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Effects of including different energy sources in the diet supplemented with small peptides of cottonseed on *in vitro* rumen fermentation, digestibility and microbial enzymes activity

Asieh Shabanzadeh, Ayoub Azizi, Amir Fadayifar and Arash Azarfar*

Department of Animal Science, Faculty of Agriculture, Lorestan University, Khorramabad, Iran

*Corresponding author,
E-mail address:
azizi.ay@lu.ac.ir
azizi.msc.modarese@gmail.com

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ORCID
Asieh Shabanzadeh
0000-0002-8245-2478
Ayoub Azizi
0000-0001-7158-0477
Amir Fadayifar
0000-0003-2957-7623
Arash Azarfar
0000-0002-7594-3623

Abstract The aim of the present study was to investigate the effects of different sources of non-fibrous carbohydrates (NFC) in the dairy cow diet supplemented with small peptides of cottonseed (Fortide C) on *in vitro* ruminal gas production (GP), fermentation parameters, substrate disappearance and microbial enzyme activity. Four iso-energetic and iso-nitrogenous diets were fed, containing 1) maize, 2) barley, 3) wheat or 4) a maize+barley mixture as the main sources of NFC. Each diet was supplemented with 7.05 g Fortide C/kg dry matter (DM) and incubated with media containing rumen liquor for 96 h *in vitro*. Dietary supplementation of the Fortide C in the wheat grain diet yielded greater gas production (GP) at 16 h of incubation, total GP and potential GP (b) than those containing maize ($P<0.05$), but similar to barley-containing diet ($P>0.05$). Other GP parameters including GP at 24, 48 and 72 h of incubation and constant rate of GP (c) were similar among the experimental diets. The highest and lowest DM disappearance, apparently degraded substrates, organic matter disappearance, estimated metabolizable energy, short chain fatty acids and microbial protein synthesis (MPS) were observed in the Fortide C-supplemented wheat and maize diets, respectively ($P<0.05$). Using wheat in the diet decreased NH_3-N compared to the maize diet ($P<0.05$). Inclusion of wheat in the diet supplemented with Fortide C increased carboxymethyl cellulase and α-amylase activities compared to the maize diet ($P<0.05$), while it was similar to the barley diet ($P>0.05$). However, microcrystalline cellulase and filter paper-degrading activities were unchanged among the dietary treatments. Overall, using wheat as the main source of NFC in the dairy cow diet supplemented with Fortide C improved *in vitro* ruminal fermentation profile, substrate disappearance, MPS and microbial enzyme activity compared to the maize or maize+barley-based diets.

Keywords: feed additive, gas production, non-fiber carbohydrates, ruminants

Introduction

Bioactive peptides, defined as specific protein fragments having a primary structure with an active sequence varying from 2 to 20 amino acid residues are produced from proteolytic hydrolysis of different plant protein sources (Srinivas and Karim, 2009). Recent studies on the use of these biological agents as synergistic feed additives in ruminant nutrition indicate their role as important intermediates in ruminant protein metabolism (Ives et al., 2002; Karimi et al., 2018). Unfortunately, ruminant nutritionists have yet not considered these materials in routine nutritional studies. Small peptides have many positive biological activities such as modulating digestive and gastrointestinal function, enhancing growth performance and nitrogen retention and offering a more cost-effective way of supplementing amino acids (Dabrowski et al., 2003; Kotzamanis et al., 2007; Srinivas and

Karim, 2009). However, their effects depend on their sequence of amino acids (Srinivas and Karim, 2009). In the rumen, smaller length peptides are converted to volatile fatty acids (VFAs) while those of the longer length are incorporated into microbial cells, giving rise to increased microbial protein synthesis (MPS) (Srinivas and Karim, 2009). Zhang et al. (2007) demonstrated that supplementing goat diets with small peptides derived from soybean (unprotected in the rumen) improved nitrogen balance, protein biological value and nutrient digestibility compared to those fed diets containing free amino acids from hydrolyzed soybean small peptides (obtained from Sanle Bio-Engineering Inc.). Although dietary small peptide supplementation was shown to improve rumen microbial growth and fiber digestibility in some experiments (Carro and Miller, 1999; Russi et al., 2002), no positive responses were reported in other works (Cruz Soto et al., 1994; Jones et al., 1998).

