the content of utilisable protein at the duodenum ($p = 0.07$) with cows fed the diet with LA-treated barley showing improved balances. In conclusion, feeding diets containing LA- or LAH-treated barley had no influence on performance, milk composition and blood metabolites, but LA treatment without heat seems to improve the energy balance of cows.

ARTICLE HISTORY
Received 18 August 2016
Accepted 19 October 2016

KEYWORDS
Acidification; barley; dairy cows; digestibility; energy balance; heat treatment; lactic acid; performance

1. Introduction

Feeding of grain-rich diets has become a common practice in dairy cows. Compared to forages, grains are richer sources of metabolisable energy (ME) and their inclusion in

CONTACT Qendrim Zebeli Qendrim.Zebeli@vetmeduni.ac.at
*Leonhard Gruber and Annabella Khol-Parisini contributed equally to this work.

the diet increases the energy intake, supporting high milk production and enhancing cost efficiency. Feeding practices based on grain, however, increase the risk of rumen digestive disorders in ruminants. One approach to alleviate the risk of rumen digestive disorders while maintaining high energy, and hence grain levels in the diet, is to feed cereal grains containing starch with lowered ruminal degradability (Silveira et al. 2007). Depending on region, availability and feeding purpose, maize, barley, wheat, sorghum and triticale are the most important grains used in the feeding of dairy cows. Compared with maize starch, the starches of barley, wheat and triticale have greater ruminal degradability (Benninghoff et al. 2015; Krieg et al. 2016), resulting in greater risk of rumen fermentation disorders when fed in large amounts (Zebeli et al. 2010). Substantial research has been conducted over the years to modify ruminal starch degradation patterns of easily fermentable grains including barley, using various technological settings (reviewed by Deckardt et al. 2013).

Organic acids including lactic acid (LA) have been shown to modify the chemical composition and the nutritive value of grains in multiple experiments. More specifically, it has been shown that LA treatment modified starch and dietary fibre content of the treated cereals (Harder et al. 2015), accelerated phytate degradation (Haraldsson et al. 2004; Metzler-Zebeli et al. 2014; Khol-Parisini et al. 2015), reduced the activity of β-glucanases (Haraldsson et al. 2004) and starch degradation during the first few hours of ruminal incubation (Khol-Parisini et al. 2015). Earlier studies have reported increased propionate in the rumen fluid of sheep and cattle by LA treatment (Morgan and L'Estrange 1976, 1977), suggesting an improved gluconeogenesis and energy status of the animals. In line with those findings, Iqbal et al. (2010) also reported greater glucose, insulin and cholesterol in plasma of dairy cows by feeding them rolled barley treated with LA.

In this study, it was hypothesised that treating barley with LA and heat induces changes in ME content of the treated barley, enhancing energy and protein metabolism as well as performance of cows around the peak of lactation and during the mid-lactation. The aim of this study therefore was to evaluate long-term feed intake, performance, changes in body weight (BW) and energy and protein balance from week 3 until 17 of lactation in dairy cows fed diets differing in the processing of grain. The ME content of unprocessed and processed barleys was determined *in vivo* using adult wethers.

2. Materials and methods

2.1. *Animals and experimental design*

This study was conducted at the Dairy Research Facilities of the Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Styria, Austria. Data of ruminal pH and temperature dynamics, and liver health reactants have been published previously (Humer et al. 2015). A total of 30 cows (12 primiparous and 18 multiparous, 25 Holstein and 5 Simmental), were included in this longitudinal trial. Cows were blocked by expected calving date, breed and parity and were allocated to three different feeding groups (n = 10) according to a block-randomised design. All cows were included in the experiment on day 21 post-partum and sampled until day

120. The three diets differed in barley grain; the control diet (CON) contained untreated ground barley grain, whereas the other two diets consisted of the same barley grain treated with 1% LA alone (Diet LA) or 1% LA plus oven-heating (Diet LAH) prior to feeding. The barley grain used in all diets was ground with a hammer mill (Ultra 110/150, Gruber Machineries, Gaspoltshofen, Austria). The treated barley was soaked daily (1:1, wt:vol) in 1% concentration of L-LA (Brenntag, Vienna, Austria). The LAH treatment included an additional heating in an oven (Memmert Universal oven UF 800, Linder Labortechnik, Vienna, Austria) for 24 h, whereby barley was kept in plastic buckets, covered with a lid during the heat treatment, and the temperature was gradually increased to a target temperature of 55°C, which was kept constant for 12 h. The total soaking time of barley in the LA solution for LA and LAH treatments was 24 h prior to the morning feeding, whereby in the case of LAH treatment, half of this time was at a temperature of 55°C.

Cows were located in a free stall, fed in Calan gates and milked twice daily (04:00 and 15:00 h) in a 6 × 6 tandem milking parlour located adjacent to the barn. All procedures involving animal handling and treatment were in accordance with national regulations for animal use in research and the national authority approved the study according to §9ff of Law for Animal Experiments (GZ FA10A-78Gu-19/2012).

2.2. *Diets, feed intake and BW*

All diets were formulated for early lactation dairy cows according to GfE (2001) guidelines. The diets consisted on dry matter (DM) basis of 50% forages (2nd cut meadow hay, grass silage and maize silage) and 39% barley grain (either CON, LA or LAH) and 11% protein–mineral–vitamin supplement (PMV). The chemical composition and energy content of the diet ingredients are given in Table 1. Average ingredient proportions and the chemical composition of the diets are shown in Table 2. All cows had free access to water. Feed was offered *ad libitum*, at 110% of the average amount of feed, the individual cow consumed on the three preceding days (real average daily feed refusals of 5–10%). Fresh feed was offered twice a day (04:30 and 16:00 h), whereby the cows received the roughage (hay and grass silage) portion first, followed by their barley allowance and the maize silage. Thereafter, the feedstuffs were offered again for a minimum of 30 min or until finished. A computer-operated central feeding station was located in the free stall of the animals and offered their PMV allowance throughout the day. The BW of the cows was recorded daily after milking in an electronic scale integrated in the automatic feeders and the BW change between week 3 and 17 was calculated.

2.3. *In vivo digestibility trial with wethers*

Digestibility trials were carried out according to GfE (1991) guidelines for determination of crude nutrient digestibility in ruminants, using four adult wethers per dietary ingredient (Gruber et al. 2014). The digestibility of concentrates was determined according to the so-called difference method described by Giger and Sauvant (1983), using concentrate levels of 0% and 50% of DM, respectively. The wethers were fed near maintenance level (1 kg DM per day, 2 meals per day). Feed intake and excretion of

Table 1. Analysed chemical composition (n = 10) and *in vivo* digestibility of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF) and non-fibre carbohydrates (NFC), determined in four wethers/ingredient, as well as energy concentration and protein value of the different dietary ingredients.

					Barley grain[♦]		
	Hay	Maize silage	Grass silage	PMV[Δ]	CON	LA	LAH
DM[◊] [%]	88.6	30.8	32.0	90.8	87.1	48.1	48.2
OM [% of DM]	88.6	95.1	86.9	84.1	96.7	97.3	97.4
CP [% of DM]	13.5	6.9	13.9	39.5	13.2	11.6	11.7
EE[‖] [% of DM]	2.4	3.3	2.7	2.0	2.6	2.1	2.2
aNDFom[$] [% of DM]	45.0	44.9	47.1	16.7	19.6	20.3	20.7
ADFom[¶] [% of DM]	28.6	26.7	33.1	13.4	5.9	7.1	6.0
ADL[+] [% of DM]	3.5	3.1	4.2	4.7	0.9	1.1	0.9
NFC* [% of DM]	27.7	40.1	22.1	30.8	61.4	63.2	62.7
Digestibility [%]							
OM	68.4	71.5	69.4	81.6	80.7	85.2	83.7
CP	59.7	46.0	56.8	85.2	72.2	73.1	73.5
NDF	65.6	59.1	68.3	59.3	39.7	55.9	49.1
NFC	80.3	90.7	82.3	93.8	95.8	96.8	96.7
ME[†] [MJ/kg DM]	9.17	10.46	9.57	11.07	12.12	12.72	12.58
NE_L[‡] [MJ/kg DM]	5.40	6.26	5.65	6.77	7.52	8.01	7.89
uCP[#] [g/kg DM]	123.2	127.1	126.1	231.8	157.9	159.9	158.7
RNB[§] [g/kg DM]	0.74	−7.82	2.34	26.3	−4.16	−7.07	−6.66

Notes: [Δ]PMV, protein–mineral–vitamin premix, contained 45.48% soybean meal, 45.48% rapeseed meal, 5.04% limestone, 2% salt and 2% mineral–vitamin supplement (contained per kg: Ca 6%, P 12%, Mg 10%, Na 8%, Mn 1500 mg, Zn 5700 mg, Cu 800 mg, Fe 150 mg, Se 30 mg, I 113 mg, Co 50 mg, vitamin A 750,000 IU, vitamin D_3 75,000 IU, vitamin E 3000 mg); [♦]barley grain was either untreated (CON), treated with 1% lactic acid (LA), or treated with 1% lactic acid and heat (LAH); [◊]DM, dry matter; [‖]EE, ether extract; [$]aNDFom, NDF (treated with α-amylase and exclusive of residual ash); [¶]ADFom, acid detergent fibre, exclusive of residual ash; [+]ADL, acid detergent lignin; *NFC, non-fibre carbohydrates = 100 − (NDF [%] + CP [%] + ether extract [%] + ash [%]); [†]ME, metabolisable energy, based on *in vivo* digestibility coefficients and calculated according to GfE (1995); [‡]NE_L, net energy lactation, based on *in vivo* digestibility coefficients and calculated according to GfE (1995); [#]uCP, utilisable crude protein at duodenum (GfE 2001); [§]RNB, ruminal N balance (GfE 2001).

Table 2. Ingredients and chemical composition of diets based on untreated barley (CON), barley steeped in 1% lactic acid (LA) solution (Diet LA) or barley steeped in 1% lactic acid solution with heat (LAH) (n = 10).

	Experimental diets		
	CON	LA	LAH
Ingredients [% of DM[◊]]			
Hay	15	15	15
Maize silage	19	19	19
Grass silage	16	16	16
Barley grain (CON, LA or LAH)	39	39	39
Protein–mineral–vitamin mix	11	11	11
Chemical composition			
DM [%]	66.1	50.7	50.7
Organic matter [% of DM]	92.6	92.7	92.9
Crude protein [% of DM]	15.2	14.6	14.6
Ether extract [% of DM]	2.8	2.7	2.7
$aNDF_{OM}$[$] [% of DM]	32.8	33.2	33.4
ADF_{OM}[¶] [% of DM]	18.4	18.9	18.6
ADL[+] [% of DM]	2.4	2.5	2.5
NFC* [% of DM]	42.0	42.5	42.2
NE_L[†] [MJ/kg DM]	6.58	6.76	6.71
uCP[‡] [g/kg DM]	149.7	150.6	150.0
RNB[#] [g/kg DM]	0.37	−0.73	−0.64

Notes: [◊]DM, dry matter; [$]aNDFom, neutral detergent fibre (treated with α-amylase and exclusive of residual ash); [¶]ADFom, acid detergent fibre, exclusive of residual ash; [+]ADL, acid detergent lignin; *NFC, non-fibre carbohydrates = 100 − (NDF [%] + CP [%] + ether extract [%] + ash [%]); [†]NE_L, net energy lactation, based on *in vivo* digestibility coefficients of individual feedstuffs and calculated according to GfE (1995); [‡]uCP, utilisable crude protein at duodenum (GfE 2001); [#]RNB, ruminal N balance (GfE 2001).

faeces were measured daily while animals were kept in metabolism cages throughout the trial. The experiment lasted for 4 weeks, with the first 2 weeks serving for diet adaption and the last 2 weeks for sampling the excretion. Nitrogen of faeces was determined in fresh material to avoid N losses during drying. Feed and faeces were analysed according to the standard procedure outlined below.

2.4. *Feed sampling, chemical analyses and energy and protein evaluation*

Feed and orts samples were collected daily and pooled for a 4 week sample. The DM was determined after oven-drying for 72 h at 55°C and then ground through a 1-mm screen. The underestimation of the DM content due to the loss of volatile substances (volatile fatty acids, LA and ammonia) occurring during oven drying was accounted for by applying equations of Weissbach and Kuhla (1995). The ground samples were analysed for N using the Dumas method, ether extracts (EE) and ash using the official methods (Naumann and Bassler 2012). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using the methods described by Van Soest et al. (1991), with heat stable α-amylase used in the NDF procedure. The NDF and ADF were expressed exclusive of residual ash (i.e. aNDFom and ADFom). The content of ME and net energy of lactation (NE_L) of the feedstuffs was calculated based on their chemical composition and the *in vivo* digestibility coefficients of individual ingredients determined in the digestibility trial with wethers as described above using the respective equations as outlined by GfE (1995). The content of utilisable protein at the duodenum (uCP) and ruminal N balance (RNB) was estimated according to GfE (2001).

2.5. *Milk production and composition*

To collect data of milk yield and composition, the cows' milk yields were recorded and milk samples were collected daily during the morning and afternoon milking and preserved using sodium dichromate. Milk samples were analysed for fat, protein, lactose, milk urea nitrogen (MUN) and somatic cell counts (SCC) by Milkoscan (Foss Electric, Hillerød, Denmark).

2.6. *Blood sampling and analysis*

Blood samples were collected shortly before the morning feeding from the coccygeal vein on experimental days 21 (baseline), 40 and 90 using 10-ml serum vacutainer tubes (Vacuette, Greiner bio-one, Kremsmünster, Austria). Blood samples were stored at room temperature for 30 min to allow clotting and were subsequently centrifuged at 3000*g* and 4°C for 20 min (Eppendorf centrifuge, Hamburg, Germany) and stored at −20°C until analyses.

The concentrations of glucose, lactate, cholesterol, blood urea-N, total protein, β-hydroxybutyric acid (BHBA), non-esterified fatty acids (NEFA), insulin, Ca, P and cortisol were measured on a conventional large-scale analyser for clinical chemistry at the laboratory of the Central Clinical Pathology Unit, University of Veterinary Medicine, Vienna. Standard enzymatic colourimetric analyses with a fully automated

autoanalyser for clinical chemistry (Cobas 6000/c501; Roche Diagnostics GmbH, Vienna, Austria) were used.

2.7. *Estimated balance of NE_L and uCP*

The estimated daily energy and protein balance was calculated by subtracting requirements for maintenance and milk yield (based on GfE 2001) from the respective individual measures of energy and protein intake, as outlined in detail by Gruber et al. (2014).

2.8. *Statistical analyses*

All data, except of blood variables, were averaged on weekly basis for the statistical analysis. The statistical analysis was performed using PROC MIXED of SAS (SAS, version 9.4). The model included the fixed effects of diet and sampling/measurement time (day or week), as well as the two-way interaction, whereas breed and lactation number were considered as random effects in the analysis. The baseline measurements taken the week before starting the feeding experiment were considered as covariates in the model of the analysis of covariance (ANCOVA). The measurements taken on the same cow but at different times were considered as repeated measures in the model of ANCOVA with various variance–covariance matrixes implemented in SAS used to account for the time dependency. For repeated measures, a first-order autoregressive (AR-1) covariance structure was chosen; regarding the random effects, the variance components covariance structure yielded the lowest AIC and was therefore selected. Degrees of freedom were estimated with the method of Kenward–Roger. The significance level was set at $p \leq 0.05$, and trends were considered at the $0.05 < p \leq 0.10$ level.

3. Results

3.1. *Nutrient composition of grains and diets, digestibility and intakes of DM, NE_L and uCP*

The analysed chemical composition, digestibility *in vivo* as well as energy concentration and protein value of the different dietary ingredients are presented in Table 1. The nutrient profile of unprocessed and processed barley showed some differences. The contents of ash, CP and EE were lower after LA treatments, whereas the ADFom was elevated by 20% after LA but not after LAH treatment. Especially, the digestibility of OM and NDF was increased by LA and less by LAH treatment. Moreover, the ME and NE_L contents were 0.4–0.6 MJ/kg higher after the treatments, whereby, as a consequence, the negative RNB became more pronounced after the treatments, too.

The dry matter intake (DMI) and the intake of NEL and uCP, and the BW of cows are shown in Table 3. The intake was not influenced by diet, only by the week postpartum, whereby the cows increased the intake and BW with distance from parturition. Barley treatments did not affect the final BW and only numerically enhanced the weight gain of the cows by an average of 15 kg ($p = 0.59$).

Table 3. Effects of feeding diets containing either untreated barley (CON), barley steeped in 1% LA solution (Diet LA), or treated with 1% LA and heat (Diet LAH) and time (week) on the intake of dry matter, energy and protein, as well as BW change, milk production and composition of cows from week 4 to 17 post-partum (n = 10).

	Experimental diet				*p*-Value		
	CON	LA	LAH	SEM[◊]	Diet	Time	Diet × Time
Total DMI* [kg/d]	20.2	20.6	20.1	0.76	0.64	<0.01	0.86
Concentrate DMI [kg/d]	10.0	10.3	10.0	0.43	0.56	<0.01	0.85
Total NE_L[†] intake [MJ/d]	132.9	138.8	134.5	4.87	0.34	<0.01	0.83
Total uCP[‡] intake [kg/d]	2.98	3.09	2.99	0.10	0.47	<0.01	0.87
Initial BW[#] (week 4) [kg]	692	660	675	65.2	0.25	–	–
Final BW (week 17) [kg]	713	697	709	59.4	0.55	–	–
BW gain [kg]	21.0	37.1	34.5	11.7	0.59	–	–
Milk yield [kg/d]	28.9	28.5	27.8	0.95	0.46	<0.01	0.95
ECM[$] [kg/d]	27.7	27.9	27.2	0.69	0.50	0.08	0.67
Milk fat [%]	3.81	3.92	3.96	0.082	0.43	<0.01	0.96
Milk protein [%]	3.21	3.32	3.35	0.052	0.10	<0.01	0.24
Milk lactose [%]	4.71	4.71	4.76	0.034	0.15	<0.01	0.07
Milk fat [kg/d]	1.10	1.09	1.06	0.076	0.82	0.49	0.79
Milk protein [kg/d]	0.92	0.92	0.89	0.081	0.89	<0.01	0.76
Milk lactose [kg/d]	1.36	1.34	1.32	0.031	0.71	<0.01	0.96
Fat:protein ratio	1.20	1.20	1.18	0.025	0.77	<0.01	0.96
Milk urea nitrogen [mg/dl]	15.4	15.5	16.2	1.45	0.76	<0.01	0.95
Somatic cell counts [cells/µl]	89.5	104.9	66.6	29.62	0.66	0.09	0.89

Notes: [◊]SEM, standard error of the mean; *DMI, dry matter intake; [†]NE_L, net energy lactation; [‡]uCP, utilisable crude protein at duodenum; [#]BW: body weight; [$]ECM, energy-corrected milk.

3.2. *Milk production and composition*

The highest milk production was observed during week 5–6 of the lactation, ranging between 29 and 32 kg/d. Data showed no overall effect of the diet on milk production and composition (Table 3). The treatment only tended to increase the milk protein content (p = 0.10). The average milk fat percentage ranged from 3.81% to 3.96%, without any effects of diet but influenced by the week post-partum. With advancing time after parturition, most of the milk constituents decreased.

3.3. *Serum variables of energy, protein and mineral metabolism*

The different dietary treatments did not influence most of the measured blood serum traits (Table 4). Blood variables were affected by the week post-partum. The only effect of diet was a tendency to increase Ca concentration in the serum with experimental diets LA and LAH (p = 0.06).

3.4. *Estimated energy and protein balance*

Changes of the energy and uCP balances are shown in Figure 1. The estimated energy balance was influenced by diet and week post-partum [p < 0.01; Figure 1(a)]. Comparisons among treatments revealed significant differences between the Control group of cows and their LA counterparts (p < 0.01), but not with Diet LAH. Diet tended to influence the estimated balance of uCP (Figure 1(b)). Again, lower values were found

Table 4. Effects of feeding diets containing untreated barley (CON), barley steeped in 1% LA solution (Diet LA), or treated with 1% LA and heat (Diet LAH) and sampling time (day) on selected blood metabolites, minerals and hormones in dairy cows (n = 10).*

	Experimental diets				*p*-Value		
	CON	LA	LAH	SEM♦	Diet	Time	Diet × Time
Glucose [mg/dl]	67.2	66.7	68.7	1.87	0.46	<0.01	0.31
Insulin [µU/dl]	7.64	7.45	7.26	0.675	0.92	0.01	0.44
BHBA† [mmol/l]	0.49	0.43	0.41	0.054	0.64	0.92	0.22
NEFA‡ [mmol/l]	0.24	0.26	0.20	0.036	0.48	<0.01	0.08
Cholesterol [mg/dl]	176	168	166	9.24	0.43	<0.01	0.06
Lactate [mmol/l]	0.81	0.97	1.00	0.284	0.73	0.11	0.57
Cortisol [µg/dl]	1.46	1.70	1.74	0.266	0.40	–	–
BUN# [mg/dl]	19.6	18.3	21.4	2.77	0.29	0.96	0.56
Total protein [g/dl]	6.77	6.91	6.96	0.129	0.25	0.02	0.75
Calcium [mmol/l]	2.34	2.36	2.42	0.057	0.06	0.48	0.69
Phosphorus [mmol/l]	1.87	1.96	1.82	0.093	0.54	0.02	0.99

Notes: *Blood metabolites were measured on days 40 and 90 post-partum, cortisol only on day 40 post-partum; ♦SEM, standard error of the mean; †BHBA, β-hydroxybutyrate; ‡NEFA, non-esterified fatty acids; #BUN, blood urea nitrogen.

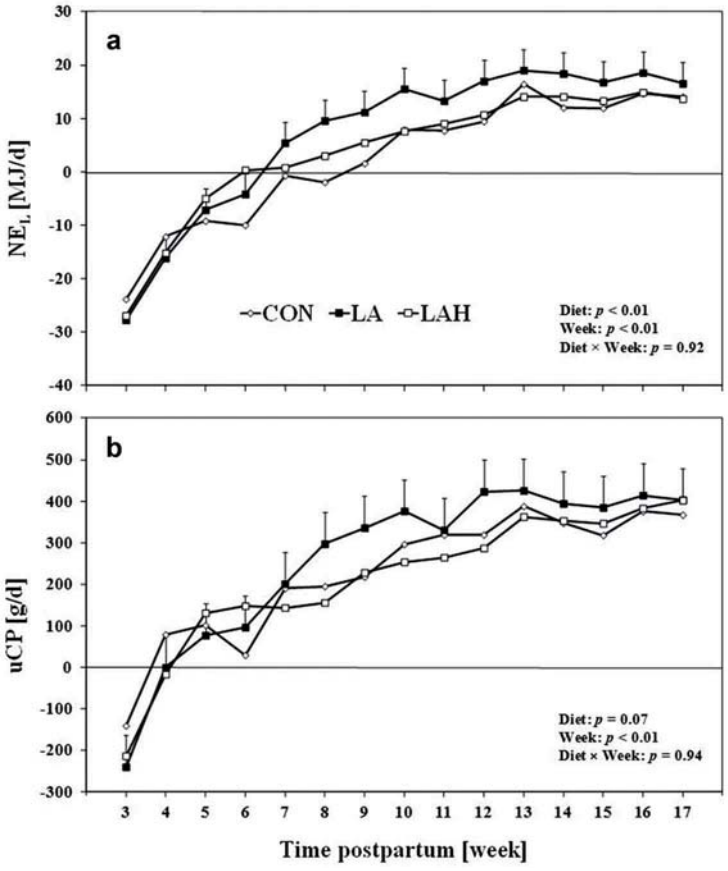

Figure 1. Estimated balances of net energy lactation (panel a) and utilisable crude protein (uCP) at the duodenum (panel b) from week 3 to 17 post-partum in cows of the 3 experimental groups [fed diets containing untreated barley (CON), barley steeped in 1% lactic acid solution (Diet LA), or steeped in 1% lactic acid solution with heat (Diet LAH)]; error bars represent pooled standard error of the means for all treatments (n = 10).

for the Control group of cows compared to their LA counterparts (p = 0.07), but not compared with LAH cows, which did not differ from CON.

