



## ***In vitro* and Lactation Responses in Mid-lactating Dairy Cows Fed Protected Amino Acids and Fat**

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**ABSTRACT:** The objective of this study was to evaluate the effect of ruminally protected amino acids (RPAAs) and ruminally protected fat (RPF) supplementation on ruminal fermentation characteristics (*in vitro*) and milk yield and milk composition (*in vivo*). Fourteen mid-lactating Holstein dairy cows (mean weight 653±62.59 kg) were divided into two groups according to mean milk yield and number of days of postpartum. The cows were then fed a basal diet during adaptation (2 wk) and experimental diets during the treatment period (6 wk). Dietary treatments were i) a basal diet (control) and ii) basal diet containing 50 g of RPAAs (lysine and methionine, 3:1 ratio) and 50 g of RPF. In rumen fermentation trial (*in vitro*), RPAAs and RPF supplementation had no influence on the ruminal pH, dry matter digestibility, total volatile fatty acid production and ammonia-N concentration. In feeding trial (*in vivo*), milk yield ( $p<0.001$ ), 4% fat corrected milk ( $p<0.05$ ), milk fat ( $p<0.05$ ), milk protein ( $p<0.001$ ), and milk urea nitrogen ( $p<0.05$ ) were greater in cows fed RPAAs and RPF than the corresponding values in the control group. With an index against as 0%, the rates of decrease in milk yield and milk protein were lower in RPAAs and RPF treated diet than those of basal diet group ( $p<0.05$ ). In conclusion, diet supplemented with RPAAs and RPF can improve milk yield and milk composition without negatively affecting ruminal functions in Holstein dairy cows at mid-lactating. (**Key Words:** Rumen Protected Amino Acids, Rumen Protected Fat, Fermentation, Milk Yield)

### INTRODUCTION

Feed additives such as methionine or fat are an appropriate method to increase milk yield and milk composition. Dairy cows need optimum energy sources through diet formulation to increase their performance as dairy farmers in Korea and many other countries generally receive a milk price based on milk yield and fat content. Dairy cows need the same essential amino acids (AA) like methionine (Met) or lysine (Lys) as nonruminants (Fuller et al., 1989). Progress in the field of individual AA nutrition of dairy cows has often been studied. Amino acids are a nutritional source for increasing protein content in milk and milk yield of dairy cows. However, Lys and Met have been determined as the most limiting AA for lactating cows fed a variety of corn-based diets (Schingoethe et al., 1988; Izumi

et al., 2000).

Non-protected dietary fat reduces forage consumption and inhibits rumen fermentation (Bines et al., 1978). Therefore, ruminally protected fat (RPF) as feed supplementation has been examined in many studies (Macleod et al., 1977; Yang et al., 1978; Sharma et al., 1978; Wrenn et al., 1978).

Chalupa et al. (1986), Mattias et al. (1982) and Veira et al. (1991) reported that the aims of supplementing RPFs to dairy cattle were to increase milk yield, milk fats, arginine and glutamine acid concentrations in blood and to enhance nitrogen retention. Previous studies indicated the different roles of supplementation of ruminally protected amino acids (RPAAs) and RPFs of dairy cows. Supplementing RPAAs and RPFs have many positive effects on lactating performance except decreasing protein (when added RPFs only) and fatty acids (when added RPAAs only) contents in milk (Palmquist and Conrad, 1978; Mattias et al., 1982; Donkin et al., 1989; Robinson et al., 1992). Addition of RPAAs and RPFs together into generally used feed may recover those above problems. However, there are no

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reports regarding the effects of supplementing RPAA and RPF together on *in vitro* ruminal fermentation and lactation responses in mid-lactating Holstein dairy cows.

Therefore, our study consisted with two experiments in which RPAA and RPF have been supplemented into the diet to evaluate the rumen fermentation and lactation performances in mid-lactating Holstein dairy cows. First experiment (*in vitro*) was conducted to evaluate the effects on fermentation characteristics by supplementation of RPAA and RPF. Second experiment (*in vivo*) was to compare the changes in milk yield and milk composition between cows fed with supplemented RPAA and RPF and cows fed without supplemented RPAA and RPF.

