Korean Journal Of Agricultural Science

pISSN : 2466-2402
eISSN : 2466-2410

ANIMAL

Effects of rumen-protected amino acid prototypes on rumen fermentation characteristics *in vitro*

Gyeongjin Kim[1], Tabita Dameria Marbun[1], Jinhyun Park[1], Sang Moo Lee[1], Hong Gu Lee[2], Jun Ok Moon[3], Jin Seung Park[3], Eun Joong Kim[1,*]

[1]Department of Animal Science and Biotechnology, Kyungpook National University, Sangju 37224, Korea
[2]Department of Animal Science and Technology, Konkuk University, Seoul 05029, Korea
[3]CJ CheilJedang Research Institute of Biotechnology, Suwon 16495, Korea

*Corresponding author: ejkim2011@knu.ac.kr

Abstract

This study was conducted to evaluate the effects of rumen-protected amino acid (RPAA) prototypes, which were chemically synthesized, on *in vitro* rumen fermentation and protection rate outcomes. Several RPAA prototypes were incubated with timothy hay and concentrate. Treatments consisted of 1) control (CON; no RPAA prototype supplement), and prototypes of 2) 0.5% RP-methionine (RPMet), 3) 0.5% RP-tryptophan (RPTrp), 4) 0.5% RP-valine (RPVal), 5) 0.5% RP-phenylalanine (RPPhe), 6) 0.5% RP-leucine (RPLeu), 7) 0.5% RP-histidine (RPHis), 8) 20% RPMet, and 9) 20% RPTrp (w·w^{-1} feed). The inoculum (50 mL) prepared with rumen fluid and McDougall's buffer (1 : 4) was dispensed in individual serum bottles and was anaerobically incubated for 0, 6, and 24 h at 39°C in triplicate. The dry matter degradability did not differ among the groups, except for the 20% RPMet and the 20% RPTrp treatments at 6 and 24 h. The total volatile fatty acid concentration in the 20% RPMet was higher ($p < 0.05$) than the rest of the groups at 6 h, and 20% RPMet showed the highest molar proportion of acetate, whereas the lowest proportion of propionate was found at 6 h ($p < 0.05$). The protection rate of the RPAA prototypes ranged from 29.85 to 109.21%. at 24 h. In conclusion, the chemically synthesized RPAA prototypes studied here had no detrimental effects on rumen fermentation parameters. Further studies using animal models are needed for more accurate evaluations of the effectiveness of RPAA.

Keywords: amino acids, fermentation, *in vitro*, rumen-protected

Check for updates

OPEN ACCESS

Citation: Kim G, Marbun TD, Park J, Lee SM, Lee HG, Moon JO, Park JS, Kim EJ. Effects of rumen-protected amino acid prototypes on rumen fermentation characteristics *in vitro*. Korean Journal of Agricultural Science 48:669-679. https://doi.org/10.7744/kjoas.20210056

Received: July 13, 2021
Revised: August 30, 2021
Accepted: September 02, 2021

Copyright: © 2021 Korean Journal of Agrcultural Science

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http: //creativecommons.org/licenses/by-nc/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Ruminants have lower nitrogen efficiency than monogastric species, as dietary crude protein (CP) consumed by them is converted into ammonia by rumen microorganisms and is absorbed in the portal blood (Bach et al., 2005). Only 25% of the nitrogen derived from dietary CP is utilized by the host (Spek et al., 2013), whereas ammonia is changed to urea in the liver and excreted in urine and feces, causing nitrogen waste and adverse environmental effects (Chase et al., 2012).

For decades, scientists made various efforts to improve the nitrogen utilization efficiency, including studies to decrease dietary CP (Frank and Swensson, 2002; Colmenero and Broderick, 2006), feeding protected protein, and feeding protected amino acid (AA) that escape from the degradation of microorganism (Nursoy et al., 2018; Liang et al., 2019). A decreasing dietary CP increases the nitrogen efficiency of ruminants; however, it may compromise animal production (Colmenero and Broderick, 2006; Barros et al., 2017). Although feeding protected protein or increasing rumen-undegradable protein (RUP) meet specific criteria of CP content, it may limit the essential AAs required in the small intestine. Such a phenomenon occurs because AAs composition in the small intestine is largely different depending on the types of protein or the microbial degradation of dietary protein used to synthesize microbial protein (Santos et al., 1998). Recently, studies reported that the protection of the limited AAs improves nitrogen efficiency (Nursoy et al., 2018; Liang et al., 2019).