A recently introduced product derived from proteolytic hydrolysis of cottonseed (Fortide C) is another biological small peptide source. Karimi et al. (2018) observed that *in vitro* incubation of fattening lambs diet when supplemented with unprotected small peptides produced from proteolytic hydrolysis of cottonseed (Fortide C; Mytech Biotech Co. China) at 7.05 g/kg dry matter (DM) improved MPS and estimated metabolizable energy (ME).

On the other hand, synchronizing the degradation rate of rumen degradable proteins and non-fiber carbohydrates (NFC) may enhance the efficiency by which rumen microorganisms utilize nitrogen and ATP for their growth, and may improve animal performance (Azizi-Shotorkhoft et al., 2013). Digestion of starch in the rumen is closely related to changes in ruminal pH, VFA, MPS, microbial activity and consequently fibrolytic enzyme activity (Lanzas et al., 2007). Many factors such as dietary starch source, composition and protein content of the diet, protein degradability, mechanical and chemical processing of cereal grains as well as the degree of adaptation of rumen microorganisms to the diet determine the rate and extent of starch digestion in the rumen (Huntington, 1997). It has been shown that cereal grains differ in their starch contents with wheat having the highest [77% of DM], followed by 72% for maize and 57-58% for barley or oats (Huntington, 1997). In addition to starch content, grains also differ in their rate of ruminal starch degradability, as ground oat has the highest rate of ruminal starch degradability, followed by ground wheat, ground barley and ground maize (Tomankova and Homolka, 2004). Supplementing different NFC sources to the Holstein cow's diet did not affect ruminal pH, VFAs and NH3-N concentrations and total tract apparent nutrient digestibility; however, total tract apparent digestibility of starch in cows fed the oats-based diet was higher compared with those fed the corn- and wheat-based diets (Gozho and Mustvngwa, 2008). In the study of Khezri et al. (2009), replacing corn starch with sucrose in the diet of Holstein cows did not affect ruminal pH, VFAs concentration and nutrient digestibility, but reduced ruminal NH3-N concentration.

Because cereal grains differ in their rates and extents of ruminal degradation, we hypothesized that incorporation of maize, barley or wheat in dairy cow's diets containing a source of small peptides may modify ruminal responses, particularly the activities of microbial enzymes. Therefore, the objective of the present study was to investigate the effects of Fortide C, as a source of small peptides, in the diets containing different NFC sources (maize, barley or wheat) on gas production (GP) and fermentation profile, substrate disappearance and rumen microbial enzyme activities.

Materials and methods

Small peptides of Fortide C and experimental diets

The Fortide C, produced from proteolytic hydrolysis of cottonseed and used as a source of small peptides, was derived from a single production batch from the manufacturers (Mytech Biotech Co. China). The chemical composition of Fortide C is presented in Table 1.

Table 1. Chemical composition (% of DM) of Fortide C (n = 4)

Item	Fortide C
Dry matter	97.5
Organic matter	93.9
Crude protein (CP)	48.6
True protein (% of total CP)	82.5
Non-protein nitrogen (% of total N)	17.5
Neutral detergent fiber	1.18
Acid detergent fiber	0.40
Ether extract	0.05

Four experimental diets containing 1) maize, 2) barley, 3) wheat, or 4) maize+barley mixture as the main NFC sources were formulated for *in vitro* incubations. The ingredients and nutrient composition of the diets, formulated according to NRC (2001), are shown in Table 2.

