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Investigation of Dietary Lysophospholipid (Lipidol^{TM}) to Improve Nutrients Availability of Diet with *In Vitro* Rumen Microbial Fermentation Test

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ABSTRACT

This study was conducted to investigate the effect of biological membrane transfer modifier, lysophospholipd (LPLs) on the parameters from *in vitro* rumen simulated fermentation. Commercially available LPLs product (Lipidol^{TM}) was supplemented into experimental diets which consisted of orchard grass and concentrate diet (60:40) in different levels (0.1%, 0.3% and 0.5%). Then *in vitro* rumen simulated fermentation was performed. Although, a declining trend of pH was found in treatments, all pH values were detected in a range relevant to normal rumen fermentation. Gas production, ammonia nitrogen and total VFA production were greatly influenced by the supplementation of LPLs. All parameters were increased along with increased levels of LPLs in diet. As a result, 0.1% of Lipidol^{TM} is recommended based on the determined *in vitro* rumen fermentative parameters in this study. (**Key words :** *In vitro* rumen fermentation, Feed value, Lysophospholipid (Lipidol^{TM}))

Ⅰ. INTRODUCTION

Recently, there are lots of interests in improving the availability of dietary sources for ruminants such as low quality grasses, crop residues, and agro-industrial by-products due to high feedstuff prices and lacks of dietary grains and forages. Also, many efforts have been tried to improve nutritional value of feedstuff using various supplementations such as direct fed microorganism (Kung, 1999; Yoon and Stern, 1995), enzymes (Templeton and Dyer, 1967), monensin (Felix and Loerch, 2011), non-ionic surfactants (Lee et al., 2003). There was little study, however, for the investigation of the efficiency of an additive affecting the permeability of biological membranes particularly in the ruminant.

Based on the previous findings (Hishikawa et al., 2008), lysophopholipids (LPLs) have recently become focus of special attention in field of animal industry due to their biological activities. Lysophospholipids (LPLs) are metabolites produced by many cells and are widely distributed in different cells and tissues. LPLs are glycerophospholipids in which one acyl chain is lacking and then only one hydroxyl group of the glycerol backbone is acylated. And LPLs can diffuse rapidly into the lipid portion of membranes easily. This is their site of action, where they alter membrane fluidity and permeability (Shier et al., 1976). Therefore LPLs are known to be potent membrane transport modifiers. The application of LPLs in animal nutrition has been investigated, though there are few reports. LPLs were reported to change biological membrane fluidity and increase the permeability of macromolecules via membrane (Tagesson et al., 1985). The benefit of using LPLs as feed additives is increased nutrient utilization rate. Results from several researches showed that supplementation of LPLs in diets could increase nutrient digestibility and weight gain in pigs and broilers (Xing et al., 2004). One of its possible beneficial activities is rumen microbial cell permeability, and it could stimulate the release of enzymes to degrade dietary nutrients. However, there are little reports for the effect of LPLs on rumen fermentation.

Fermentation parameters from the *in vitro* rumen simulated fermentation have been used as indicators for the estimation of the quality and the potential of the feed components to supply nutrients to rumen microbes and the host and those

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parameters have been generally considered as sufficient approximates of *in vivo* fermentation (Krishnamoorthy et al., 1991). Gizzi et al. (1998) also noted that *in vitro* fermentation is a useful tool in evaluating individual feedstuffs.

The present study hypothesized that the availability of dietary nutrients in the rumen can be improved due to LPLs supplementation. Therefore, the changes of rumen parameters induced by LPLs supplementation were investigated using *in vitro* rumen simulated fermentation technique in this study.

