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Effect of Processed Barley Grain on *in vitro* Rumen Fermentation and Fate of Nitrogen Metabolism

Research Article

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ABSTRACT

Rapid degradation of barley grain (BG, *Hordeum vulgare*) starch in the rumen can seriously impair rumen fermentation efficiency. Some strategies to curb the negative effects of grain feeding and hamper dysfermentation rely on the usage of phytogenic substances or organic acids. In order to process BGs, they were steeped in 5% lactic acid (BGLA), oregano (BGORE) or thyme (BGTHY) extracts for 48 h. Therefore, an in situ study was conducted to assess the effect of either processed BG or unprocessed BG (control; BGCTRL) on ruminal degradation kinetics (a; soluble fraction, b; potential degradable fraction, c; fractional degradation rate) and effective rumen degradability (ERD) of dry matter (DM), crude protein (CP) and starch. *In vitro* trials with a 2 × 2 × 4 factorial design were also used to assess the effect of diets which contained intact or processed BGs with different CP [160 (P16) *vs*. 170 (P17)] g/kg DM,) and ruminal protein degradability percentages [90 (low degradability; LD) *vs*. 115 (high degradability; HD) g/kg DM] on rumen gas production characteristics (b; asymptotic gas volume, c; the constant rate of gas production), yield of microbial crude protein (MCP) and effective utilizable crude protein in the duodenum (EuCP). *In situ* data demonstrated that BG_{LA} compared with BG_{CTRL} had significantly lower fractions of "a" (0.22 *vs*. 0.26, P=0.03) and "c" (0.10 *vs*. 0.17, P<0.01) and ERD of starch (0.53 *vs*. 0.64, P=0.01). The treatment of BGs with the plant extracts, however, was not able to change the *in situ* parameters relatively to BG_{CTRL}. Results of the *in vitro* trials indicated that diets containing processed BG had higher MCP when compared with BG_{CTRL} (19.74 *vs*. 15.85 mg/250 mg DM, P<0.01). Lactic acid and ORE-treated barley decreased the gas production constant rate (c; mL/h) and gas volume after 2 h compared with BG_{CTRL} (P≤0.05). Our study revealed that processed BG can alter the rumen starch degradation pattern, and rumen gas production parameters and increase MCP and EuCP.

KEY WORDS barley, fermentation gas, lactic acid, oregano, thyme.

INTRODUCTION

High-producing dairy cows have higher energy requirements, especially during early and mid-lactation periods. To meet these requirements, nutritionists formulate high-energy diets which are rich in cereals (e.g. barley, wheat, corn, etc.). These are the source of rapidly fermentable carbohydrates for the rumen microbes (Nocek, 1997). Barley grain (BG) has been used worldwide in the ruminants' diet to support the demands for high milk production (Iqbal *et al*. 2009). The majority of the starch (80 to 90%) present in BGs is rapidly fermented in the rumen, which might lead to

serious problems for the animal through increasing gas production and lowering the fermentation efficiency. As a consequence, rapidly-fermented starch has a negative influence on fermentation products, such as microbial crude protein (MCP) (Zebeli *et al.* 2008). Protein requirements for dairy cows are fulfilled in the form of dietary rumen non-degradable and rumen-synthesized MCP, both of which correspond to effective utilizable crude protein in the duodenum (EuCP) (Edmunds *et al.* 2012). Approximately, 50 to 80% of MCP flows from the rumen to the lower part of the digestive tract and its specific amino acid profiles are essential for milk and meat protein production (Schwab and Broderick, 2017). It has been well documented that microbial protein synthesis can be impacted by several factors such as the amount and source of carbohydrates and protein (Fébel and Fekete, 1996) and rumen function synchronization (Malekjahani *et al.* 2017). The amount and rate of organic matter and starch degradation in the rumen are known to be the main limiting factors in MCP yield (Fébel and Fekete, 1996). Therefore, to reach the optimum MCP, both solubility and degradability of barley starch must be modified. Processing of BG has been used for decades with the main purpose of reducing the rate of starch degradability and availability for the rumen microorganisms (Deckardt *et al.* 2014). Recently, lactic acid (LA), a mild organic acid and inoffensive chemical substance has become the focus of researchers as a promising strategy for barley processing (Iqbal *et al.* 2009; Deckardt *et al.* 2014). In addition to organic acids, plant extracts such as alfalfa and sugar beet pulp were investigated as potential candidates to manipulate starch digestion in the rumen (Naseroleslami *et al.* 2018). However, most of the studies conducted so far focused mainly on the effects of BG processing on starch degradation rate and alleviation of deleterious effects of ruminal acidosis (Iqbal *et al.* 2009). While investigations evaluating the effect of processed BG on the fate of nitrogen in the rumen are lacking. Thymol and carvacrol are active components in thyme (THY) and oregano (ORE) essential oils, respectively, these compounds with phenolic structures known for their antimicrobial properties (Calsamiglia *et al.* 2007). The non-covalent interaction between starch and phenolic compounds has been previously suggested to influence starch physicochemical and digestion characteristics. A possible explanation for this effect could be alternation in starch structure mainly increasing amylose proportion in α-glucan of starch (Zhu, 2015). Duval *et al.* (2007) suggested that a blend of essential oils supplementation declined the degradation rate of rolled BG starch. In general, plant-derived components through the selective effect on certain rumen bacteria and the attachment and pattern of their colonization of starch-rich substrates may lower starch degradation (Hart *et al.* 2008).

We hypothesized that the processing of BG with LA or plant extracts may alter the *in situ* degradation rate of its nutrients. Besides, the dietary inclusion of the processed BG has the potential to influence rumen responses by changing the gas production parameters and higher yield of MCP and EuCP. Therefore, the aims of the current study were to 1) investigate the ruminal degradation kinetics of dry matter (DM), crude protein (CP) and starch of BG as intact (BG_{CTRL}) or treated with LA 5% (BG_{LA}), THY (BG_{THY}) and ORE (BG_{ORE}) using an *in situ* technique and 2) to include the processed grains in various experimental diets containing two levels of dietary CP (160 *vs*. 170 g/kg DM) and two levels of rumen protein degradability (RDP, 90 *vs*. 115 g/kg DM, using xylose processed soybean meal or a commercially available soybean meal) for assessing the effect of processed grains, CP and RDP levels on rumen fermentation responses, MCP and EuCP using an *in vitro* gas production technique.

MATERIALS AND METHODS

Solution preparation

In order to process barley grain (*Hordeum vulgare*), various experimental solutions of 5% LA (lactic acid 85% excellent grade, Qingdao Derun Chemical CO., LTD), THY and ORE extracts were used. As described by Naseroleslami *et al.* (2018) 50 g of dried and ground THY or ORE were mixed with 1000 mL of 70% v/v ethanol and shook for 10 minutes at 120 rpm. After extraction, the solutions were filtered through Whatman No. 1 paper (Whatman Ltd., Maidstone, England). To eliminate the ethanol, the filtrated solution was evaporated under reduced pressure at 38 ˚C using a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany).