Several types of rumen-protected AAs (RPAAs) have been developed to improve nitrogen efficiency by resisting the degradation of microorganisms in the rumen, categorized as physical or chemical methods. Physical rumen protection methods include lipid encapsulation (Batistel et al., 2017; Ahn et al., 2019) and pH-sensitive polymers that protect their core at a pH of 5.5 to 7.0 in the rumen and release AAs at a pH of 2 to 3 in the abomasum (Schwab, 1995). These physical methods are characterized by resisting rumen microbial digestive enzymes with low rumen solubility and without AAs interaction (Wu and Papas, 1997). The chemical method includes analog and derivatives of AAs, where the α-amino group is replaced with a non-nitrogenous group or is modified with a chemical blocking to minimize the access by microorganism or enzyme (Schwab, 1995). Recently, interest in RPAA have been increased as some domestic companies are developing RPAA. Most RPAA products are developed through physical methods, however, attempts to protect amino acids using the chemical method are rare. In this study, several AAs were protected based on the chemical methods that were derivatives from a Korean company (CJ CheilJedang, Seoul, Korea). Therefore, the objective of this study was to evaluate the effects of newly developed RPAA prototypes created using chemical methods on rumen fermentation characteristics and examine their protection rate *in vitro*.

Materials and Methods

All experimental procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of the Kyungpook National University (Approved No. 2020-0129).

Experimental diets and treatments

In this study, timothy hay and a commercial concentrate ground to 1 mm were used as substrate diets. The RPAA prototypes consisted of methionine (RPMet), tryptophan (RPTrp), valine (RPVal), phenylalanine (RPPhe), leucine (RPLeu), and histidine (RPHis) obtained from CJ CheilJedang, Korea. Each RPAA prototype has a similar chemical structure, acetylated AA, with an acetyl group chemically conjugated to the amine group (NH_2-) of relevant AA. This acetyl moiety acts as a protection group that can inhibit AA utilization by rumen microbes or enzymes. Proximate analysis of substrate feed and RPAAs are shown in Table 1. The AA compositions of the substrate feed are shown in Table 2.

Table 1. Chemical composition of experimental diets (% of dry matter basis unless otherwise stated).

Item	Experimental diet							
	Timothy	Concentrate	RPMet	RPTrp	RPVal	RPPhe	RPLeu	RPHis
Dry matter	92.98	90.46	99.97	99.77	99.36	99.97	99.95	99.48
Organic matter	90.13	91.69	99.71	99.28	99.77	99.68	99.91	99.94
Crude protein	10.05	22.10	76.17	78.44	72.27	79.12	74.66	70.70
Ether extract	0.93	5.12	-	-	-	-	-	-
Neutral detergent fiber	71.14	21.33	-	-	-	-	-	-
Acid detergent fiber	42.59	9.82	-	-	-	-	-	-

RPMet, rumen-protected methionine prototype; RPTrp, rumen-protected tryptophan prototype; RPVal, rumen-protected valine prototype; RPPhe, rumen-protected phenylalanine prototype; RPLeu, rumen-protected leucine prototype; RPHis, rumen-protected histidine prototype.

Table 2. Amino acid composition of experimental diets ($mg \cdot g^{-1}$).

Item	Timothy	Concentrate
EAA ($mg \cdot g^{-1}$)		
Arginine	3.27	13.71
Histidine	1.31	5.53
Iso-leucine	2.94	8.40
Leucine	5.23	17.58
Lysine	3.92	10.72
Methionine	0.87	2.76
Tryptophan	-	2.32
Phenylalanine	4.14	9.84
Threonine	3.49	8.40
Valine	4.36	10.61
Total EAA	29.53	89.87
NEAA ($mg \cdot g^{-1}$)		
Alanine	4.91	11.72
Aspartic acid	9.38	19.46
Cystine	0.98	4.31
Glycine	3.60	9.73
Glutamic acid	8.72	40.02
Proline	6.87	14.26
Serine	3.49	10.28
Tyrosine	1.96	5.97
Total NEAA	39.91	115.75
Total AA	69.44	205.62

EAA, essential amino acid; NEAA, non-essential amino acid; AA, amino acid.

Measures of 0.24 g of timothy hay and 0.16 g (as-fed basis) of a commercial dairy cow concentrate (FARMSCO Inc., Anseong, Korea) were placed in each serum bottle. This ratio is referred to as the ratio used in typical Korean dairy farms. Nine experimental conditions were designed to have different RPAA prototypes and the level of supplementation: 1) control (CON; no RPAA prototype supplement), 2) 0.5% RPMet, 3) 0.5% RPTrp, 4) 0.5% RPVal, 5) 0.5% RPPhe, 6) 0.5% RPLeu, 7) 0.5% RPHis, 8) 20% RPMet, and 9) 20% RPTrp. A high supplementation level (i.e., 20% of the substrate used) was set as an experimental treatment to examine the obvious effect of the specific RPAAs.