Ⅱ. MATERIALS AND METHODS

1. Diets, LPLs and experimental design

Orchard grass and a commercial formulated concentrate diet were used and nutritional compositions of those two diets were shown in Table 1. Two diets were mixed in 60:40 rations and used for experimental diet. The experimental diet was dried and ground (<1 mm in size) prior to *in vitro* fermentation and then used. A commercial product (Lipidol™, Pathway Intermediates, Ltd. UK) is used as LPLs source. Lipidol™ had total 7.85% of LPLs and there were 4 different LPLs constituents, LPC (lysophosphatidylcholine), LPI (lysophosphatidylinositol), LPE (lysophosphatidylethanolamine), and LPA (Lysophosphatidic acid). Total 4 experimental groups were prepared according to the addition levels of LPLs as 0% (control), 0.1%, 0.3% and 0.5% in diet.

2. Preparation of rumen fluid

Rumen fluid was collected before morning feeding from one rumen cannulated Hanwoo steer weighing about 350 kg. The steer was being fed a commercial TMR (rate of forage to concentrate was 60:40) individually in a metabolic pen twice a day, at 0900 and 1700 h. The rumen fluid was strained through 4 layers of cheese clothes into a flask previously filled with N_2 gas. The flask was then transported to the laboratory in less than 1 hour. The rumen fluid was further strained through another 4 layers of cheese clothes on arrival at the laboratory. The strained rumen fluid was then mixed with McDougall's buffer prepared according to Troelsen and Donna (1966), at a rate of 1:4 (rumen fluid: buffer). The buffer (pH6.5) contained 9.8 g of $NaHCO_3$, 4.62 g of $Na_2HPO_4 \cdot 2H_2O$, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4 \cdot 7H_2O$ and 4 g $CaCl_2$ per 1 L of distilled water.

Table 1. Nutritional content of experimental feed stuffs

Item	Concentrate	Orchard grass (%)
DM	89.81	89.28
CP	13.25	7.22
EE	4.04	1.64
CF	10.27	30.86
NDF	ND	58.25
ADF	ND	31.86
Ash	6.04	6.77

ND, Not determined.

3. Experiment procedure

In vitro rumen simulated fermentation was performed in 250 mL serum bottle under the stream of N_2 gas to maintain anaerobic condition. Aliquot 0.5 g of diet containing different addition levels of LPLs according to experimental design was mixed with 50 mL of buffered rumen fluid in the serum bottle whilst gassing with N_2 gas. The bottles were then sealed and capped using aluminum sealed rubber stopper and they were placed in an incubator at 39°C. All treatments were performed in triplicates. The procedure followed the method outlined by Tilley and Terry (1963). At each sampling time (0 h, 3 h, 6 h, 9 h, 12 h, 24 h), total gas production was measured by way of displacing a glass syringe. The bottles were then uncapped and the pH was measured using a pH meter (S20 Seven Eazy™, Mettler-Toledo). A solid phase and diet particle free supernatant were separated by centrifugation (10,000 rpm for 15 min at 4°C). The liquid phase was stored at −20°C until analysis. Ammonia nitrogen concentration was analyzed using a spectrophotometer (Optizen UV2120, Mecasis, Korea) according to the method of Chaney and Marbach (1962). Volatile fatty acids (VFAs) were determined using gas chromatography (Agilent 6890, Agilent Technology Inc., USA) according to Erwin et al. (1961).

4. Statistical analysis

The data generated were subjected to analysis of variance

(ANOVA) using the general linear model procedures in SPSS (version 18, IBM, USA) and multiple comparisons were performed using Duncan's posthoc test.

Ⅲ. RESULTS AND DISCUSSION

The pH values were ranged within an optimal rumen pH for microbial environmental condition across the treatments during the whole incubation times (Table 2). Although pH values were appears to be decreased as increasing LPLs supplementation levels, the significant differences across the treatments is regardless of rumen microbial fermentation due to their optimal ranges. As shown in Table 2, the pH was within the optimum range (6.3 to 6.7) and the result was found to be similar to other studies (Chanjula et al., 2004 Wanapat et al., 2009). In addition, rumen pH is one of the most critical determinants for rumen function as cellulolytic bacteria fail to grow below pH 6.0. While, early studies have reported that low rumen pH has negative effects on appetite (Shinozaki, 1959), microbial yield (Oliveira et al., 1997; Russell and Dombrowski, 1980), methane production (Lee et al., 2003), dry matter intake (Dewhurst et al., 2001), milk yield (Wanapat et al., 2009) and fiber digestion (Smith et al., 1973; Stewart, 1977; Terry et al., 1969).