Grain processing and diet preparation

Intact BGs were treated with 5% LA, THY, and ORE extracts while unprocessed BG was considered as control (BG_{CTRL}). One kg of BG (DM=89%, and CP=132 and starch=620 g/kg DM) was soaked using the experimental solutions (1:1 weight/volume) for 48 h (Deckardt *et al.* 2014; Naseroleslami *et al.* 2018). The samples were then dried at 55 ˚C for 48 h using an air-forced oven. This resulted in three different treatments: 5% LA processed BG (BG_{LA}), THY processed BG (BG_{THY}) and ORE processed BG (BG_{ORE}). The experimental diets (n=16) were formulated to meet the requirement of lactating Holstein dairy cows according to NRC (2001) guidelines, including 53.36% forage (alfalfa hay, corn silage, and wheat straw) and 46.65% concentrate including different processed barely grains and soybean meal (a high degradable protein source, 115 g/kg DM; HD) or a xylose protected soybean

meal; Yasminomaxsoy® (a low degradable protein source, 90 g/kg DM; LD), with two protein concentrations (160 (P16) and 170 (P17) g/kg DM, (Table 1).

***In situ* technique**

In order to assess the rumen degradation kinetics of DM, CP, and starch, three ruminally cannulated Holstein lactating dairy cows (BW=640±8 kg, DIM=210±12) were used. Animals were fed a diet consisting of corn silage (250 g/kg DM), alfalfa hay (250 g/kg DM), and concentrate (500 g/kg DM consisted of 150 g/kg DM barley grain, 100 g/kg DM corn grain, 50 g/kg DM cotton seed meal, 50 g/kg DM soybean meal, 55 g/kg DM sugar beet pulp, 90 g/kg DM wheat bran and 5 g/kg DM vitamin and mineral supplement). Cows had free access to fresh water during the experiment. The *in situ* procedure was similar to that described by Iqbal *et al*. (2009). Briefly, polyester bags (12×17 cm, 50 µm pore size) were filled with 6 g DM of 2 mm ground sample. To limit movement, all bags were tied to 60 cm bale by using plastic cable ties. To mimic saliva stimulation, the bags were put in warm water (circa 39 ˚C) for 5 minutes and placed in the rumen ventral sac prior to the morning feeding. Baseline samples were taken at time 0.0 (water-washed, but not incubated in the rumen). After 2, 4, 8, 12, 24, 48 and 72 h incubation, the bags were removed and washed under cold tap water using a washing machine. The *in situ* bags were dried at 60 ˚C for 48 h, weighed, and used to determine DM, CP, and starch quantity. This experiment was done with 3 runs and 3 bags for each sample at different time points.

***In vitro* rumen fermentation and MCP yield**

In vitro trials were performed using the Hohenheim gas production technique (Menke and Steingass, 1988) to evaluate rumen fermentation, MCP, and EuCP (23, run=3, n=4). Prior to the morning feeding, ruminal contents were collected from the different part of the rumen of 3 rumen cannulated lactating dairy Holstein cows previously used in the *in situ* study. The solid part was squeezed to obtain the particle-associated bacteria and immediately blended with the fluid part. The mixture was strained through four layers of cheesecloth to remove coarse feed particles and then moved to the laboratory in a pre-warmed container. To prepare media (buffered ruminal fluid), filtrated rumen fluid was mixed with carbonated buffer, macro-mineral solution, and deionized water in a ratio of 1:1:0.5:1.5, respectively. The media was reduced by the addition of 41.7 mL reduction solution (40 mL deionized water, 1 mL 1 N NaOH and 1 g Na_2S $9H_2O$) per liter of medium. Incubation bottles (125 mL serum bottle) containing 250 mg DM experimental diet were poured with 20 mL of reduced medium. Anaerobic conditions were maintained through a CO_2 stream.

The bottles were sealed with a rubber stopper and aluminum cap and placed in a 39 ˚C water bath for 96 h. Cumulative gas production pressure was detected by an electronic pressure transducer (Pressure Sensor, PSA-01, Autonics) at 2, 4, 8, 12, 24, 48, 72, and 96 hours of incubation time the converted to the volume using an experimentally calibrated curve (Theodorou *et al.* 1994). For all experiments, blanks (reduced medium without experimental diets) were used for correcting MCP, EuCP, and gas parameters. Bottles were treated in the same manner as the samples and handling of ruminal inoculums were performed under a constant stream of CO_2. Corrected gas volume data based on the blank was fitted to an exponential model Ørskov and McDonald (1979) as:

$$y= b \times (1-e^{-ct})$$

Where:
y: cumulative volume of gas produced at time "t" (h).
b: asymptotic gas volume.
c: constant rate of gas production.

Half time of gas production ($t_{1/2}$) [i.e., the time (h) when half of the asymptotic gas volume (b; mL) was produced] was calculated as:

$$t_{1/2}= \ln 2/ c$$

After the initial gas production assay, to calculate $t_{1/2}$ for each experimental diet, the second incubation was performed to determine rumen MCP yield at substrate-specific times (i.e., $t_{1/2}$ for each experimental diets, Grings *et al.* 2005). The incubations were stopped at the diet-specific "$t_{1/2}$" and the MCP at "$t_{1/2}$" was determined by using the "N balance" equation, which is given as follows:

Microbial N Production at $t_{1/2}$= Diet N + (ΔNH_3–N) - NDFN at $t_{1/2}$
ΔNH_3–N= NH_3–N in blanks (0h) − NH_3–N in diet incubations at $t_{1/2}$

Where:
N: nitrogen content of the experimental diets.
NH_3–N in 0.0 h blanks: average amount (mg) of ammonia N in the blanks prior to incubation (0.0 h).
NH_3–N in diet incubations at $t_{1/2}$: amount (mg) of ammonia N in the incubation bottles from each sample at "$t_{1/2}$".
NDFN at $t_{1/2}$: N content of truly non-degraded residue at "$t_{1/2}$".

After calculation, Microbial N Production at "$t_{1/2}$" is converted to MCP using a 6.25 coefficient.