Dietary treatments were similar in type and proportion of ingredients except for the source of cereal grains (*i.e.,* maize, barley or wheat) in order to avoid the effects of feed ingredient characteristics on rumen fermentation kinetics (Seo et al., 2010). Each experimental diet was supplemented with 7.05 g Fortide C/kg DM (see Karimi et al., 2018).

Animals as rumen inoculum donors

Rumen liquor was collected from two adult, dry and non-pregnant Lori cows fitted with permanent rumen cannula. All experimental procedures were conducted according to The Care and Use of Agricultural Animals in Research and Teaching Guidelines (FASS, 2010) and approved by the Ethical Committee of Lorestan University. Animals were fed a total mixed ration (TMR) based on wheat stra-

w, 300; alfalfa hay, 100; maize silage, 275; maize grain, 110; wheat bran, 200; urea, 5; calcium carbonate, 5; salt 2.5 and mineral/vitamin premix, 2.5 (g/kg DM). The TMR was formulated to meet all maintenance nutrient requirements (NRC, 2001). Cows were fed twice daily at 0900 and 1700 h in equal amounts and had free access to fresh drinking water. A two-week diet adaptation period was followed by collection of the rumen contents before the morning feeding, which was placed in a pre-warmed (39°C) thermal container. Rumen contents were transported to the laboratory before being mixed, homogenized and strained through 4 layers of linen cloth under O_2-free CO_2. The length of time between collection of rumen contents and incubation never exceeded 60 min.

Table 2. Feed ingredients, chemical composition (% of DM) and energy of experimental diets containing different sources of non-fiber carbohydrates supplemented with small peptides of cottonseed (Fortide C)

	Source of carbohydrate in the diet			
	Barley	Corn	Wheat	Corn/barley
Ingredients				
Alfalfa hay	24.8	24.8	24.8	24.8
Corn silage	12.0	12.0	12.0	12.0
Corn, ground	0.0	38.0	0.0	19.4
Barley, ground	40.30	0.0	0.0	19.4
Wheat, ground	0.0	0.0	39.8	0.0
Soybean meal	6.55	8.55	6.55	7.55
Wheat bran	7.20	7.50	7.70	7.70
Fish meal	4.80	4.80	4.80	4.80
Fat calcium-salt	1.65	1.65	1.65	1.65
Vitamin-mineral premix[1]	1.20	1.20	1.20	1.20
NaCl	0.5	0.5	0.5	0.5
$NaHCO_3$	1.0	1.0	1.0	1.0
Chemical composition				
Dry matter	81.1	79.1	79.2	79.6
Organic matter	91.9	91.5	91.1	91.0
Crude protein	18.1	18.1	18.5	18.0
Rumen degradable protein	12.3	11.6	12.4	11.8
Rumen undegradable protein	5.80	6.50	6.10	6.20
Neutral detergent fiber	30.6	26.2	27.8	28.5
Acid detergent fiber	17.8	16.4	16.8	17.2
Ether extract	4.05	4.80	4.03	4.40
Non fiber carbohydrates	39.15	42.4	40.77	40.1
Starch	29.6	34.3	31.8	31.9
Metabolizable energy (Mcal/kg DM)	2.54	2.57	2.57	2.55
Net energy of lactation (Mcal/kg DM)	1.61	1.64	1.63	1.67

[1]Contained (per kg): 8067 mg Mn, 18333 mg Fe, 14667 mg Zn, 4583 mg Cu, 92 mg I, 55 mg Se, 1000000 IU vitamin A, 200000 IU vitamin D and 6000 IU vitamin E (Roshd-Daneh, Karaj, Iran).