The patterns of total gas production were significantly increased in LPLs supplementation levels compared with that of control during entire incubation times (Table 3). During incubation, 0.1% LPLs treatment has relatively higher total gas production compared with 0.3% or 0.5% treatments. It has been reported that gas production is positively related to microbial protein synthesis (Krishnamoorthy et al., 1991). Also, similar to previous other study (Calabrò et al., 2001), there are linear correlations between total gas and VFA production, and this is related to dietary fiber digestion.

Ammonia nitrogen (ammonia-N) increased in LPLs treatments compared with control for whole incubation times (Table 4). At 24 h of incubation, the ammonia-N production was higher than other incubation times. In addition, ammonia-N production was not particularly different among LPLs supplement levels. While, a consistent increasing of ammonia-N concentration during *in vitro* rumen simulated fermentation may indicate poor utilization of ammonia driven from diet or may be due to the absence of ammonia absorption system at *in vitro* fermentation. Therefore, elevated ammonia-N result in this study can be referred for

Table 2. Effect of LPLs supplement on pH of *in vitro* rumen simulated fermentation

Incubation time	Control	Levels of LPLs in diet			SEM	p value
		0.1 %	0.3 %	0.5 %		
0	7.12^{a}	7.06^{bc}	7.02^{c}	7.10^{ab}	0.067	0.010
3	6.81^{a}	6.78^{b}	6.76^{c}	6.78^{b}	0.007	<0.001
6	6.72	6.70	6.71	6.70	0.005	0.219
9	6.65^{a}	6.65^{a}	6.63^{b}	6.61^{b}	0.006	0.048
12	6.64^{a}	6.59^{b}	6.56^{b}	6.56^{b}	0.012	0.003
24	6.57^{a}	6.58^{a}	6.55^{b}	6.56^{b}	0.005	0.002

Different superscripts in same row mean significantly different ($p<0.05$).

Table 3. Effect of LPLs supplement on gas production of *in vitro* rumen simulated fermentation

Incubation time	Control	Levels of LPLs in diet			SEM	p value
		0.1 %	0.3 %	0.5 %		
	.....		mL	.....		
3	13.67^{d}	24.67^{a}	21.00^{c}	22.33^{b}	1.252	<0.001
6	41.00^{c}	47.67^{b}	47.67^{b}	50.33^{a}	1.250	<0.001
9	63.33^{c}	71.67^{a}	66.67^{bc}	69.00^{bc}	1.156	<0.001
12	76.67^{b}	82.67^{a}	79.33^{ab}	80.00^{ab}	0.981	<0.001
24	87.33^{d}	95.00^{a}	90.00^{c}	93.00^{b}	1.048	<0.001

Different superscripts in same row mean significantly different ($p<0.05$).

Table 4. Effect of LPLs supplement on ammonia nitrogen production of *in vitro* rumen simulated fermentation

Incubation time, h	Control	Levels of LPLs in diet			SEM	p value
		0.1 %	0.3 %	0.5 %		
	·····	mg/100 mL	·····			
0	1.78^{c}	2.51^{b}	2.76^{a}	2.50^{b}	0.067	<0.001
3	2.06	2.20	2.20	2.23	0.169	0.133
6	1.51^{b}	2.01^{a}	2.05^{a}	2.01^{a}	0.250	<0.001
9	0.44^{c}	1.26^{b}	1.41^{a}	1.48^{a}	0.080	<0.001
12	1.70^{c}	3.20^{b}	3.48^{a}	3.68^{a}	0.822	<0.001
24	9.02^{b}	10.89^{a}	10.52^{a}	10.82^{a}	0.912	<0.001

Different superscripts in same row mean significantly different ($p<0.05$).

increment of potentially available protein precursors in the rumen.