Table 1 Ingredients and chemical composition of the experimental diets

Components	Experimental diets															
	P16								P17							
	HD				LD				HD				LD			
	BG_{CTRL}	BG_{LA}	BG_{THY}	BG_{ORE}	BG_{CTRL}	BG_{LA}	BG_{THY}	BG_{ORE}	BG_{CTRL}	BG_{LA}	BG_{THY}	BG_{ORE}	BG_{CTRL}	BG_{LA}	BG_{THY}	BG_{ORE}
Ingredient, % of DM																
Corn silage	21.74	21.74	21.74	21.74	21.74	21.74	21.74	21.74	21.05	21.05	21.05	21.05	21.05	21.05	21.05	21.05
Alfalfa hay	25.51	25.51	25.51	25.51	25.51	25.51	25.51	25.51	24.70	24.70	24.70	24.70	24.70	24.70	24.70	24.70
Wheat straw	6.11	6.11	6.11	6.11	6.11	6.11	6.11	6.11	5.92	5.92	5.92	5.92	5.92	5.92	5.92	5.92
Corn grain	19.57	19.57	19.57	19.57	19.57	19.57	19.57	19.57	18.95	18.95	18.95	18.95	18.95	18.95	18.95	18.95
BG_{CTRL}	15.32	0.00	0.00	0.00	15.32	0.00	0.00	0.00	14.83	0.00	0.00	0.00	14.83	0.00	0.00	0.00
BG_{LA}	0.00	15.32	0.00	0.00	0.00	15.32	0.00	0.00	0.00	14.83	0.00	0.00	0.00	14.83	0.00	0.00
BG_{THY}	0.00	0.00	15.32	0.00	0.00	0.00	15.32	0.00	0.00	0.00	14.83	0.00	0.00	0.00	14.83	0.00
BG_{ORE}	0.00	0.00	0.00	15.32	0.00	0.00	0.00	15.32	0.00	0.00	0.00	14.83	0.00	0.00	0.00	14.83
Soybean meal	8.97	8.97	8.97	8.97	0.00	0.00	0.00	0.00	11.84	11.84	11.84	11.84	0.00	0.00	0.00	0.00
Yasmi-nomax®	0.00	0.00	0.00	0.00	8.97	8.97	8.97	8.97	0.00	0.00	0.00	0.00	11.84	11.84	11.84	11.84
Rapeseed meal	2.79	2.79	2.79	2.79	2.80	2.80	2.80	2.80	2.70	2.70	2.70	2.70	2.70	2.70	2.70	2.70
Chemical composition									(% of DM)							
CP	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
NDF	35.60	35.60	35.60	35.60	35.80	35.80	35.80	35.80	35.00	35.00	35.00	35.00	35.30	35.30	35.30	35.30
NFC	40.20	40.20	40.20	40.20	42.80	42.80	42.80	42.80	39.40	39.40	39.40	39.40	42.90	42.90	42.90	42.90
RDP	11.50	11.50	11.50	11.50	9.00	9.00	9.00	9.00	11.50	11.50	11.50	11.50	9.00	9.00	9.00	9.00
RUP	5.30	5.30	5.30	5.30	7.60	7.60	7.60	7.60	5.30	5.30	5.30	5.30	7.60	7.60	7.60	7.60

BG_{CTRL}: unprocessed barley grain; BG_{LA}: lactic acid 5% processed barley grain; BG_{THY}: thyme processed barley grain; BG_{ORE}: oregano processed barley grain; CP: crude protein; LDP: low degradable protein (RDP=9%); HDP: high degradable protein (RDP=11.5%); P16 and P17: CP concentration 160 and 170 g/kg DM, respectively; NDF: neutral detergent fibre; NFC: non-fiber carbohydrates; RDP: rumen degradable protein and RUP: rumen undegradable protein.

Effective utilizable CP in the duodenum

Regarding the measurement of the utilizable CP in the duodenum, the modified Hohenheim gas production technique (Edmunds *et al.* 2012) was conducted with 3 runs and 12 replicates. The procedure and buffer preparation were the same as the Menke and Steingass (1988) method, except for the incubation time. Rumen fluid collection and preparation were exactly similar to that of gas production technique. The filtrated rumen fluid was mixed with buffered mineral solution as 1:2 of rumen fluid to mineral buffer ratio (V/V). Sampling was performed at the 8, 24, and 48 h of the incubation. One-third of the replicates (n=4) of each treatment (and blanks) at 8 h, half of the remained bottles (n=4) at 24 h and the last 4 bottles 48 h post incubation were transferred to a water-ice mixture to stop fermentation. Afterwards, the bottles were opened and 5 mL of liquid phase was pipetted into 50 mL serum bottles that were containing 5 mL of 0.2 N HCl. Then, these bottles were sealed with a rubber stopper and aluminum cap and stored in at 4 ˚C to measure the ammonia N concentration.

For each of the feed samples the uCP value was calculated according to this equation (Edmunds *et al*. 2012):

$$uCP\ (g/kg\ DM) = (NH_3N_{blank} + N_{sample} - NH_3N_{sample}/weight\ (mg\ DM)) \times 6.25 \times 1000$$

Where:

NH_3N_{blank}: average amount (mg) of ammonia N in the blanks.

N: amount (mg) of diets nitrogen.

NH_3N_{sample}: average amount (mg) of ammonia N in the diets.

Weight: amount of feed sample weighted into bottles.

To calculate EuCP for each feed sample, uCP values of 8, 24, and 48 h incubation were plotted against a log time (ln(t)) scale, where "t" is the time of incubation. Thereafter, the slope (a) and intercept (y) of the resulted regression equation placed in the following equation (Edmunds *et al.* 2012):

$$EuCP\ (g/kg\ DM) = y + a \times \ln(1/kp)$$

Where:

kp: assumed fractional passage rate at 0.03, 0.06 and 0.09/h.

Chemical analysis

All samples were ground using a hammer mill (1 mm, Toos Shekan Khorasan, Mashhad, Iran). Ash (535 ˚C; method 942.05), DM (95 ˚C for 24 hours as per method 930.15), and N (Kjeltec Analyzer, CP=N×6.25, method 990.03) were measured based on AOAC (1990). Ammonia nitrogen concentration was measured in the supernatant using a phenol-hypochlorite reaction (Weatherburn, 1967). The method used to evaluate neutral detergent fiber (NDF; without sodium sulfite and alpha amylase, expressed inclusive of the residual ash) and acid detergent fibers (ADF) were based on

(Van Soest *et al*. 1991). Starch was analyzed by an anthrone/sulphuric acid method using glucose as standard and estimated as 0.9 × glucose content (Aaman and Hesselman, 1984).

Statistical analysis

Variables of *in situ* kinetics of nutrient (DM, CP, and starch) disappearance of the samples were estimated using the NLIN procedure of SAS (2004) based on the following model (McDonald, 1981):

$$Y = a + b\ (1 - e^{-kdt})$$

Where:
Y: nutrient disappearance at a certain time after incubation "a" is the soluble fraction.
b: insoluble but potentially degradable fraction.
kd: fractional rate of disappearance (/ h).
t: incubation time (h).