In vitro rumen simulation study

Rumen fluid was collected from two Hanwoo cows using a stomach tube at 07:00 h, before the morning feeding (07:30 h) at the experimental farm of the university. The rumen fluid was pooled and immediately transported to a laboratory. Then, it was filtered through eight layers of muslin purged with CO_2 to maintain anaerobic conditions. The filtered rumen fluid was mixed with McDougall's buffer (McDougall, 1948) at a ratio of 1 : 4. Then, 50 mL of the rumen inoculum was filled into each serum bottle containing substrate diets. Later, each serum bottle was capped using a rubber stopper and an aluminum cap. The serum bottles were incubated in triplicates for 0, 6, and 24 h at 39℃.

Analysis of experimental diets and rumen fermentation

Samples were analyzed for dry matter (method 934.01), organic matter (method 942.05), ether extract (method 920.39A), CP (method 2001.11), neutral detergent fiber (method 2002.04) with alpha-amylase and sodium sulfite, and acid detergent fiber (method 973.18), according to AOAC (AOAC, 2019). At the end of each incubation, the serum bottles were opened and poured into a 5 × 10 cm nylon bag (pore size 50 μm, R510, ANKOM Technology, USA), and the pH of the rumen fluid filtered from the nylon bag was analyzed using a pH meter (ST210, Ohaus, New Jersey, USA).

Residues that remained in the serum bottles were poured into nylon bags to determine their dry matter digestibility (DMD). The nylon bag was rinsed with distilled water until the water became clear and dried overnight at 105℃. The ammonia-N was analyzed according to the method described in Chaney and Marbach (1962). A total of 20 μL of sample was mixed with 1 mL of phenol color regent (phenol 50 g and sodium nitroferricyanide 0.25 g per 1 L) and 1 mL of alkali hypochlorite reagent (sodium hydroxide 25 g and sodium hypochlorite 16.8 mL per 1 L). Then, the mixture reacted for 30 min at room temperature. The ammonia-N concentration was analyzed colorimetrically using a spectrophotometer (Optizen pop, Mecasys, Daejeon, Korea) at 630 nm wavelength.

Volatile fatty acids (VFAs) were analyzed with the method by Erwin et al. (1961) using gas chromatography (450-GC, Bruker Inc., Billerica, USA) equipped with BR-Wax fame (BR87503, Bruker Inc., Billerica, USA). Injector, detector (flame ionization detector), and oven temperature were 250, 250, and 100℃, respectively. The airflow for nitrogen, hydrogen, and air were set at 29, 30, and 300 $mL \cdot min^{-1}$, respectively.

Calculation of rumen protection rate

The rumen protection rate of the RPAA prototypes was calculated based on the relative concentration ratio (%) between 0 h and 6 h or 24 h. The RPAA concentration was analyzed at the CJ CheilJedang company. Briefly, the incubated rumen fluid was centrifuged (1730MR, Labogene, Seoul, Korea) for 10 min at 9,425 × *g*, and the supernatant was separated and filtered using a 0.45 μm syringe filter (RJN1345NH, Rephile, Shanghai, China). Then, the RPAA prototype concentration in rumen fluid was analyzed with an HPLC (CBM-20A, Shimadzu, Kyoto, Japan) equipped with a Purospher® STAR RP-18 end-capped 5 μm column (EMD Millipore, Darmstadt, Germany) at 230 nm wavelength. Distilled water, 0.2% H_3PO_4, C_2H_3N, and 0.2% H_3PO_4 were used as the pump solvents.

Statistical analysis

All data were subjected to analysis of variance, and further mean comparisons were performed with Duncan's multiple range test to determine significant differences using SPSS (IBM Corporation, 2017). The treatment effect was analyzed; however, the incubation time effect was not statistically examined. Differences were considered significant at $p < 0.05$, and p-values between 0.05 and 0.10 were considered to represent a tendency.

Results and Discussion

The effects of the RPAA prototypes developed by a chemical method were evaluated on *in vitro* rumen fermentation and protection rate in this study. Due to the lack of derivatives *in vitro* rumen fermentation research, we referred to 2-hydroxy-4-(methylthio) butanoic acid (HMB) and isopropyl ester form of HMB (iHMB), which was one of the chemical rumen-protected methods. Briefly, 50% of these are bypassed in the rumen and absorbed in the small intestine, converted into keto methylthio butyrate that is intermediate to synthesize Met and transaminated to L-Met that the host animal can utilize for maintenance, growth, and production (Firkins et al., 2015).