Generally, the high VFA concentration may reflect that the availability of dietary energy source is improved via improved metabolic efficiency. As expected, total VFA and all individual fatty acids concentration were higher in LPLs treatments than in control for entire incubation times. And two major VFA concentrations such as acetate and propionate were noticeable across LPLs treatments with different levels during incubation. Acetic acid productions were significantly higher in treatments than in control at all of incubation times ($p<0.05$, Fig. 1A). Significant differences, however, among treatments were not detected ($p>0.05$) at all of incubation time, except 12 h incubation when 0.1% treatment showed significantly higher than others ($p<0.05$). In propionate production, higher productions were found in treatments than in control for all of incubation times (Fig. 1B). Among treatments, propionate production in 0.3% and 0.5% treatments showed significantly higher than 0.1% treatment ($p<0.01$) at all incubation times, except 12 h incubation time. As propionate is used for generating energy via gluconeogenesis in host animals (Murray et al., 2006; Yost et al., 1977), the increment in propionate production should improve feed efficiency and leads to decrease methane production. Butyric and valeric acid showed similar patterns with acetate and propionate productions. All treatments showed higher butyrate and valerate productions than control for entire incubation time (Fig. 2). A/P ratios reflect the acetate and propionate production patterns. A/P ratio was decreased as levels of LPLs supplement were increased (Fig. 3A). Total VFA production was greatly increased by increasing LPLs

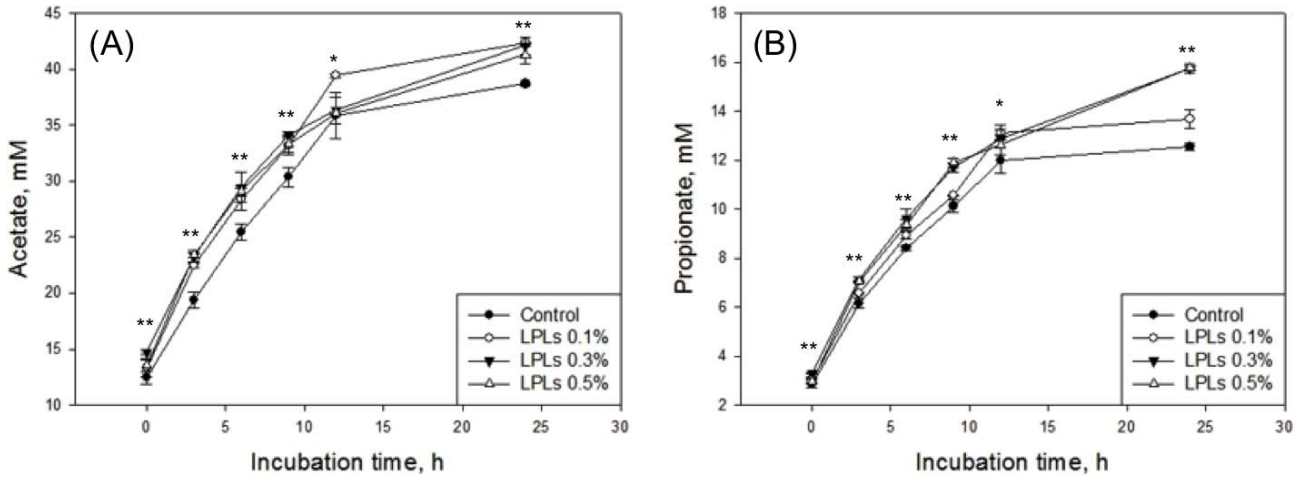

Fig. 1. Effect of LPLs supplement on acetate and propionate productions of *in vitro* rumen simulated fermentation. (A) and (B) denote acetic acid and propionic acid productions, respectively. Filled circle is control. Empty circle, filled and empty triangles are 0.1%, 0.3% and 0.5% LPLs supplement treatments, respectively. Probabilities at each incubation times were marked as * ($p<0.05$) and ** ($p<0.01$).