## MATERIALS AND METHODS

### Rumen fermentation, *in vitro*

Ruminal fluid was collected from a ruminally fistulated Holstein-Friesian cow fed 53.3% of total mixed ratio, 16.7% of timothy 10% or rice straw and 20% of concentrate supplement once per day. Immediately following collection it was squeezed through four layers and eight layers of cheesecloth into an Erlenmeyer flask with an O<sub>2</sub>-free headspace. The flask was not disturbed for 30 min incubation in a 39°C water bath to permit feed particles to rise to top of flask. Particle-free ruminal fluid was anaerobically transferred to a buffer (pH 7.0) containing 7.5 g of NaHCO<sub>3</sub>, 0.824 g of Na<sub>2</sub>HPO<sub>4</sub> anhydrous, 0.31 g of KH<sub>2</sub>PO<sub>4</sub> anhydrous, 0.03 g of MgSO<sub>4</sub> 7H<sub>2</sub>O, 3.25 mg of CaCl<sub>2</sub> anhydrous, 2.5 mg of MnCl<sub>2</sub> 4H<sub>2</sub>O, 0.25 mg of COCl<sub>2</sub> 6H<sub>2</sub>O and 2.0 mg of FeSO<sub>4</sub> 7H<sub>2</sub>O/L. Ruminal fluid and buffer were mixed (1:1 ratio), and 200ml rumen

samples (100 mL ruminal fluid+100 mL buffer) were anaerobically transferred to 250 mL bottles containing 4 g of control and treatment diet samples. The bottles were capped with butyl-rubber stoppers containing gas regulator and placed at shaking incubator (Vision, Deajeon, Korea) at 39°C, 100 rpm. Samples were incubated at 3, 6, 12, 24, and 48 h. Experimental diet was ground to pass through a 2 mm screen using a Wiley Mill. 2.71% of RPLys and 0.90% RPMet (3:1 ratio) and 3.62% of RPF were then mixed. The chemical composition of experimental diets was analyzed as shown in Table 1.

### Lactation responses, *in vivo*

Fourteen multiparous (2nd to 3rd lactation) Holstein dairy cows (mean body weight, 653±62.59) of mid-lactating stages were fed as shown in Table 1. The cows were divided into two groups of seven cows each according to mean milk yield (35 kg/d) and mean number of days postpartum (145 d). They were fed a basal diet during adaptation period (2 wk) and the basal diet with RPAA and RPF during treatment periods (6 wk).

The ingredients and chemical composition of the experimental diet are shown in Table 1. Dietary treatments were i) the control (basal diet; no addition of RPAA and RPF), and the treatment group fed basal diet with 50 g of RPAA and 50 g of RPF twice a day (total 100 g of each RPAA and RPF/d). Dairy dry matter intake (DMI) was recorded and water was provided *ad libitum*.

Cows were milked twice daily starting at 06:00 and 18:00 h, and milk yield was recorded weekly and milk samples were taken weekly from each cow during the experimental period (6 wk). Milk samples were refrigerated

**Table 1.** Ingredient and chemical composition of experimental diets fed for lactating cows (*in vitro*, *in vivo* %, DM basis)

Composition (%)	Ingredients <sup>1,2</sup>					
	Concentrate mix <sup>3</sup>	Beet pulp	Cotton seed	Oat	Alfalfa hay	Mineral mix <sup>4</sup>
Dry matter	89.50	89.29	90.36	94.79	82.86	Free choice
Crude protein	21.33	10.66	20.27	9.45	16.14	
Crude fat	5.62	0.94	21.94	3.57	2.41	
Crude fiber	10.34	25.80	34.06	35.43	41.11	
Crude ash	6.07	6.09	6.02	5.73	5.85	
NDF	50.07	56.42	55.42	72.01	54.30	
ADF	13.85	29.90	45.79	44.07	46.08	
NFE	46.14	45.81	8.07	40.61	27.34	
Mixing rate <sup>5</sup>	42.19	7.07	5.72	31.50	13.53	

DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; NFE, nitrogen free extract; RPAA, ruminally protected amino acids; RPF, ruminally protected fat; RPLys, ruminally protected lysine; RPMet, ruminally protected methionine.

<sup>1</sup> *In vitro* study: control group incubated 4 g of basal diet only (no addition of RPAA and RPF), and the treatment group incubated basal diet with 2.71% RPLys and 0.90% RPMet (3:1 ratio) and 3.62% RPF.