In vitro ruminal incubation

For all experimental diets (four treatments), a total of 129 bottles [(4 treatments × 10 replicates + 3 blanks = 43) in 3 separate runs (43 × 3= 129)] were incubated; 48 bottles for *in vitro* GP and 72 for estimation of the fermentation parameters. Kinetics of *in vitro* GP were measured for 96 h as described by Marten and Barnes (1980). Approximately 250 mg sample (on a DM basis and particle size of 1 mm) were accurately weighed into 100 mL-serum bottles. Each bottle was filled separately with 5 mL strained rumen fluid and 20 mL Marten and Barnes (1980) buffer solution, and immediately flushed with O_2-free CO_2 before being closed with a butyl rubber stopper, sealed with aluminum crimp, shaken and placed in a water-bath at 39°C. The Marten and Barnes (1980) buffer contained 1 L of solution A [(per liter): 10.0 g KH_2PO_4, 0.5 g $Mg_2SO_4.7H_2O$, 0.5 g NaCl, and 0.1 g $CaCl_2$. $2H_2O$] and 20 mL of solution B [(per 100 mL): 15.0 g Na_2CO_3, and 1.0 g $Na_2S.9H_2O$] and the pH was adjusted to 6.8 with solution B Marten and Barnes (1980). Gas volume produced was recorded at 3, 6, 8, 12, 16, 24, 48, 72 and 96 h using a digital pressure transducer (Tracker 200, Baley and Mackey, Ltd., Birmingham, UK) (Theodorou et al., 1994).

Fermentation parameters were determined after 16 h of incubation (Vercoe et al., 2010). Bottles were placed in an ice-bath to stop fermentation and gradually warmed up to 25°C. The volume of gas produced was recorded and bottles were uncapped for immediate measurement of pH (GLP22+ pH meter; Crison Instruments SA, Spain). Samples of supernatant (5 mL) from each bottle were immediately preserved with 5 mL of 0.1 N HCl and stored at -20°C for NH_3-N analysis. Concentration of NH_3-N was

determined as described by Broderick and Kang (1980). Fermentation residues were oven-dried at 60°C for 48 h for estimation of DM disappearance (DMD).

Calculations

Kinetic parameters throughout the *in vitro* fermentation period were estimated using the exponential equation of Ørskov and McDonald (1979) as:

$$Y = b\ (1\text{-}e^{-ct})$$

where, Y is the volume of gas produced at time t; a is the gas produced from soluble fractions (mL); b is the asymptotic gas production (mL); c is the rate of gas production (/h) and t is the incubation time (h).

The DMD at 16 h of incubation was calculated as the difference between its content in the sample before incubation and its amount in the residue after ruminal incubation.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* organic matter disappearance (IVOMD) were estimated according to Menke et al. (1979) as:

ME = 2.20+ 0.136 GP + 0.0057 CP (g/kg DM)
IVOMD = 148.8 + 8.89 GP + 0.45 CP (g/kg DM) + 0.65 ash (g/kg DM)

where, GP is the net gas production in mL from 200 mg dry sample after 16 h of incubation (Vercoe et al., 2010) and CP is crude protein.

Concentration of short chain fatty acids (SCFA) was calculated according to Getachew et al. (2002) as:

SCFA (mmol/g DM) = 0.0222 GP − 0.00425

where, GP is the 16 h net gas production (mL/g DM).

Microbial protein (MP) production was calculated according to Blümmel et al. (1997) as:

MP (mg/g DM) = mg ADS − (mL gas × 2.2 mg/mL)

where, ADS is apparently degraded substrate and 2.2 mg/mL is a stoichiometric factor which expresses mg of C, H and O required for the SCFA gas associated with the production of one mL of gas according to Blümmel et al. (1997).