4. Discussion

The main aim of the present study was to evaluate the long-term influence of feeding barley grain processed with LA, with or without heat treatment, on feed intake, performance, BW, as well as energy and protein balance in dairy cows during the entire early lactation and mid-lactation. The treatment of barley with LA and LAH resulted in changes of the chemical composition of barley, including an elevation of its OM content, at the expense of ash, and also of the carbohydrate fractions (aNDFom plus NFC), at the expense of CP and EE. These findings corroborate our previous investigations (Deckardt et al. 2014; Harder et al. 2015; Khol-Parisini et al. 2016) and suggest a modification of chemical composition and nutritive value of barley as well as leakage of various nutrients after the treatment with LA and LAH. For example, lower CP levels can be explained by the washing out effect of 1% LA on soluble protein fractions of barley including non-protein–nitrogen, an effect which was associated with lowered RNB of the treated barleys. Furthermore, an enhanced energy content of the barley after LA treatments by almost 0.4–0.7 MJ ME per kg DM was observed, which is mainly explained by the elevated OM digestibility of the treated barleys, especially with LA. Indeed, the OM digestibility increased from 80.7% in CON to 85.2 and 83.7% in LA and LAH barley, respectively, which might be partly explained by the enhanced OM content of the treated barleys due to leaching of ash. Furthermore, the improvement might be due to more favourable ruminal fermentation processes due to a shift of the site of nutrient degradation (i.e. starch) from the rumen to the small intestine (Khol-Parisini et al. 2015), thus relieving the rumen metabolism. Detailed information regarding the starch degradation kinetics of the different barleys is published in a companion paper (Khol-Parisini et al. 2015). In brief, lower starch disappearance was found in barley processed with LA and LAH after 2 and 4 h of incubation. The lag time of starch degradation as well as the fractional disappearance of starch tended to be decreased in the LA- and LAH-treated barleys, too. The speculation of improved ruminal fermentation processes in the wethers used in the present study is further supported by the enhanced total tract NDF digestibility, showing an increase from 39.7% to 55.9% and 49.1% in barley processed with LA and LAH, respectively. Moreover, the additional energy input through the inclusion of LA in the soaking solution (i.e. 10 g LA/kg grain) has to be taken into account. Assuming an energy content of 15.1 kJ/g LA, the LA treatment provided additional 0.151 MJ/kg DM of treated barley. Thus, the additional LA energy does only partly explain the increase of the energy content of the treated barleys. Summing up, treating barley with LA increases energy content by 5.0–6.5% which is mainly due to enhanced OM digestibility, a shift in nutrient composition and the addition of LA. However, due to differences between sheep fed at maintenance level and high yielding dairy cows, especially with regard to retention time, the measured effects on digestibility in wethers cannot be directly transferred to dairy cows. Nevertheless, it was also expected to observe positive effects of the barley treatments on the digestibility of nutrients in dairy cows. Although, in the present study, the total tract digestibility in the cows was not determined, this was performed with the same

experimental diets in a study with fistulated cows (Khol-Parisini et al. 2015). Indeed, in that study, enhanced apparent total tract digestibility of DM was found in cows fed the diets containing LA- or LAH-treated barleys compared to cows fed the control diet.

The data showed that the processing of barley did not affect DMI, and the daily increases in NE_L and uCP did not reach significant levels. Earlier studies performed in sheep and cattle have shown little effect of LA on DMI using LA dosages ranging from 600, 800 or 1000 mmol/kg DM (Morgan and L'Estrange 1977); thus, LA doses comparable to the concentrations used in this study. Other studies where barley grain was treated with 0.5% LA at room temperature for 48 h (Iqbal et al. 2009) or 1% LA at 55°C for 48 h (Iqbal et al. 2012) and fed at about 30% of DM as TMR also reported no difference in DMI among groups of dairy cows. However, by increasing the concentration of LA to 5%, Khol-Parisini et al. (2016) observed a decreased DMI in cows during early lactation.

The diet also showed no overall effect on milk yield and composition in this study. Earlier studies by Iqbal et al. (2009, 2012) reported an elevation of the milk fat content by LA treatments. In their control animals, the authors observed low milk fat percentages of around 2.6% and 3.1%, which were accompanied by a significant risk of developing subacute rumen acidosis (SARA), a condition which was alleviated by the treatments of grains with LA, that showed significant effects on both, milk fat and ruminal pH. In contrast, the cows of the study reported here were not at risk of developing SARA, and the effect of LA treatment on ruminal pH was minimal (Humer et al. 2015). Furthermore, in contrast to the studies reported by Iqbal et al. (2009, 2012), in the present study, the milk fat content of cows fed control barley was relatively high (3.7%), which might have been the reason that it did not show any further response to the treatments.

No effects of treatments on serum metabolites were detected in this study. Due to the reported changes in the ruminal metabolism (i.e. enhanced propionate concentration) and increased by-pass starch of treated barleys (Khol-Parisini et al. 2015; Metzler-Zebeli et al. 2015), corresponding effects on blood metabolic traits, like higher glucose or insulin levels, or lowered BHBA and NEFA in the treatment groups were expected in this study. However, the relatively low milk yield of the cows in this study and the lack of mobilisation (i.e. low values of both NEFA and BHBA, as well as the increase of BW gain) might have contributed to the fact that changes of blood metabolites are missing. The tendency for greater Ca levels in the blood of cows receiving diets with LA- and LAH-treated barley is an interesting finding of the study, although the Ca levels were within normal range and the increase is of limited physiological relevance. The exact mechanisms behind this linear increase of blood Ca after feeding the LA diet (2.34, 2.36 and 2.42 mmol/l for Groups CON, LA and LAH, respectively) are not understood and need further investigations. It is hypothesised that an improved hydrolysis of phytate by these treatments (Metzler-Zebeli et al. 2014; Khol-Parisini et al. 2015, 2016), with barley having almost 60% of its P as phytate-P (Metzler-Zebeli et al. 2014), might have increased the availability of dietary Ca.

The estimated energy balance showed differences between the Control group of cows and their LA counterparts. An improved energy balance is vital for cows, especially during the weeks before the peak of lactation, when cows have not achieved their maximum DMI levels yet. The cows receiving diets containing LA-treated barley were in a positive energy balance starting from week 6 to 7 post-partum (i.e. peak of lactation), whereas the cows of

Group CON reached a positive NE_L balance on week 8–9 post-partum. In addition, the uCP balance changed with an overall tendency of an enhancing effect of the LA treatment when compared to CON. The fact that the LA treatment enhanced energy balance but not milk production of the cows indicates that this treatment stimulates energy retention in form of body reserves rather than milk production, possibly because of the low milk production potential of the cows (Roche et al. 2009) in the present study. Besides increasing the energy content of barley by roughly 0.5 MJ NE_L/kg, other mechanisms by which the LA treatment resulted in an improved energy status enhanced the discrepancy between DMI and milk yield (e.g. numerically higher energy intake with similar or lower milk production). Furthermore, the effects of the LA treatment on rumen fermentation, most importantly on higher propionate production observed previously (Morgan and L'Estrange 1976, 1977; Iqbal et al. 2009; Khol-Parisini et al. 2015; Metzler-Zebeli et al. 2015), have to be taken into account. Such differences in the rumen fermentation could have had an impact on the gluconeogenesis, stimulating the energy balance of the cows during and after the peak of the lactation. However, when interpreting the current findings, one has to consider that the balances of NE_L and uCP are estimations. Nevertheless, to improve the goodness of the estimations, the NE_L contents of the feeds were determined using an *in vivo* digestibility trial and BW, separate intake of each feed component, milk yield as well as milk constituents were evaluated on a daily basis.

Overall, the additional heat treatment seemed to lessen the positive effects on estimated NE_L and uCP balances compared to the pure LA treatment. In general, the LAH-treated barley showed similar energy contents compared to the LA-treated barleys, and also comparable effects on nutrient degradation characteristics, ruminal fermentation profiles and pH dynamics have been observed previously (Humer et al. 2015; Khol-Parisini et al. 2015; Metzler-Zebeli et al. 2015). Therefore, differences between those two treatments likely derive from the numerically lower DMI as well as differences in initial BW of the cows fed LAH diets compared to LA diets. In considering the costs of heating, the present results do not provide any practical support for additional heating treatment of grain in dairy cattle feeding.

5. Conclusion

In conclusion, treating barley grain with LA or LAH modulated the carbohydrate fractions, lowered ash, CP and RNB contents. Most importantly, the study showed an increased digestibility of OM and NDF, and thereby greater ME and NE_L contents of the treated barleys. These results suggest an improved feeding value of the treated barleys. The treatments did not affect long-term performance and feed intake of the cows, but the treatment with LA alone enhanced the estimated balances of uCP and particularly of NE_L. Further research is warranted to elucidate the mechanisms behind the partitioning of the energy in cows fed the LA-treated diet in favour of energy retention rather than milk production.

Acknowledgements

The authors thank S. Gschaider and A. Rudischer (Institute of Animal Nutrition and Functional Plant Compounds, Vetmeduni Vienna) and the staff of the research station at AREC Raumberg-

Gumpenstein, especially A. Schauer and A. Adelwöhrer, for their excellent organisation of the animal trials and care to the cows. This research was supported by the project "optGerste" of the Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW, Austria).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW, Austria).

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J. Dairy Sci. 98:6433–6448
http://dx.doi.org/10.3168/jds.2015-9522

Long-term reticuloruminal pH dynamics and markers of liver health in early-lactating cows of various parities fed diets differing in grain processing

E. Humer,*[1] A. Khol-Parisini,*[1] L. Gruber,† J. Gasteiner,† Sh. M. Abdel-Raheem,*[2] and Q. Zebeli*[3]
*Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, Vetmeduni Vienna, Veterinaerplatz 1, 1210 Vienna, Austria
†Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Raumberg 38, 8952 Irdning, Austria

ABSTRACT

The present study aimed to investigate the long-term effect of feeding barley grain steeped in lactic acid (La) with or without thermal treatment on reticuloruminal pH dynamics and metabolic activity of the liver in 12 primiparous and 18 multiparous early-lactating dairy cows. All cows were included on d 21 postpartum and sampled until d 90 postpartum. Cows were fed a diet based on differently processed ground barley grain: untreated grain (control diet, CON), or grain treated with 1% La alone for 24 h before feeding (La), or with an additional oven-heating at 55°C for 12 h (LaH). The reticuloruminal pH and temperature were measured via indwelling sensors that allowed for continuous (every 10 min) and long-term measurement from d 21 to 80 postpartum. Blood samples were taken on d 21, 40, and 90 of lactation and analyzed for liver enzymes aspartate aminotransferase (AST), gamma-glutamyltransferase, and glutamate dehydrogenase, as well as bilirubin, bile acids, and serum amyloid A. Dry matter intake was higher in multiparous cows (20.7 ± 0.27 kg/d) compared with primiparous cows (18.2 ± 0.33 kg/d), but was not affected by dietary treatment. Overall, the relatively short duration (51 ± 5 min/d) of reticuloruminal pH <5.8 suggests low risk of subacute ruminal acidosis throughout the experiment. Results indicated that La treatment of barley, with or without heat, lowered the time duration of pH <5.8 compared with CON, but only in primiparous cows (from 118 ± 13 to 46 ± 11 and 25 ± 11 min/d for CON, La, and LaH, respectively). In multiparous cows, the opposite effect of feeding the La-treated barley on time duration of pH <5.8 (11 ± 8 vs. 46 ± 9 vs. 57 ± 9 min/d for CON, La, and LaH, respectively) was observed. Multiparous cows generally showed higher pH readings and shorter periods in which the ruminal pH dropped below the threshold of pH 5.8. The reticuloruminal temperature was not affected by dietary treatment, whereas parity affected the time duration of reticuloruminal temperature >39.5°C, being 60 ± 19 min/d shorter in primiparous cows. The measured activities of the liver enzymes AST, gamma-glutamyltransferase, and glutamate dehydrogenase, as well as bilirubin, bile acids, and the acute phase protein serum amyloid A, were not affected by grain feeding. Additionally, only one small effect of parity on investigated serum variables was noticed, showing slightly but significantly higher values of AST in multiparous (80.5 ± 1.4 U/L) compared with primiparous cows (76.0 ± 1.7 U/L). In conclusion, our results indicate greater risk for primiparous cows to develop subacute ruminal acidosis-like conditions during early lactation than multiparous cows. The study also suggests limited benefits of feeding processed barley grain with La with or without thermal treatment to modulate ruminal tolerance of grain feeding, whereby differing effects in primiparous cows were observed compared with multiparous cows.

Key words: rumen pH, parity, liver health, grain processing, lactic acid

INTRODUCTION

Inclusion of large amounts of grain in the diet is a common practice in dairy cows during early lactation. This feeding practice is useful to alleviate the energy deficit but also keeps the cows at high risk of developing rumen fermentation disorders such as SARA. Subacute ruminal acidosis is characterized by intermittent and moderate drops of ruminal pH in response to highly fermentable diets (Plaizier et al., 2008; Zebeli and Metzler-Zebeli, 2012). Because this disorder lacks overt clinical signs, its diagnosis relies mostly on ruminal pH measurements; however, single-point measurements of rumen pH are not reliable for predicting the

Received March 3, 2015.
Accepted June 7, 2015.
[1]These authors contributed equally to this paper.
[2]Present address: Department of Animal Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.
[3]Corresponding author: Qendrim.Zebeli@vetmeduni.ac.at

risk of SARA compared with measuring the duration of pH below certain SARA thresholds (Penner et al., 2007), which can be obtained by continuous monitoring of rumen pH (Penner et al., 2007; Klevenhusen et al., 2014). Subacute ruminal acidosis is a prevalent metabolic disorder of cattle, and metabolic stress associated with SARA primarily afflicts the energy and lipid metabolism in different body tissues, most importantly in the liver and the mammary gland (Bionaz et al., 2007; Zebeli and Ametaj, 2009; Chang et al., 2015). A long duration of a low ruminal pH may result in the activation of the acute phase response (**APR**), commonly perceived as increased concentrations of acute phase proteins such as serum amyloid A (**SAA**) in the blood of affected cows (Plaizier et al., 2008).

Dairy cow diets should contain sufficient forages rich in physically effective NDF (**peNDF**) to prevent SARA (Yang and Beauchemin, 2007; Zebeli et al., 2012). However, during early lactation, diets typically contain lower proportion of forage compared with late-lactation diets due to the energy deficit and the need to alleviate it by highly fermentable, energy-rich diets (Zebeli et al., 2010). One promising approach to prevent SARA without the need to change the diet's forage proportion is to feed cereal grains or cultivars with low ruminal degradability (Silveira et al., 2007a,b; Nasrollahi et al., 2012). Studies conducted by Iqbal et al. (2009, 2012) found, for the first time in cattle, that treating barley grain with lactic acid (**La**) alleviated the risk of SARA when fed to late-lactation cows. Processing of barley grain with La has also been shown to enhance the content of slowly digestible starch and fibers and to trigger the hydrolysis of phytate P in more recent studies (Deckardt et al., 2014, 2015; Metzler-Zebeli et al., 2014). An additional thermal processing of barley with 50 to 55°C and La showed synergistic effects in terms of starch composition, its microscopic structure, and fiber fractions as well as in the hydrolysis of phytate P (Metzler-Zebeli et al., 2014; Harder et al., 2015a,b). However, less is known about feeding La-treated grains in early-lactating dairy cows. Several events such as the sudden reduction of dietary peNDF and the lack of an adequate adaptation of the rumen to a highly fermentable diet make early-lactating dairy cows more susceptible to SARA (Penner et al., 2007; Zebeli et al., 2008; Bannink et al., 2012). Apart from the stage of lactation, SARA susceptibility may also be influenced by parity, with first production heifers being differently sensible to dietary changes than multiparous cows that have experienced more high-grain diets.

Because of the aforementioned beneficial effects of La and thermal treatments on the carbohydrate fractions of barley such as greater content of slowly digestible starch and fibers, we hypothesized that feeding of treated barley modulates reticuloruminal pH dynamics in early-lactating dairy cows. Another hypothesis of the study was that the effects of barley processing on reticuloruminal pH and metabolic activity of the liver are different in primiparous vs. multiparous dairy cows due to their presumably different sensibility against fermentable diets. Therefore, the aim of this study was to evaluate long-term dynamics of reticuloruminal pH and temperature, and the metabolic activity of the liver in early-lactating primiparous and multiparous dairy cows fed differently processed barley grain.

MATERIALS AND METHODS

Animals and Experimental Design

The longitudinal trial was conducted at the Dairy Research Facilities of the Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Austria, from June 2012 to March 2013. A total of 30 (12 primiparous and 18 multiparous) early-lactating cows, including 25 Holstein and 5 Simmental cows, were used. At the beginning of the trial, at d 20 postpartum, Holstein cows weighed 599 ± 56.4 kg (mean ± SD) and produced 28.4 ± 5.7 kg milk/d, whereas Simmental cows weighed 708 ± 73.4 kg and produced on average 25.3 ± 4.2 kg milk/d. Cows were blocked by expected calving date, breed and parity and were randomly allocated to 1 of 3 different feeding groups (10 cows in each group) using a longitudinal block-randomized design. The feeding experiment started at d 21 postpartum, and cows were sampled for this study until d 90 postpartum. The 3 experimental diets were alike, except for the processing of the barley, which was ground with a hammer mill (Ultra 110/150, Gruber Machineries, Gaspoltshofen, Austria) at 3-mm screen and fed as such for the untreated control group (**CON**), ground and steeped 1:1 (wt/vol) in 1% L-lactic acid (Brenntag, Vienna, Austria) for 24 h at room temperature (i.e., La), or ground and steeped in L-lactic-acid plus heating at 55°C for 12 h (**LaH**). Preparation of La solutions and chemical treatment of barley grain has been described previously (Deckardt et al., 2014). The chemical composition of the untreated and treated barley grains is shown in Table 1.

All diets were formulated to meet the energy and nutrient requirements of dairy cows of 650 kg of BW, producing 35 kg milk/d with 4% fat, and 3.3% protein (GfE, 2001). Diets were designed to provide (DM basis) 50% forage (15% meadow hay, 15% grass silage, 20% corn silage), and 50% concentrate [38.5% barley grain (CON, La, or LaH), and 11.5% of a protein-mineral-vitamin mix (**PMV**)]. The PMV contained extracted soybean- and rapeseed meal in equal proportions,

Table 1. Analyzed chemical composition of barley grains untreated (CON), or treated with 1% lactic acid (La), or with 1% lactic acid and heat (LaH)

	Barley grain		
Item[1]	CON	La	LaH
% of DM unless stated			
DM, % fresh matter	87.1	48.1	48.2
OM	96.7	97.3	97.4
CP	13.2	11.6	11.7
Ether extract	2.6	2.1	2.2
aNDFom	19.6	20.3	20.7
Starch	61.1	59.3	60.7
ME, MJ/kg of DM	12.1	12.7	12.6
NE_L, MJ/kg of DM	7.52	8.01	7.89

[1]The contents of ME and net NE_L of the barley grains were calculated according to Gruber et al. (2014). aNDFom = amylase-treated NDF determined on an organic matter basis.

limestone, salt, and a commercial mineral-vitamin premix for lactating dairy cows. The analyzed chemical composition of the forages and the PMV is shown in Table 2, and the chemical composition and particle size (**PS**) distribution of the experimental diets (accounting for different intakes by each group) are shown in Table 3. All cows had free access to water. Cows were fed ad libitum in individual Calan gates, being offered 110% of the average amount of feed consumed on the 3 preceding days (realized average daily feed refusals of 5 to 10%). Fresh feed was offered twice a day (0430 and 1600 h), whereby the cows received their roughage (hay and grass silage) portion first, followed by their barley allowance and corn silage, subsequently being offered each component again for at least 30 min or until finished. A computer-regulated feeding station was centrally located in the stable to individually and continuously offer the proportion of PMV.

During the experiment, cows were located in a free stall. All procedures involving animal handling and treatment were in accordance with national regulations for animal use in research and approved by the authority according to §9ff, Law for Animal Experiments (GZ FA10A-78Gu-19/2012–2).

Feed Sampling and Chemical Analyses

Amounts of feed offered and refused were recorded daily to determine the feed intake of cows by difference. Feed and orts samples were collected weekly. They were mixed and aliquots were used for analyses of nutrient composition and PS distribution. Dry matter was determined after oven drying for 72 h at 55°C. The ground samples (1-mm screen) were analyzed for N using the Kjeldahl method, and ether extract and ash were measured according to the official methods (Naumann and Bassler, 2012). The NDF and ADF were determined using the methods described by Van Soest et al. (1991), with heat-stable α-amylase used in the NDF procedure. The starch contents of the barley grains were determined enzymatically according to the analysis procedure [AOAC (2002) Official Method 2002.02; AACC (2002) 101 Method 32–40] provided by the Megazyme Kit (Megazyme International Ireland Ltd. Co., Wicklow, Ireland). Calculation of energy concentration of feedstuffs was based on their crude nutrient composition and in vivo digestibility coefficients of individual ingredients determined with the method described by Gruber et al. (2014). The PS distribution was determined using a Penn State Particle Separator with 3 sieves (Kononoff et al., 2003). Particles retained on each fraction were oven dried at 55°C. The physical effectiveness factors (**pef**, i.e., the cumulative proportion of feed DM retained on sieves of the Penn State Particle Separator) were nominated as $pef_{>8}$ and $pef_{>1.18}$, respectively. The physically effective NDF of 2 (**peNDF$_{>8}$**) and 3 sieves (**peNDF$_{>1.18}$**) were calculated by multiplying the fraction on $pef_{>8}$ and $pef_{>1.18}$ with the NDF content (Kononoff et al., 2003), respectively.

Table 2. Analyzed chemical composition of forages and protein-mineral-vitamin mix (PMV)

Item,[1] % of DM unless otherwise stated	Hay	Corn silage	Grass silage	PMV[2]
OM	88.6	95.1	86.9	84.1
CP	13.5	6.9	13.9	39.5
Ether extract	2.4	3.3	3.8	2.0
aNDFom	45.0	44.9	47.1	16.7
ADFom	28.6	26.7	33.1	13.4
NFC	27.7	40.0	22.1	25.9
NE_L, MJ/kg of DM	5.40	6.26	5.65	6.77

[1]NFC [= 100 − (% NDF + % CP + % ether extract + % ash)] and NE_L according to (Gruber et al., 2014). aNDFom = amylase-treated NDF determined on an organic matter basis; ADFom= ADF determined on an organic matter basis.

[2]The PMV contained 45.48% soybean meal, 45.48% rapeseed meal, 5.04% limestone, 2.00% salt and 2.00% mineral-vitamin supplement for lactating dairy cows (contained per kg: Ca 6%, P 12%, Mg 10%, Na 8%, Mn 1,500 mg, Zn 5,700 mg, Cu 800 mg, vitamin A 750,000 IU, vitamin D_3 75,000 IU, vitamin E 3,000 mg).

Table 3. Analyzed nutrient composition and particle size distribution of the diets fed to primiparous (Prim) and multiparous (Mult) early-lactating dairy cows from d 21 to 80 pospartum[1]

	CON		La		LaH	
Item,[2] % of DM unless stated	Prim	Mult	Prim	Mult	Prim	Mult
DM, % of fresh matter	67.9	66.9	52.6	52.9	52.8	53.5
OM	91.9	92.4	92.2	92.6	91.4	92.3
CP	15.3	14.6	14.5	15.2	14.5	14.6
Ether extract	2.8	2.8	2.6	2.6	2.6	2.7
aNDFom	32.0	33.2	32.5	33.2	32.7	33.3
ADFom	18.3	19.2	18.9	19.4	18.6	19.0
NFC	41.8	41.8	42.6	41.6	41.6	41.7
NE_L, MJ/kg of DM	6.55	6.51	6.75	6.70	6.70	6.64
Particle size distribution, g/kg of DM retained on sieves						
19 mm	352	324	312	286	311	276
8 mm	114	132	120	141	105	131
1.18 mm	449	453	529	530	527	539
Pan	85	92	39	43	58	55
$pef_{>8}$	0.47	0.46	0.43	0.43	0.42	0.41
$pef_{>1.18}$	0.92	0.91	0.96	0.96	0.94	0.95
$peNDF_{>8}$, % of DM	15.0	15.3	14.0	14.3	13.7	13.7
$peNDF_{>1.18}$, % of DM	29.4	30.2	31.2	31.9	30.7	31.6

[1]Experimental diets differed in barley grain processing, being either unprocessed (CON), or processed with 1% lactic acid (La), or with 1% lactic acid and heat (LaH); Diet components are shown in Table 4.