The rumen fermentation *in vitro* results are shown in Tables 3 and 4. The pH was lower in the CON (6.88 and 6.79 at 6 and 24 h, respectively) than the other treatments at 6 h and 24 h ($p < 0.05$; Table 3). Dry matter digestibility was higher in the 20% RPMet and the 20% RPTrp treatments than the other groups at 6 h and 24 h ($p < 0.05$). However, except for the 20% RPMet and the 20% RPTrp treatments, there was no significant difference among groups in DMD (Table 3). The DMD is used as a fermentation indicator in feed evaluation when using *in vitro* rumen simulation. At the end of each fermentation process, filter crucible (Aderao et al., 2018), filter paper (Navarro-Villa et al., 2011), or Ankom filter bag (Hart et al., 2009) were often used to filter the substrate residues and further to dry it in an oven to determine the dry matter of the residues in the serum bottle. In our study, the DMD was determined using nylon bags (Guo et al., 2019). The DMD of all treatments was not significant, except for the RPMet 20% and the RPTrp 20% treatments (Table 3). The DMD results were similar to those observed in previous research (Vazquez-Anon et al., 2001), with the addition of the HMB concentration (0, 0.20, 0.77, and 1.43% in the diet) in *in vitro* rumen fermentation, except for the RPMet 20% and the RPTrp 20% treatments. Nevertheless, both the RPMet 20% and the RPTrp 20% treatments had higher DMDs than the rest of the groups ($p < 0.05$). The reason behind this result may be that the nylon bag used for determining the DMD had a 50 μm pore size, and the RPAAs used here were high solubility in water and had a small particle size, such as fine granules. In the *in vitro* rumen fermentation, the residue in nylon bags is considered as indigestion. Thus, the DMD was calculated by subtracting the residue (%) from the total percentage (100%). Probably, when the rumen fluid was distributed in bottles of the RPMet 20% and the RPTrp 20% treatments, their high concentration level occurred because the RPAA prototypes were either soluble in the rumen fluid or passed through the nylon bag during the filtering process. Therefore, the DMD was likely to be overestimated (Table 3). Our results indicate that using nylon bags or any other filtering methodology during *in vitro* rumen simulation may not be an appropriate method to estimate the effect of RPAA on DMD, especially when the solubility or particle size of the RPAA is being evaluated. In line with the above-mentioned hypothesis, Bach et al. (2005) also noted that high solubility in rumen fluid did not result in high rumen degradability, and the rumen degradability degree depended on molecular structure.

Table 3. Effect of rumen-protected amino acid prototypes on ruminal fermentation *in vitro*.

Time (h)	Experimental group (n = 3)										
	Control	RPMet 0.5%	RPTrp 0.5%	RPVal 0.5%	RPPhe 0.5%	RPLeu 0.5%	RPHis 0.5%	RPMet 20%	RPTrp 20%	SEM	p-value
pH											
6	6.88e	6.92d	6.94bcd	6.96abc	6.96abc	6.98a	6.97ab	6.93d	6.94cd	0.009	< 0.01
24	6.79e	6.81d	6.85abc	6.86a	6.86ab	6.87a	6.87a	6.83cd	6.83bcd	0.008	< 0.01
Dry matter digestibility (%)											
6	44.91b	43.78b	46.09b	44.63b	44.94b	44.91b	44.68b	54.32a	54.10a	0.941	< 0.01
24	71.02b	71.06b	70.29b	71.54b	72.39b	71.92b	71.93b	77.53a	75.66a	0.972	< 0.01
Ammonia-N (mg·100 mL^{-1})											
6	3.66c	4.01abc	3.90abc	3.83bc	4.19ab	4.03abc	4.35a	2.32d	3.92abc	0.137	< 0.01
24	1.96d	2.03cd	2.35bcd	2.06cd	2.30bcd	3.05ab	3.43a	2.66bcd	2.78abc	0.230	< 0.01

RPMet, rumen-protected methionine prototype; RPTrp, rumen-protected tryptophan prototype; RPVal, rumen-protected valine prototype; RPPhe, rumen-protected phenylalanine prototype; RPLeu, rumen-protected leucine prototype; RPHis, rumen-protected histidine prototype; SEM, standard error of the mean.

a - e: Means in a row with different letters are significantly different ($p < 0.05$).

The ammonia-N of the CON treatment showed the lowest level among the groups ($p < 0.05$), except for 20% RPMet at 6 h ($p < 0.05$). The protection rate of 20% RPMet at 6 h showed higher rate (93.75%). The lowest ammonia-N concentration in 20% RPMet at 6 h was one of the peculiar phenomena in this study. It was expected that with high protection rate of RPMet, the ammonia-N concentration of the supernatant would be similar or a little higher compared with the control and yet the results were somewhat different. It is not clear why such results were obtained. Further experiments are planned to elucidate this phenomenon.