<sup>2</sup> *In vivo* study: control group fed basal diet only (no addition of RPAA and RPF), and the treatment group fed basal diet with 50 g of RPAA (50% of RPLys and RPMet, [3:1 ratio]) and 50 g of RPF (98% of RPF) twice a day (total 100 g of each RPAA and RPF/d).

<sup>3</sup> Commercial concentrate which was manufactured for lactating cows producing 30 to 40 kg milk per day.

<sup>4</sup> Containing 200 mg manganese, 100 mg cobalt, 4,000 mg sulfur, 150 mg iodine, 2,000 mg iron, 100 mg zinc, 100 mg copper, 50 mg nickel, 2,000 mg calcium, 3,000 mg magnesium, 40 mg selenium.

<sup>5</sup> The rate of forage and concentrate was 6:4.

at 4°C for the analysis of milk composition.

### Chemical analysis

*In vitro* samples (50 mL) were removed through the butyl rubber stopper using a 60 mL syringe at 0, 3, 6, 12, 24, and 48 h of incubation. Samples were immediately centrifuged (14,000×g at 4°C for 15 min), and supernatant fluids were stored at -20°C for analysis. Cell wall contents (neutral detergent fiber and acid detergent fiber) were estimated as per the method of Goering and Van Soest (1970). Nitrogen free extract, crude protein, ether extract, and total ash contents of diets were analyzed according to AOAC (1984) procedures. The pH values of fermented samples were determined according to the method of Briggs et al. (1957). *In vitro* dry matter digestibility (DMD) was determined using the method of Tilley and Terry (1963). The total volatile fatty acid (VFA) in samples of supernatant fluid were measured by a gas chromatograph (Hewlett Packard 6890, USA), (column temperature: 120°C, injector temperature: 265°C, detector temperature: 240°C) equipped with an autosampler and crosslinked polyethylene glycol,  $\phi$ 0.53 mm×30 m size FFAP column (Hewlett Packard, USA). The concentration of ammonia-N was measured by a color-metric method Chaney and Marbach (1962).

Milk samples refrigerated at 4°C were analyzed for fat, protein and MUN by automatic milk analyzer (Automatic IR 4000/5000 Milk Analyzer, Foss Electric, Hillerød, Denmark).

### Statistical analysis

All data were analyzed using the general linear models of Statistical Analysis System (SAS, 1996). The last significant differences between means were calculated using a *t*-test procedure.

## RESULTS AND DISCUSSION

### *In vitro* rumen fermentation

The effects of adding of RPAA and RPF to the diet on ruminal pH of *in vitro* fermentation are shown in Table 2.

The pH value measurement can be used as a tool to evaluate the fermentation in the rumen (Kumar et al., 2013). Ruminal pH was not affected by supplementing RPAA and RPF in the diet ( $p>0.05$ ). In present study, the pH is similar between control and treatment, this observation is in agreement with previous *in vivo* studies (Sutton et al., 1983; Canale et al., 1990; Wang et al., 2004), which reported that pH was not significantly affected by supplementing RPAA or RPF.

Supplementation of RPAA and RPF also did not affect ( $p>0.05$ ) the DMD compared to control (Table 2). Hill and West (1991) and Canale et al. (1990) observed that RPAA and RPF supplementation of ruminant diets did not affect DMD. Ngidi et al. (1990) reported that RPF supplementation of beef finishing diets did not affect DMD or energy digestibility. Our study is in agreement with above results and indicated that the RPAA and RPF supplementation did not influence pH and DMD in the rumen.

Results of the *in vitro* fermentation on total VFA and ammonia-N concentrations of RPAA and RPF by mixed rumen microorganisms are summarized in Table 3. The pattern of total VFA concentrations between cows fed with basal diet and RPAA and RPF diet were similar. The cows fed diet containing RPAA and RPF diet trended a higher VFA at 24 h (86.05 mM) and 48 h (101.30 mM) compared to controls (24 h: 67.98 mM, 48 h: 99.01 mM). Ammonia-N concentrations between basal diet group and RPAA and RPF treated group were not affected. Diets containing 7.5% tallow fed to finishing cattle (Bogges et al., 1987) tended to lower in total VFA concentrations. In contrast, Sutton et al. (1983) reported no change in the concentration of total VFA and ammonia-N in sheep fed supplemental fat (3% linseed oil, 3% coconut oil). Our study observed no great effect on total VFA and ammonia-N concentrations by supplementing RPAA and RPF.