[2]NFC [= 100 − (% NDF + % CP + % ether extract + % ash)] and NE_L according to (Gruber et al., 2014); aNDFom = amylase-treated NDF determined on an organic matter basis; ADFom= ADF determined on an organic matter basis; pef = physically effective factor; peNDF = physically effective NDF (Kononoff et al., 2003).

Reticuloruminal pH and Temperature

To monitor rumen pH continuously, all cows received an indwelling wireless pH-transmitting unit (smaXtec Animal Care Sales GmbH, Graz, Austria). These units (3.5 cm i.d., 12 cm long, and weighing 210 g) were manually inserted into the reticulorumen via the esophagus (Gasteiner et al., 2012) in all cows on d 17 postpartum. Prior to this, the units were calibrated by a 2-point calibration using buffers of pH 4.0 and 7.0 following the company's instruction protocol. The units measured pH and temperature every 10 min and transmitted the data in real time to a basis station using the ISM band (433 MHz). Antennas installed in the barn and milking parlor picked up the sensor signals. Both data of pH and temperature were collected using an analog to digital converter and stored in an external memory chip. As the guaranteed working time of the sensors was 60 d, the measurements were taken continuously until d 80 postpartum from each cow. One concern of the indwelling sensors is that they provide data of pH only in the site they are located, namely in the reticulum as they are located in the reticulum within 24 h after administration (Gasteiner et al., 2009). The sensors used in this study were recently evaluated and have shown to satisfactorily reflect the pH of the free rumen liquid (Klevenhusen et al., 2014). Thus, the term reticuloruminal pH is used here. Daily mean, minimal, and maximal pH values, standard deviation, and duration during which the pH was below thresholds of 5.8 and 6.0 were calculated. Furthermore, diurnal changes (measurements throughout 1 d taken at 10-min intervals) were analyzed separately. Data of temperature were analyzed for diurnal changes. In accordance to the pH data, daily mean temperature and duration during which temperature was above a threshold of 39.5°C were computed and analyzed separately.

Blood Sampling and Analyses

Blood samples were collected shortly before the morning feeding from the coccygeal vein on experimental d 21 (baseline), 40, and 90. Serum 10-mL evacuated tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) were used to collect blood samples. Immediately after collection, blood samples were allowed to clot for 30 min at 25°C; subsequently serum was separated by centrifuging at 3,000 × *g* at 4°C for 20 min. Serum samples were stored at −20°C until analyses. Concentrations of SAA were determined by a commercially available ELISA bovine kit according to the method described previously (Klevenhusen et al., 2013). In brief, serum samples for SAA were initially diluted 1:200, and samples with optical density values above the range of the standard curve were diluted further (1:400 or 1:250) and reanalyzed. All samples were tested in duplicate and the optical density values were read by an iMark microplate absorbance reader (Bio-Rad Laboratories

GmbH, Vienna, Austria) at 450 nm. The concentrations of bile acids, bilirubin, and liver enzymes such as aspartate aminotransferase (**AST**), glutamate dehydrogenase (**GLDH**), and gamma-glutamyltransferase (**GGT**) in the serum were measured by a conventional large-scale analyzer for clinical chemistry at the laboratory of the Central Clinical Pathology Unit, University of Veterinary Medicine, Vienna. The standard enzymatic colorimetric analyses with a fully automated autoanalyzer for clinical chemistry (Cobas 6000/c501; Roche Diagnostics GmbH, Vienna, Austria) was used. The intraassay variation was controlled by limiting the coefficient of variation to ≤10% for SAA and <5% for other blood variables.

Statistical Analyses

Statistical analyses were performed using PROC MIXED of SAS (version 9.2, SAS Institute Inc., Cary, NC). To evaluate the effect of cow's parity, cows were classified in 2 different lactation groups: primiparous (n = 12) or multiparous (n = 18). The model included the fixed effects of block, diet, parity, sampling time (i.e., day or time of the day at 10-min intervals), as well as the diet × time and diet × parity interaction. The baseline measurements were considered as covariate in the model. The data gained from the same cow but at different times were considered as repeated measures in the model with a first-order autoregressive variance-covariance matrix. Comparisons among treatments were evaluated by Tukey's test. Degrees of freedom were estimated with the method of Kenward-Roger. Furthermore, the significance of the overall effect of the La treatment was tested for all variables (orthogonal contrast involving the average of the 2 La-treated groups vs. control). Associations among pH and temperature measurements were studied by performing a Pearson correlation (PROC CORR of SAS). The significance level was set at $P \leq 0.05$, and a trend was considered up to $0.05 < P < 0.10$ level.

RESULTS

Nutrient Composition of Barleys and Diets, and Feed Intake

The processing of barley with La and LaH resulted in minor changes in the composition of crude nutrients and energy content (Table 1). Treatments with La and LaH slightly reduced ash, starch, and CP contents, but increased amylase-treated NDF determined on an organic matter basis (**aNDFom**) and NE_L contents of the treated barleys; however, these differences in nutrient and energy contents were less visible in the consumed diets (Table 3).

Effects of barley feeding on DMI from d 21 to 80 of lactation are shown in Table 4. Total DMI was not affected by barley treatment ($P = 0.44$), whereas primiparous cows consumed less ($P < 0.01$) feed (18.2 kg of DM/d), including barley grain ($P < 0.01$), compared with their multiparous counterparts (20.7 kg of DM/d). Relative intake of the different dietary ingredients did not differ among feeding groups either (Table 4). However, primiparous cows consumed a higher ($P = 0.03$) proportion of barley (38.8%) compared with multiparous cows (36.5%), and this happened at the expense of hay (14.7% in primiparous cows compared with 16.0% in multiparous cows, Table 4).

Reticuloruminal pH and Temperature Changes

Effects of barley feeding on the reticuloruminal pH and temperature changes from d 21 to d 80 of lactation are shown in Table 5. In cows receiving LaH barley, the mean, maximum, and minimum reticuloruminal pH values were lowered by 0.05 units compared with CON and La ($P < 0.01$). Figure 1a shows daily mean pH values of the 3 dietary treatment groups. Slightly lower pH-values were measured in LaH cows ($P < 0.01$), whereas no differences between lactation days were observed ($P = 0.71$). As shown in Figure 1b, mean reticuloruminal temperature was 0.07°C higher in La cows compared with CON and LaH ($P < 0.01$), whereas no effect of lactation day was recorded ($P = 0.65$). Diurnal variations of mean reticuloruminal pH as well as temperature on 2 exemplary days (d 40 and 60) are shown in Figure 2. Values were averaged over all treatment groups as no effect of the barley processing method was observed. However, reticuloruminal pH as well as temperature were affected by time ($P < 0.01$). The pH profile showed that the pH maximum occurred just before the offering of fresh feed and declined until 6 to 7 h thereafter. Additionally, abrupt drops in temperature were noticed when fresh feed was offered. The same was true for water intake, when observing temperature curves of single cows (data not shown). The duration of pH below the threshold of 6.0 was not influenced by barley treatment and averaged 231 min/d (Table 5). In contrast, the daily period of pH values <5.8 was 21 min shorter ($P = 0.02$, Figure 3a) in the La groups. The time of pH below both thresholds was not different between lactation days ($P > 0.10$), whereas time below 5.8 was interactively affected by lactation day and treatment ($P = 0.03$). Interestingly, an interaction between parity and treatment group was observed for the minimum pH, showing the lowest

Table 4. Diet components and DMI in primiparous (Prim) and multiparous (Mult) cows fed diets based on untreated barley grain (CON), or barley grain steeped in 1% lactic acid solution (La), or steeped in 1% lactic acid solution with heat (LaH) for 12 h from d 21 to 80 postpartum

	CON		La		LaH			*P*-value[1]			
Item	Prim	Mult	Prim	Mult	Prim	Mult	SEM	Diet	Par	Diet × Par	Con
Diet component, % of DM											
Barley grain	39.3	37.3	39.1	36.2	38.1	36.0	1.21	0.59	0.03	0.92	0.37
Corn silage	18.4	19.7	18.9	20.2	19.9	19.4	0.83	0.76	0.32	0.50	0.46
Grass silage	16.4	17.1	16.3	16.6	15.9	16.5	0.76	0.68	0.28	0.95	0.43
Hay	14.5	16.4	14.7	15.3	15.0	16.4	0.66	0.27	<0.01	0.32	0.76
Protein-mineral mix	11.4	9.5	11.0	11.7	11.1	11.7	0.93	0.49	0.83	0.42	0.24
DMI, kg/d											
Barley grain	7.30	7.81	6.77	7.85	6.73	7.31	0.236	0.10	<0.01	0.43	0.07
Corn silage	3.45	4.02	3.28	4.29	3.53	3.93	0.333	0.97	<0.01	0.48	0.93
Grass silage	3.05	3.49	2.82	3.55	3.55	3.33	0.109	0.14	<0.01	0.22	0.10
Hay	2.72	3.34	2.56	3.26	2.67	3.30	0.161	0.66	<0.01	0.94	0.48
Protein-mineral mix	2.06	1.90	1.88	2.46	2.32	2.32	0.184	0.29	<0.01	0.01	0.13
Total	18.5	20.6	17.3	21.4	18.8	20.1	0.50	0.44	<0.01	0.13	0.33

[1]Effect of dietary treatment (Diet), effect of parity (Par), interactive effect of dietary treatment and parity (diet × Par), and overall effect of lactic acid (Con).

values in primiparous cows of the CON group ($P < 0.01$), resulting in the longest duration of pH <6.0 and 5.8 in these cows ($P < 0.01$). Finally, in primiparous CON cows the duration of the reticuloruminal pH <5.8 was on average 83 min/d longer when compared with the 2 other treatment groups (Table 5, $P < 0.01$). On the contrary, in multiparous cows the opposite effect was observed, showing on average a 41 min/d shorter duration of reticuloruminal pH <5.8 in CON cows compared with the cows of the 2 other treatments ($P < 0.01$). Time duration of temperature >39.5°C was neither affected by barley treatment ($P = 0.31$) nor by lactation day ($P = 0.27$, Figure 3b). However, an interaction was observed between lactation day and treatment ($P = 0.04$).

Parity affected certain pH and temperature variables. Although the mean pH was not affected (Figure 4a), the maximum pH was higher in multiparous cows compared with first-lactation heifers ($P < 0.01$). The duration of pH <6.0 was on average 66 min/d shorter in multiparous cows ($P = 0.02$). Additionally, the time during which pH was below the threshold of 5.8 was reduced by 25 min/d in cows in second or higher lactation ($P < 0.01$, Figure 5a). Mean reticuloruminal temperature was 0.06°C higher in multiparous cows compared with primiparous cows ($P < 0.01$, Figure 4b). Furthermore, the time during which temperature was >39.5°C was on average 60 min/d longer in multiparous cows compared with first-lactation heifers ($P < 0.01$, Figure 5b).

Serum Variables

Barley treatment and parity showed minor effects on liver-associated variables (Table 6). No effect of the barley processing technique was observed. Moreover, only small effects of parity on investigated serum variables were noticed. Aspartate aminotransferase showed 6% higher values in cows in the second or higher lactation compared with primiparous cows ($P = 0.05$). Concentrations of bilirubin decreased with sampling day ($P < 0.01$), whereas none of the investigated serum variables was interactively affected by sampling day and diet.

DISCUSSION

This study was primarily undertaken to monitor long-term reticuloruminal pH dynamics and metabolic activity of the liver in early-lactating primiparous and multiparous dairy cows fed a diet based on barley grain processed with La, with or without thermal treatment. All cows were included on d 21 postpartum and sampled until d 90 postpartum, whereby pH and temperature data were analyzed from d 21 to d 80 postpartum as

Table 5. Effect of dietary treatment and parity (Prim = primiparous; Mult = multiparous) on reticuloruminal pH and temperature in cows from d 21 to d 80 of lactation fed untreated barley (CON), barley steeped in 1% lactic acid solution (La), or steeped in 1% lactic acid solution with heat (LaH)

	CON		La		LaH			*P*-value[1]			
Item	Prim	Mult	Prim	Mult	Prim	Mult	SEM	Diet	Par	Diet × Par	Con
pH											
Mean	6.20	6.26	6.23	6.23	6.18	6.17	0.004	<0.01	0.28	0.13	0.15
Maximum	6.51	6.57	6.53	6.59	6.48	6.52	0.004	<0.01	<0.01	0.81	0.38
Minimum	5.79^{c}	5.91^{a}	5.89^{a}	5.86^{ab}	5.83^{bc}	5.81^{bc}	0.005	<0.01	0.21	<0.01	0.97
SD[2]	0.155^{b}	0.142^{c}	0.135^{d}	0.161^{a}	0.138^{d}	0.156^{a}	0.14	0.95	<0.01	<0.01	0.81
Time <6.0, min/d	370^{a}	97^{d}	246^{bc}	221^{bc}	177^{c}	277^{b}	0.37	0.98	0.02	<0.01	0.92
Time <5.8, min/d	118^{a}	11^{c}	46^{b}	46^{b}	25^{bc}	57^{b}	0.97	0.07	<0.01	<0.01	0.02
Temperature											
Mean	38.78	38.84	38.87	38.89	38.75	38.86	0.004	<0.01	<0.01	0.11	0.07
Time >39.5°C, min/d	207	275	218	266	180	243	4.65	0.31	<0.01	0.91	0.99

[a-d]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]Effect of dietary treatment (diet), effect of parity (Par), interactive effect of dietary treatment and parity (diet × Par), overall effect of lactic acid (Con).

[2]Standard deviation of measurements within a day.

the indwelling sensors allowed for measurement for as many as 60 d.

Depression of ruminal pH occurs early postpartum due mainly to dramatic dietary changes, whereby SARA is considered a prevalent metabolic disorder during this period (Plaizier et al., 2008). The durations of pH below thresholds of 5.6 or 5.8 for longer than 3 or 5.4 h/d, respectively, are considered as safer indicators of SARA than single measurements of pH (Penner et al., 2007; Plaizier et al., 2008; Zebeli and Metzler-Zebeli, 2012). Overall, the short duration (average time: 51 min/d) of reticuloruminal pH <5.8 suggests a low risk of SARA in the cows of this study. Despite the fact that cows were in early lactation and fed almost 50% concentrate in the diet, this could be explained by low DMI and a relatively high concentration of peNDF (>1.18) in all diets, averaging 30.8%, which is assumed to be sufficient in maintaining a mean ruminal pH of above 6 (Zebeli et al., 2008).

Overall results showed limited effects of feeding barley processed with Laor LaH on reticuloruminal pH. Accordingly, the differences of 0.05 pH units between LaH compared with CON and La is in fact below the pH accuracy level (±0.2 units). However, data revealed important differences when analyzed separately by parity. Indeed, an interesting finding of the study was that La and LaH treatments of barley shortened the time duration of pH <5.8 by 83 min/d in primiparous cows compared with their CON counterparts. This result confirms, at least in part, findings reported by Iqbal et al. (2009), who also observed a slightly shorter duration in which rumen pH was <5.8 (2.4 vs. 3.9 h/d) in primiparous late-lactating cows fed barley steeped in 0.5% La compared with cows fed control barley grain. The reason(s) why this grain treatment improved the ruminal tolerance of barley in primiparous cows by shortening their time duration of rumen pH <5.8 compared with control cows, but not in multiparous cows, is not clear. Nevertheless, these results suggest that primiparous cows may benefit from La treatments. It is also possible that primiparous cows had a greater SARA challenge than multiparous cows, as evidenced by a longer duration of pH <5.8 in those cows. The processing of barley with La and LaH resulted in few changes in the composition of crude nutrients such as reduced ash, total starch, and CP contents, and also increased the aNDFom content of the treated barleys, although these chemical changes of barleys were leveled up in the whole diets, which provided almost similar crude nutrient composition to the cows. Most importantly, treatments of barley grain with La and LaH have been shown to reduce the rate of enzymatic starch digestion, alter its microscopic structure (Deckardt et al., 2014; Harder et al., 2015a,b), and also modulate

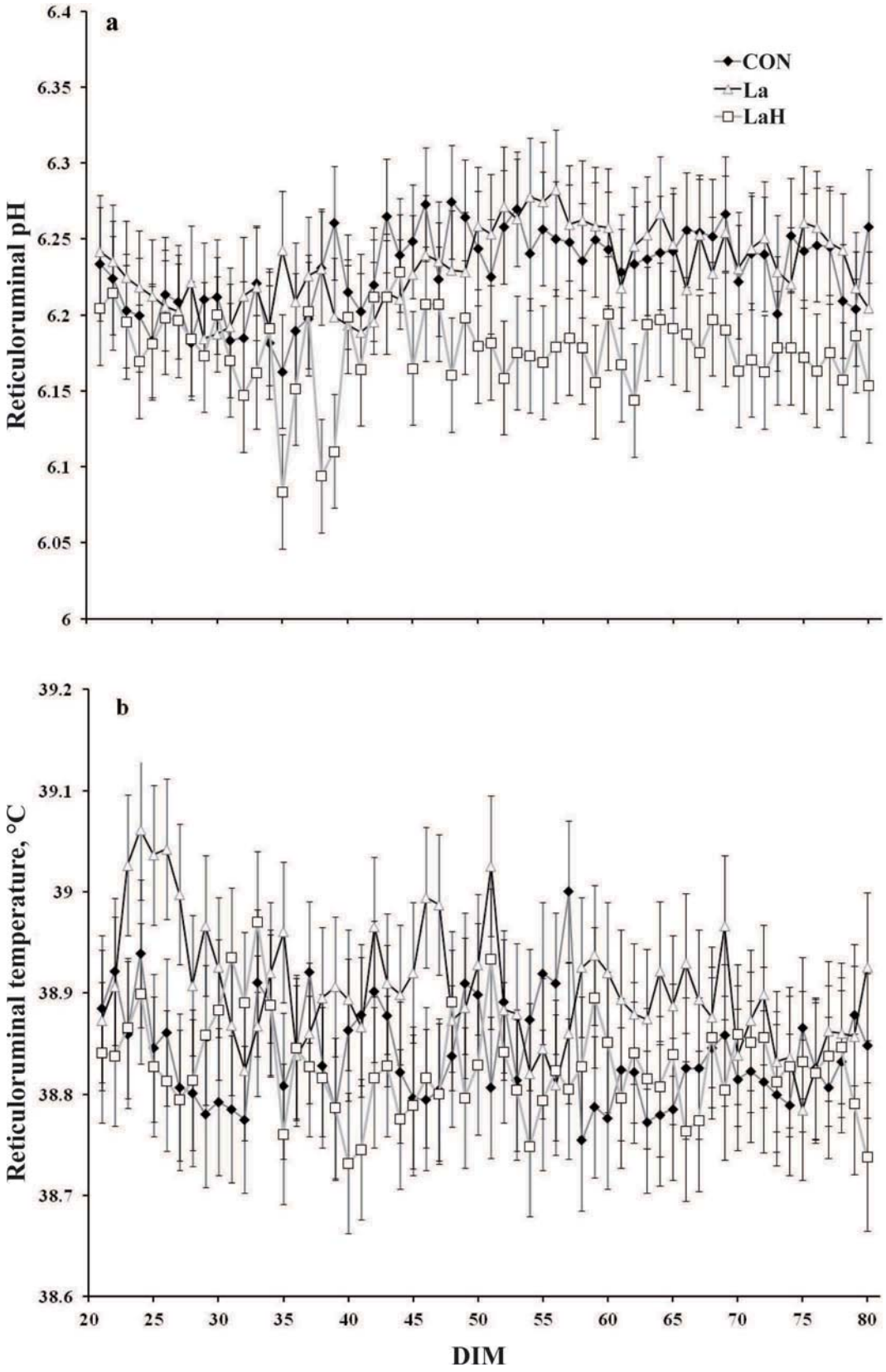

Figure 1. Effect of dietary treatment on mean reticuloruminal pH (a) and temperature (b) in lactating cows fed barley (CON), barley steeped in 1% lactic acid solution (La), or steeped in 1% lactic acid solution with heat (LaH). Error bars represent SEM.