The effective rumen degradability (ERD) was calculated assuming a fractional passage rate (kp) of 0.06/h using the following equation:

$$ERD = a + b \times kd / (kd+kp)$$

The complete randomized design (4 treatments with three replicates) was applied to analyze the *in situ* parameters according to the following model:

$$Y = \mu + T_i + \varepsilon_{ij}$$

Where:
Y: analyzed variable.
μ: overall mean.
T_i: effect of the BG processing.
ε_{ij}: experimental error.

Statistical analysis of the *in vitro* results was performed using the mixed procedure of SAS (2004). The experimental design consisted of 2 × 2 × 4 factorial arrangement of treatments with 2 different types of degradable protein sources (90 and 115 g/kg DM), two protein levels (160 and 170 g/kg DM) and 4 barley types (BG_{CTRL}, BG_{LA}, BG_{ORE}, and BG_{THY}). The effects of degradability, protein level, barley and their interaction were considered as fixed effects, while run was defined as random effect. As the F-value of the all interaction effects were not significant, the results of the main effects were almost considered in this paper. Differences among least square means were tested using tukey's multiple comparison test. Significance was declared at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$. In order to obtain the surface responses, data were visualized by using Sigma Plot (version 11.0).

RESULTS AND DISCUSSION

***In situ* degradation of nutrients**

Degradation fractions of DM, CP, and starch along with effective rumen degradation are presented in Table 2. Processed BG did not change *in situ* degradation fractions of DM and CP. However, the fractions of (a) and (c) of starch decreased (P=0.001) due to LA treatment of BG. The fractional rate of degradation (c) of starch varied from 0.1/ h for BG_{LA} to 0.18/h for BG_{THY}. The fractional rate of starch degradation for untreated BG was approximately 70 percent higher than the average of BG_{LA} (P<0.05). The decrease in the fractional rate of degradation of starch when BG was processed by LA was accompanied by a similar decrease in ERD. However, the effective degradability of starch of BG_{ORE} and BG_{THY} was similar with that of untreated BG (P<0.001).

Gas production parameters of the experimental diets

The effect of CP and RD levels on ruminal cumulative gas production from the experimental diets are shown in Figure 1. The estimated parameters describing the *in vitro* rumen fermentation pattern are shown in Table 3. During the early hours (2 h) of incubation, BG_{THY} and BG_{ORE} in comparison to the BG_{CTRL} decreased the gas volume (15.10 *vs*. 16.06 mL/250 mg DM, (P<0.05), Table 3). All ruminal fermentation parameters differ significantly between the processed BG treatments (P<0.05). The fraction "b" was the highest for BG_{ORE}, while BG_{THY} exhibited the lowest "b" fraction (P<0.05, Table 3).

In addition, the fraction of "b" differed significantly (P<0.05) between the CP percentage which was higher in P17 group. *In vitro* ruminal gas produced from P16 was notably lower than that of P17. Processing BG with LA caused a significant reduction in "c" fraction (P<0.05), followed by BG_{ORE}, whereas BG_{THY} and BG_{CTRL} did not differ significantly. Regarding "$t_{1/2}$", the values were higher for both BG_{LA} and BG_{ORE} when compared with BG_{CTRL}. Furthermore, the "$t_{1/2}$" of P16 was significantly higher than that of P17 (P<0.05).

***In vitro* MCP and EuCP of experimental diets**

In vitro results of barley processing, CP, and RDP levels on DM disappearance and ruminal fate of nitrogen metabolism including MCP, NH_3-N, and EuCP are presented in Table 4. *In vitro* MCP yield (mg/250 mg DM) was significantly affected by the type of grain processing, all processed BG (BG_{LA}, BG_{ORE}, and BG_{THY}), compared with BG_{CTRL} increased the ruminal MCP yield (P≤0.05).

Table 2 Rumen degradation fractions (a, b and c) of dry matter (DM), crude protein (CP) and starch of unprocessed barley grain (BG_{CTRL}) and processed barley grain with 5% lactic acid (BG_{LA}), oregano extract (BG_{ORE}) and thyme extract (BG_{THY})

Item	Treatment				SEM	P-value
	BG_{CTRL}	BG_{LA}	BG_{ORE}	BG_{THY}		
DM						
a	0.37	0.35	0.38	0.36	0.006	0.79
b	0.53	0.52	0.53	0.53	0.006	0.72
c	0.16	0.17	0.16	0.17	0.006	0.68
ERD	0.66	0.64	0.67	0.66	0.014	0.30
CP						
a	0.47	0.47	0.50	0.46	0.008	0.84
b	0.45	0.44	0.43	0.45	0.009	0.98
c	0.09	0.07	0.09	0.09	0.006	0.97
ERD	0.55	0.49	0.56	0.55	0.025	0.84
Starch						
a	0.26[a]	0.22[b]	0.28[a]	0.25[a]	0.010	0.05
b	0.61	0.62	0.61	0.62	0.010	0.32
c	0.17[a]	0.10[b]	0.18[a]	0.17[a]	0.006	0.001
ERD	0.64[a]	0.53[b]	0.67[a]	0.64[a]	0.022	< 0.001

BG_{CTRL}: unprocessed barley grain; BG_{LA}: lactic acid 5% processed barley grain; BG_{THY}: thyme processed barley grain; BG_{ORE}: oregano processed barley grain; DM: dry matter; CP: crude protein; a: soluble fraction; b: potential degradable fraction; c: fractional degradation rate and ERD: effective rumen degradability.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