### *In vivo* dairy dry matter intake, milk production and milk composition

Dairy dry matter intake of Holstein dairy cows fed

**Table 2.** Effects of supplementation of RPAA and RPF on ruminal pH and DMD, *in vitro*

Time (h)	Ruminal pH				DMD (%)			
	Control <sup>1</sup>	Treatment <sup>2</sup>	SEM	p value	Control	Treatment	SEM	p value
3	6.33	6.38	0.02	0.12	29.93	30.93	1.08	0.55
6	6.10	6.13	0.04	0.64	33.97	37.07	1.05	0.30
12	5.74	5.73	0.01	0.59	43.78	44.28	0.56	0.58
24	5.48	5.46	0.02	0.52	53.12	53.95	0.68	0.53
48	5.43	5.44	0.01	0.69	55.21	56.11	0.69	0.46

RPAA, ruminally protected amino acids; RPF, ruminally protected fat; DMD, dry matter digestibility; SEM, pooled standard error of mean; RPLys, ruminally protected lysine; RPLMet, ruminally protected methionine.

<sup>1</sup> Without supplementation of RPAA and RPF.

<sup>2</sup> 2.71% of PRLys, 0.90% of RPLMet, and 3.62% of RPF was incubated with 200 mL rumen inoculums at 39±0.5°C incubator for 48 h.

**Table 3.** Effects of supplementation of RPAA and RPF on ruminal total VFA and ammonia-N concentrations, *in vitro*

Time (h)	Total VFA(mM)				Ammonia-N (mg/dL)			
	Control <sup>1</sup>	Treatment <sup>2</sup>	SEM	p value	Control	Treatment	SEM	p value
3	50.29	49.60	22.18	0.74	6.42	6.40	0.30	0.96
6	87.10	86.71	34.68	0.35	9.01	9.72	0.35	0.27
12	58.64	56.52	29.10	0.43	11.85	12.25	1.15	0.82
24	67.98	86.05	45.03	0.12	18.88	17.15	0.61	0.13
48	99.01	101.30	51.55	0.42	14.59	15.53	0.16	0.35

RPAA, ruminally protected amino acids; RPF, ruminally protected fat; VFA, volatile fatty acid; SEM, pooled standard error of mean; RPLys, ruminally protected lysine; RPMet, ruminally protected methionine.

<sup>1</sup> Without supplementation of RPAA and RPF.

<sup>2</sup> 2.71% of PRLys, 0.90% of PRMet, and 3.62% of RPF was incubated with 200 mL rumen inoculums at 39±0.5°C incubator for 48 h.

control diet (basal diet) and treatment diet (basal diet+ RPAA and RPF) were not significantly different (Table 4). Wang et al. (2004) reported that DMI linearly decreased with dietary supplementation of fat in lactating cows. Schauff and Clark (1992) also found a similar tendency of decreasing DMI when cows were fed rations containing 3, 6, or 9% of protected fat. However, the rations supplemented with fat would not necessarily cause the decrease of DMI during early lactation. According to Lee et al. (2008), the dietary supplementation with RPAA did not affect DMI in lactating cows ( $p>0.05$ ). Under the condition of this study, DMI was also not affected by supplementation of RPAA and RPA.

Milk yield and 4% fat corrected milk (FCM) were higher when cows were fed a diet containing RPAA and

RPF in this study ( $p<0.001$ ,  $p<0.05$ ) (Table 4). Depeters and Cant (1992) reported that supplementation of dairy rations with more than 2% added fat often improved milk yield. Sarwar et al. (1991) reported an increase of milk yield and 4% FCM in cows fed diets supplemented with Ca salts of fatty acid. Many researchers (Schwab et al., 1989; Chapoutot et al., 1992; Robinson et al., 1992) have indicated that milk yield and 4% FCM increased with dietary supplementation of RPAA.

The average fat ( $p<0.05$ ), protein ( $p<0.001$ ) and MUN ( $p<0.05$ ) contents in milk from cows fed RPAA and RPF were higher than those of control (Table 4).