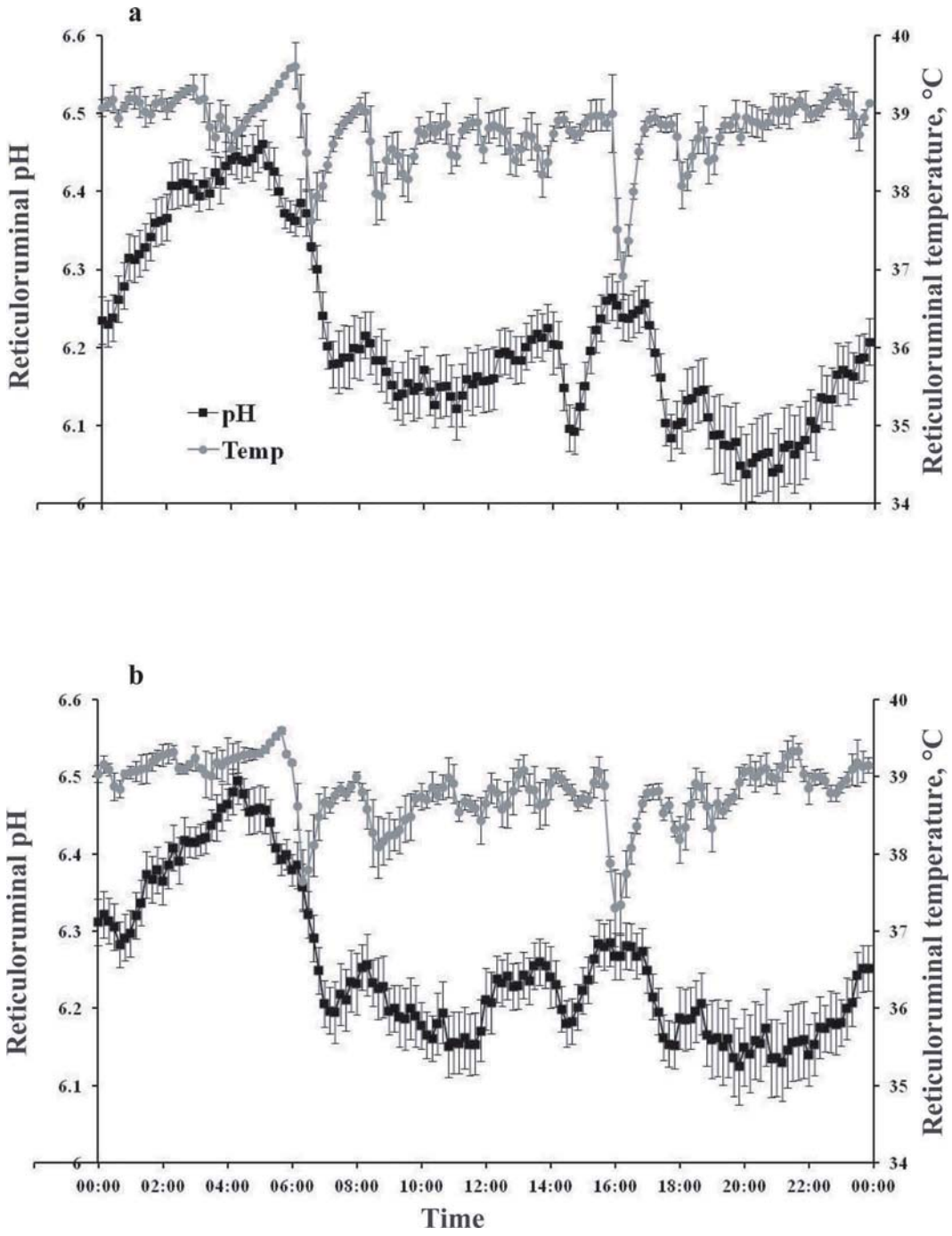

Figure 2. Diurnal variation of reticuloruminal pH and temperature in lactating cows, averaged across all treatment groups on d 40 (a) and d 60 (b) of lactation. Error bars represent SEM.

rumen microbial activity, and lower the concentration of VFA in a semi-continuous culture fed 60% concentrate (Deckardt et al., 2015). Grain processing might be more effective in modulating ruminal pH when cows are fed grain-rich diets, challenging them to SARA conditions. Healthy cows are assumed to be less responsive to dietary treatments compared with SARA-challenged cows, an argument that is further strengthened by the observed interaction between parity and treatment in this study. Evaluation of this hypothesis requires more research with cows fed diets richer in grain. However, it has to be emphasized that the opposite effect to that

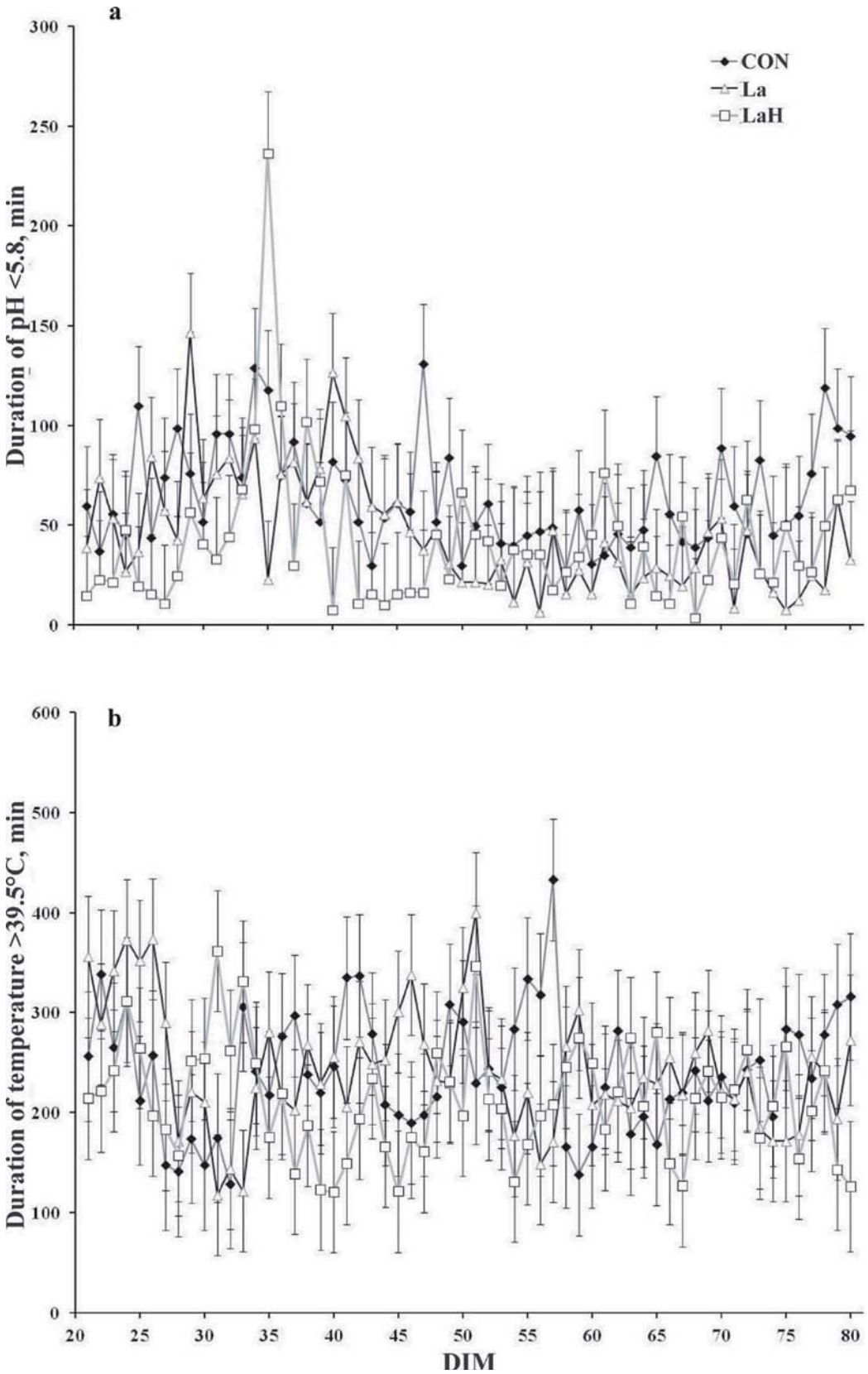

Figure 3. Effect of dietary treatment on time reticuloruminal pH < 5.8 (a) and temperature > 39.5°C (b) in lactating cows fed barley (CON), barley steeped in 1% lactic acid solution (La), or steeped in 1% lactic acid solution with heat (LaH). Error bars represent SEM.

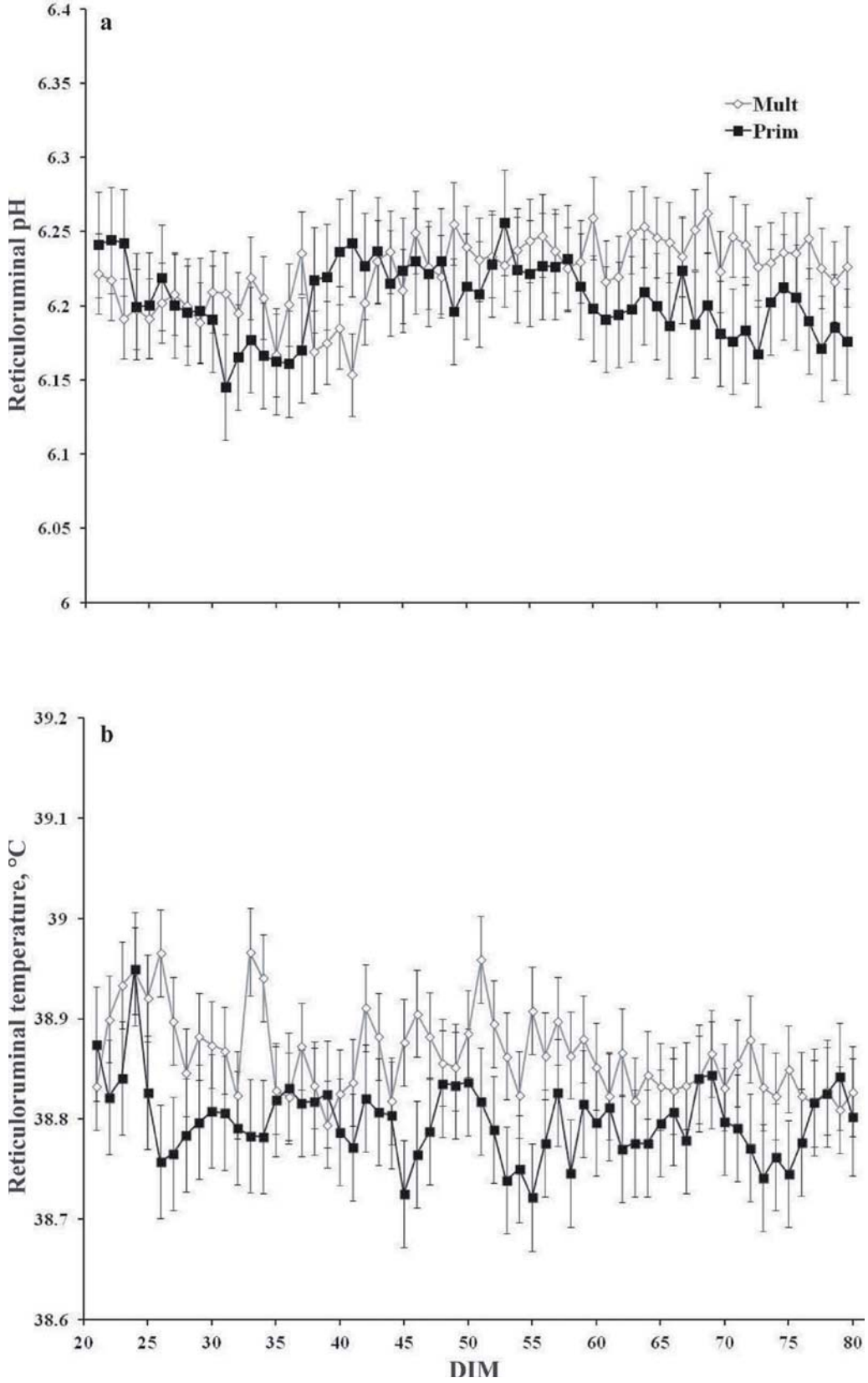

Figure 4. Effect of parity (Mult = multiparous, Prim = primiparous) on reticuloruminal pH (a) and temperature (b) in lactating cows. Error bars represent SEM.

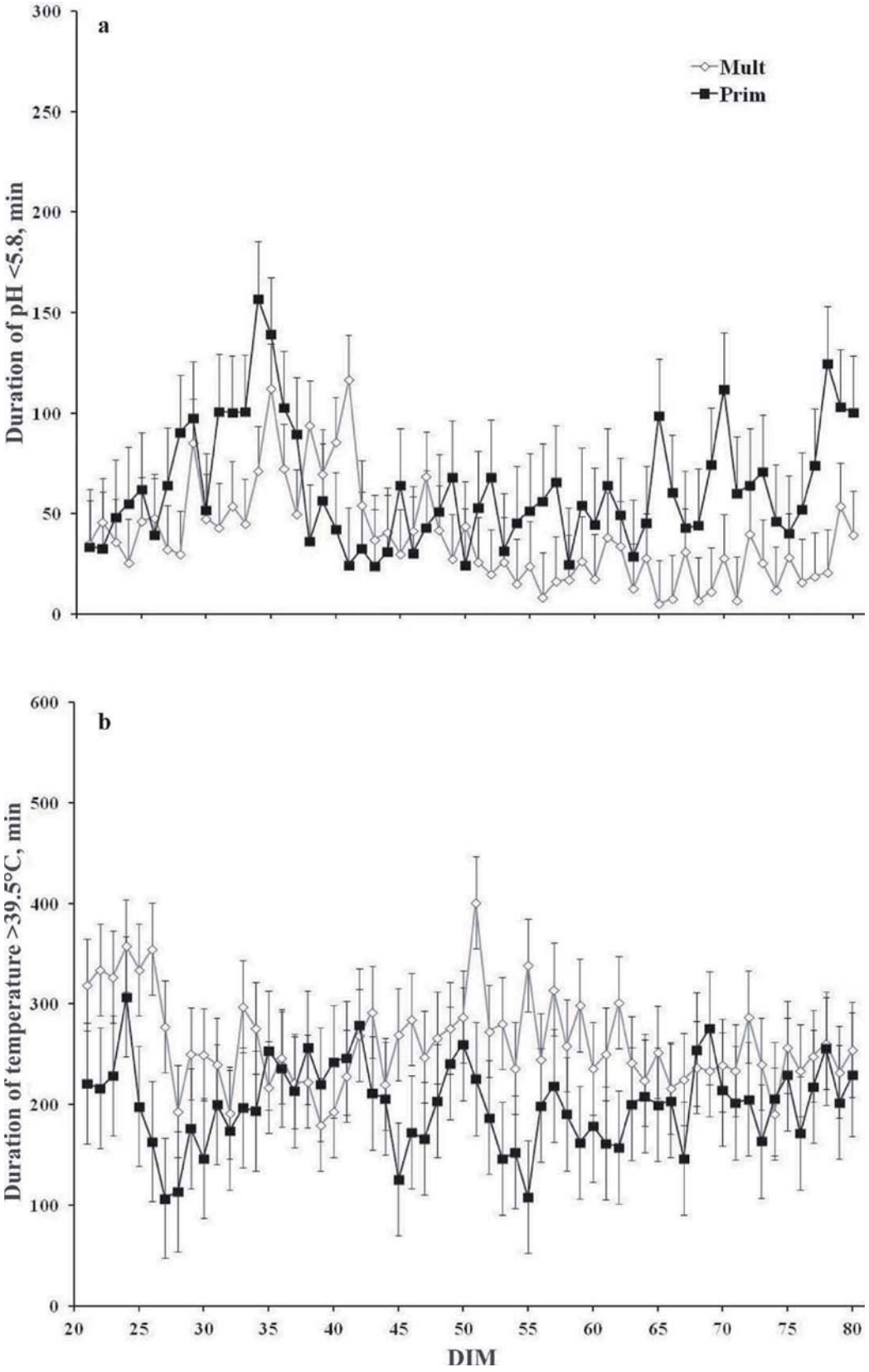

Figure 5. Effect of parity (Mult = multiparous, Prim = primiparous) on time duration of reticuloruminal pH < 5.8 (a) and temperature > 39.5°C (b) in lactating cows. Error bars represent SEM.

Table 6. Effect of dietary treatment and parity (Prim = primiparous; Mult = multiparous) on serum variables in lactating cows fed barley (CON), barley steeped in 1% lactic acid solution (La), or steeped in 1% lactic acid solution with heat (LaH)

	CON		La		LaH			*P*-value[2]			
Item[1]	Prim	Mult	Prim	Mult	Prim	Mult	SEM	Diet	Par	Diet × Par	Con
AST, U/L	74.92	79.53	75.10	83.66	77.99	78.40	1.43	0.74	0.05	0.31	0.52
GGT, U/L	22.10	21.92	25.17	26.26	26.26	22.20	0.81	0.19	0.63	0.66	0.14
GLDH, U/L	11.65	12.59	15.39	13.54	21.51	13.69	1.49	0.14	0.20	0.27	0.11
Bile acids, μmol/L	47.99	51.65	49.58	58.39	67.17	53.99	4.50	0.68	0.98	0.63	0.49
Bilirubin, mg/dL	0.04	0.07	0.07	0.08	0.10	0.08	0.01	0.18	0.65	0.35	0.11
SAA, mg/L	2.34	1.55	14.30	24.85	1.65	7.99	3.66	0.30	0.71	0.68	0.71

[1]AST = aspartate aminotransferase; GGT = gamma-glutamyltransferase; GLDH = glutamate dehydrogenase; SAA = serum amyloid A.
[2]Effect of dietary treatment (Diet), effect of parity (Par), interactive effect of dietary treatment and parity (diet × Par), overall effect of lactic acid (Con).

found for primiparous was observed in multiparous cows, whereby La and LaH cows showed an on average 41 min/d longer duration of pH <5.8 compared with the CON group. This contradictory observation of grain processing in primiparous versus multiparous cows is difficult to explain with the current data of pH dynamics. Clarification of the mechanisms behind the latter effects requires further investigations aiming to establish the effects of barley processing on reticuloruminal fermentation profile such as the dynamics of release, neutralization, and metabolism of VFA and lactate, and also chewing activity, events which are known as determinants of intra-reticuloruminal pH regulation (Allen, 1997; Zebeli et al., 2012).

Besides monitoring the effect of La-treated barley on pH dynamics, this study further aimed at investigating possible differences between first-production heifers and multiparous cows. The role of the parity in the risk of developing SARA has not been clarified yet. For example, Krause and Oetzel (2006) and Bramley et al. (2008) observed a decreased risk of developing SARA in multiparous cows compared with primiparous cows, but other studies have reported an opposite trend (Maekawa et al., 2002), or no relation between risk of ruminal acidosis and parity (Gröhn and Bruss, 1990). Generally, one could expect that primiparous cows should be at lower risk of SARA, due to their lower production potential and DMI (Krause and Oetzel, 2006). Indeed, primiparous cows consumed approximately 2.5 kg/d less feed DM than multiparous cows in this study. Although multiparous cows had on average 2% less barley grain at the expense of more hay and corn silage in their diets, these cows in general consumed significantly greater amounts of easily fermentable substrates (14.0 vs. 12.4 kg of DM/d), contained in concentrates and corn silage, with almost unchanged $peNDF_{1.18}$ contents. Because of these feeding patterns, a greater risk of SARA for multiparous cows was expected. It is known that for each unit increase in the DMI, and hence in fermentable substrates and fiber, acid generation rather than acid neutralization processes are stimulated in the rumen of dairy cows (Allen, 1997). However, contrary observations were made in the present study. Multiparous cows showed shorter periods in which pH decreased below 6.0 and 5.8, respectively. The occurrence of SARA may be exacerbated in first-production heifers when compared with multiparous cows, because primiparous cows have not had previous long-term exposure to a highly fermentable lactation diet yet. One possible explanation might be that they may have fewer rumen papillae and less bacterial adaption than mature cows (Penner et al., 2007; Bramley et al., 2008). This is probably the reason why feeding additional concentrate prepartum is suggested to al-

leviate the risk of postpartum SARA in cows aiming to help them in increasing the absorptive capacity of rumen papilla and adapt better to high grain feeding postpartum (Stone, 2004). However, this was not supported by Penner et al. (2007), who were not able to detect ameliorative effects of feeding additional concentrate in primiparous cows on postpartum SARA development. Another reason for the differing SARA susceptibility might be related to higher chewing time in multiparous cows compared with primiparous cows. Thus, a lower daily saliva output in heifers compared with multiparous cows may result in reduced rumen buffering and consequently in lower pH compared with older cows (Maekawa et al., 2002; Bowman et al., 2003). Furthermore, Oetzel (2007) assumed that heifers need more time to self-regulate their own ruminal pH after they start consuming large amounts of cereal grains after parturition compared with multiparous cows. Additionally, first-lactation cows represent a largely unculled population. Thus, it is also possible that the heifers that are unable to learn to self-regulate their own ruminal pH have not yet been removed from the herd, but will be removed in the following lactations due to complications of SARA (Oetzel, 2007).

In addition, parity affected reticuloruminal temperature. Although the differences of the mean reticuloruminal temperature of +0.06°C is below the measurement accuracy (±0.25°C), older cows spent 1 h more per day with a reticuloruminal temperature >39.5°C, when compared with primiparous animals. Thus, it seems that the obtained longer duration of high reticuloruminal temperature in the present study was less directly associated with a higher risk of SARA, than rather to a higher heat production due to higher fermentation resulting from higher feed intake in multiparous cows. This is supported by the observation that no correlation was found between ruminal temperature and ruminal pH in this study ($r = -0.06$).

The present study also focused on liver-associated variables, as it is well known that metabolic stress associated with low ruminal pH during early lactation primarily affects liver function (Bionaz et al., 2007). For instance, in cattle affected by SARA, APR is typically expressed as an increase of the acute phase protein SAA (Plaizier et al., 2008; Iqbal et al., 2010). Furthermore, in early-lactation cows, the liver has to adequately adapt to cope with the increased flow of NEFA resulting from excessive lipolysis (Goff and Horst, 1997). In case of an increased lipid deposition in the liver, lesions of the liver tissue can be the consequence, and levels of enzymes that indicate liver injury (AST, GGT, and GLDH) are generally augmented (Bobe et al., 2004).

The measured activities of AST, GGT, and GLDH corresponded with previous reports (Wille et al., 2010; Lee et al., 2012). In general, liver-associated variables were not affected by barley feeding in this study. Because the values of presented enzyme activities were within reference intervals (Wille et al., 2010), no clear sign is found of impaired liver function of the present cows, which is also explained by low level of SARA and the cows' low production level. Thus, the present data suggest that the process of lipomobilization in these cows was not affected by diet, and was not strong enough to cause liver lesions in early-lactating cows. Although treatment with La and LaH enhanced energy content of barleys by almost 0.4 to 0.5 MJ/kg of DM, compared with CON barley, the total NE_L contents of the ingested diets were almost similar. The increase of NE_L content of La-treated barleys can be mainly explained by changes in the profile of degradation of its nutrients such as ash, CP, starch, phytate P, and soluble fiber (Deckardt et al., 2014; Metzler-Zebeli et al., 2014; Harder et al., 2015a). Also, the addition of 1% La in the soaking solution (resulting in 10 g of La/kg of barley) might have contributed to greater energy value of the treated barley. Assuming energy content of 15.1 kJ per g of La, the La treatment provided additional 0.15 MJ per each kg of barley (i.e., 1% La solution with ratio 1:1). Because the DMI of concentrate and forages remained unchanged, resulting in no difference in overall DMI among feeding groups, it is reasonable to assume that total NE_L intake also was similar among treatments. The similar energy intakes together with no SARA conditions likely explain no effect of diets on the process of lipomobilization, APR, and liver health in the cows of our study. Because the intake of barley grain was not significantly affected by treatment, it seems that treatment of grain with La and LaH does not negatively affect the palatability of the treated grain.

The concentrations of the acute phase protein SAA were not affected by dietary treatment and generally below recommended cutoff levels for identification of an APR in postparturient dairy cows (Humblet et al., 2006). Thus, the absence of SARA and its related inflammatory state in all treatment groups is further supported. Most liver-associated variables were not affected by parity, too. However, 6% higher activities of AST were found in multiparous cows compared with primiparous cows. This is supported by Wille et al. (2010) and Cozzi et al. (2011) who found only minor effects of the parity on enzyme activity. In general, elevated levels of certain liver enzymes in multiparous cows compared with primiparous cows could be the

result of an elevated productive stress compared with primiparous cows.

CONCLUSIONS

In conclusion, the present study showed contradictory effects of steeping barley grain in La with or without thermal treatment on ruminal tolerance of barley grain feeding, determined by long-term reticuloruminal pH dynamics; the treatment reduced the duration that reticuloruminal pH was <5.8 in primiparous cows but increased it in multiparous cows. As the risk of SARA seems to be greater in primiparous cows, our data indicate that more care should be taken in the practice in terms of feeding and management of this group of cows. The lack of pronounced effects of dietary treatment on liver-associated variables may be explained by the low production level of the cows and the fact that cows in the present study were not challenged by SARA conditions. Thus, it would be of interest to test the effects of feeding La-treated barley grain on dairy cows challenged with SARA conditions in future studies.

ACKNOWLEDGMENTS

The contribution of S. Gschaider, A. Rudischer, and A. Dockner (Institute of Animal Nutrition and Functional Plant Compounds at Vetmeduni, Vienna) with their help with the animal trial and the analysis of SAA as well as the staff of the laboratory of the Central Clinical Pathology Unit (Vetmeduni, Vienna) for blood analyses is highly acknowledged. The authors thank the staff of the research station at AREC Raumberg-Gumpenstein for their care of the cows as well as A. Schauer and A. Adelwöhrer for excellent help in data collection and preparation. This research was supported by a project of the Federal Ministry of Agriculture, Forestry, Environment and Water Management (BML-FUW, Austria, Vienna; project no. 100824), which is highly acknowledged.

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Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Ruminal disappearance of phosphorus and starch, reticuloruminal pH and total tract nutrient digestibility in dairy cows fed diets differing in grain processing

A. Khol-Parisini[a,1], E. Humer[a,1], Ö. Sizmaz[a,2], Sh.M. Abdel-Raheem[a,3], L. Gruber[b], J. Gasteiner[b], Q. Zebeli[a,*]

[a] *Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, Vetmeduni Vienna, Veterinaerplatz 1, 1210 Vienna, Austria*
[b] *Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Raumberg 38, 8952 Irdning, Austria*

ARTICLE INFO

Article history:
Received 5 May 2015
Received in revised form
29 September 2015
Accepted 29 September 2015

Keywords:
Barley grain processing
Dairy cow
Lactic acid
Rumen fermentation
Rumen undegradable starch
Phosphorus

ABSTRACT

Ruminal disappearance kinetics of nutrients such as phosphorus (P), starch and protein in cereal grains is determinant of their nutritional and health value in ruminants. The objective of the present in vivo and in situ trials was to evaluate whether feeding barley grain processed with lactic acid (LA), with or without thermal treatment, affects in situ degradation kinetics of nutrients, reticuloruminal pH dynamics, and apparent total tract digestibility (ATTD) in dairy cows. The experiment involved 6 rumen-fistulated Holstein cows in a double 3 × 3 Latin square design fed a diet based on differently processed barley grain: untreated grain as control (CON), or grain treated with 1% LA alone (LA diet), or 1% LA plus oven-heating (LAH diet) prior to feeding. Each experimental period lasted 21 d, with the first 11 d used for adaptation to the diets. The in situ trial consisted in the incubation of the differently treated barley grains (i.e., CON, LA, LAH) up to 48 h in the rumen of cows fed diets containing the respective barleys. The reticuloruminal pH and temperature were continuously measured throughout the experiment via wireless indwelling sensors. In situ data demonstrated increased ($P < 0.001$) disappearance of barley P, being on average more than twice as high during the first 24 h of incubation with LA and LAH compared to CON barley. In contrast, the degradation of starch decreased ($P < 0.05$) after 2 h and 4 h of the ruminal incubation, but not thereafter. Feed intake remained unchanged among treatment groups, whereas ATTD of dry matter was slightly greater ($P = 0.05$) in cows fed the LA-treated barley. There was no effect of diet on reticuloruminal pH and temperature dynamics either.