Estimation of rumen enzyme activity

The pellet obtained after centrifugation of the contents of each bottle (4 bottles per treatment in each run) were used to estimate the enzyme activity in the particulate material (PM) fraction (Nogueira Filho et al., 2000). The activities of fiber-degrading enzymes viz. carboxymethyl cellulase (CMCase), microcrystalline cellulase (MCCase), filter paper-degrading (FPD) activity and α-amylase were estimated according to the procedure of Agarwal (2000). For the extraction of enzymes, pellets containing microbial biomass were suspended in a volume of 0.1 M phosphate buffer (pH 6.8) equal to the extracellular liquid. Lysozyme solution (4 g/L) and carbon tetrachloride were separately added to the suspension at the rate of 5 mL/30 mL cell suspension. Lysozyme treatment was followed by sonication in an ice bath for 6 min with a 30 s pulse rate and power supply of 0.5 W. The suspension was then incubated for 3 h at 39°C and centrifuged at 27000 × g for 30 min at 4°C. The clear supernatant (CS) was collected and used as the enzyme source for the PM fraction.

For estimation of CMCase, the reaction mixture containing 0.1 M phosphate buffer (pH 6.8) 1 mL, CS 0.5 mL and 1% carboxymethyl cellulose 0.5 mL, was incubated at 39°C for 60 min. To determine MCCase, the reaction mixture containing 0.1 M phosphate buffer (pH 6.8) 1 mL, CS 1 mL and 1% microcrystalline cellulose 1 mL, was incubated at 39°C for 60 min. To measure α-amylase, the reaction mixture containing 0.1 M phosphate buffer (pH 6.8) 1 mL, CS 0.5 mL and 1% starch 0.5 mL was incubated at 39°C for 30 min. The reaction mixture which contained 1 mL buffer (0.1 M, pH 6.8), 1 mL CS and 0.5 g Whatman No. 1 filter paper was incubated at 39°C for 60 min to estimate the FPD activity. In these assays, the reaction was stopped by adding 3 mL of 1% dinitrosalicylic acid solution. Glucose liberated due to enzyme activities was quantified according to the method described by Miller (1959), using glucose as the standard. Enzyme activities were calculated considering that one unit of enzyme produced 1 µmol of glucose/min/mL under the assay conditions.

Chemical analyses

Samples were analyzed for DM (#930.15), ash (#924.05) and N (#954.01) using standard methods as described in AOAC (1995). Neutral detergent fiber (NDF; inclusive of residual ash) was measured without the use of sodium sulfite or amylase (Van Soest et al., 1991). Acid detergent fiber (ADF; #973.18) was determined and expressed inclusive of residual ash (AOAC, 1995). The NFC content of experimental diets was calculated as:

NFC (g/kg DM) = 1000 – [NDF (g/kg DM) + CP (g/kg DM) + EE (g/kg DM) + ash (g/kg DM)]

Statistical analysis

The data were analyzed as a balanced completely randomized design using the GLM procedure of SAS (2002) according to the model:

$$Y_{ijk} = \mu + T_i + R_j + e_{ijk}$$

where, Y_{ijk} is the measured value, µ the general mean, T_i the effect of treatment on measured parameters, R_j the random effect of run and e_{ijk} the residual error. Means were compared by the Duncan's multiple range test $P \leq 0.05$.

Results

In vitro gas production and fermentation parameters

The addition of wheat in the Fortide C-supplemented diet yielded greater gas at 16 h of incubation, GP_{96} and potential of GP (b) than the diets containing maize (P<0.05), but similar to the diet containing barley (P>0.05). Other *in vitro* GP parameters including GP at 24, 48 and 72 h of incubation and GP rate constant (c) were similar among the experimental diets (P>0.05; Table 3).

Regarding fermentation parameters (Table 4), the highest and lowest *in vitro* DMD, ADS, IVOMD, estimated ME, SCFA and MPS were observed in diets supplemented with wheat and maize, respectively (P<0.05) while there was no significant differences (P>0.05) between wheat or barley-containing diets.