The total VFA concentration of the 20% RPMet (27.95 and 52.56 mmol at 6 h and 24 h, respectively) was higher than the rest of the groups at 6 and 24 h ($p < 0.05$). The 20% RPMet supplementation had the highest molar proportion of acetate but the lowest proportion of propionate compared to other groups at 6 h and 24 h ($p < 0.05$). Consequently, acetate : propionate (A : P) ratio was highest in the 20% RPMet (Table 4). Volatile fatty acid, one of the *in vitro* rumen fermentation indicators, supplies more than 70% of the energy to ruminants (Bergman, 1990). The acetate level, which accounts for the largest percentage of VFA, was relatively higher in the 20% RPMet and the 20% RPTrp treatments. In the case of branched-chain VFA (BCVFA), it is produced when protein and AAs, especially branched-chain AAs (BCAAs), are degraded (Allison and Bryant, 1963). Therefore, the BCVFA concentration has been used as another indicator in RPAA-related studies (Fowler et al., 2015; Baghbanzadeh-Nobari et al., 2017). Previous research showed that BCVFA promotes the growth of cellulolytic microbes in the rumen (Dehority et al., 1967) and increases total VFA and acetate concentrations (Liu et al., 2009). However, in this study, although the concentration of BCVFAs in the 20% RPMet treatment was not higher than other treatments, and acetate was the highest in this treatment ($p < 0.05$). The results of studies to evaluate the effects of RPAA on VFA are limited and controversial. The study by Vazquez-Anon et al. (2001), who supplemented 0, 0.2, 0.77, and 1.43% HMB in *in vitro* rumen fermentation, did not find significant differences in the total VFA and individual VFA. In agreement with this study, Baghbanzadeh-Nobari et al. (2017), who supplemented 1.8 g·kg^{-1} HMBi to ewe, found an increase in the total VFA, acetate, and A : P ratio. In addition, Noftsger et al. (2003), who supplemented 0, 0.055, and 0.11% HMB in *in vitro* rumen fermentation, found a significant propionate decrease. Notably, it was confirmed that the propionate concentration decreased as the RPAA concentrations increased. The effect of RPAAs on VFA concentration was not apparent.

Table 4. Effect of rumen-protected amino acid prototypes on volatile fatty acids *in vitro*.

Time (h)	Experimental group (n = 3)										
	Control	RPMet 0.5%	RPTrp 0.5%	RPVal 0.5%	RPPhe 0.5%	RPLeu 0.5%	RPHis 0.5%	RPMet 20%	RPTrp 20%	SEM	p-value
Total VFA (mmol)											
6	24.87b	25.91ab	25.01b	24.03b	24.19b	20.73c	23.54b	27.95a	24.34b	0.726	< 0.01
24	44.31de	43.38de	43.77de	41.40e	53.81a	47.02cd	42.89de	52.56ab	49.37bc	1.312	< 0.01
Acetate (molar proportion)											
6	69.36d	69.75cd	69.74cd	70.49c	69.83cd	69.86cd	70.51c	75.21a	72.57b	0.265	< 0.01
24	67.32c	66.61cd	66.25d	66.63cd	67.02cd	67.43c	67.31c	72.33a	68.69b	0.252	< 0.01
Propionate (molar proportion)											
6	19.73a	19.66a	19.49a	19.06a	19.22a	19.33a	19.40a	16.40c	17.82b	0.207	< 0.01
24	22.79b	23.37a	23.52a	23.06ab	22.70b	22.22c	21.91cd	19.17e	21.55d	0.151	< 0.01
Butyrate (molar proportion)											
6	8.50a	8.32a	8.45a	8.24a	8.53a	8.41a	7.91b	6.60d	7.47c	0.102	< 0.01
24	7.29c	7.42bc	7.61bc	7.54bc	7.71b	7.64bc	8.05a	6.44d	7.40bc	0.114	< 0.01
BCVFA (molar proportion)											
6	1.82abc	1.72bcd	1.76abcd	1.68cd	1.89a	1.85ab	1.64d	1.37e	1.64d	0.044	< 0.01
24	1.50c	1.51c	1.53bc	1.72a	1.51c	1.64ab	1.62abc	1.21e	1.36d	0.040	< 0.01
Acetate/propionate ratio											
6	3.52d	3.55cd	3.58cd	3.70c	3.63cd	3.61cd	3.63cd	4.59a	4.08b	0.053	< 0.01
24	2.95de	2.85ef	2.82f	2.89ef	2.95de	3.03cd	3.07c	3.77a	3.19b	0.040	< 0.01

RPMet, rumen-protected methionine prototype; RPTrp, rumen-protected tryptophan prototype; RPVal, rumen-protected valine prototype; RPPhe, rumen-protected phenylalanine prototype; RPLeu, rumen-protected leucine prototype; RPHis, rumen-protected histidine prototype; SEM, standard error of the mean; VFA, volatile fatty acid; BCVFA, branched chain volatile fatty acid.

a - f: Means in a row with different letters are significantly different ($p < 0.05$).

Table 5 shows the *in vitro* rumen respective AA contents in the different incubation hours and the rumen and protection rate of the AAs at 6 h and 24 h. The *in vitro* rumen protection rate of the 0.5% RPMet and the 0.5% RPTrp treatments were 29.85% and 40.28%, whereas the 20% RPMet and the 20% RPTrp treatments showed 90.70% and 109.21% protection rates at 24 h, respectively. Other prototypes showed 75.05% (RPVal), 63.77% (RPPhe), 73.41% (RPLeu), and 78.74% (RPHis) of protection rates at 24 h. The protection rates of the RPAAs prototypes were not significantly different among groups at 6 h, except for the 0.5% RPMet and the 20% RPTrp treatments, and the 20% RPTrp showed the highest protection rates among the RPAA prototypes at 24 h.