Abbreviations: ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive residual ash; ATTD, apparent total tract digestibility; BW, body weight; CP, crude protein; DM, dry matter; ERD, effective rumen degradability; InsP, Inositol phosphates; $InsP_6$, *myo*-inositol hexakisphosphate; k_d, fractional disappearance rate; *L*, lag time; LA, lactic acid; LAH, lactic acid plus heat treatment; ME, metabolizable energy; NEL, net energy of lactation; NFC, non-fibre carbohydrates; OM, organic matter; P, phosphorus; PMV, protein-mineral-vitamin; RUP, rumen undegradable protein; RUS, rumen undegradable starch; UP, unreleasable phosphorus.

* Corresponding author at: Department for Farm Animals and Veterinary Public Health, Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, 1210 Vienna, Austria.
E-mail address: Qendrim.Zebeli@vetmeduni.ac.at (Q. Zebeli).
[1] These authors contributed equally to this paper.
[2] Present address: Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, University of Ankara, 06110 Ankara, Turkey.
[3] Present address: Department of Animal Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.

http://dx.doi.org/10.1016/j.anifeedsci.2015.09.028

In conclusion, the present study indicates that treatment of barley grain with LA and LAH lowers rumen degradability of starch early after incubation, without affecting reticuloruminal pH and the ATTD of most nutrients. The improved ruminal disappearance of barley P suggests an improved solubility and faster ruminal availability of organic P of the treated grain, and warrants further investigations to tap its potential with regards to improved P nutrition and minimization of P excretion in dairy cows.

1. Introduction

Cereal grains are important ingredients in the diets of high-producing dairy cows. Besides energy and protein, grains also are valuable sources of minerals, especially of phosphorus (P). Phosphorus plays a crucial role not only with respect to cow's health and production but also regarding ecological and economic aspects of cattle production (Knowlton et al., 2004, 2007; Humer and Zebeli, 2015). In cereal grains, P is primarily stored in form of phytates (*myo*-inositol hexakisphosphate, $InsP_6$; Harland and Morris, 1995), thus potentially reducing its ruminal solubility and consequently availability for ruminants. Although rumen microbes are assumed to hydrolyze nearly all the feed $InsP_6$ into phosphate (Raun et al., 1956; Morse et al., 1992), new data in high-producing cows indicate limitation of ruminal $InsP_6$ degradation due likely to high feed intake, faster passage rate, and suboptimal rumen fermentation conditions (Brask-Pedersen et al., 2013; Jarrett et al., 2014). For example, a recent study by Brask-Pedersen et al. (2013) showed that the ruminal degradability of $InsP_6$ was increased by supplementing exogenous phytase to cow diets, resulting in a higher P availability for ruminal microbiota. These findings indicate that dairy cows can still benefit from native plant $InsP_6$ hydrolysis.

In previous studies, treatment of cereal grains with lactic acid (LA), a mild organic acid used in food and feed technology (Haraldsson et al., 2004; Iqbal et al., 2009, 2012; Deckardt et al., 2013), has shown to be effective in the hydrolysis of $InsP_6$ in grains (Haraldsson et al., 2004; Metzler-Zebeli et al., 2014). Furthermore, these studies have indicated a synergistic effect of LA and the additional heat treatment on the hydrolysis of $InsP_6$ (Haraldsson et al., 2004; Metzler-Zebeli et al., 2014). Although these data suggest a potential role for LA treatment to improve the solubility of native P in cereal grains, this has not been evaluated in the rumen yet.

On the other hand, treatment of cereal grains with LA and temperature modulated other key nutrients of barley grain such as starch and fibre. For example, treating barley with graded concentrations of LA (0.5–5%) enhanced the content of slowly degradable starch and fibre fractions, and modulated the microscopic structure of the starch (Deckardt et al., 2014; Harder et al., 2015a), increasing resistance to ruminal degradation (Deckardt et al., 2015); however, it is not clear if this modification of the structure of starch might also increase its resistance against the microbial degradation in the rumen. Modulation of the degradation of barley starch in the rumen is beneficial because this starch is rapidly fermentable in the rumen (Offner et al., 2003), and its inclusion in the diet in large amounts increases the risk of rumen disorders (Zebeli et al., 2012). Studies conducted by Iqbal et al. (2009, 2012) reported improved fermentation with higher ruminal pH in cows fed barley treated with LA and heat.

The hypothesis of this study was that the treatment of LA alone or with heat might improve ruminal P release and modulate starch degradation characteristics of barley grain in the rumen. Therefore, an in situ trial as well as an in vivo experiment with rumen-cannulated dairy cows was conducted, aiming to evaluate the kinetics of disappearance of nutrients, including starch and P, by treating barley grain with LA alone or with LA and heat. Besides rumen disappearance characteristics, also the apparent total tract digestibility (ATTD) of nutrients and reticuloruminal pH and temperature dynamics were investigated.

2. Materials and methods

2.1. Animals, diets, and experimental design

The experiment was conducted at the Dairy Research Facilities of the Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Austria. Six ruminally cannulated (100 mm i.d.; Bar Diamond, Parma, ID, USA) Holstein cows were used in a double 3 × 3 Latin square design ($n = 6$ per treatment). Before the start of the trial, cows produced on average (mean ± SD) 26.4 ± 6.6 kg milk/d with 4.1 ± 0.3% fat and 3.3 ± 0.4% protein in the milk. Each experimental period lasted 21 d, with the first 11 d for adaptation to the diets and the last 10 d for experimental measurements. The diets of the three groups were identical with the only difference in the treatment of barley grain included in the ration. The control diet (CON) contained untreated barley grain, whereas the other dietary treatments consisted of barley grain treated with 1% LA or 1% LA plus oven-heating (LAH) prior to feeding. The barley grain used in all diets was ground to pass a 3 mm screen using a hammer mill (Ultra 110/150, Gruber Machineries, A-4673 Gaspoltshofen, Austria). The barley of the CON diet was fed as such, whereas the barley of the LA diet daily was soaked 1:1 (w/v) in 1% LA (v/v; Brenntag, Austria) at room temperature. The barley of the LAH treatment underwent the same procedure of soaking as the LA barley but coupled with an additional oven-heating at 55 °C for 12 h. The soaking time for LA and LAH treatments was the same and lasted for 24 h prior to the morning feeding. Analyzed chemical composition and energy values of the barley grains are shown in Table 1.

Table 1
Analyzed chemical composition and energy contents of barley grain and protein-mineral-vitamin (PMV) premix used as concentrate components in the diet.[a]

Variables[b]	Barley grain[c]			PMV[d]
	CON	LA	LAH	
% of dry matter (DM) unless stated				
DM (% of fresh feed)	87.1	48.1	48.2	90.8
Ash	2.47	2.46	2.36	16.7
CP	11.5	11.5	11.3	39.5
$aNDF_{om}$	19.6	20.3	20.7	16.7
ADF_{om}	5.9	7.1	6.0	13.4
Starch	61.3	60.0	60.8	ND
P (g/kg DM)	3.72	4.04	3.67	13.8
ME (MJ/kg DM)	12.1	12.7	12.6	11.1
NE_L (MJ/kg DM)	7.52	8.01	7.89	6.77

[a] The ratio of barley grain and PMV in the concentrate proportion was 77:23 (on DM basis).
[b] ME, metabolizable energy; NE_L, net energy of lactation (calculated according to Gruber et al., 2014).
[c] Barley grain was either untreated (CON), or treated with 1% lactic acid (LA), or with 1% lactic acid and heat (LAH) for 24 h.
[d] The PMV contained 45.48% soybean meal, 45.48% rapeseed meal, 5.04% limestone, 2.00% salt and 2.00% mineral-vitamin supplement for lactating dairy cows (contained per kg: Ca 6%, P 12%, Mg 10%, Na 8%, Mn 1500 mg, Zn 5700 mg, Cu 800 mg, vitamin A 750,000 IU, vitamin D3 75,000 IU, vitamin E 3000 mg); ND, not determined.

All diets were formulated to meet or exceed the energy and nutrient requirements of a dairy cow of 650 kg body weight (BW), producing 30 kg milk/d with 4% fat, and 3.3% protein (GfE, 2001). All diets (DM-basis) were designed to have 15% meadow hay, 15% grass silage, 20% maize silage, and 50% concentrate, consisting of 77% barley grain (either CON, LA, or LAH), and 23% protein-mineral-vitamin (PMV) supplement. The PMV supplement contained solvent-extracted soybean- and rapeseed meals in equal proportions, limestone, salt, and a commercial mineral-vitamin premix for lactating dairy cows. Components and analyzed chemical composition of the PMV supplement are shown in Table 1. Analyzed nutrient composition and energy contents of forages and consumed diets (taking into account the consumed feed amounts and refusals) are shown in Table 2.

All cows were fed for ad libitum intake being offered 110% of the average amount of feed consumed on the 3 preceding days (real average daily feed refusals of 5–10%). Fresh feed was offered twice a day, whereby the cows received roughage (hay and grass silage) portion first (04:00 h and 16:00 h), followed by their barley, PMV feed allowance and the maize silage (07:00 h and 19:00 h). Feed was freely available until the next fresh feed was offered. Amounts of feed offered and refused were recorded to determine the feed intake of cows by difference. Feed and orts samples were collected daily. They were mixed and aliquots were used to determine DM and nutrient composition of feeds and orts. The cows were housed in individual tie stalls bedded with rubber mats plus sawdust and had free access to fresh water. All procedures involving animal handling and treatment were in accordance with national regulations for animal use in research and approved by the authority according to §9ff of Law for Animal Experiments (GZ FA10A-78Gu-19/2012).

Table 2
Analyzed chemical composition and energy contents of forages and consumed diets.[a]

Variables[b]	Forages			Diets[c]		
	Hay	MS	GS	CON	LA	LAH
% of dry matter (DM) unless stated						
DM (%)	88.6	30.8	32.0	68.5	53.1	53.4
Organic matter	88.6	95.1	86.9	92.3	92.8	92.6
CP	13.5	6.9	13.9	15.1	15.4	15.1
Ether extract	2.4	3.3	2.7	2.8	2.5	2.6
$aNDF_{om}$	45.0	44.9	47.1	32.0	31.6	32.4
ADF_{om}	28.6	26.7	33.1	18.1	18.0	18.1
NFC	27.7	40.1	22.1	42.5	43.9	43.1
ME (MJ/kg DM)	9.2	10.5	9.6	10.9	11.1	11.0
NE_L (MJ/kg DM)	5.40	6.26	5.65	6.60	6.79	6.74

[a] Diets were designed to contain (DM basis) 15% hay, 20% maize silage (MS), 15% grass silage (GS) and 50% concentrate. The concentrate proportion consisted of 77% barley grain and 23% protein-mineral-vitamin premix (45.48% soybean meal, 45.48% rapeseed meal, 5.04% limestone, 2.00% salt and 2.00% mineral-vitamin supplement for lactating dairy cows (contained per kg: Ca 6%, P 12%, Mg 10%, Na 8%, Mn 1500 mg, Zn 5700 mg, Cu 800 mg, vitamin A 750,000 IU, vitamin D3 75,000 IU, vitamin E 3000 mg)).
[b] NFC (nonfiber carbohydrates) = 100 − (% aNDFom + % CP + % ether extract + % ash); ME, metabolizable energy; NE_L, net energy of lactation (calculated according to Gruber et al., 2014).
[c] Experimental diets differed in barley grain processing, being either untreated (CON), or treated with 1% lactic acid (LA), or with 1% lactic acid and heat (LAH) for 24 h.

2.2. Reticuloruminal pH and temperature

To monitor reticuloruminal pH and temperature continuously, all cows received an indwelling wireless pH-transmitting unit (smaXtec animal care sales GmbH, Graz, Austria). These units (3.5 cm i.d., 12 cm long, and weighing 210 g) were manually inserted into the reticulum via the cannula. Prior to this, the units were calibrated by a two point calibration using a buffer of pH 4.0 and one of pH 7.0 following the company's instruction protocol. The use of the wireless pH-transmitting units has been validated in a previous cow experiment (Klevenhusen et al., 2014). The units measured pH and temperature every 10 min and transmitted the data in real-time to a basis station using the ISM-Band (433 MHz). Antennas installed in the barn and milking parlour picked up the sensor signals. Both data of pH and temperature were collected using an analogue to digital converter (A/D converter) and stored in an external memory chip. Besides diurnal changes, data of pH were analyzed as daily mean pH, daily minimal and maximal pH values, standard deviation and duration during which the pH was below the thresholds of 5.8 and 6.0. Because wireless sensors are located in the reticulum (Gasteiner et al., 2009) and little differences are observed between pH measured by wireless units in the reticulum and in the ventral rumen sac (Klevenhusen et al., 2014), the term "reticuloruminal pH" is used here. Data of temperature were analyzed for diurnal changes, as well as for the daily mean temperature, and the duration during which the temperature was above a threshold of 39.5 °C.

2.3. Investigation of ruminal nutrient degradability using the in situ incubation technique

To evaluate the effects of the diet on ruminal nutrient degradation characteristics, differently treated barley grain samples (coarsely ground using a hammer mill without a sieve) were incubated in the rumen of the respective cows fed the CON, LA and LAH diets, and parameters of the in situ degradation kinetics were evaluated. The total incubation time in the rumen was 48 h. Barley samples of approximately 5 g (DM basis) were transferred into polyester bags (10 × 20 cm; Ankom, USA) with a pore size of 50 ± 15 μm (mean ± SD) and incubated in the rumen of each cow (Ørskov et al., 1980). The polyester bags used for the determination of disappearance at 0 h were placed into 39 °C warm water for 15 min. The other bags were located into the ventral rumen sac at 06:00 h on day 15 and incubated for 2, 4, 8, 12, 18, 24, 32, and 48 h on days 15, 16, and 17 of each experimental period. Samples of 0–4 h were incubated in duplicate, those of 8 and 12 h in triplicate, those of 18 and 24 in quadruplicate, and samples of 32 and 48 in quintuplicate to obtain enough sample residues for chemical analysis from each diet and each cow. In addition, one empty bag was incubated for 48 h as blank in each incubation to correct for changes in the weight of the polyester bag itself. After incubation, samples were removed from the rumen and directly put into ice water to stop fermentation processes. The same procedure was applied with samples from 0 h. Samples were thoroughly rinsed with water and washed in the washing machine. Afterwards, all sample bags were dried at 55 °C for 48 h, weighed, and stored at 4 °C until analyses.

2.4. Apparent total tract digestibility

The ATTD was determined using TiO_2 as an external marker. From d 12 to 20 of each experimental run for each cow 20 g of TiO_2 were dosed twice daily at 07:00 h and 15:00 h (40 g per animal and day) through the rumen cannula. Fecal grab samples containing approximately 200 g of fresh feces were taken from the rectum twice during each 24-h period in the last 7 d of each experimental run and combined by animal at the end of each collection period. Feces were mixed thoroughly and 20% of it was sampled and dried at 60 °C for 72 h using a forced air oven.

2.5. Chemical analysis and calculation of energy contents

All samples were ground through a 0.5-mm screen (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany). Dry matter was determined by oven-drying at 103 °C and ash by combustion over night at 580 °C. The ground samples were analyzed for N using the Kjeldahl method, ether extract, ash, aNDFom and ADFom were analyzed according to the German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA, 2012). Heat stable α-amylase was used in the aNDFom procedure (Van Soest et al., 1991) and both fibre fractions were expressed exclusive of residual ash (i.e., aNDFom, ADFom). Starch contents were determined using commercial enzymatic kits (K-TSTA; Megazyme International Ireland Ltd., Bray, Ireland). The P content was determined photometrically using the vanado-molybdate method, which measures the colour intensity at 436 nm after wet-ashing the lyophilized samples in HNO_3 and H_2O_2 via microwave (MLS-ETHOS plus Terminal 320, Leutkirch, Germany). The approach for the photometrical determination of the TiO_2 concentration followed the specifications of Brandt and Allam (1987) and the modification of Glindemann et al. (2009). Analysis of InsP in barley grains was performed using a modified method of Blaabjerg et al. (2010). HPIC was used for separation of $InsP_2$-$InsP_6$ and isomers of $InsP_3$-$InsP_5$. In brief, 0.5 g of ground samples were extracted with 5 mL of 0.5 M HCl for 3 h and centrifuged at 5.000 rpm for 30 min using ultracentrifugation (Amicon® Ultra-2.0, Merck Millipore, Austria). Separation was performed on a HPIC CarboPac PA-1 analytical column combined with a CarboPac PA-1 guard column (Dionex, Thermo Scientific, Austria). Inositol phosphates (InsP) were eluted with a gradient of 5–100% CH_3SO_3H at a column temperature of 30 °C. The eluate was monitored using UV detection after post-column reaction with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ in 2% $HClO_4$ using a Dionex mixing tee and a reaction coil (7 m, 0.25 mm i.d.). The flow rates of the eluents and the post-column reaction solution were 0.5 and 0.33 mL min^{-1}, respectively. A reference sample for peak identification and verification of retention times was prepared by

Table 3
Concentrations of various Inositol phosphates (InsP) in untreated barley grain (CON), or barley grain soaked in 1% lactic acid solution without (LA) or with heat (LAH).

InsP-P (g/kg DM)	CON	LA	LAH
$InsP_2$ [b]	ND[a]	0.61	ND
$InsP_3$ [c]	ND	0.09	0.11
Ins (1,5,6) P_3	ND	0.06	0.42
Ins (1,2,5,6) P_4	0.13	0.36	0.64
Ins (2,4,5,6) P_4	ND	0.13	0.12
Ins (1,4,5,6) P_4	ND	0.03	ND
Ins (1,2,4,5,6) P_5	0.12	ND	ND
Ins (1,2,3,4,6) P_5	0.07	ND	ND
Ins (1,2,3,4,5) P_5	0.07	ND	ND
$InsP_6$	2.61	0.03	0.06

[a] ND, not detectable.
[b] No further differentiation between isomers possible.
[c] No further differentiation between isomers possible due to coelution of Ins(1,2,6)P_3, Ins(1,4,5)P_3 and Ins(2,4,5)P_3.

partial hydrolysis of phytic acid by boiling 4 g of phytic acid solution with 74 mL 0.5 M HCl for 20 h at 120 °C. Due to the lack of lower InsP standards, the phytic acid dodecasodium salt hydrate was used as standard for $InsP_6$ as well as for the lower InsP. Correction factors for difference in detector response for $InsP_5$-$InsP_2$ isomers compared to $InsP_6$ were derived from literature (Blaabjerg et al., 2010). Energy contents [metabolizable energy (ME) and net energy of lactation (NE_L)] of all feeds including barley were calculated from their analyzed chemical composition and in vivo digestibility coefficients obtained from each feed, as described previously (Gruber et al., 2014).

2.6. Calculations and statistical analyses

Variables of the in situ kinetics of nutrient disappearance were estimated using the NLIN procedure of SAS (SAS Institute Inc., Cary, NC, version 9.2) according to the following model (McDonald, 1981):

$$Y = a + b \times (1 - \exp^{-k_d \ (t-L)}) \quad \text{for} \quad t > L,$$

where Y is the disappearance of nutrients in % at a certain time after incubation; a is the soluble fraction (%); b is the insoluble, potentially degradable fraction (%), k_d is the fractional rate of disappearance (%/h); L is the lag time (h); and t is the time of incubation (h). The effective rumen degradability (ERD) was calculated assuming a fractional passage rate (k_s) of 6%/h using the following equation: $ERD = a + b \times k_d/(k_d + k_s)$.

General statistical evaluation of the data was done using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, version 9.2). For each response variable tested, the model included the fixed effects of period, measurement time (i.e., day or hour), and treatment as well as the resulting 2-way interaction of the latter 2 factors. Measurements collected at different times on the same cow were considered as repeated measures in the ANOVA, with a first-order autoregressive variance-covariance structure. The PDIFF option was used in each of the comparisons. Degrees of freedom were approximated by the method of Kenward–Roger. Furthermore, the significance of the overall effect of the LA treatment was tested for all variables (linear contrast involving the average of the two lactic acid treated groups vs. control) with the CONTRAST statement of SAS. The least squares means and the respective standard error of means were computed. Significance was declared at $P \leq 0.05$, whereas a tendency was considered for $0.05 < P \leq 0.10$.

3. Results

3.1. Chemical composition and energy content of barleys and diets and concentration of different inositol phosphates of barleys

The treatment of barley with LA and LAH had minimal effects on the composition of crude nutrients (Table 1). Treatments with LA and LAH resulted in slightly lower starch but higher aNDFom contents. Treatments with LA and LAH increased the energy contents (both ME and NE_L) of the treated barleys. However, these differences in energy content of barleys did not play a role in the total NE_L content of the whole diets (6.6 vs. 6.8 vs. 6.7 MJ NE_L/kg DM) (Table 2). The content of other crude nutrients of barley did not apparently change due to treatment (Table 1). Determination of InsP revealed a strong reduction in $InsP_6$-P in LA (0.03 g/kg) and LAH (0.06 g/kg) compared to CON (2.61 g/kg) (Table 3). While the predominate $InsP_5$ isomer was Ins(1,2,4,5,6)P_5, in CON, $InsP_5$ was not detectable in LA and LAH. On the opposite, higher concentrations of $InsP_4$ were measured in LA and LAH, with Ins(1,2,5,6)P_4 as the prevailing isomer. Furthermore, $InsP_3$ was not detectable in CON, whereas small amounts were found in LA and higher amounts in LAH with InsP(1,5,6)P_4 as the dominating form. While no $InsP_2$ isomers were detectable in CON and LAH, 0.61 g $InsP_2$-P was measured in LA.

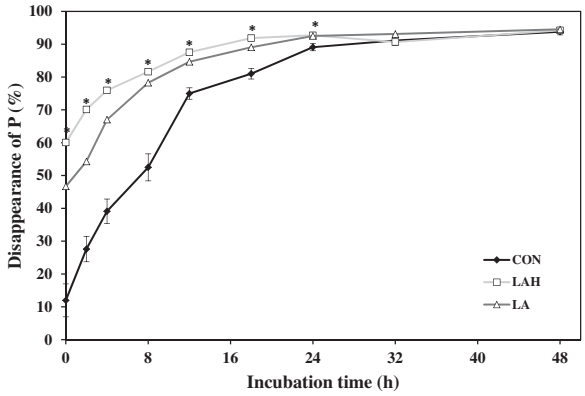

Fig. 1. The kinetics of disappearance of phosphorus in untreated barley (CON), or barley grain soaked in 1% lactic acid solution without (LA) or with heat (LAH) in Holstein cows fed diets based on corresponding barley grains, * differences at $P \leq 0.05$.

Table 4
In situ kinetics parameters of the disappearance of phosphorus, starch, and crude protein in untreated barley grain (CON), or barley grain soaked in 1% lactic acid solution without (LA) or with heat (LAH).