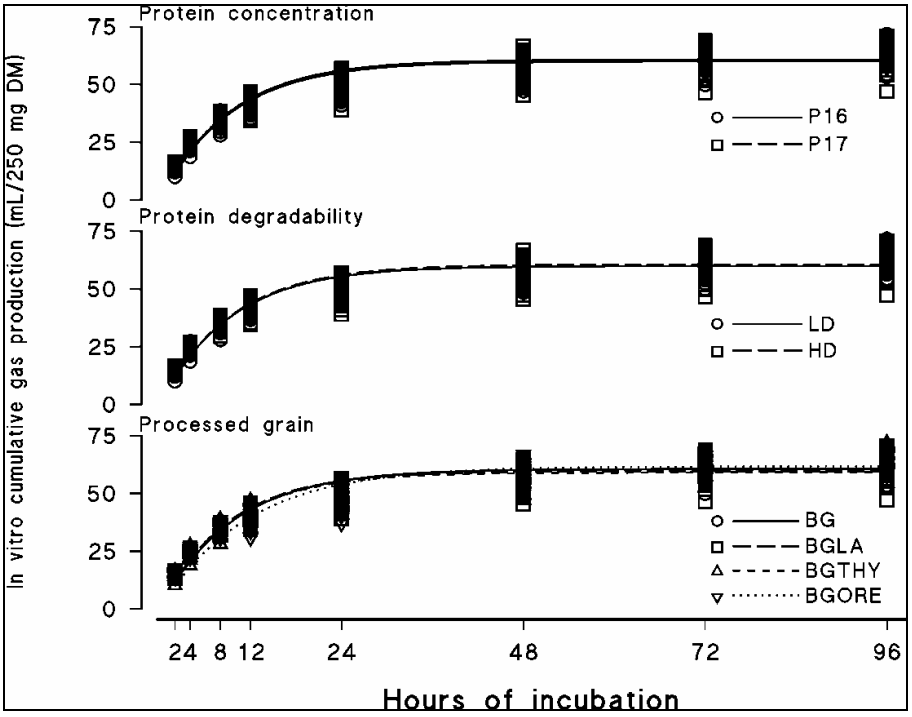

Figure 1 *In vitro* cumulative gas production (mL/250 mg DM) from the experimental diets. BG_{CTRL}: unprocessed barley grain; circle, BG_{LA}: lactic acid 5% processed barley grain; square, BG_{THY}: thyme processed barley grain; triangle, BG_{ORE}: oregano processed barley grain; inverted triangle, protein degradability: low degradability (LD; circle) and high degradability (HD; square), protein concentration: 160 g/kg DM crude protein (P16; circle) and protein 170 g/kg DM crude protein (P17; square) throughout the different time points (2, 4, 8, 12, 24, 48, 72 and 96 h after incubation)

Table 3 *In vitro* gas produced during 2 h after the fermentation and rumen fermentation fractions of the experimental diets

Item	Processing				Degradability		Protein concentration (g/kg DM)		SEM	P-value		
	BG_{CTRL}	BG_{LA}	BG_{ORE}	BG_{THY}	High	Low	160	170		Pro.	Deg.	CP
Gas volume[a]	16.06[a]	14.53[bz]	15.03[b]	15.10[by]	15.14	15.22	14.83[b]	15.53[a]	0.216	< 0.01	0.69	0.002
b	60.62[b]	60.92[b]	62.18[a]	58.88[c]	61.47[a]	59.83[b]	60.46[z]	60.84[y]	0.188	< 0.01	< 0.01	0.06
c	0.100[a]	0.088[c]	0.094[b]	0.100[a]	0.095	0.096	0.094	0.096	0.001	< 0.01	0.23	0.11
$t_{1/2}$	6.68[c]	7.74[a]	7.11[b]	6.76[c]	7.04	7.21	7.15[a]	7.00[b]	0.069	< 0.01	0.37	0.04

BG_{CTRL}: unprocessed barley grain; BG_{LA}: lactic acid 5% processed barley grain; BG_{THY}: thyme processed barley grain and BG_{ORE}: oregano processed barley grain.
High: high degradability of crude protein and Low: low degradability of crude protein.
[a] volume of produced gas in 2 hours; b: asymptotic gas volume (mL/250 mg DM); c: gas production constant (mL/ h) and $t_{1/2}$: half time of gas production (h).
[a, b and c]: Least square means with different superscripts within a row at processing, degradability and crude protein concentration separately indicate significant differences (P≤0.05).
[y, z]: Indicate trends at processing, degradability, and CP level separately (0.05<P≤0.1).
SEM: standard error of the means.

Table 4 Microbial crude protein (mg/250 mg DM), NH_3-N (mg/250 mg DM), dry matter disappearance at $t_{1/2}$ (g/kg DM), effective utilizable crude protein at the duodenum (g/kg DM) at different fractional passage rates (0.03, 0.06 and 0.09 /h) of experimental diets

Item	Processing				Degradability		Protein concentration (g/kg DM)		SEM	P-value		
	BG_{CTRL}	BG_{LA}	BG_{ORE}	BG_{THY}	High	Low	160	170		Pro.	Deg.	CP
MCP	15.85[b]	19.21[a]	20.56[a]	19.45[a]	18.97	18.56	18.40	19.13	0.846	0.003	0.64	0.40
NH_3-N	1.45[a]	1.44[a]	1.26[ab]	1.16[b]	1.37	1.29	1.15[b]	1.51[a]	0.092	0.09	0.40	0.001
DMD	571.5[ab]	561.2[b]	583.9[a]	589.6[a]	576.7	576.4	574.3	578.8	7.290	0.04	0.97	0.54
EuCP												
r=0.03/h	91.84[b]	96.44[a]	97.94[a]	93.08[b]	93.77	95.89	93.57[b]	96.08[a]	2.440	0.05	0.09	0.004
r=0.06/h	94.33[c]	98.43[ab]	100.53[a]	95.74[bc]	96.87	97.64	97.05	97.47	1.986	0.004	0.52	0.72
r=0.09/h	95.79[c]	99.59[ab]	101.72[a]	97.30[bc]	98.69	98.50	98.91	98.28	1.197	0.01	0.87	0.60

BG_{CTRL}: unprocessed barley grain; BG_{LA}: lactic acid 5% processed barley grain; BG_{THY}: thyme processed barley grain and BG_{ORE}: oregano processed barley grain.
MCP: microbial crude protein (mg/250 mg DM); NH_3-N: ammonia-N (mg/250 mg DM); DMD: dry matter disappearance at $t_{1/2}$ and EuCP: effective utilizable crud protein at the duodenum at different passage rates (0.03, 0.06 and 0.09 /h).
High: high degradability of crude protein and Low: low degradability of crude protein.
SEM: standard error of the mean.
[a, b and c]: Least square means with different superscripts within a row at processing, degradability and crude protein concentration separately indicate significant differences (P≤0.05).

A significant effect of CP level on *in vitro* ruminal NH_3-N concentration was also found (P<0.05). *In vitro* rumen NH_3-N concentration was significantly higher in P17 when compared with that of P16. Treating BG with THY extract resulted in a decrease in NH_3-N as compared to BG_{LA} and BG_{CTRL} (P<0.05). *In vitro* DM disappearance was significantly affected by the type of processing of BG (P<0.05). Processing BG with LA decreased DM disappearance, while both ORE and THY led to an increase in this parameter. *In vitro* values for EuCP were influenced by the type of BG processing (P<0.05). At any ruminal outflow rate, BG_{ORE} had a higher EuCP compared with that of BG_{THY} (P=0.01) and BG_{CTRL} (P=0.001). Relatively to BG_{CTRL}, treating BG with LA caused an increase in EuCP, while no significant differences were detected between LA and the plant extracts.

Reciprocal effects of processing and degradability

All parameters which were significantly affected by the interaction of degradability and processing are shown in Table 5. The highest value of "b" was recorded for BG_{CTRL} and BG_{ORE} in HD and the lowest value was for BG_{CTRL} in LD (P<0.05).

In both HD and LD groups, BG_{LA} caused a significant decrease in "c" relative to the others (P<0.05), while BG_{THY} in HD and BG_{CTRL} in LD have the highest "c". BG_{LA} and BG_{ORE} in HD and BG_{LA} and BG_{THY} in LD group had higher "$t_{1/2}$" in comparison with other diets (P<0.05). Processing BG with LA or plant extracts increased the MCP concentration in both degradability levels (P<0.05), however processing BG with THY was not able to improve MCP relative to BG_{CTRL} in HD group.