This is supported by an animal trial (Canale et al., 1990) where fat and protein contents in milk were noted higher in dairy cows fed the diets containing RPAA and fat. Similar

**Table 4.** Effect of RPAA and RPF supplementation on DM intake, milk yield and milk composition in mid-lactating Holstein-Frisian dairy cows

Items	Control <sup>1</sup>	Treatment <sup>2</sup>	SEM	p value
----- Milk yield and milk composition -----				
DM intake (kg)	23.06	22.92	1.08	0.082
Milk yield (kg)	30.52	33.29**	0.34	0.0002
4% FCM (kg)	29.73	32.63*	0.55	0.007
Milk fat (%)	3.73	3.87	0.07	0.16
Milk fat (kg)	1.14	1.28*	0.02	0.002
Milk protein (%)	3.02	3.05	0.02	0.47
Milk protein (kg)	0.92	1.02**	0.01	0.0001
MUN (mg/dL)	12.27	16.68*	0.86	0.005
----- Index against 0% -----				
DM intake (kg/%)	-5.32	-6.21	1.41	0.42
Milk yield (kg/%)	-5.61	-1.86*	1.05	0.03
4% FCM (kg/%)	-4.54	-0.98	1.81	0.22
Milk fat (%)	+5.78	+5.05	1.62	0.77
Milk fat (kg/%)	-0.28	+3.54	1.87	0.19
Milk protein (%)	+0.99	+1.76	0.84	0.58
Milk protein (kg/%)	-4.88	+0.04*	1.11	0.01
MUN (mg/dL/%)	-13.55	-6.49	6.43	0.46

RPAA, ruminally protected amino acids; RPF, ruminally protected fat; DM, dry matter; SEM, pooled standard error of mean; FCM, fat corrected milk; MUN, milk urea nitrogen; RPLys, ruminally protected lysine; RPMet, ruminally protected methionine.

<sup>1</sup> Without supplementation of RPAA and RPF (-, decrease rates; +, increase rates).

<sup>2</sup> Control group fed basal diet only (no addition of RPAA and RPF), and the treatment group fed basal diet with 50 g of RPAA (50% of RPLys and RPMet, [3:1 ratio]) and 50 g of RPF (98% of RPF) twice a day (total 100 g of each RPAA and RPF/d).

Significantly difference between control and treatment (\*  $p<0.05$ , \*\*  $p<0.001$ ).

results were reported by Sloan et al. (1989). MUN concentration were higher (16.68 mg/dL) in dairy cows fed RPAA and RPA compared to control (12.27 mg/dL), ( $p < 0.05$ ). However, according to Roseler et al. (1993), the suitable MUN concentration for Holstein dairy cow is between 12 mg/dL to 18 mg/dL which is where the present results ranged.

With an index against as 0% (Table 4), decrease rate of milk yield (-1.86%) and milk protein (0.04%) were significantly lower ( $p < 0.05$ ) in cows fed RPAA and RPF than when cows were fed basal (Control) diet (milk yield, -5.61%; milk protein, -4.88%). The variations of milk yield, 4% FCM, milk fat, milk fat yield, MUN, milk protein, milk protein yield in dairy cows fed diets containing RPAA and RPF or basal diet are shown in Figure 1A, 1B, 1C, 1D, 1E, 1F, and 1G. Cows fed with diet containing RPAA and RPF were linearly higher in milk yield (kg/%), milk fat yield (kg/%), milk protein yield (kg/%) and MUN (mg/dL) during whole experimental period (6 wk), (Figure 1A, 1D, 1F and 1G), ( $p > 0.05$ ). It is known that the milk fat content is higher when cows are fed RPAA (Robinson et al., 1992). In addition, supplementation of RPF to lactating cows