Table 3. *In vitro* gas production (GP) and estimated parameters of experimental diets containing different sources of non-fiber carbohydrates supplemented with small peptides of cottonseed (Fortide C)

Item	Source of carbohydrate in the diet				SEM	P-value
	Barley	Corn	Wheat	Corn/barley		
IVGP (mL/250 mg DM)						
GP_{16}	67.7[a]	55.5[b]	68.4[a]	60.8[b]	1.78	<0.01
GP_{24}	91.1	80.9	91.4	90.4	3.27	0.14
GP_{48}	109	104	113	111	3.53	0.34
GP_{72}	122	114	126	119	5.78	0.58
GP_{96}	126[a]	120[b]	134[a]	124[ab]	3.38	0.04
b	128[a]	122[b]	135[a]	126[ab]	3.41	0.25
c	0.079	0.72	0.81	0.75	0.006	0.73

GP, gas production; b, GP from the fermentable fraction (mL); c, fractional rate of GP (/h); SEM, standard error of the mean.
[a-b] Within rows, means with common superscript(s) do not differ (P>0.05).

Dietary supplementation of wheat decreased *in vitro* ruminal NH_3-N compared to maize (P<0.05) while there was no significant difference between diets containing wheat or barley (P>0.05). *In vitro* ruminal pH was similar (P>0.05) among the experimental Fortide C-supplemented diets containing different NFC sources.

Enzyme activity

The effects of experimental diets on rumen microbial enzyme activities are presented in Table 5. *In vitro* rumen CMCase and α-amylase activities were higher in wheat-containing diet compared to maize (P<0.05) but were similar to diet containing barley (P>0.05). However, MCCase and FPD activities were similar among the dietary treatments (P>0.05).

Table 4. Fermentation parameters and substrate disappearance of experimental diets containing different sources of non-fiber carbohydrates supplemented with small peptides of cottonseed (Fortide C)

Item	Source of carbohydrate in the diet				SEM	P-value
	Barley	Corn	Wheat	Corn/barley		
IVDMD	744[a]	642[b]	754[a]	686[ab]	20.3	0.02
ADS	186[a]	161[b]	188[a]	172[ab]	5.09	0.02
IVOMD	746[a]	656[b]	771[a]	703[b]	15.7	<0.01
ME	11.5[a]	9.87[b]	11.6[a]	10.5[b]	0.24	<0.01
SCFA	6.02[a]	4.91[b]	6.05[a]	5.38[b]	0.157	<0.01
MPS	154[ab]	148[b]	169[a]	152[ab]	6.12	0.04
pH	6.19	6.30	6.15	6.20	0.063	0.19
NH_3-N	18.1[bc]	19.9[a]	17.4[c]	18.8[b]	0.336	<0.01

IVDMD, *in vitro* dry matter disappearance (%); ADS, apparently degraded substrate; IVOMD, *in vitro* organic matter disappearance (%); ME, estimated metabolizable energy (MJ/kg DM); SCFA, short chain fatty acids (mmol/g DM); MPS, microbial protein synthesis (mg/g DM); NH_3-N, ammonia nitrogen (mg/dL); SEM, standard error of the mean.
[a-c] Within rows, means with common superscript(s) do not differ (P>0.05).

Discussion

Gas production kinetics and fermentation parameters

In terms of nutrient synchrony, most of the previous studies focused on the ruminal synchronization between energy with different dietary protein sources (Chamberlain et al., 1993; Azizi-Shotorkhoft et al., 2013) but information regarding the synchronous supply of different NFC sources with small peptides is not available. Tables 3 and 4 indicate that the cumulative GP at 16 h of incubation, TGP, ADS, IVDMD, IVOMD, ME, SCFA and MPS were greatest when wheat was included in the diet supplemented with Fortide C. This was probably due to the availability of more rapidly fermentable starch supplied by wheat with mono-and di-peptides of Fortide C than the other grains (Huntington,