In all passage rates (0.03, 0.06, and 0.09/h) BG_{ORE} led to a decrease in HD and an increase in LD when compared with the other groups (P<0.05). No significant reciprocal effect of processing and protein concentration, degradability and protein concentration, and triple interaction (processing, protein concentration, and degradability) was observed in the current study.

In this study, the *in situ* degradation of DM, CP, and starch of BG_{LA}, BG_{ORE}, and BG_{THY} were evaluated. Our results showed that the *in situ* ruminal starch degradation parameters have been influenced by the BG processing method. Organic acids like LA are advantageous in modifying the structure of starch toward more contents of resistant and less degradable starch (Deckardt *et al.* 2014).

Table 5 Reciprocal effect between processing and degradability on rumen fermentation fractions (a, b and $t_{1/2}$), microbial crude protein (mg/250 mg DM) and effective utilizable crude protein at the duodenum (g/kg DM) at different fractional passage rates (0.03, 0.06 and 0.09 /h) of experimental diets

Item	High degradable protein source				Low degradable protein source				SEM	P-value		
	BG_{CTRL}	BG_{LA}	BG_{ORE}	BG_{THY}	BG_{CTRL}	BG_{LA}	BG_{ORE}	BG_{THY}		Pro.	Deg.	Deg. × Pro.
B	63.76	60.10	63.19	58.83	57.49	61.75	61.17	58.93	0.26	< 0.01	< 0.01	< 0.01
C	0.097	0.088	0.092	0.104	0.104	0.088	0.097	0.096	0.001	0.23	< 0.01	< 0.01
$t_{1/2}$	6.97	7.72	7.34	6.40	6.40	7.76	6.90	7.12	0.10	0.37	< 0.01	< 0.01
MCP	17.79	19.60	21.32	17.16	13.91	18.81	19.80	21.75	1.56	0.64	0.003	0.011
EuCP												
r=0.03/h	95.83	96.36	90.85	96.07	94.57	96.54	105.03	87.36	1.69	0.09	0.05	< 0.01
r=0.06/h	99.32	97.68	96.02	98.52	97.75	99.18	105.04	92.97	1.61	0.52	0.004	0.001
r=0.09/h	101.36	98.46	99.04	99.95	99.61	100.72	104.39	94.64	1.56	0.87	0.01	< 0.01

BG_{CTRL}: unprocessed barley grain; BG_{LA}: lactic acid 5% processed barley grain; BG_{THY}: thyme processed barley grain and BG_{ORE}: oregano processed barley grain.
b: asymptotic gas volume (mL/250 mg DM); c: gas production constant (mL/h) and $t_{1/2}$: half time of gas production (h); MCP: microbial crude protein (mg/250 mg DM) and EuCP: effective utilizable crud protein at the duodenum at different passage rates (0.03, 0.06 and 0.09/ h).
Pro: processing effect; Deg: degradability and Deg × Pro: interaction effect of processing and degradability.
SEM: standard error of the mean.

In the current study, the soluble fraction of starch of BG in response to LA was decreased. This result confirmed the finding of Iqbal *et al.* (2009), who reported that treating rolled BG with 1% LA significantly increased the lag time and slightly decreased the soluble fraction of DM. Also, they noted that steeping rolled BG in the 1% LA for 48 h, compared to 0.5% LA, increased the fractional degradation rate of DM and suggested the importance of LA concentration on the degradation of BG. Our ruminal kinetics of starch showed that BG_{LA} compared to the others, had a lower soluble fraction, fractional degradation rate, and ERD. Results of a study indicated that treating BG with 1% LA compared with intact BG tended to decline the fractional degradation rate, and chemical analysis of LA treatment also showed 8% reduction in the content of soluble starch and 17.7% elevation in resistance starch (Iqbal *et al.* 2009).

Moreover, Deckardt *et al.* (2014) processed different genotypes of BG with LA and tannic acid (three concentrations; 0.5, 1, and 5% of DM) at either room temperature or 55 ˚C for 24 or 48 h and reported that processed BG with 5% LA for 48 h at room temperature is the most effective way to increase the resistant starch proportion, which was five folds higher than that of resistant starch of intact BG (3.1 *vs*. 0.57% of DM). Electron micrographs of treated BG revealed that LA treatment had no pronounced effects on starch granule surface, suggesting that the effect of LA on BG starch might have occurred at the molecular level (Deckardt *et al.* 2014). In addition, previous research has also reported that LA could slow down the amylase activity in BG, causing a reduction in starch degradability in human and *in vitro* studies (Östman *et al.* 2002). The interaction between BG gluten and LA may provide a barrier for enzymatic activity attack, which could be another explanation for decreasing BG starch degradability in response to LA processing (Östman *et al.* 2002).

Contrary to our expectations, processing BG with plant extracts (THY and ORE) was not able to change the *in situ* ruminal degradation parameters of DM, CP, and starch. Plant extracts have long been used by industries for many years, mainly as feed additives in ruminants (Calsamiglia *et al.* 2007) and as pharmaceuticals and food preservative agents for human consumption (McCue *et al.* 2004). Their main effects in the rumen are associated with a reduction in starch and protein degradation and inhibition of amino acid deamination (Duval *et al.* 2007). However, their decreasing effect on CP and starch degradation seems to be highly selective and diet- and protein sources- dependent (Molero *et al.* 2004). Molero *et al.* (2004) reported that ruminal incubation of different protein sources in sheep that received a blend of essential oils had various effects on ruminal kinetics degradation of DM and CP. Diet supplementation with a blend of essential oils tended to decrease fraction soluble fraction of DM in soybean meal and sunflower meal, and increased the potential degradable fraction of soybean meal DM relative to an un-supplemented diet, whereas both soluble and potential degradable fraction of CP in sunflower meal was influenced by the supplementation of essential oils. Interaction between starch and phenolic components (as a pure form or plant extracts) may alter the starch structure, increasing amylose proportion in α-glucan of starch (Zhu, 2015) and lowering starch degradation. Recently, our research team used alfalfa and sugar beet pulp extracts to process BG. According to them, the site and extent of protein and starch degradation were modified in response to BG processing with a variety of organic extracts (Naseroleslami *et al.* 2018). However, in the current study, the processing of BG with the plant extracts did not change the ruminal degradation of starch. Together, the relatively low temperature (room temperature), concentration, nature, and active components of the extracts and the barley genotype could be possible reasons for the absence

of a more pronounced effect of the extracts on ruminal kinetics of DM, CP, and starch disappearance (Wenk, 2003).