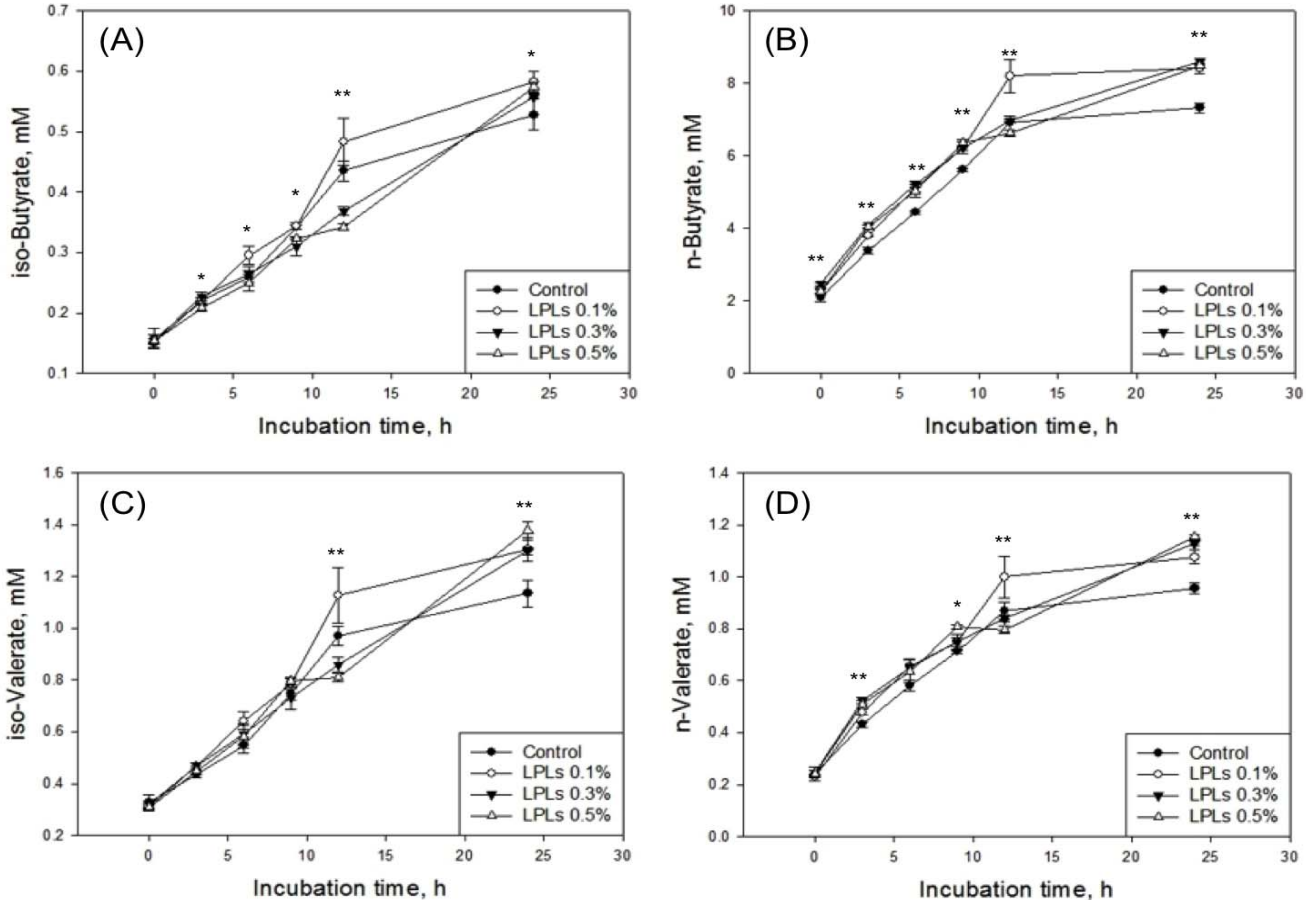

Fig. 2. Effect of LPLs supplement on iso/n-butyrate and iso/n-valerate productions of *in vitro* rumen simulated fermentation. (A), (B), (C) and (D) denote iso-butyrate, n-butyrate, iso-valerate and n-valerate productions, respectively. Filled circle is control. Empty circle, filled and empty triangles are 0.1%, 0.3% and 0.5% LPLs supplement treatments, respectively. Probabilities at each incubation times were marked as * ($p< 0.05$) and ** ($p<0.01$).