1997; Tomankova and Homolka, 2004). Additionally, in starchy feeds, starch degradation is linearly related to GP which might be another reason for our results (Chamberlain et al., 1993; Chai et al., 2004). On the other hand, in our work the reduction in GP and nutrient digestibility when maize was the grain in the Fortide C-supplemented diet may have been due to the lower ruminal degradability of maize starch compared to the other starchy feeds, leading to some degree of asynchrony in the provision of protein and energy nutrients for rumen microorganisms (Tomankova and Homolka, 2004). In agreement with the results obtained in the present study, Herrera-Saldana et al. (1990) and Galloway et al. (1993) ranked cereal grains based on their ruminal starch degradability with wheat having the fastest degradation rate followed by barley, maize and sorghum. Seo et al. (2010) also showed that the synchronous supply of energy and protein in the diet of Holstein steers enhanced MPS and VFA production and decreased nitrogen excretion, while total tract nutrient digestibility was unaffected. Research has suggested that biological small peptides have a positive effect on protein utilization in ruminants. In agreement with our results, Pan and Webb (1998) reported that dietary supplementation with ruminally-unprotected soybean small peptides in ruminants improved protein digestibility and protein biological value compared to the other protein sources. Moreover, in a study conducted by Zhang et al. (2007), N balance and nutrient digestibility increased by supplementing goat diets with unprotected small peptides of soybean compared to goats fed diets containing supplemental free amino acids.

Table 5. Rumen microbial enzyme activity of experimental diets containing different sources of non-fiber carbohydrates supplemented with small peptides of cottonseed (Fortide C)

Item	Source of carbohydrate in the diet				SEM	P-value
	Barley	Corn	Wheat	Corn/barley		
Enzyme activity (U/min/mL)						
CMCase	2.84^{ab}	2.71^{b}	3.51^{a}	2.97^{ab}	0.221	0.04
MCCase	1.17	1.19	1.34	1.30	0.138	0.78
FPD activity	0.927	0.940	1.04	0.91	0.094	0.78
α-amylase	18.8^{ab}	16.9^{c}	19.7^{a}	17.9^{bc}	0.413	<0.01
Enzyme activity (µg glucose/min/mL)						
CMCase	256^{ab}	244^{b}	316^{a}	268^{ab}	19.93	0.04
MCCase	211	214	242	234	24.9	0.78
FPD activity	83.4	84.6	93.3	81.9	8.48	0.72
α-amylase	424^{ab}	381^{c}	443^{a}	404^{bc}	9.28	<0.01

CMCase, Carboxymethyl cellulase; MCCase, Microcrystalline cellulase; FPD, Filter paper-degrading; SEM, standard error of the mean.
$^{a-c}$ Within rows, means with common superscript(s) do not differ (P>0.05).

In none of the experimental diets, ruminal pH was affected by NFC source, and the mean ruminal pH values were within the normal physiological range of 6.1–6.8 (Van Soest, 1994). Similar results were reported by Khezri et al. (2009) when the diet of Holstein dairy cows was supplemented with increasing levels of sucrose at the expense of corn starch. Additionally, dietary supplementation with maize, barley, wheat or oats had no effect on ruminal pH in Holstein dairy cows (Gozho and Mustvngwa, 2008). However, it has been reported that feeding high rumen fermentable carbohydrates to ruminants decreased their rumen pH (Sahoo et al., 1999; Azizi-Shotorkhoft et al., 2013).

In all incubated diets from the present study, the mean concentration of ruminal NH_3-N was sufficient (more than 5 mg/dL) for optimum microbial growth (Satter and Slytor, 1974). Decreased NH_3-N concentration in diets containing wheat compared to maize or maize+barley diets was probably due to increased incorporation of ammonia into microbial biomass. Stimulatory effects of small peptides on the growth rate of rumen microorganisms and production of rumen microbial mass when energy is not limiting would prove this claim (Srinivas and Karim, 2009). It has been suggested that when energy supply is insufficient or the rate of peptide lysing exceeds the rate of amino acid used for MPS, peptide catabolism in the rumen increases and this may lead to increased NH_3-N production (Wallace, 1996). Similar findings were reported by Obara and Dellow (1993) and Araba et al. (2002) in different ruminant classes. Additionally, in Holstein dairy cows, when the diet was supplemented with high levels of rumen fermentable carbohydrates (i.e. sucrose), NH_3-N concentration fell while the rumen concentration of N peptides increased compared to the starchy grains (Khezri et al., 2010). Our findings concerning the effects of NFC on NH_3-N concentration are in line with those of Sannes et al. (2002) and Broderick et al. (2008) but contradict the data of Gozho and Mustvngwa (2008) who reported no effects of the NFC sources (maize, barley, wheat or oats) on ruminal NH_3-N concentration in Holstein cows; this contradiction may be due to differences in feed ingredients in the diet, animal species or small peptide used in the diet.