In the current study, several dairy diets that included processed BG were evaluated to determine *in vitro* ruminal fermentation response, MCP and EuCP. In the present study, BG_{LA} caused a reduction in fractions asymptotic gas volume and gas production constant, indicating a lower solubility and gas production constant of BG in response to LA treatment as reported previously (Iqbal *et al.* 2009; Deckardt *et al.* 2014). Treating BG with acids (organic acids and formaldehyde) led to a decrease in the availability of starch for microorganisms (Table 1). Therefore, it can decrease the asymptotic gas volume and gas production constant of gas production relative to the intact BG (Colkesen *et al.* 2005). It has been previously claimed that fermentation gases result from degradation of the highly soluble fraction of substrates followed by that of the slowly degradable and insoluble part (Groot *et al.* 1996). So, gas production in the early hours may indicate the degradation of the soluble part (Groot *et al.* 1996), as the early fermentation process may be influenced by the availability and solubility of substrates (e. g., starch) in the rumen (Downing *et al.* 2008). Our *in situ* results revealed that processing BG with LA decreased the soluble fraction and fractional degradation rate of starch, and therefore, BG_{LA} inclusion in the experimental diets can decrease the availability of the nutrients for ruminal microorganisms during the first hours of incubation. This is in line with the lowered gas production during the initial hours and lowered gas production constant in our study and previous experiments (Colkesen *et al.* 2005). In the second phase of gas production, slowly degradable substrates have a key role. A negative association was observed between gas production constant and "$t_{½}$". By decreasing the gas production constant, "$t_{½}$" was increased. On the other hand, half of the total gas produced during the incubation lasted longer with decreasing the gas production constant, BG_{LA} increased the "$t_{½}$" and decreased the fermentation rate (Fadaee *et al.* 2013). P17 had higher gas volume and asymptotic gas volume than P16, which clearly shows that a higher level of CP in the diets led to higher output. However, P16 had a higher "$t_{1/2}$" which could be in favor of ruminal microbiome and fermentation efficiency. Carvacrol (circa 69%) and thymol (circa 45- 47%) are the main compounds in ORE and THY, respectively (Calsamiglia *et al.* 2007). These are generally classified according to their similar antimicrobial activity, which is related to their hydroxyl group in the phenolic structure (Ultee *et al.* 2002) and may thus be used as a rumen microbial modifier (Jahani-Azizabadi *et al.* 2011). In a study by Temizkan *et al.* (2011), with different concentrations of ORE (500, 1000, and 2000 mg/L) in the medium with different substrates (alfalfa hay, maize silage, and BG), a negative correlation between the concentration of plant extracts and gas production was reported. The authors concluded that this negative correlation might be associated with their antimicrobial activity. In the current study, during the early hours (2 h) of incubation, BG_{THY} and BG_{ORE} relatively to the BG_{CTRL} decreased the gas volume, which is in agreement with Jahani-Azizabadi *et al.* (2011). It is reported that supplementation of various plant extracts (such as ORE, THY, coriander, rosemary, and cinnamon) in the medium caused a significant reduction in the gas volume during the 24 h of *in vitro* incubation. This reduction may occur due to low DM and CP digestibility and manipulation of fermentation patterns through antimicrobial activity (Jahani-Azizabadi *et al.* 2011).

In the present study, diets containing processed BG showed better responses in increasing *in vitro* rumen MCP (Table 4). Microbial metabolism may be influenced by several factors, such as the amount and rate of organic matter or carbohydrate degradation in the rumen (Fébel and Fekete, 1996). The efficiency of MCP yield may be improved by balancing energy and nitrogen availability in the rumen (Trevaskis *et al.* 2001; Malekjahani *et al.* 2017). *In situ* results showed that treating BG with LA increased the slowly degradable starch while decreasing the rumen's starch degradation rate (Table 2). Indeed, processing BG with LA aimed to slow the energy release from barley starch degraded in the rumen which may promote rumen synchronizing and increase MCP yield. Naseroleslami *et al.* (2018) reported that changing the site and extension of starch digestion may also positively impact the MCP yield in the rumen, which is highly dependent to the provision of energy from the fermentation of the organic matter to the rumen microbes. Protein concentration only had a significant effect on NH_3-N concentration, it is well documented that by increasing the protein level of diets the concentration of NH_3-N could increase (Bach *et al.* 2005). Oh *et al.* (2008) results are in the same direction as well, increasing the CP concentration and degradability visibly increased highly- available nitrogen concentration. With regards to protein concentration level effect on gas fermentation parameters and NH_3-N and no effect on MCP, it could be concluded that in the present study the diets with higher protein concentration level (P17) decreased the efficiency of fermentation (Hristov *et al.* 2004). Decreasing protein degradation rate and soluble part of protein can positively affect MCP in the rumen (Parand *et al.* 2015). However, our data did not show any effect of protein degradability (HD *vs.* LD) on MCP, and it might refer to the protein processing method. Plant extracts used in the current study to process BG had a significant effect on *in vitro* rumen MCP and NH_3-N. As reported by (Satter and Slyter, 1974), ammonia concentration has an important role in MCP yield;

by decreasing the ammonia concentration, MCP increased. Generally, there are two suggested action modes for plant extracts: one is an effect on the pattern of bacterial colonization in particular starch rich substrates, and the second is their inhibition of "hyper ammonia producing bacteria" involved in amino acid deamination (Hart *et al.* 2008). Borchers (1965) was the first to report the potential benefits of essential oils on rumen microbial fermentation and reported that *in vitro* incubation of casein in the rumen fluid with thymol supplementation led to amino acids accumulation and reduction in NH_3-N, which is explained by the inhibition of hyperammonemia-producing bacteria in response to thymol supplementation. *In vitro* supplementation of carvacrol (2.2 mg/L) decreased large ruminal peptide concentrations and increased NH_3-N concentrations 2 h after feeding, suggesting that carvacrol inhibited proteolysis or stimulated peptide lysis (Busquet *et al.* 2005). Therefore, it can improve the flow of dietary proteins to the lower part of the digestive tract and improve nitrogen usage by the microorganisms to produce MCP (Demeyer and Van Nevel, 1986). However, some *in vitro* (Spanghero *et al.* 2008) and *in vivo* (Khorrami *et al.* 2015) studies reported that dietary supplementation of essential oils did not change the ruminal NH_3-N concentration. This discrepancy might refer to the action of essential oils being dose-dependent and the selective effect of essential oils on substrate degradation through the selective rumen microorganisms' alternation (Khorrami *et al.* 2015).