Table 5. Concentration ($mg \cdot mL^{-1}$) and protection rate (%) of rumen-protected amino acid (RPAA) prototypes.

Time (h)	Experimental groups (n = 3)									
	RPMet 0.5%	RPTrp 0.5%	RPVal 0.5%	RPPhe 0.5%	RPLeu 0.5%	RPHis 0.5%	RPMet 20%	RPTrp 20%	SEM	p-value
Targeted RPAA concentration ($mg \cdot mL^{-1}$)										
0	0.043c	0.059c	0.057c	0.054c	0.053c	0.053c	2.176a	1.425b	0.1335	< 0.01
6	0.022e	0.050cd	0.063c	0.060cd	0.056cd	0.048d	1.947a	1.547b	0.0040	< 0.01
24	0.013e	0.024d	0.043c	0.034c	0.038c	0.041c	1.883a	1.520b	0.0033	< 0.01
Protection rate (%)										
6	52.72b	85.83a	110.03a	110.57a	105.17a	93.27a	93.75a	111.17a	9.018	< 0.01
24	29.85d	40.28cd	75.05b	63.77bc	73.41b	78.74b	90.70ab	109.21a	8.276	< 0.01

RPMet, rumen-protected methionine prototype; RPTrp, rumen-protected tryptophan prototype; RPVal, rumen-protected valine prototype; RPPhe, rumen-protected phenylalanine prototype; RPLeu, rumen-protected leucine prototype; RPHis, rumen-protected histidine prototype; SEM, standard error of the mean.

a - e: Means in a row with different letters are significantly different ($p < 0.05$).

The methods used for evaluating the protection rate of RPAAs were different depending on the protection methods. In the case of coating-based RPAA, for example, *in situ* experiments (Berthiaume et al., 2000; Koenig and Rode, 2001), which uses nylon bags, are regarded as a standard approach. However, the RPAA prototypes used here have some unique characteristics compared to conventional coating-based RPAAs, such as 1) powder form, 2) relatively soluble in rumen fluid, and 3) split of prototypes in NH_2-blocking group and AA in the small intestine by enzyme (Endo, 1980; Windschitl and Stern, 1988). Using nylon bags via an *in situ* methodology to estimate the protection rate of RPAA developed by the chemical method is likely to underestimate the actual protection rate. Because the RPAA solubility was different (data not shown), it is improbable that the protection rate of RPAA prototypes was analyzed using residue in nylon bag (Wilman and Adesogan, 2000; Schwab and Ordway, 2003). Therefore, our study was conducted using batch culture *in vitro* to evaluate the rumen protection rate. *In vitro* rumen protection rates of the 0.5% RPMet and the 0.5% RPTrp treatments were 29.85% and 40.28%, whereas the 20% RPMet and the 20% RPTrp treatments were 90.70% and 109.21% at 24 h, respectively. Especially, the protection rate of RPMet and RPTrp was affected by the level of supplementation (0.5% or 20%). It is not clear why the protection rate was different depending on its supplementation level. Velle et al. (1997) reported that rumen degradation was reduced as methionine dosage increased (75, 150, 300, and 600 mmol), although unprotected methionine was used in the study. They further discussed that such discrepancy has occurred because the liberation of sulfur inhibits deaminating enzymes. Another study has also reported that derivatives were not adapted to rumen microbes as AAs (Witt et al., 1998). Thus, it may be feasible to conclude that a high derivatives level caused rumen microbes to fail to degrade, resulting in high protection rate. In the case of RPAA products on the market, they have a different level of rumen protection: 90% of coating RPAA with pH-sensitive polymer (Robert and Williams, 1997), 50% of HMBi, another type of AA analog (St-Pierre and Sylvester, 2005), and the 66% or 78% of coating RPAA based on ethyl-cellulose (Berthiaume et al., 2001; Koenig and Rode, 2001). Therefore, the results of this study showed a reasonable range of rumen protection compared to other RPAAs in the literature with 75.05% for RPVal, 63.77% for RPPhe, 73.41% for RPLeu, and 78.74% for RPHis at 24 h.

Conclusion

In this study, the effects of RPAA prototypes, which were chemically synthesized, were evaluated on *in vitro* rumen fermentation and protection rate. Differences among treatments in terms of ammonia-N and VFA were observed. No DMD differences were noted among treatments, except for the RPMet 20% and the RPTrp 20% supplementations. The *in vitro* ruminal protection rate of 0.5% of PRMet, PRTrp, PRVal, PRPhe, PRLeu, PRHis, and 20% of RPMet, RPTrp prototypes averaged 29.85, 40.28, 75.05, 63.77, 73.41, 78.74, 90.70, and 109.21% respectively. Especially, the ruminal protection rate of Met and Trp was affected by the level of supplementation (0.5% and 20%), although the reason for such discrepancy remains unclear. Given the *in vitro* protection rate of RPAA prototypes, derivatives developed in this study have potential as rumen-protected amino acids. However, *In vivo* studies are needed for the accurate evaluation of the RPAA prototypes.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This research was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Agri-Bio industry Technology Development-Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (117030-3). This research was also supported by Kyungpook National University Development Project Research Fund, 2018.