In dairy cows, MPS accounts for more than 50% of total amino acids absorbed in the small intestine (Seo et al., 2010). Increased MPS in diets containing wheat and Fortide C might be due to improved synchrony between small peptides and wheat starch energy for ruminal microbial utilization. It has been shown that rumen microbes use peptides more rapidly, and that peptides are also integrated into microbial cells more efficiently

than the corresponding free amino acids (Argyle and Baldwin, 1989). Additionally, the Cornell model suggests that the supply of pre-formed amino acids and small peptides for rumen non-cellulolytic bacteria increases MPS by 18.7% due to lower ATP requirements for biosynthesis of amino acids (Russell et al., 1992). In similar work, replacing different levels of a maize+barley mixture with molasses increased urinary excretion of allantoin, uric acid and xanthine+hypoxanthine and consequently improved MPS in sheep fed recycled poultry bedding as a source of rumen degradable protein (RDP) (Azizi-Shotorkhfot et al., 2013). On the contrary, in a study conducted by Gozho and Mustvngwa (2008), adding different NFC sources to the diets of Holstein cows had no effect on microbial N flow to the small intestine.

Rumen enzymatic activity

In most published studies, the effects of synchronous supply of ruminal fermentable energy and RDP have mainly focused on animal performance, but the activity of rumen microbes (measured as the activities of rumen hydrolytic enzymes) has received less attention. Rumen microbial enzyme activities are related to the capacity of carbohydrate fermentation by the mixed microbial population, and they have been considered as an index of microbial colonization (Nogueira Filho et al., 2000). Our study clearly revealed that CMCase activity (as an index for ruminal fiber degradation) was increased in the presence of when wheat grain and Fortide C (Table 5). It has been shown that when energy and NH_3-N are sufficient, supplying peptides and amino acid improves microbial fermentation rate and growth yield (Merry et al., 1990; CruzSoto et al., 1994; Chikunya et al., 1996). This may explain why ruminal IVDMD, IVOMD, estimated ME and MPS (Table 4) improved when the more rapidly fermentable starch (wheat diet) was supplemented with Fortide C in comparison with maize or a mixture of maize and barley. Recently, Azizi-Shotorkhoft et al. (2018) showed that the activities of rumen cellulolytic enzymes (and consequently NDF digestibility) were enhanced in sheep when the diet was supplemented with fermentable carbohydrate and RDP. The role of α-amylase in the digestion of dietary starch in the rumen is well established (Engvall, 1980). Increased α-amylase activity in the wheat diet was probably due to availability of more substrate (starch) giving rise to greater activity and proliferation of amylolytic bacteria in the rumen. A positive correlation has also been shown between the amount of starch fed to sheep and rumen α-amylase activity (Nasr, 1950).

Conclusion

The results of the present study indicated that adding wheat grain to the dairy cow diet containing Fortide C, as a source of bioactive small peptides, improved *in vitro* digestibility, nitrogen metabolism, CMCase and α-amylase activity compared to the diets containing maize or maize+barley while it was not different to barley. Further *in vivo* studies are required to elaborate the effects of dietary supplementation of dairy cow diets containing different sources of NFC with bioactive small peptides on production performance.

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