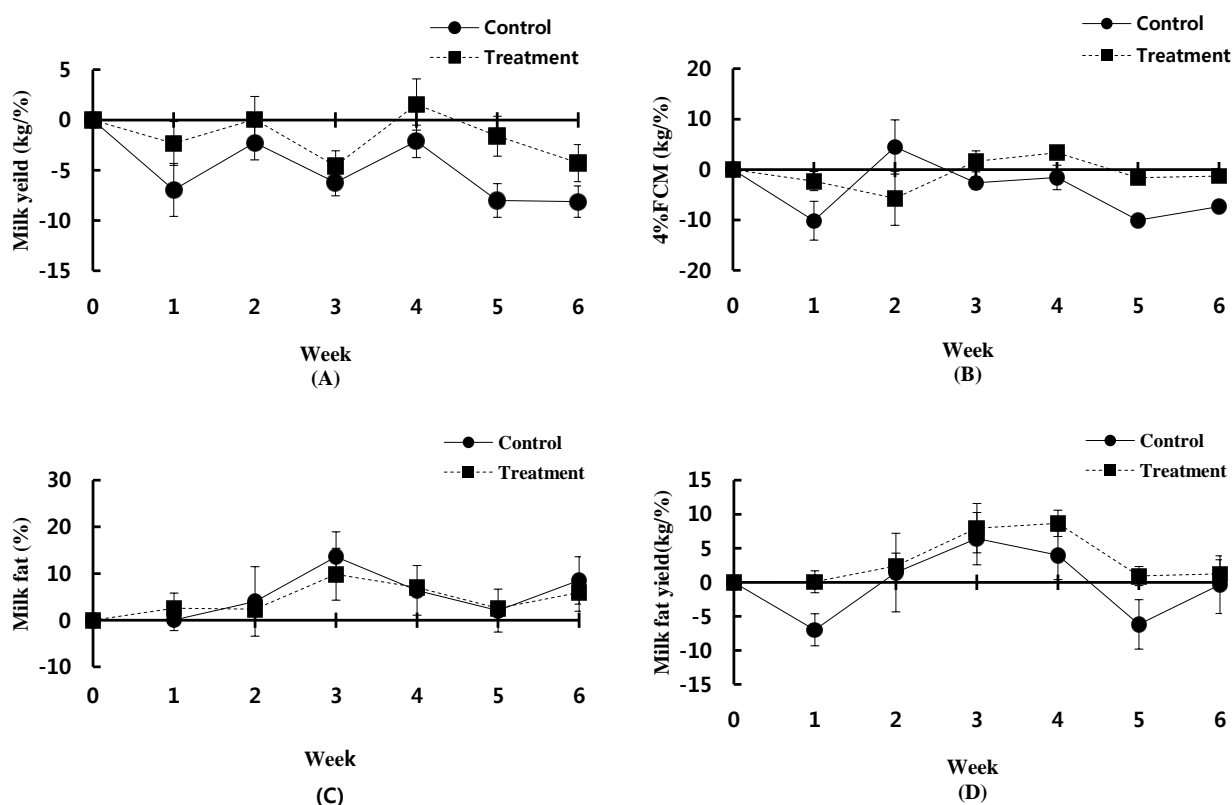
increased milk yield and fat content in milk (Mattias et al., 1982).

The results of this study demonstrate that dietary supplementation of RPAA and RPF has a positive effect on milk yield and milk composition including 4% FCM, fat, protein in mid-lactating dairy cows without adversely affecting ruminal fermentation.