Authors Information

Gyeongjin Kim, https://orcid.org/0000-0003-2202-126X
Tabita Dameria Marbun, https://orcid.org/0000-0002-3360-9715
Jinhyun Park, https://orcid.org/0000-0001-6328-4125
Sang Moo Lee, https://orcid.org/0000-0003-2510-2591
Hong Gu Lee, https://orcid.org/0000-0002-0679-5663
Jun Ok Moon, https://orcid.org/0000-0002-2118-3833
Jin Seung Park, https://orcid.org/0000-0002-4333-1024
Eun Joong Kim, https://orcid.org/0000-0002-5962-6994

References

Aderao GN, Sahoo A, Bhatt RS, Kumawat PK, Soni L. 2018. *In vitro* rumen fermentation kinetics, metabolite production, methane and substrate degradability of polyphenol rich plant leaves and their component complete feed blocks. Journal of Animal Science and Technology 60:1-9.

Ahn JS, Kwon EG, Shin JS, Kim MJ, Son GH, Choi CS, Lee CW, Park JK, Park BK. 2019. Changes in growth performance, carcass characteristics, and meat properties of late fattening Hanwoo steers according to supplementation of rumen protected methionine and lysine. Korean Journal of Agricultural Science 46:671-682. [in Korean]

Allison MJ, Bryant MP. 1963. Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria. Archives of Biochemistry and Biophysics 101:269-277.

AOAC (Association of official analytical chemists). 2019. Official method of analysis, 21th ed. AOAC, Washington, D.C., USA.

Bach A, Calsamiglia S, Stern MD. 2005. Nitrogen metabolism in the rumen. Journal of Dairy Science 88:E9-E21.

Baghbanzadeh-Nobari B, Taghizadeh A, Khorvash M, Parnian‐Khajehdizaj F, Maloney SK, Hashemzadeh-Cigari F, Ghaffari AH. 2017. Digestibility, ruminal fermentation, blood metabolites and antioxidant status in ewes supplemented with DL-methionine or hydroxy-4 (methylthio) butanoic acid isopropyl ester. Journal of Animal Physiology and Animal Nutrition 101:e266-e277.

Barros T, Quaassdorff MA, Aguerre MJ, Colmenero JJO, Bertics SJ, Crump PM, Wattiaux MA. 2017. Effects of dietary crude protein concentration on late-lactation dairy cow performance and indicators of nitrogen utilization. Journal of Dairy Science 100:5434-5448.

Batistel F, Arroyo JM, Bellingeri A, Wang L, Saremi B, Parys C, Trevisi E, Cardoso FC, Loor JJ. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein dairy cows. Journal of Dairy Science 100:7455-7467.

Bergman EN. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews 70:567-590.

Berthiaume R, Dubreuil P, Stevenson M, McBride BW, Lapierre H. 2001. Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. Journal of Dairy Science 84:194-203.

Berthiaume R, Lapierre H, Stevenson M, Cote N, McBride BW. 2000. Comparison of the *in situ* and *in vivo* intestinal disappearance of ruminally protected methionine. Journal of Dairy Science 83:2049-2056.

Chaney AL, Marbach EP. 1962. Modified reagents for determination of urea and ammonia. Clinical Chemistry 8:130-132.

Chase LE, Higgs RJ, Van Amburgh ME. 2012. Feeding low crude protein rations to dairy cows-What have we learned. pp. 32-42. In Proceedings of the 23rd Ruminant Nutrition Symposium, University of Florida, Gainsville, FL, USA.

Colmenero JJO, Broderick GA. 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. Journal of Dairy Science 89:1704-1712.

Dehority BA, Scott HW, Kowaluk P. 1967. Volatile fatty acid requirements of cellulolytic rumen bacteria. Journal of Bacteriology 94:537-543.

Endo Y. 1980. *In vivo* deacetylations of n-acetyl amino acids by kidney acylases in mice and rats a possible role of acylase system in mammalian kidneys. Biochimica et Biophysica Acta (BBA)-General Subjects 628:13-18.

Erwin ES, Marco GJ, Emery EM. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. Journal of Dairy Science 44:1768-1771.

Firkins JL, Fowler CM, Devillard E, Bequette BJ. 2015. Kinetics of microbial methionine metabolism in continuous cultures administered different methionine sources. Journal of Dairy Science 98:1178-1194.