Item[a]	Dietary treatment			SEM	*P*-values[b]	
	CON	LA	LAH		Treatment	Contrast
Phosphorus						
a (%)	35.9	39.9	39.7	0.87	0.01	<0.01
b (%)	60.3	54.0	54.0	1.45	0.01	<0.01
k_d (%/h)	10.5	14.4	15.4	1.15	0.23	0.09
L (h)	3.3	−0.8	−0.47	1.27	<0.01	<0.01
ERD (%)	73.1	77.7	77.0	1.72	0.17	0.07
UP (%)	26.9	22.3	23.0	1.72	0.17	0.07
Starch						
a (%)	37.7	36.3	35.6	1.17	0.41	0.23
b (%)	54.4	57.0	58.1	1.27	0.16	0.07
k_d (%/h)	22.6	17.2	15.1	2.71	0.17	0.08
L (h)	1.56	1.91	1.96	0.16	0.14	0.06
ERD (%)	69.5	66.2	64.2	2.05	0.23	0.12
RUS (%)	30.5	33.8	35.8	2.05	0.23	0.12
Crude protein						
a (%)	16.2	16.4	16.9	1.32	0.92	0.78
b (%)	74.5	74.6	73.6	1.50	0.86	0.85
k_d (%h)	14.3	12.0	10.2	1.63	0.37	0.22
L (h)	−0.24	0.07	−0.97	0.23	0.07	0.51
ERD (%)	67.5	65.7	62.9	1.51	0.21	0.16
RUP (%)	32.5	34.3	37.1	1.51	0.21	0.16

[a] *a* is the soluble fraction; *b* is the insoluble, potentially degradable fraction, k_d is the fractional rate of disappearance; *L* is the lag time; ERD, effective rumen degradability, calculated assuming a fractional passage rate (k_s) of 6%/h using the following equation: $ERD = a + b \times k_d/(k_d + k_s)$; UP, unreleasable phosphorus in the rumen: 100 − ERD of phosphorus; RUS, rumen undegradable starch: 100 − ERD of starch; RUP, rumen undegradable protein: 100 − ERD of crude protein.

[b] Effect of dietary treatment (Treatment), overall effect of lactic acid (Contrast).

3.2. *In situ ruminal degradation kinetics*

Data of the disappearance of P in untreated and treated barley samples are shown in Fig. 1. These data showed higher P disappearance at 0, 2, 4, 8, 12, 18 and 24 h of incubation ($P < 0.05$) in LA and LAH compared to CON barley. Additionally, the analysis of P disappearance kinetics revealed a higher soluble fraction *a* ($P < 0.01$) and ERD ($P = 0.07$), as well as lower *b* fraction and shorter lag time ($P < 0.01$) in the LA and LAH treatments (Table 4).

Starch degradation at various incubation time points is presented in Fig. 2a. After 2 h of incubation, the starch disappearance was lower ($P < 0.05$) in barley processed with LA and LAH (45.5% in CON, 39.6% in LA, 34.5% in LAH). Almost similar effect was observed at 4 h of incubation (decrease from 62.6 to 53.8 and 53.6%), but the difference minimized thereafter (Fig. 2a). Kinetics data of starch disappearance showed a tendency for LA-treatments to prolong ($P = 0.06$) the lag time of degradation (from 1.56 to 1.91 and 1.96 h for CON, LA and LAH, respectively) and lower fractional disappearance rate of starch (k_d) from 22.6 to 17.2 and 15.1%/h was observed ($P = 0.08$; Table 4).

The degradation of CP, DM and OM at various incubation times is shown in Fig. 2b–d. The disappearance of barley CP was higher ($P = 0.01$) at 0 h for the LAH treatment (23.0% compared to 17.1 and 15.9% in CON and LA, respectively) but after 24 h of incubation it was significantly lower ($P = 0.04$) for this treatment group compared to CON (82.9 vs. 87.1%) while an

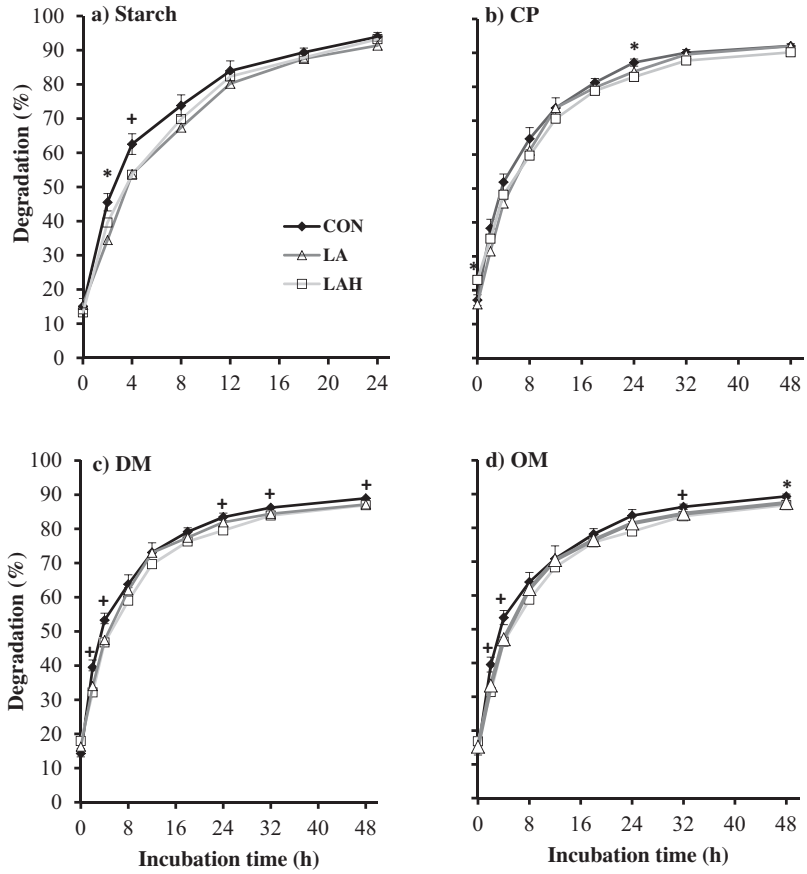

Fig. 2. The kinetics of in situ degradation of starch (a), crude protein (CP) (b), dry matter (DM) (c) and organic matter (OM) (d) of untreated barley grain (CON), or barley soaked in 1% lactic acid solution without (LA) or with heat (LAH) in Holstein cows fed diets based on corresponding barley grains, * differences at $P \leq 0.05$, + differences at $0.05 < P \leq 0.10$.

intermediate value was obtained with LA (84.5%). Lag time tended to be lower in LAH compared to LA ($P = 0.07$), whereas other kinetics variables of CP were not affected by treatment (Table 4). In situ DM disappearance was on average 7% lower at 2, 4, 24, 32 and 48 h in barley processed with LA or LAH ($P < 0.10$, Fig. 2c). Similarly, disappearance of OM was on average 11% lower at 2, 4, 32 and 48 h of incubation with the barley grains treated with LA or LAH ($P < 0.10$, Fig. 2d). Nevertheless, no significant effect of treatment on in situ DM and OM degradation kinetics was observed.

3.3. Feed intake and apparent total tract digestibility of nutrients

Data of DM intake of concentrates, maize silage, grass silage, and hay as well as the ATTD of nutrients are listed in Table 5. No differences in DM intake were observed among treatment groups ($P > 0.10$).

The ATTD of the whole diet DM was slightly greater in cows fed LA (66.6%) and LAH (67.4%) than in cows fed the control diet (65.0%, $P = 0.05$). No differences could be detected for the ATTD of OM, crude protein (CP), ether extract, aNDFom and non-fibre carbohydrates (NFC) between treatment groups ($P > 0.10$). The lowest DM of feces was found in the LAH group ($P = 0.05$; Table 5).

3.4. Reticuloruminal pH and temperature changes

The treatments showed no effect on minimum, mean and maximum reticuloruminal pH as well as on time that the pH was below pH 6.0 and pH 5.8, respectively (Table 6). The mean reticuloruminal pH ranged from 6.34 to 6.40 and the time below pH 6.0 from 29.1 to 108.8 min/d, respectively. In general, pH values showed higher variations in the cows receiving barley grains treated with lactic acid ($P < 0.05$). The mean reticuloruminal temperature and the mean time above 39.5 °C were not affected by treatment (Table 6). Diurnal variations of mean reticuloruminal pH and temperature on an exemplary day (d 15 of the respective experimental periods) are shown in Fig. 3. Values were averaged over all treatment groups as no significant effect of the barley processing method was observed. However, reticuloruminal pH and temperature were significantly affected by time ($P < 0.01$). The pH profile showed that the maximal pH was recorded just before offering the

Table 5
Dry matter (DM) intake of dietary components and total-tract apparent digestibility (ATTD) in dairy cows fed diets based on untreated barley grain (CON), or barley soaked in 1% lactic acid solution without (LA) or with heat (LAH).

Item[a]	Dietary treatment			SEM	*P*-values[b]	
	CON	LA	LAH		Treatment	Contrast
Intake (kg DM/d)						
Concentrate	9.7	9.4	9.3	0.71	0.91	0.70
Maize silage	4.2	3.7	4.0	0.30	0.11	0.08
Grass silage	2.4	2.0	2.2	0.15	0.41	0.28
Hay	2.9	2.7	2.8	0.09	0.37	0.19
Overall	19.3	17.9	18.3	1.33	0.76	0.50
ATTD (%)						
DM	65.0 b	66.6 a	67.4a	0.08	0.05	0.03
Organic matter	68.0	69.6	70.1	0.39	0.25	0.17
Crude protein	80.6	81.0	82.1	0.38	0.32	0.28
Ether extract	61.8	63.1	61.8	0.41	0.35	0.41
aNDFom	45.8	46.2	48.9	0.40	0.17	0.18
NFC	88.8	90.0	89.7	0.37	0.38	0.25
DM of feces, g/kg	159.5 a	161.0 a	149.9 b	0.41	0.05	0.08

Means within a row with different letters (a and b) differ ($P < 0.05$).
[a] NFC, non-fibre carbohydrates = 100 − (% aNDFom + % crude protein + % ether extract + % ash).
[b] Effect of dietary treatment (Treatment), overall effect of lactic acid (Contrast).

Table 6
Data of reticuloruminal pH and temperature in dairy cows fed diets based on untreated barley grain (CON), or barley soaked in 1% lactic acid solution without (LA) or with heat (LAH).

Item[a]	Treatment			SEM	*P*-value[b]	
	CON	LA	LAH		Treatment	Contrast
Reticuloruminal pH						
Minimum	6.03	5.95	5.99	0.02	0.49	0.33
Mean	6.38	6.34	6.40	0.02	0.64	0.77
Maximum	6.66	6.70	6.73	0.01	0.29	0.17
Standard deviation	0.14 b	0.16 a	0.16 a	0.004	0.04	0.01
Time <6.0, min/d	29.05	108.81	40.24	12.09	0.18	0.25
Time <5.8, min/d	1.19	30.95	5.95	4.54	0.22	0.27
Reticuloruminal temperature						
Mean	38.72	38.82	38.78	0.01	0.82	0.30
Time >39.5 °C, min/d	127.14	176.43	142.14	14.12	0.55	0.65

Means within a row with different letters (a and b) differ ($P < 0.05$).
[a] Data of pH and temperature are averaged across d 15 to d 21 of experimental periods.
[b] Effect of dietary treatment (Treatment), overall effect of lactic acid (Contrast).

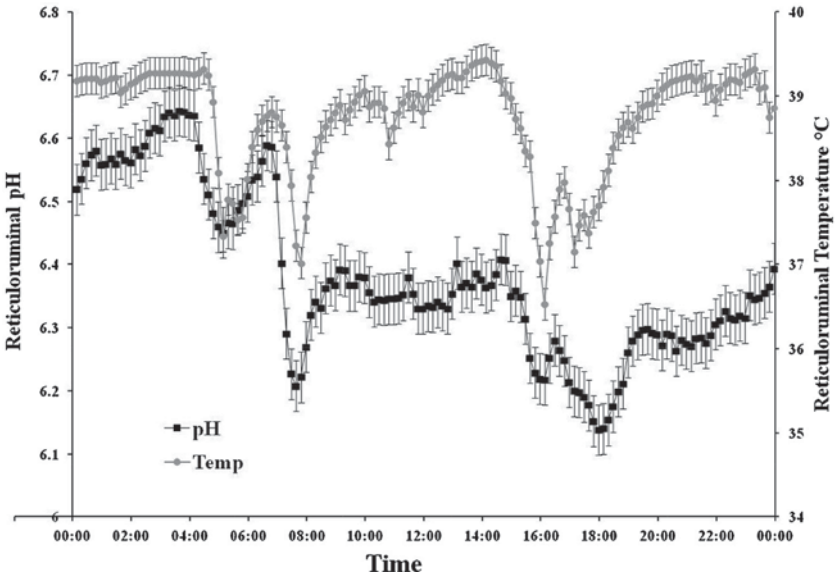

Fig. 3. Diurnal variation of reticuloruminal pH and temperature in Holstein cows averaged across all treatment groups on day 15 of the experimental periods.

fresh feed and declined rapidly after the concentrate was fed. Additionally, abrupt drops in temperature were noticed when fresh feed was offered.

4. Discussion

4.1. In situ ruminal disappearance of P

The current in situ results suggest that treatment with 1% LA and LAH enhanced the kinetics of ruminal disappearance of P of barley grain. As P utilization largely depends on its solubility in the rumen (Field, 1981), the increased P disappearance is an important prerequisite to improve its utilization in ruminants. In barley, the $InsP_6$ makes almost 60% of the total P (Metzler-Zebeli et al., 2014), which is mainly located in the aleurone layers and the outer bran (Humer et al., 2015). The release of P in cereal grains depends upon the hydrolysis of $InsP_6$ (Humer and Zebeli, 2015). The underlying mechanism(s) by which LA treatment increased the disappearance of P of the treated barleys is likely the potential of LA and LAH to promote the hydrolysis of $InsP_6$ in barley (Haraldsson et al., 2004; Metzler-Zebeli et al., 2014). The efficacy of LA to promote the hydrolysis of $InsP_6$ of cereal grains has been described by Haraldsson et al. (2004), using low concentrations of LA. More recently, using a large array of combinations of LA concentrations (0.5, 1 and 5%) and heat treatments, a linear increase in the hydrolysis of $InsP_6$ by 0.5 to 1 g in barley was observed (Metzler-Zebeli et al., 2014), making 20–30% of total P of the cereal grains available. Treating barley with LA and additional 55 °C thermal treatment was the most efficient method to reduce the content of $InsP_6$, and this might explain why LAH was a more efficient treatment to enhance the P disappearance compared to LA in this study. Indeed, LAH treatment changed the abundance of $InsP_4$ and $InsP_5$ isomers with Ins(1,2,5,6)P_4 and Ins(1,2,3,4,5)P_5 as the dominating isomers (Metzler-Zebeli et al., 2014). A strong reduction of $InsP_6$ was also visible in the current study and treatment with LA and LAH also changed the abundance of $InsP_2$-$InsP_5$ isomers, with Ins(1,2,5,6)P_4 as the main isomer, resembling to the predominating $InsP_4$ isomer when microbial 3-phytase is supplied (Schlemmer et al., 2001).

As the inherent phytase activity of rumen microbes can hydrolyze nearly all $InsP_6$ into inorganic P (Raun et al., 1956), previous studies considered the whole $InsP_6$-P fully available to ruminants (Morse et al., 1992). In fact, the in situ data presented here also indicate that after 24 h of incubation almost 90% of barley P disappeared, suggesting a relatively high $InsP_6$ degradation in the rumen of the cows. However, rather than total disappearance of P from barley grain aleurone matrix, the speed by which $InsP_6$-P is hydrolyzed is more important for the availability of P for rumen microbes and the host. Indeed, new studies in high-producing dairy cows question the fact that $InsP_6$ is completely available in ruminants due mainly to greater DM intake, faster passage rate and suboptimal rumen fermentation in these cows (Jarrett et al., 2014). Such conditions may limit ruminal $InsP_6$ degradation due to short-duration exposure of the $InsP_6$ molecule to microbial phytase. Accordingly, recent studies have indicated that the ruminal degradation is incomplete and is influenced by the processing of feedstuffs and supplementation with exogenous phytase (Konishi et al., 1999; Brask-Pedersen et al., 2013). In addition, high-grain diets are also associated with reduced secretion of saliva, possibly decreasing salivary P available for microbial use and for absorption in the small intestine (Scott and Buchan, 1985).

4.2. In situ ruminal degradation of starch

Another finding of the study is the evidence that treatment of barley grain in LA and LAH modulated in situ rumen degradation kinetics of starch in cows. Indeed, results obtained from the in situ trial showed a reduced starch disappearance after 2 and 4 h of incubation of on average 17%, as well as a tendency for lower fractional disappearance rate of starch in LA and LAH treated barley. Additionally, a tendentially prolonged degradation lag time was observed in the LA-treated groups, which suggests an increased resistance of treated barley starch against the amylolytic microbial degradation. In support of these findings it has been shown that treating barley with 1 and 5% LA can reduce the rate of enzymatic starch digestion (Deckardt et al., 2014) and modulate rumen microbial activity in semi-continuous culture (Deckardt et al., 2015). Additionally, heating barley grain has been shown to decrease rumen digestibility of the starch in dairy cows (Ljøkjel et al., 2003). Studies conducted by Iqbal et al. (2009, 2012) also found lowered ruminal fermentation intensity when late-lactation dairy cows were fed diets treated with LA and heat; however, in these studies this effect was associated with a slightly improved ruminal pH (Iqbal et al., 2009, 2012).

4.3. In situ ruminal degradation of crude protein

When high amounts of barley are included in the diet, barley proteins constitute a significant part of total dietary protein. However, due to its high rumen degradation, its contribution to post-ruminally digestible protein is low (Prestløkken, 1999). Thus, several treatments aimed at reducing rumen degradation of barley protein, thereby increasing post-ruminal protein value. In the present study, the decrease in degradation of CP after 24 h of incubation for the LAH treatment was counterbalanced by an apparent increase in the water soluble fraction. Thus, no higher rumen undegradable protein was obtained. Deckardt et al. (2015) and Harder et al. (2015b) found a decrease in CP degradation and lower ammonia when barley grain was treated with LA, citric acid, tannic acid and heat (55 °C for 48 h) in an in vitro rumen simulation technique. Other studies using steam flaking, flame roasting and expander treatment demonstrated that most of the reduction in rumen

degradable protein in barley can be obtained by modest heat at 100 °C for 5 min (Ljøkjel et al., 2001). Even though several previous studies have shown that heat treatment is an effective method to decrease rumen CP degradability, several factors may influence the effect, including temperature, time, moisture level and presence of reducing sugars (Wallace and Falconer, 1992; Martins et al., 2001). Together, the relatively low temperature during the treatment could be the reason for the absence of a more pronounced reduction in rumen degradability of the LAH diet in this study.

4.4. *In situ ruminal degradation of dry matter and organic matter*

In situ rumen disappearance of DM and OM was lowered at 2, 4, 32 and 48 h of incubation with the LA-treated diets compared to CON. Nevertheless, these differences did not significantly affect rumen degradation kinetics. However, Iqbal et al. (2009) found greater ERD of DM of barley grain treated with 1% LA compared to barley soaked in an equal quantity of tap water. On the other side, soaking of barley in 0.5% LA reduced the ERD of DM. These results emphasize the importance of LA concentrations on the degradation of barley grain in the rumen, as observed previously, too (Deckardt et al., 2014, 2015).

4.5. *Feed intake and apparent total tract digestibility of nutrients*

Although treatment with LA and LAH enhanced energy content of barleys by about 0.5 MJ/kg DM, the total NE_L content of the diets was similar. Also, the DM intake of concentrate and forages remained unchanged, resulting in no differences in overall DM intake among groups. This is in agreement with results by Iqbal et al. (2009, 2012) who also recorded a lack of response to LA and LAH treatments and a similar range of DM intake. It seems that treatment of grain with LA and LAH does not affect the palatability of the treated grain.

Theoretically, reducing starch degradation in the rumen would allow for more starch to be digested in the intestine, which in turn would decrease the microbial protein output (Silveira et al., 2007). The barley treated with LA or LAH required a longer time to be degraded in the rumen, suggesting that more starch would be available for intestinal absorption. In general, the efficacy of increasing small intestinal glucose absorption depends on reducing ruminal starch degradation without hampering overall digestion of starch in the gastrointestinal tract (Harmon et al., 2004). Indeed, although reduced in situ starch degradation in the rumen was determined, no diminishing effect on ATTD of NFC was measured. Because starch was the main component of NFC, it is reasonable to assume that the absence of treatment effects on ATTD of NFC is indicative of a shift in the site of starch digestion from the rumen to the intestine. Determination of energy content of barley indicated an increase of ME and NE_L content by about 0.5 MJ/kg DM. Both these units were measured taking into account both the analyzed crude nutrient composition and in vivo digestibility coefficients of barley in a previous experiment with sheep (Gruber et al., 2014). Because crude nutrients did not significantly change, the increases in energy values of the treated barley can be attributed to enhancement in DM digestibility and modifications in the degradation profile of its nutrients, and likely also the inclusion of 10 g LA per kg of barley.

4.6. *Reticuloruminal pH changes*

A prolonged degradation lag time of starch and lowered degradation at 2 and 4 h after incubation, observed in cows fed the LA-treated diets in this study, was expected to contribute to higher reticuloruminal pH values in these cows. However, these effects were not reflected in the reticuloruminal pH dynamics of the respective cows. One reason for this discrepancy might be that the cows in the present study were not at eminent risk for developing a subacute rumen acidosis (SARA), wherefore they likely would have been more responsive to dietary treatments than in this case. The fact that cows of the present study were not challenged by SARA conditions is supported by the short duration of the reticuloruminal pH below the threshold of 5.8 (12.7 min/d on average). Normally, a SARA condition is described when ruminal pH drops below thresholds of 5.6 (Plaizier et al., 2009) or 5.8 (Zebeli et al., 2012) for 3 or 5–6 h, respectively. Another reason for the lacking effect of lowered starch degradation on ruminal pH could also be that modifications in starch degradation due to LA treatment of barley were not pronounced enough to cause significant effects on pH and fermentation. Studies conducted by Silveira et al. (2007) have shown that selection of barley grain varieties with pronounced lower ruminal degradation of starch can greatly affect ruminal fermentation and pH in dairy cows.

5. Conclusion

Taken together, the results of the present study provide evidence that soaking of barley grain in 1% LA with or without heat lowered ruminal degradation of barley starch shortly after incubation; however, feeding of LA and LAH-treated barley did not affect reticuloruminal pH and ATTD of most nutrients in dairy cows. We observed an enhanced solubility and ruminal disappearance of native barley P by treating barley with 1% LA and LAH. The latter finding warrants further investigations in particular to establish the extent to which organic P is available to the animal and also to evaluate the potential for reducing P excretion in cattle.

Acknowledgements

We acknowledge the assistance of S. Gschaider, A. Rudischer, A. Dockner, M. Wild and S. Leiner (Institute of Animal Nutrition and Functional Compounds, University of Veterinary Medicine, Vienna) with the animal trial and laboratory analyses. The authors thank the staff of the research station at AREC Raumberg-Gumpenstein for their care to the cows as well as A. Schauer and A. Adelwöhrer for their excellent help in data collection and preparation. The authors are very grateful to the Institute of Animal Nutrition, Livestock Products and Nutrition Physiology, University of Natural Resources and Life Sciences Vienna, especially to A. Busch-Petersen, for analysis of Inositol phosphates. Ö. Sizmaz was a recipient of the postdoctoral fellowship of the Scientific and Technological Research Council of Turkey (Tübitak), which is gratefully acknowledged. This research was supported by project "optGerste" of BMLFUW which is highly acknowledged.

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Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Microbial populations and fermentation profiles in rumen liquid and solids of Holstein cows respond differently to dietary barley processing

B.U. Metzler-Zebeli[1,2], A. Khol-Parisini[2,3], L. Gruber[4] and Q. Zebeli[2,3]

1 Department for Farm Animals and Veterinary Public Health, University Clinic for Swine, University of Veterinary Medicine Vienna, Vienna, Austria
2 Research Cluster "Animal Gut Health", Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria
3 Department for Farm Animals and Veterinary Public Health, Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna, Austria
4 Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Irdning, Austria

Keywords
barley processing, dairy cow, fermentation, lactic acid, rumen microbiota.