Information about the effect of processed BG with plant extracts and LA on EuCP is limited and needs to be more investigated. Estimation of EuCP in the *in vitro* condition is essential in many novel protein evaluation systems, which considered the interaction between carbohydrates and proteins degradation in the rumen (Van Duinkerken *et al.* 2011). Effective uCP is calculated from the uCP, which is estimated from the end product of fermentation (i. e., MCP) and NH_3-N (Edmunds *et al.* 2012). By increasing the passage rate, the concentration of EuCP increased means that the non-degradable dietary protein flow to the intestine increased. Treating BG with ORE presents the highest MCP and consequently the highest EuCP as well, which can be related to the protective effect of plant extracts on protein degradation (Naseroleslami *et al.* 2018), their microbiome modifier effect in the rumen and their effects on protein degradation in the rumen (Calsamiglia *et al.* 2007). The surface responses of EuCP to the interaction of fractional degradation rate of CP (%) and fractional degradation rate of DM (%), the fractional degradation rate of CP (%) and fractional degradation rate of starch (%), effective rumen degradable CP (%) and effective rumen degradable DM (%), and effective rumen degradable CP (%) and effective rumen degradable starch (%) are depicted in Figure 2A-D.

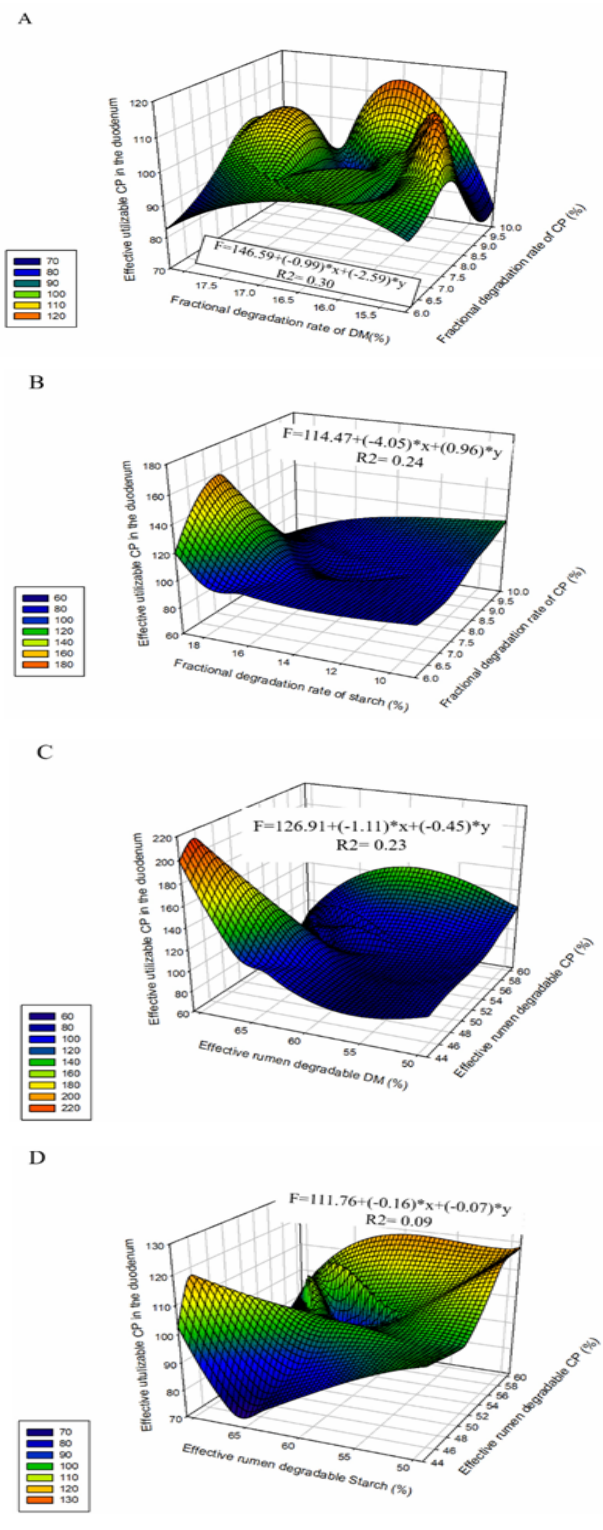

Figure 2 Graphs showing results of response surface analysis relating effective utilizable crude protein in the duodenum (EuCP) to interaction of A: fractional degradation rate of crude protein (%) and fractional degradation rate of dry matter (%); B: fractional degradation rate of crude protein (%) and fractional degradation rate of starch (%); C: effective rumen degradable crude protein (%) and effective rumen degradable dry matter (%) and D: effective rumen degradable crude protein (%) and effective rumen degradable starch (%)

An interesting 3 D response (R2=0.31) was observed in EuCP to the interaction of the fractional degradation rate of CP and starch. This response was depending on the ruminal starch degradation rate; while EuCP was better when ruminal starch fractional degradation rate increased. An interaction (R2=0.23) was also observed between effective rumen degradable DM (%) and effective rumen degradable CP (%), where a higher response in EuCP was identified with higher effective rumen degradable DM and lower values of effective rumen degradable CP. These responses suggest that to provide an optimum level of EuCP, a combination of effective rumen degradable starch and DM with effective rumen degradable CP should be considered.

In the diets with higher protein degradability including BG_{ORE} led to an increase in MCP concentration, while in the LD group processing BG either with LA or plant extracts increased the MCP yield relative to BG_{CTRL}. It is recommended that including of processed BG with LA or plant extracts in the diets with different protein degradability could pose various effects on fermentation gas kinetics and subsequently MCP yield and EuCP. The reason(s) for this apparent diversity is unclear. A potential reason for these different effects could be related to the antimicrobial and rumen modulatory characteristics of plant extracts (Calsamiglia *et al.* 2007; Hart *et al.* 2008). Hence, further research is warranted to better understand the reciprocal effects of LA or plant extracts processed BG and protein degradability.

CONCLUSION

Our results support a hypothesis that the method of prospecting barley grain is an important factor for altering rumen degradation parameters of DM, CP, and starch. In addition, gas production kinetics and the fate of nitrogen metabolism (presented by NH_3-N, MCP, and EuCP) of the diets used in the present study can be based solely on the method applied to the grain. Lactic acid represented a considerable effect only on rumen starch degradation, additionally, an adulteration of barley grain using thyme extract could not change EuCP when compared with the intact grain. Overall, *in situ* estimates of the rumen degradation kinetics and ERD of starch in barley grain were influenced by lactic acid treatment, while the plant extracts were not able to change these parameters. Whereas *in vitro* ruminal microbial protein yield was greater in plant extract processed barley grains included in the diets, and this should be considered when oregano and thyme extracts are used in dairy cow rations. Our results suggest that the responses of rumen fermentation and fate of nitrogen metabolism within different dietary CP and RUP levels were minor. However, the effects on *in vitro* ruminal NH_3-N concentration and gas produced during 2 h of fermentation caused by CP level is important and should be considered in future experiments. Therefore, possible effects on ruminal responses including fermentation and nitrogen metabolism caused by the different organic acids and various plant extracts treated barely grain still need to be further investigated.

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