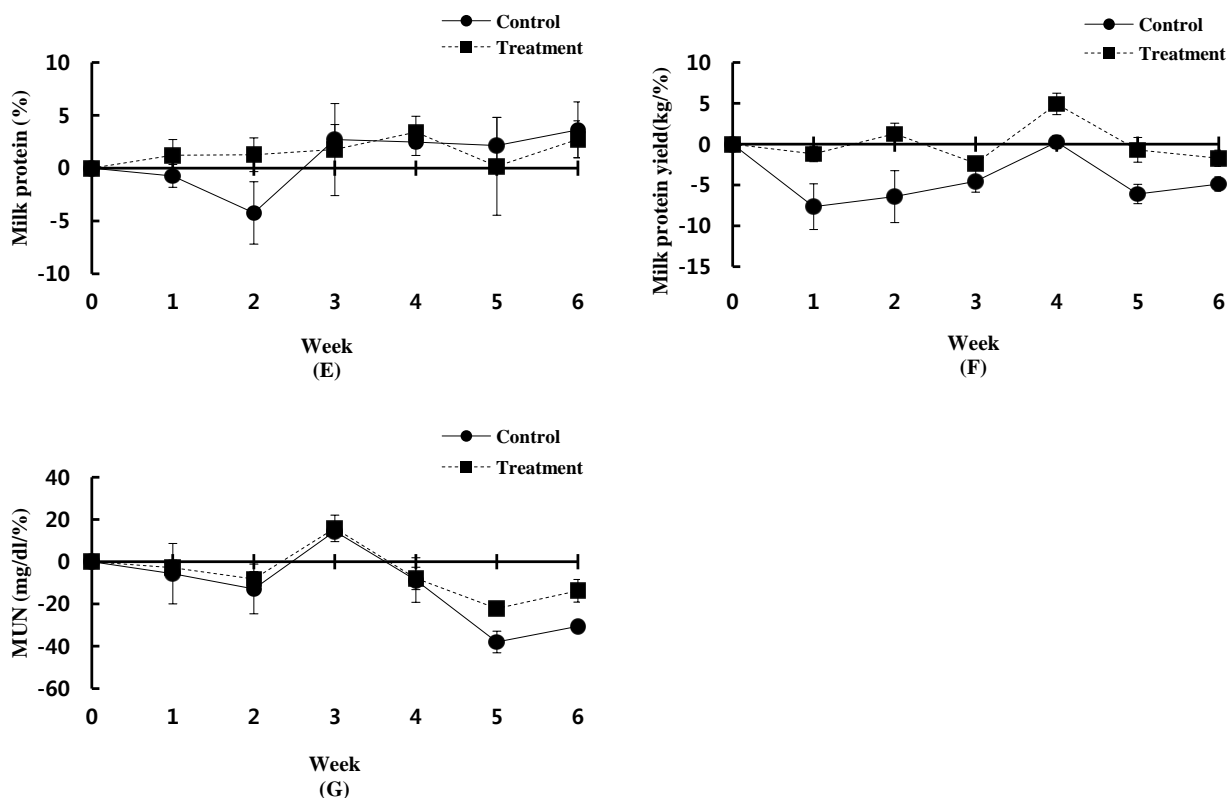
According to previous studies, supplementation of RPAA or RPF may positively affect milk yield and milk composition. However, no report has been published on the effect of supplementing RPAA and RPF together on ruminal performances and lactation responses in mid-lactating dairy cows. In particular, we found that the milk protein content was higher when cows were fed RPAA and RPF together compared to supplementing RPAA or RPF individually.

However, it is not yet known what percentage of RPAA and RPA are required to affect milk yield and milk composition. Therefore, the question of digestion and absorption of RPAA and RPF in the abomasum requires further study.

The best strategy for dairy farmers to increase their



**Figure 1.** i) Effect of RPAA and RPF supplementation on milk (A) yield (kg/%), (B) 4% fat corrected milk (FCM) (kg/%), (C) fat (%), (D) fat yield (kg/%), (E) protein (%), (F) protein yield (kg/%), (G) MUN (milk urea nitrogen) yield (mg/dL/%) of Holstein-Frisian dairy cows (Index against 0%). Control group fed basal diet only (no addition of RPAA and RPF), and the treatment group fed basal diet with 50 g of RPAA (50% of RPLys and RPMet [3:1 ratio]) and 50 g of RPF (98% of RPF) twice a day (total 100 g of each RPAA and RPF/d). RPAA, ruminally protected amino acids; RPF, ruminally protected fat; RPLys, ruminally protected lysine; RPMet, ruminally protected methionine.



**Figure 1. ii)** Effect of RPAAs and RPF supplementation on milk (A) yield (kg/%), (B) 4% fat corrected milk (FCM) (kg/%), (C) fat (%), (D) fat yield (kg/%), (E) protein (%), (F) protein yield (kg/%), (G) MUN (milk urea nitrogen) yield (mg/dL/%) of Holstein-Frisian dairy cows (Index against 0%). Control group fed basal diet only (no addition of RPAAs and RPF), and the treatment group fed basal diet with 50 g of RPAAs (50% of RPLys and RPMet [3:1 ratio]) and 50 g of RPF (98% of RPF) twice a day (total 100 g of each RPAAs and RPF/d). RPAAs, ruminally protected amino acids; RPF, ruminally protected fat; RPLys, ruminally protected lysine; RPMet, ruminally protected methionine.

income is through genetic improvement and the optimization of nutrient utilization of their cows. Feed additives are one of the quicker methods to increase milk yield and milk composition. Therefore, supplementation of RPAAs and RPF together might increase the profits from dairy farms.

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