Correspondence
Qendrim Zebeli, Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria.
E-mail: qendrim.zebeli@vetmeduni.ac.at

2015/1515: received 28 May 2015, revised 12 September 2015 and accepted 16 September 2015

doi:10.1111/jam.12958

Abstract

Aims: To evaluate the effects of treating barley grain with lactic acid (LA) and heat on postprandial dynamics of 19 microbial taxa and fermentation in the rumen of dairy cows.
Methods and Results: This study was designed as a double 3 × 3 Latin square with six rumen-cannulated cows and three diets either containing untreated control barley or barley treated with 1% LA and 1% LA and heat (LAH, 55°C). Microbial populations, pH and volatile fatty acids were assessed in rumen liquid and solids during the postprandial period. Propionate increased and butyrate decreased in rumen solids of cows fed LA and LAH treated barley compared to the control barley. The LA but not LAH treatment depressed *Fibrobacter succinogenes* in rumen liquid and solids, whereas the opposite effect was observed for *Ruminococcus albus* in both fractions and *Ruminococcus flavefaciens* in rumen solids. LA promoted *Ruminobacter amylophilus* with the effect being more pronounced with LAH. The *Lactobacillus* group and *Megasphaera elsdenii* increased in both fractions with LA but not with LAH.
Conclusions: LA and LAH treatment of barley differently altered ruminal abundance of certain bacterial taxa and fungi and increased propionate fermentation in rumen solids, whereby LA and LAH effects were consistent and mostly independent of the rumen fraction and time after barley feeding.
Significance and Impact of the Study: Results provided evidence that LA and LAH treatment of barley can enhance rumen propionate fermentation without adversely affecting rumen pH. As propionate is the major contributor to gluconeogenesis in ruminants, the present barley treatment may have practical application to enhance energy supply in dairy cows.

Introduction

Integration of knowledge gained from culture-based studies and modern molecular-based techniques rapidly advances our understanding of the extremely diverse and dynamic rumen microbiota and their function (Mullins *et al.* 2013; Golder *et al.* 2014; Seedorf *et al.* 2015). The bacterial microbiome is dominated by bacteria of the *Firmicutes* and *Bacteroidetes* phyla, whereas *Proteobacteria* seem to be the third most abundant bacterial phylum (Mohammed *et al.* 2012; Petri *et al.* 2012; Kang *et al.* 2013; Golder *et al.* 2014). It is common knowledge that rumen microbial populations respond to changes in diet and environmental conditions (Khafipour *et al.* 2009;

Metzler-Zebeli *et al.* 2013a; Golder *et al.* 2014) and display substantial host specificity (Weimer *et al.* 2010). Feeding high-grain diets at the onset of lactation to meet the high energy demand may impact physiological microbial dynamics of cows due to intensification of ruminal fermentation and the subsequent pH drop (Tajima *et al.* 2001; Khafipour *et al.* 2009; Fernando *et al.* 2010). Acidic ruminal conditions alter bacterial abundances and metabolic activity mostly at the expense of fibrolytic and in favour of amylolytic and lactic acid-utilizing microbes (Tajima *et al.* 2001; Khafipour *et al.* 2009; Fernando *et al.* 2010). Suboptimal fibre degradation in the rumen is therefore one of the key reasons for poor feed efficiency and production in dairy cattle (Khiaosa-ard and Zebeli 2014).

Substantial research efforts have been made over the past years to develop feed management practices to maintain and foster the rumen microbial homeostasis and thus to promote optimal production efficiency (de Menezes *et al.* 2011). We could recently show that processing of barley (*Hordeum vulgare* L.) with various concentrations of organic acids such as lactic acid (LA) and an optional heat treatment increased the content of fibre and resistant starch (RS) which is defined to be less degradable for host α-amylases as well as reduced the *myo*-inositol hexakisphosphate content in the treated grain (Deckardt *et al.* 2014; Metzler-Zebeli *et al.* 2014; Harder *et al.* 2015). Due to less soluble carbohydrates, LA-treated barley appears to be less ruminally degradable and thus may have fewer consequences for microbial homeostasis than the native barley (Deckardt *et al.* 2015). Despite some benefits for dairy cow's ruminal pH, energy status and innate immune response were reported before (Iqbal *et al.* 2010, 2012), little is known about the ecological dynamics in the ruminal microbial community after feeding LA-treated grain. Recently, we screened increasing concentrations (0·5–5%) of various acids including LA, with or without an additional heat treatment, *in vitro* (Deckardt *et al.* 2015). This study suggested a concentration of 1% LA combined with an additional heat treatment of 24 h to enhance microbial activity and fibre degradation *in vitro*. In acknowledging that observations made *in vitro* may not necessarily mirror the situation *in vivo*, our present research was based on the hypothesis that treating barley with 1% LA and heat would support the abundance of fibrolytic and hemicellulolytic microbes in the solid and fluid rumen digesta fractions due to increased substrate availability and slower starch fermentation; thereby supporting our recent *in vitro* findings (Deckardt *et al.* 2015).

The objective of this study was therefore to examine the postprandial dynamics of 19 well-characterized ruminal microbial taxa and the fermentation patterns in dairy cows in response to barley treated with 1% LA. Another aim was to evaluate the effects of an additional heat treatment of barley that goes beyond the LA processing alone. For this, barley was treated with 1% LA and heat (LAH). Due to different species abundances in liquid and solid fractions of the rumen, both fractions were investigated separately. We selected a qPCR based approach to detect well-characterized ruminal taxa. These taxa only represent a minor proportion of the rumen microbiota; however, many of them are well accepted sentinel populations in response to dietary changes (Mullins *et al.* 2013). Evaluating dietary responses of well-studied populations may better allow predicting metabolic consequences of changes in rumen ecology when compared to assessing the many often uncharacterized bacterial species in the rumen (Mullins *et al.* 2013).

Materials and methods

Diets

Diets were formulated according to actual nutritional recommendations for dairy cattle of 650 kg BW, producing 30 kg milk day^{-1} with 4·0% fat, and 3·3% protein (GfE 2001) comprising 20% corn silage, 15% grass silage, 15% meadow hay, 38·5% barley grain and 11·5% protein–mineral–vitamin premix. The protein–mineral supplement contained extracted soybean- and rapeseed meal in equal proportions, limestone, salt and a commercial mineral–vitamin premix for lactating dairy cows. The chemical composition of the feeds is given in Table S1. Diets were identical with the only difference in the treatment of barley grain. The barley grain used in the control (CON) diet was untreated chemically and thermally, whereas the other two diets contained barley grain that was treated with 1% LA or 1% LAH prior to feeding. The barley grain used for the CON diet and for LA and LAH treatment was ground to pass a 3 mm screen using a hammer mill (Ultra 110/150, Gruber Machineries, Gaspoltshofen, Austria). The processing of barley consisted of soaking barley grain 1 : 1 (w/v) in a 1% LA solution (v/v; Brenntag, Vienna, Austria) at room temperature alone or in the presence of oven-heating at 55°C for 24 h. The treated barley was freshly prepared daily, prior to the morning feeding.

Experimental design and rumen digesta sampling

The experiment was conducted at the Dairy Research Facilities of the Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Austria. All procedures involving animal handling and treatment were in accordance with national regulations for

animal use in research and approved by the authority according to §9ff of Law for Animal Experiments (GZ FA10A-78Gu-19/2012). The experiment was designed as a replicated 3 × 3 Latin square design using six healthy ruminally cannulated (100 mm i.d.; Bar Diamond, Parma, ID) Holstein cows to evaluate responses to LA and LAH treatment of barley grain. Two cows were fed one diet during each experimental period, resulting in a total of six observations per treatment. Before the start of the trial, the cows produced on average (mean ± SD) 26·4 ± 6·6 kg milk day^{-1} with 4·1 ± 0·3% fat and 3·3 ± 0·4% protein in the milk. The cows were housed in individual tie stalls bedded with rubber mats plus sawdust and had free access to fresh water. All cows were fed *ad libitum* being offered about 110% of the average amount of feed consumed on the three preceding days. Fresh feed portions including the treated or untreated barley grain were provided at about 0700 and 1500 h during adaptation and sampling periods. Feed refusals were collected every day and were subtracted from the feed amount offered to determine dry matter intake (DMI).

The duration of each experimental period was 21 days. Rumen samples for microbial metabolites and taxa were collected on day 18 and 19 of each experimental run in such a way that seven time points were sampled in each cow and period, representing every 2 h of a 12-h period from 0700 h. More specifically, rumen liquid and solid samples were collected before feeding at 0700 h (0 h) and afterwards in 2-h intervals until 1900 h. The sampling at 8 h after feeding took place before feeding the second feed portion of the day. On the first sampling day, rumen samples were collected at 0, 4, 8 and 12 h, whereas on the second sampling day, rumen samples were collected at 2, 6 and 10 h. Free rumen liquid samples (200 ml) from the ventral rumen sac were collected as described before (Zebeli *et al.* 2008). Immediately after collection, ruminal liquid was filtered through four layers of medical gauze (about 1 mm pore size), and frozen at −20°C. It is well recognized that filtering rumen liquid will also remove microbes that adhere to small feed particles (Mullins *et al.* 2013). However, in order to clearly distinguish between rumen liquid and solids and to be able to compare the present data on the rumen microbial communities with previous *in vivo* and *in vitro* studies using the same sampling technique for rumen liquid (Khafipour *et al.* 2009; de Menezes *et al.* 2011; Mohammed *et al.* 2012; Deckardt *et al.* 2015), we decided to apply the common procedure of straining rumen liquid through gauze. Grab samples of ruminal digesta were collected from the rumen mat of the dorsal sac according to Zebeli *et al.* (2008). Briefly, grab samples were collected through the cannula 15–20 cm below the mat surface of the dorsal sac. Subsamples of rumen solids for rumen microbiota analysis were directly aseptically bagged and immediately frozen at −20°C. For SCFA and pH measurement, the rest of the rumen mat sample was squeezed through four layers of medical gauze (about 1 mm pore size) to obtain particle-associated rumen liquid (PARL) before storage at −20°C. The rumen liquid and PARL subsamples for SCFA and pH analysis were collected at all sampling times (0, 2, 4, 6, 8, 10 and 12 h), whereas subsamples for rumen microbiota community analysis were only taken at 0, 4 and 8 h.

Proximate nutrients, SCFA and pH analyses

Diet samples were ground through a 0·5-mm screen (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany). Dietary components were analysed for dry matter, nitrogen using the Kjeldahl method, ash, ether extract (Naumann and Bassler 2012), neutral detergent fibre (NDF) and acid detergent fibre (ADF), with heat stable α-amylase used in the NDF procedure (Van Soest *et al.* 1991). The NDF and ADF results were expressed exclusive of residual ash. Energy content (net energy of lactation, NE_L) of feeds was calculated based on their chemical composition and *in vivo* digestibility coefficients (Gruber *et al.* 2014). The analysed chemical composition and energy content of all dietary components are shown in Table S1.

The pH in rumen liquid and PARL was measured directly after sampling using a pH electrode (S400 Seven Excellence, Mettler-Toledo, Switzerland) calibrated for pH 4·0 and 7·0. Concentrations of SCFA (acetic, propionic, n-butyric, iso-butyric n-valeric, iso-valeric, and caproic acid) were determined using gas chromatography. Rumen liquid and PARL samples (1 ml) were mixed with 0·2 ml oxalic acid and 0·2 ml internal standard (4-methylvalerian acid). After centrifugation at 20 000 ***g*** for 20 min at 4°C, the supernatant was analysed for SCFA as described by Deckardt *et al.* (2015).

DNA isolation

In considering that the applied DNA isolation method affects the bacterial composition and DNA yield (Henderson *et al.* 2013; Weimer 2015), we used the same DNA isolation protocol as in our *in vitro* study (Deckardt *et al.* 2015) to allow direct comparison between our previous and current data. To prevent post-sampling fermentation, an effort was made to minimize the time before DNA extraction and frozen rumen liquid and solid subsamples were thawed on ice until they became pliant. Both types of rumen material were thoroughly homogenized and a subsample of the homogenized mixture was used for genomic DNA isolation using the PowerSoil DNA isolation kit

(MOBIO Laboratories Inc., Carlsbad, CA). This DNA isolation kit uses a bead-beating step to dissociate microbes from feed particles and to disrupt the bacterial cells. After mixing samples with buffer C1 which contains sodium dodecyl sulphate, a heating step at 70°C for 10 min was added to ensure proper lysis of bacteria (Metzler-Zebeli *et al.* 2013a,b; Deckardt *et al.* 2015). Thereafter, samples were bead-beaten followed by chemical removal of cell debris and PCR inhibitors and column-based isolation of total genomic DNA according to the manufacturer's instructions. Isolated genomic DNA was quantified (Nanodrop-2000 spectrophotometer; NanoDrop Technologies, Wilmington, DE) and sample volumes were adjusted to achieve similar DNA concentrations across samples (Mullins *et al.* 2013).

Quantitative PCR

Primer sets used for qPCR have been described previously (Table S2) and their currently known specificities were checked *in silico* by BLAST search in Genbank. Overall, primer sets targeted the 16S rRNA gene of eubacteria and Archaea and the 18S rRNA gene of protozoa. Due to the fact that the primer sets to detect *Butyrivibrio fibrisolvens, Ruminococcus flavefaciens* and *Selenomonas ruminantium* were not completely monospecific, the assemblage identified by these primer sets will be referred to as *B. fibrisolvens* group, *R. flavefaciens* group and *S. ruminantium* group as also suggested by Mullins *et al.* (2013) and Mohammed *et al.* (2012). The *Lactobacillus* group primer sets cover the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Weisella* and the *Enterobacteriaceae* family primer set comprises the genera *Citrobacter, Cronobacter, Enterobacter, Erwinia, Escherichia, Pantoea, Pectobacterium* and *Shigella*.

The qPCR analysis was performed with the Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). The DNA of each microbial species or group in rumen liquid and solids was quantified using the Brilliant II SYBR Green QPCR Low ROX master mix kit (Agilent Technologies). Samples and standards were assayed in 25 μl reaction mixtures containing 12·5 μl of master mix, forward and reverse primers (62·5 pmol) and 1 μl of DNA template. The amplification programme included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, optimal annealing temperature (Table S2) for 30 s, and 72°C for 30 s. Fluorescence was measured at the last step of each cycle. Melting curve analysis was performed to determine the specificity of the amplification. The dissociation of PCR products were monitored by slow heating with an increment of 0·1°C s^{-1} from 55 to 95°C, with fluorescence measurement at 0·1°C intervals. The PCR product length was additionally verified by horizontal gel electrophoresis of a 10 μl aliquot in a 2%-agarose gel in Tris-Acetate-EDTA (40 mmol l^{-1} Tris acetate, 1 mmol l^{-1} EDTA; pH 8·5). The agarose gel contained 2 μl Midori Green Advanced DNA Stain (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for visualization of DNA bands under UV light. A 2 kb ladder (Quantitas DNA Marker, Biozym Scientific GmbH) was included on each gel for confirmation of the correct size of the amplified product. Negative controls without template DNA were included in triplicate. Amplification efficiency was calculated as the negative reciprocal of the slope of the line of the standard curve: $E = -1 + 10^{-1/\text{slope}}$ (Table S2).

Standard curves for each bacterial group and species were generated using 10-fold serial dilutions (10^7–10^3 molecules μl^{-1}) of the purified and quantified PCR products generated by standard PCR using DNA from rumen fluid and digesta of the present experiment and the corresponding primer sets (Metzler-Zebeli *et al.* 2013a,b; Deckardt *et al.* 2015). The final copy number of total bacteria, total protozoa, total methanogens and fungi were calculated using the following equation: (QM × C × DV)/(S × V), where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample, DV was the dilution volume of isolated DNA, S was the DNA amount (ng) subjected to analysis and V was the weight of the sample (g) subjected to DNA extraction as previously described (Metzler-Zebeli *et al.* 2013a,b; Deckardt *et al.* 2015). To minimize errors of quantification of DNA from rumen fluid and solid samples, relative quantification methods for target microbial groups and species were used by expressing the amplification of target microbial groups and species relative to the amplification of reference primers utilizing experimentally derived amplification efficiency (Stevenson and Weimer 2007). Abundances of eubacterial, archaeal methanogens and protozoal groups and species were expressed as percentage of total eubacterial 16S rRNA gene, total archaeal 16S rRNA gene and total protozoal 18S rRNA gene respectively. Eubacteria will be referred to as bacteria hereafter, whereas Archaea will be referred to as methanogens.

Statistical analysis

Data on DMI, microbial abundances, pH and VFA were analysed using analysis of variance (ANOVA) with PROC MIXED in SAS (ver. 9.2; SAS Stat Inc.; Cary, NC). To investigate barley treatment effects, ANOVA was conducted separately for rumen fluid and solids, with treatment, time and their two-way-interaction as fixed effect and cow within experimental period as random effect. To compare the microbial community and fermentation

between rumen fluid and solids, the fixed effect was rumen site, time and their two-way interaction and the random effect of cow within period. Degrees of freedom were estimated with the Kenward–Roger method. Microbial, VFA and pH variables, measured at different times on the same subject, were considered as repeated measures with different variance-covariance methods used to model their time-dependency. The covariance structure of these repeated measures was modelled separately according to the smallest values of the fit statistics based on the Bayesian information criteria (BIC). Differences at $P \leq 0{\cdot}05$ were declared significant, and trends were considered at $0{\cdot}05 < P < 0{\cdot}10$ level. Data are presented as least squares means ± standard error of the mean (SEM). For illustration of microbial shifts related to ruminal site, time after feeding and treatment of barley grains, a linear discriminant analysis was performed using JMP (ver. 10.0.0; SAS Stat Inc.). Total microbial abundances and target groups and species were used as covariates as well as ruminal site, time after feeding and diet as categorical variable respectively.

Results

The dry matter intake of cows fed CON (19·2 kg day^{-1}), LA (17·9 kg day^{-1}) and LAH (18·3 kg day^{-1}) diets did not differ in this study (SEM = 1·33; $P = 0{\cdot}76$). Treatment with LA and LAH modified the chemical composition of barley, resulting in lower crude protein content, greater NDF, nonfibre carbohydrates, and energy contents (Table S1).

Results for the effect of sampling site (rumen liquid and solid fractions) and the overall effect of time after feeding on microbial populations can be found in Tables S3–S5.

Total bacterial, protozoal and methanogen gene copy numbers in rumen liquid (Table 1) and solids (Table 2) were not affected by barley treatment, whereas gene copy numbers of fungi tended ($P < 0{\cdot}10$) to be reduced in rumen liquid and solids in cows fed the LA-treated barley but not in cows fed the LAH treated barley. In the following, effects of barley treatment on microbial abundances as expressed as percentage of total bacterial, methanogenic and protozoal abundances are presented separately for rumen liquid and solids and significant differences between treatment groups are described. The LA, but not LAH, treated barley substantially increased the relative abundances of the *Ruminococcus albus* and *R. flavefaciens* group, *Lactobacillus* group and *Megasphaera elsdenii* in rumen liquid (Table 1). The LA-treated barley depressed the relative abundance of *Fibrobacter succinogenes* in rumen liquid at 0 h compared to the CON barley, whereas the LAH treated barley raised the abundance of *F. succinogenes* compared to cows that were fed the CON barley at 4 and 8 h. Relative abundance of the genus *Prevotella*, in turn, decreased in rumen liquid of cows fed LA-treated barley and even more in cows fed LAH treated barley at 8 h compared to the CON barley.

Barley treatment effects in rumen solids partly differed from those in rumen liquid. Similar to the rumen liquid, *R. albus* abundance in rumen solids increased with LA treatment but not with LAH treatment of barley, whereas the abundance of *Clostridium* cluster XIV was decreased in rumen solids of cows fed the LA and LAH treated barley at 8 h (Table 2). Both treatments increased the relative abundance of *Ruminobacter amylophilus*, with the promotion of its abundance being much more pronounced with LAH than with LA treatment of barley compared to the CON barley. The barley treatment effect was also similar in rumen solids for *Lactobacillus* group and *M. elsdenii* compared to the rumen liquid, being higher in rumen solids of cows fed the LA-treated barley compared to cows fed the CON and LAH treated barley. In addition, LA-treated but not LAH treated barley depressed the abundance of *F. succinogenes* and *Entodinium* spp. in rumen solids independently of the time after feeding.

Linear discriminant analysis also showed three clearly separately clustering microbial communities (Fig. 1c). Fungi, *Clostridium* cluster XIV, *Enterobacteriaceae*, *Entodinium* spp., genus *Prevotella* and *S. ruminantium* group were closely correlated with the CON diet. The LA-treated barley diet was best correlated with *M. elsdenii* and *Lactobacillus* group, whereas *F. succinogenes*, *B. fibrisolvens* group, *Methanobrevibacter* spp. and *Methanosphaera* spp. discriminated best for the LAH treated barley diet.

Ruminal pH and VFA in free rumen liquid and PARL

Ruminal pH decreased by about 0·8 and 1 log units in free rumen liquid and PARL after feeding (Fig. 2a). At 8 h after feeding, LAH treated barley raised the pH by 0·4–0·5 log units in PARL compared to LA and control treated barley. There was no overall effect of grain processing on pH of PARL and free rumen liquid. Total VFA concentrations were similar in both rumen fractions among the diets and peaked with progressing time at 4 and 10 h after feeding, whereby total VFA concentrations reached preprandial levels at 8 and 12 h after feeding (Fig. 2a). The LA and LAH treatment of barley increased the propionate proportion in PARL at all time points but not in free rumen liquid (Fig. 2b). While no effect was observed for acetate, valerate, *iso*-butyrate, *iso*-valerate and caproate (data not shown), the acetate-to-propionate ratio decreased in the PARL of cows fed LA and LAH diets, whereas no effect was observed for free rumen liquid (Fig. S1). Also, there were no

Table 1 Microbial population abundances in rumen liquid of cows fed untreated barley grain (CON), or barley treated in 1% lactic acid without (LA) or with heat (LAH)*,†

Time after feeding	0 h			4 h			8 h						
Microbial abundance	CON	LA	LAH	CON	LA	LAH	CON	LA	LAH	SEM	Time	Diet	Time × diet
Total bacteria (log_{10} gene copies ml^{-1})	9·9	9·8	9·7	9·8	9·9	10·0	10·0	9·9	10·0	0·102	0·076	0·989	0·636
Clostridium cluster XIV (%)	5·35	4·53	5·22	4·50	6·20	4·40	4·39	4·76	4·40	0·741	0·617	0·684	0·443
Butyrivibrio fibrisolvens group (%)	0·12	0·14	0·16	0·09	0·15	0·12	0·10	0·18	0·14	0·043	0·799	0·259	0·952
Clostridium cluster IV (%)	2·34	1·97	2·70	3·32	4·46	3·45	2·80	3·43	2·72	0·663	0·044	0·673	0·684
Ruminococcus albus (%)	0·046	0·060	0·039	0·074	0·163	0·080	0·052	0·084	0·041	0·025	0·014	0·034	0·058
Ruminococcus flavefaciens group (%)	0·18	0·20	0·19	0·33	0·70	0·50	0·23	0·48	0·35	0·109	0·003	0·069	0·604
Lactobacillus group (%)	0·29	2·49	0·18	0·61	5·53	0·42	1·82	3·06	1·06	1·792	0·683	0·075	0·843
Megasphaera elsdenii (%)	0·02	0·29	0·05	0·02	0·99	0·10	0·11	0·96	0·04	0·328	0·572	0·230	0·775
Selenomonas ruminantium group (%)	0·20	0·15	0·10	0·30	0·34	0·23	0·26	0·18	0·19	0·061	0·027	0·272	0·844
Ruminobacter amylophilus (%)	0·003	0·005	0·014	0·012	0·042	0·014	0·016	0·058	0·053	0·021	0·145	0·361	0·800
Fibrobacter succinogenes (%)	1·15	0·51	0·87	1·85	1·70	3·57	1·65	1·36	2·77	0·503	0·002	0·015	0·357
Genus *Prevotella* (%)	50·64	53·39	47·82	49·55	47·97	47·82	62·29	52·73	46·07	4·128	0·304	0·131	0·350
Enterobacteriaceae (%)	0·003	0·004	0·002	0·003	0·008	0·002	0·007	0·007	0·003	0·0027	0·506	0·273	0·822
Total protozoa (log_{10} gene copies ml^{-1})	7·9	7·5	7·5	7·8	8·0	7·8	7·7	8·0	8·0	0·21	0·220	0·923	0·474
Entodinium sp. (%)	60·4	41·0	69·4	90·1	103·8	82·2	70·1	58·6	59·6	24·12	0·173	0·958	0·878
Total methanogens (log_{10} gene copies ml^{-1})	7·6	7·5	7·5	7·4	7·5	7·3	7·4	7·6	7·4	0·12	0·437	0·551	0·743
Methanobrevibacter spp. (%)	96·8	107·3	109·6	105·8	97·0	105·6	107·7	95·5	107·1	4·77	0·900	0·173	0·171
Methanosphaera spp. (%)	0·25	0·39	0·43	0·13	0·23	0·85	0·16	0·08	0·18	0·085	0·001	0·285	0·723
Fungi (log_{10} gene copies ml^{-1})	5·9	5·2	5·4	6·2	5·8	6·3	6·0	5·6	6·2	0·27	0·024	0·054	0·715

*Values are least squares means ± standard error of the mean (SEM); n = 6 per barley treatment and time.
†Time after morning feeding of grain at 0700 h.