Fowler CM, Plank JE, Devillard E, Bequette BJ, Firkins JL. 2015. Assessing the ruminal action of the isopropyl ester of 2-hydroxy-4-(methylthio) butanoic acid in continuous and batch cultures of mixed ruminal microbes. Journal of Dairy Science 98:1167-1177.

Frank B, Swensson C. 2002. Relationship between content of crude protein in rations for dairy cows and milk yield, concentration of urea in milk and ammonia emissions. Journal of Dairy Science 85:1829-1838.

Guo G, Shen C, Liu Q, Zhang SL, Wang C, Chen L, Xu QF, Wang YX, Huo WJ. 2019. Fermentation quality and *in vitro* digestibility of first and second cut alfalfa (*Medicago sativa* L.) silages harvested at three stages of maturity. Animal Feed Science and Technology 257:114274.

Hart KJ, Martin PG, Foley PA, Kenny DA, Boland TM. 2009. Effect of sward dry matter digestibility on methane production, ruminal fermentation, and microbial populations of zero-grazed beef cattle. Journal of Animal Science 87:3342-3350.

IBM Corporation. 2017. IBM SPSS Statistics for Windows. In Version 25.0 ed. IBM Corporation, NY, USA.

Koenig KM, Rode LM. 2001. Ruminal degradability, intestinal disappearance, and plasma methionine response of rumen-protected methionine in dairy cows. Journal of Dairy Science 84:1480-1487.

Liang SL, Wei ZH, Wu JJ, Dong XL, Liu JX, Wang DM. 2019. Effect of N-acetyl-L-methionine supplementation on lactation performance and plasma variables in mid-lactating dairy cows. Journal of Dairy Science 102:5182-5190.

Liu Q, Wang C, Yang WZ, Zhang B, Yang XM, He DC, Zhang P, Dong KH, Huang YX. 2009. Effects of isobutyrate on rumen fermentation, lactation performance and plasma characteristics in dairy cows. Animal Feed Science and Technology 154:58-67.

McDougall EI. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43:99.

Navarro-Villa A, O'brien M, López S, Boland TM, O'kiely P. 2011. Modifications of a gas production technique for assessing *in vitro* rumen methane production from feedstuffs. Animal Feed Science and Technology 166:163-174.

Noftsger SM, St-Pierre NR, Karnati SKR, Firkins JL. 2003. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) on microbial growth in continuous culture. Journal of Dairy Science 86:2629-2636.

Nursoy H, Ronquillo MG, Faciola AP, Broderick GA. 2018. Lactation response to soybean meal and rumen-protected methionine supplementation of corn silage-based diets. Journal of Dairy Science 101:2084-2095.

Robert JC, Williams PEV. 1997. Influence of forage type on the intestinal availability of methionine from a rumen protected form. Journal of Dairy Science 80:248.

Santos FAP, Santos JEP, Theurer CB, Huber JT. 1998. Effects of rumen-undegradable protein on dairy cow performance: A 12-year literature review. Journal of Dairy Science 81:3182-3213.

Schwab CG. 1995. Protected proteins and amino acids for ruminants. In Biotechnology in Animal Feeds and Animal Feeding edited by Wallace RJ, Chesson A. pp. 115-141. VCH Publishers, Weinheim, Germany.

Schwab CG, Ordway RS. 2003. Methionine supplementation options. pp. 93-98. In Proceeding Four-State Applied Nutrition and Management Conference.

Spek JW, Dijkstra J, van Duinkerken G, Hendriks WH, Bannink A. 2013. Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in northwestern Europe and North America: A meta-analysis. Journal of Dairy Science 96:4310-4322.

St-Pierre NR, Sylvester JT. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. Journal of Dairy Science 88:2487-2497.

Vazquez-Anon M, Cassidy T, McCullough P, Varga GA. 2001. Effects of Alimet on nutrient digestibility, bacterial protein synthesis, and ruminal disappearance during continuous culture. Journal of Dairy Science 84:159-166.

Velle W, Sjaastad ØV, Aulie A, Grønset D, Feigenwinter K, Framstad T. 1997. Rumen escape and apparent degradation of amino acids after individual intraruminal administration to cows. Journal of Dairy Science 80:3325-3332.

Wilman D, Adesogan A. 2000. A comparison of filter bag methods with conventional tube methods of determining the *in vitro* digestibility of forages. Animal Feed Science and Technology 84:33-47.

Windschitl PM, Stern MD. 1988. Influence of methionine derivatives on effluent flow of methionine from continuous culture of ruminal bacteria. Journal of Animal Science 66:2937-2947.

Witt MW, Newbold CJ, Wallace RJ. 1998. Influence of dietary acetylated peptides on fermentation and peptidase activities in the sheep rumen. Journal of Applied Microbiology 84:847-851.

Wu SHW, Papas A. 1997. Rumen-stable delivery systems. Advanced Drug Delivery Reviews 28:323-334.