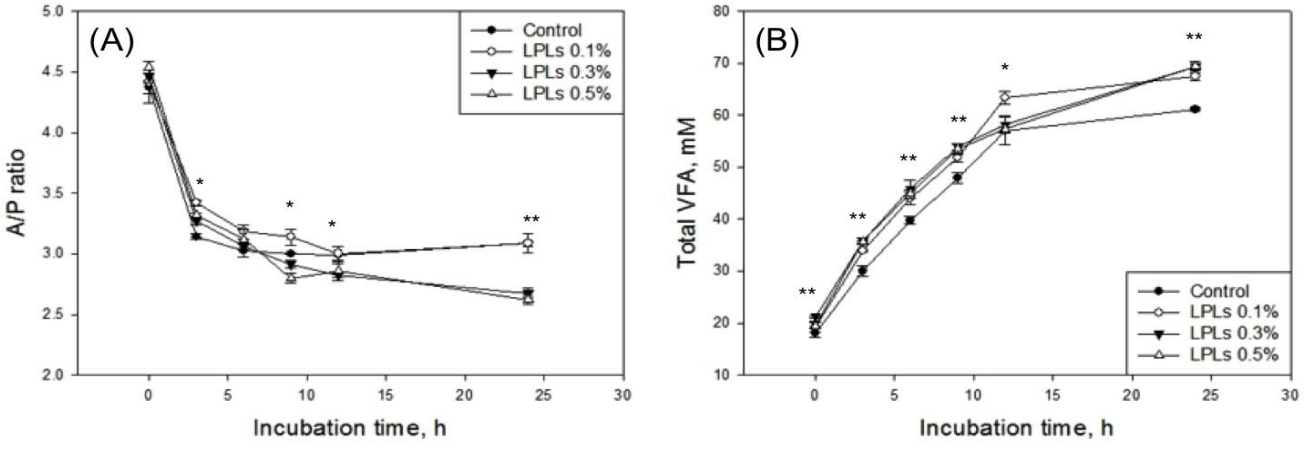

Fig. 3. Effect of LPLs supplement on acetate and propionate ratio and total volatile fatty acid production of *in vitro* rumen simulated fermentation. (A) and (B) denote acetate and propionate ratio, and total volatile fatty acid production, respectively. Filled circle is control. Empty circle, filled and empty triangles are 0.1%, 0.3% and 0.5% LPLs supplement treatments, respectively. Probabilities at each incubation times were marked as * ($p<0.05$) and ** ($p<0.01$).

supplementation levels in diet (Fig. 3B). Although, a certain trend for total VFA production among different LPLs levels was not detected at all incubation periods, total VFA was significantly increased depend on the levels of LPLs in diet at 24 h incubation time ($p<0.05$). In summary, LPLs supplementation as a Lipidol™ form had positive effects on rumen fermentation and potential nutrients availability.

Ⅳ. CONCLUSION

Compared with control where no LPLs were included, parameters from *in vitro* rumen fermentation such as pH, total gas production, ammonia-N production, and VFA concentration were beneficially improved. Particularly, the pH is one of the most critical determinants for rumen function was ranged with optimal microbial rumen fermentation. In addition, total gas and ammonia-N production and VFA concentration as indicators for the utilization of digestible nutrients were improved by the addition of LPLs in diets. Also, the present result may indicate that 0.1% of Lipidol™ level is recommendable based on *in vitro* rumen fermentative parameters. While, further research will be needed to investigate its absorption mechanism in small intestine, and practical farm experiment.

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