Table 2 Microbial population abundances in rumen solids of cows fed untreated barley grain (CON), or barley treated in 1% lactic acid without (LA) or with heat (LAH)*,†

Time after feeding	0 h			4 h			8 h						
Microbial abundance	CON	LA	LAH	CON	LA	LAH	CON	LA	LAH	SEM	Time	Diet	Time × diet
Total bacteria (log_{10} gene copies ml^{-1})	9·6	9·7	9·7	9·5	9·6	9·7	9·7	9·8	9·7	0·094	0·372	0·328	0·853
Clostridium cluster XIV (%)	8·21	7·64	9·75	10·60	8·64	11·07	10·18	8·00	7·94	0·874	0·066	0·056	0·252
Butyrivibrio fibrisolvens group (%)	0·16	0·21	0·28	0·19	0·29	0·20	0·17	0·20	0·26	0·069	0·905	0·399	0·829
Clostridium cluster IV (%)	4·86	5·04	5·18	8·22	7·65	6·85	6·05	5·09	7·88	0·934	0·007	0·649	0·316
Ruminococcus albus (%)	0·044	0·075	0·050	0·037	0·059	0·032	0·052	0·053	0·044	0·010	0·283	0·034	0·667
Ruminococcus flavefaciens group (%)	0·91	1·12	1·07	0·97	1·17	0·99	1·02	0·90	1·07	0·173	0·936	0·767	0·802
Lactobacillus group (%)	0·28	0·52	0·11	0·22	0·80	0·20	0·68	0·84	0·19	0·263	0·467	0·044	0·088
Megasphaera elsdenii (%)	0·02	0·41	0·09	0·004	0·48	0·09	0·05	0·61	0·07	0·208	0·911	0·024	0·986
Selenomonas ruminantium group (%)	0·09	0·12	0·08	0·16	0·17	0·16	0·14	0·13	0·12	0·031	0·043	0·712	0·948
Ruminobacter amylophilus (%)	0·006	0·018	0·029	0·003	0·006	0·044	0·010	0·014	0·051	0·012	0·690	0·002	0·749
Fibrobacter succinogenes (%)	1·71	1·36	2·18	1·58	1·06	1·71	1·60	1·09	1·53	0·325	0·369	0·057	0·942
Genus *Prevotella* (%)	32·07	34·04	30·92	34·10	33·40	29·47	29·64	32·75	31·64	2·340	0·838	0·372	0·725
Enterobacteriaceae (%)	0·0010	0·0008	0·0003	0·0002	0·0010	0·0003	0·0008	0·0005	0·0003	0·0003	0·134	0·526	0·294
Total protozoa (log_{10} gene copies ml^{-1})	8·2	8·1	8·0	7·9	7·7	8·1	8·0	8·0	7·8	0·14	0·195	0·678	0·267
Entodinium sp. (%)	149·2	97·3	154·4	137·2	116·8	155·5	157·1	114·7	209·9	40·11	0·668	0·160	0·950
Total methanogens (log_{10} gene copies ml^{-1})	7·5	7·7	7·7	7·4	7·4	7·5	7·5	7·5	7·5	0·10	0·054	0·302	0·971
Methanobrevibacter spp. (%)	101·3	99·7	101·6	98·8	98·9	95·2	97·6	100·9	101·7	2·87	0·367	0·968	0·671
Methanosphaera spp. (%)	0·66	0·60	0·56	0·95	1·00	0·96	0·65	0·78	0·57	0·149	0·010	0·709	0·951
Fungi (log_{10} gene copies ml^{-1})	6·6	6·4	6·7	6·6	6·4	6·8	6·8	6·4	6·7	0·19	0·881	0·081	0·893

*Values are least squares means ± standard error of the mean (SEM); $n = 6$ per barley treatment and time.
†Time after morning feeding of grain at 0700 h.

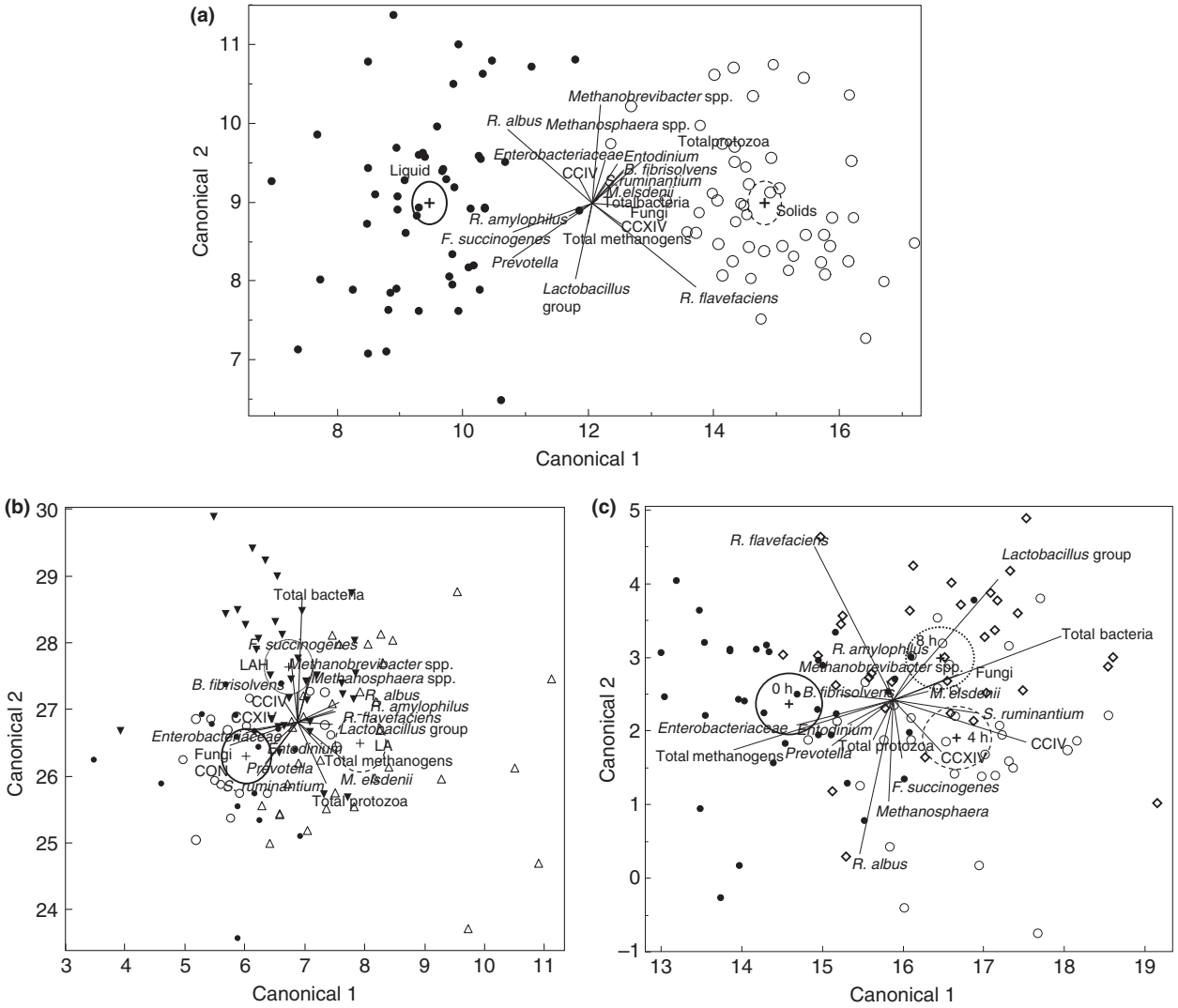

Figure 1 Linear discriminant analysis with the first two principal components of the bacterial, protozoal, archaeal and fungal populations for (a) rumen site: rumen liquid (●) and rumen solids (○), $n = 54$ per rumen fraction; (b) time after morning feeding of grain at 0700 h: 0 h (●), 4 h (○), and 8 h (◇); $n = 36$ per time; and (c) chemical treatment of barley grain: CON = untreated control barley (●), LA = lactic acid treated barley (△), LAH = lactic acid and heat treated barley (▲); $n = 36$ per barley treatment. Circles indicate 95% confidence intervals. CCIV, *Clostridium* cluster IV; CCXIV, *Clostridium* cluster XIV.

time-dependant changes in the proportions of VFA in this study. Other data of this study showed that butyrate proportion was lower in PARL of cows fed the LA and LAH diets when compared to cows fed the CON barley (Fig. 2b). However, no effect of diet was observed for butyrate in rumen liquid, whereby proportions of butyrate changed with feeding time in both rumen fractions (Fig. 2b).

Discussion

Using a targeted qPCR approach, present results showed that treatment of barley grain with 1% LA had a greater impact on targeted microbial populations in rumen liquid and solids than the LAH treatment, suggesting that microbes could differently degrade nutrients in the barley grain after the chemical treatment and the chemical and additional thermal treatment; thereby being consistent with our recent *in vitro* results (Deckardt *et al.* 2015). Effects of LA and LAH treatments were mostly seen on bacterial groups and fungi which might have been related to substrate preferences and changes in substrate availability due to the LA and LAH treatments, whereas an effect on protozoa and methanogenic Archaea was almost absent. Accordingly, the *Lactobacillus* group, *M. elsdenii*, the *R. flavefaciens* group, *R. albus* and fungi were

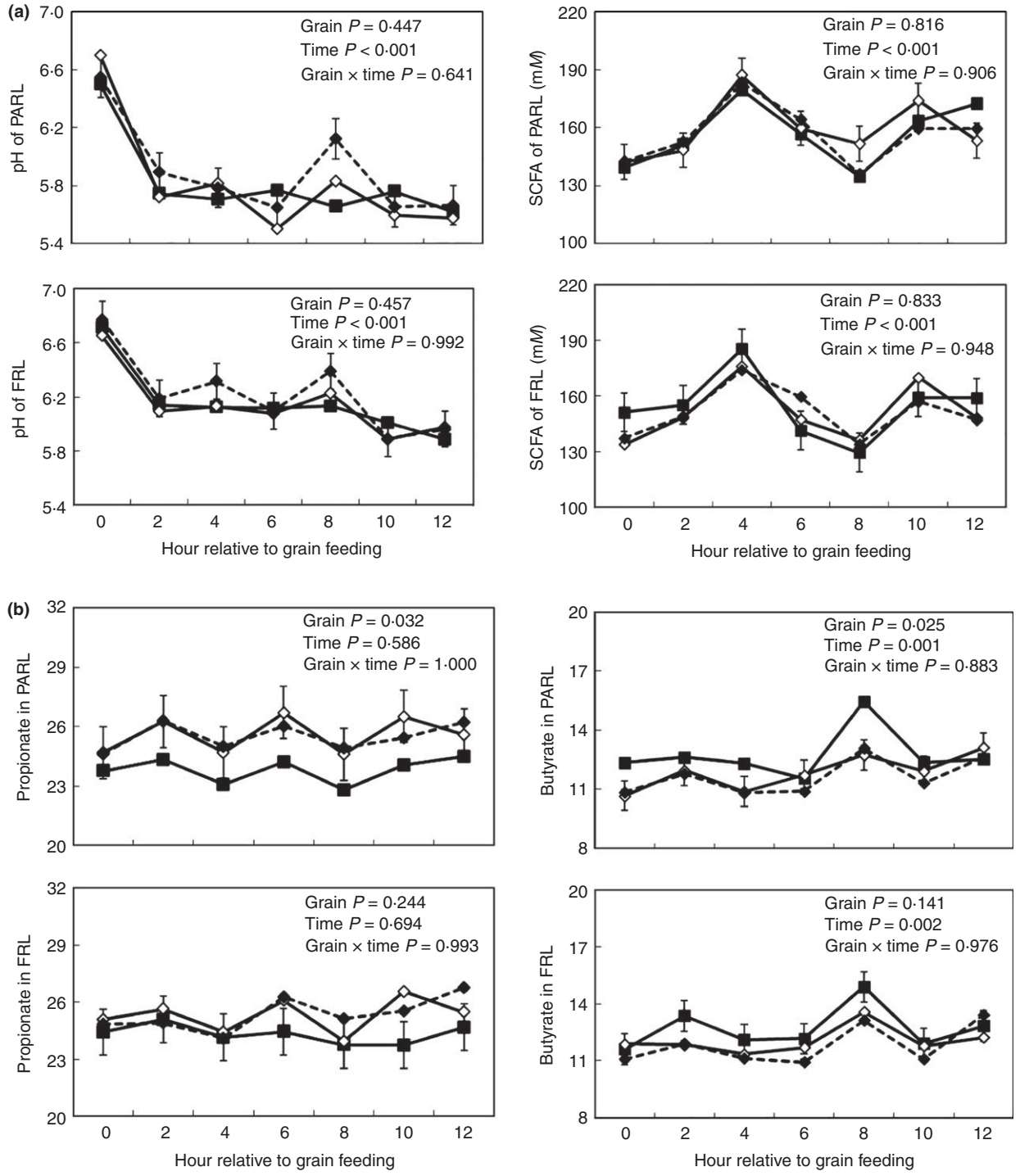

Figure 2 (a) Effects of grain processing on pH and concentration of total volatile fatty acids (VFA) and (b) proportion of propionate and butyrate (mol 100 mol^{-1} total volatile fatty acids) in particle-associated rumen fluid (PARL) and free rumen liquid (FRL) in Holstein cows fed diets containing unprocessed barley (■), barley processed with 1% lactic acid (◊), or barley processed with 1% lactic acid and heat (♦). Time after morning feeding of grain at 0700 h. Data are shown as least squares means ± standard error of the mean, $n = 6$ per barley treatment and rumen fraction.

promoted by the LA treatment, whereas *R. amylophilus* may have been more representative for the LAH treatment of barley in the present study. Besides the treatment of barley, LDA scores plots emphasized that rumen site and time after feeding were similarly important influencing factors for rumen microbial populations in the

current study. Detailed information related to the alterations in the chemical composition of the native barley grain caused by the LA and LAH treatments can be found elsewhere (Deckardt *et al.* 2014; Metzler-Zebeli *et al.* 2014; Harder *et al.* 2015).

The LA and LAH treatment of barley did not influence pH profiles in rumen liquid and PARL and likely caused similar postprandial pH-related effects on the microbial population structure and activity for all three diets. Likewise, treating barley grain with 1% LA or LAH seemed to have little influence on ruminal VFA concentrations in rumen liquid and PARL which was inconsistent with our recent *in vitro* (Deckardt *et al.* 2015) and earlier *in vivo* findings when barley grain was treated with LA concentrations of 0·5 or 1% with or without heat (Iqbal *et al.* 2009, 2012) and may be related to differences in the experimental set-up.

Bacterial, methanogenic archaeal, protozoal and fungal abundances were generally in the range previously reported for rumen liquid and solids (Stevenson and Weimer 2007; Zhou *et al.* 2009; Petri *et al.* 2012; Mullins *et al.* 2013). The relative abundance of *Entodinium* sp. and *Methanobrevibacter* spp. in rumen liquid and solids partly surpassed 100%. This can be related to different numbers of the copies of gene sequences within the protozoal 18S rRNA gene and the archaeal 16S rRNA gene that were amplified by the universal and genus-specific primers. Also, different species within *Entodinium* and the *Methanobrevibacter* may have different copy numbers of the targeted gene sequences, thereby leading to relative abundances of greater than 100% when expressed as percentage of total protozoa and total methanogens. Concentrations of VFA were greatest at 4 h after feeding which coincided with the microbial population profiles in rumen liquid and solids. In contrast to ruminal VFA concentrations which reached preprandial levels at 8 h after morning feeding, abundances of most microbial populations at 8 h remained higher than before feeding the barley grain portion at 0 h, being available for anew substrate degradation. The latter likely explains the observation that after provision of the second barley portion at 1500 h the peak VFA concentration was already reached 2 h later (corresponding to 10 h after morning feeding) and not 4 h after the second barley portion at 12 h when compared to the increase in VFA after morning feeding.

With the changes in rumen degradability of starch and chemical modification in the LA and LAH treated barley, it seemed feasible that fibrolytic species were more abundant in cows fed the LA and LAH treated barley compared to cows fed the CON barley. Yet, results showed that the LA and LAH treatment effects need to be regarded separately for the different key fibrolytic bacteria targeted here. As the most pivotal rumen cellulolytic bacterium (Koike and Kobayashi 2009; Suen *et al.* 2011; Ransom-Jones *et al.* 2012), *F. succinogenes* might have profited from the increase in the ADF (cellulose) fraction of barley due to LA treatment. In contrast to our assumption, LA treatment of barley largely depressed *F. succinogenes* abundance in rumen liquid and solids. Because *F. succinogenes* showed enhanced hydrolytic activity when in co-culture with non-fibrolytic bacteria (e.g. *S. ruminantium* and R-25 strains; Fukuma *et al.* 2015), it might be thinkable that the actual negative effect of the LA treatment was rather on other members of the fibre-associated community, thereby depressing the growth of *F. succinogenes* as well. Possible mechanisms behind the contribution of nonfibrolytic bacteria to ruminal fibre degradation are indirect and related to hydrogen transfer and cross-feeding of degradation or fermentation products derived from fibre (Flint 1997). Moreover, the contrary dynamics of *R. albus* in both fractions and *R. flavefaciens* group in rumen liquid in relation to the LA and LAH treatment of barley are hard to explain and might be related to the substrate (structural changes in the treated barley), competition among fibrolytic bacteria or to indirect mechanisms such as bacterial hydrogen transfer (Flint 1997).

Although being low abundant, starch-degrading *R. amylophilus* (Anderson 1995) was one of the bacterial taxa that profited most from the treatment of barley. In contrast to other targeted amylolytic bacteria, LAH treatment of barley promoted *R. amylophilus* in rumen solids by 5- to 15-fold, thereby depending on the time after morning feeding. This may be either linked to the enzymatic equipment of this bacterium or a growth advantage because other starch-degraders, including *Prevotella* and *Lactobacillus* species, may have less efficiently metabolized the modified barley grain. The present enhancement of the propionate proportion with LA and LAH treatment of barley was consistent with those reported previously for the liquid fraction of the rumen (Iqbal *et al.* 2009; Deckardt *et al.* 2015) and may beneficially contribute to the energy supply in high-producing dairy cows (Aschenbach *et al.* 2011). Currently, the effects were much greater and consistent over time for PARL and less pronounced and only at later hours after barley grain feeding for rumen liquid. This observation can be linked to the lower availability of soluble carbohydrates in the liquid fraction and greater availability of less soluble carbohydrates (i.e. RS and fibre) in PARL of cows fed LA and LAH treated barley compared to cows fed the CON barley. *Megasphaera elsdenii* has been recognized as an important propionate producing bacterium in the rumen (Haikara and Helander 2006). Yet, it was only low abundant and only LA but not LAH treatment enhanced its relative abundance in both rumen fractions. *Megasphaera elsdenii* is known to largely depend on primary colonizing populations of amylolytic

and lactic acid producing bacteria such as lactobacilli (Louis *et al.* 2007). Fittingly, the *Lactobacillus* group which comprises many amylolytic species showed a similar dynamic to the LA treatment than *M. elsdenii* in both rumen fractions which strongly hints at increased lactate production and cross-feeding of lactate between the two bacterial populations (Flint 1997). However, structural modification caused by the additional heat treatment seemed to have rendered starch and potentially other carbohydrates in the barley grain less degradable for lactobacilli, thereby also potentially limiting lactate as substrate for *M. elsdenii*. Further important propionate producers can be found in *Clostridium* cluster IX, such as *S. ruminantium* (Evans and Martin 1997; Louis *et al.* 2007) which were little influenced by LA and LAH treatment of barley after feeding. Likewise, the genus *Prevotella* comprises propionate producers and can utilize a broad range of carbohydrates including starch and fibre (Flint *et al.* 2012). However, results indicated that *Prevotella* species could less efficiently use the nutrient fractions in the LA and particularly in the LAH treated barley compared to the nutrient fractions in the native barley grain which is in accordance to our *in vitro* observations after 24 hours of incubation (Deckardt *et al.* 2015). Because the genus *Prevotella* was more dominantly abundant than other starch fermenting taxa, changes in this bacterial population due to LA and LAH treatment of barley might have had the greatest impact on the ruminal ecosystem as compared to *Lactobacillus* group and *R. amylophilus.*

The second major change in the fermentation acid profile was the depression in the butyrate proportion in rumen solids of cows fed LA and LAH treated barley. Butyrate can be formed by a range of bacteria including targeted *Clostridium* clusters IV and XIV, *B. fibrisolvens* group and *M. elsdenii* by primary action on the substrate or by cross-feeding of primary metabolites (Flint 1997; Louis *et al.* 2007). Because results on targeted propionate and butyrate producers were inconclusive at the different time points, which led to the conclusion that changes were more on metabolic level than on actual abundance, key bacteria were not covered by the present primer sets (Mullins *et al.* 2013), or key propionate and butyrate producers changed over time after feeding. For instance, *Clostridium* cluster XIV was indeed depressed in rumen solids of cows fed LA and LAH treated barley 8 h after feeding when the greatest difference in butyrate between the CON and the two treated barleys was observed.

In conclusion, results demonstrated that the greatest effect of processing barley grain with 1% LA or LAH was the enhancement of the propionate proportion in rumen liquid and solids which may be beneficial for cow's energy supply. The LA and LAH treatment effects on responding bacterial populations and fungi were consistent and mostly independent of the rumen fraction and time after barley feeding. Both amylolytic and fibrolytic bacteria responded to the treatment of barley, whereby more targeted populations changed with the LA treatment indicating that microbes could differently use the treated barley after the additional heat treatment. Particularly the diverging response of pivotal fibrolytic bacteria showed that more than one fibrolytic group should be used as sentinel population to categorize dietary effects on rumen homeostasis.

Acknowledgements

We acknowledge the assistance of S. Gschaider, S. Abdel Raheem, A. Rudischer, M. Finsterböck, A. Dockner, M. Wild and S. Leiner (Institute of Animal Nutrition and Functional Compounds, University of Veterinary Medicine Vienna, Vienna, Austria) with the animal trial, samplings, and laboratory analyses. The authors thank the staff of the research station at AREC Raumberg-Gumpenstein for the care to the experimental cows. This research was within the framework of the Research Cluster "Animal Gut Health" at the University of Veterinary Medicine Vienna, and was supported partly by project "optGerste" (BMLFUW), which is highly acknowledged.

Conflict of Interest

All authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Chemical composition of the diet components.

Table S2 Primer sets used for amplification of microbial populations using quantitative PCR.

Table S3 Effect of rumen site on microbial population abundance.

Table S4 Effect of time on microbial population abundances in rumen liquid.

Table S5 Effect of time on microbial population abundances in rumen solids.

Figure S1 Acetate-to-propionate ratio in particle-associated rumen fluid and free rumen liquid in Holstein cows fed diets with unprocessed barley, barley processed with 1% lactic acid or barley processed with 1% lactic acid and heat.