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Effects of direct-fed microbials and their combinations with yeast culture on *in vitro* rumen fermentation characteristics*

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ABSTRACT

This study was conducted to determine the effects of *Bacillus licheniformis* (Bl) and *Clostridium butyricum* (Cb) and their combinations with yeast culture on *in vitro* rumen fermentation in a two-way factorial design. Treatments included Bl or Cb at levels of 0, 0.5, 1, 5 and 10 mg and their combination with yeast culture at 0, 18, 27, 36 and 60 mg per 200 mg substrate, respectively. Gas production was recorded after 2, 4, 6, 9, 12, 24, 36, 48 and 72 h incubation. *In vitro* organic matter digestibility (IVOMD) was estimated by 24 h gas production. Rumen fermentation parameters were determined after 24 h of incubation. Rate constant of gas production was not influenced by Bl or Cb alone, but increased ($P<0.05$) with inclusion of yeast culture. The IVOMD was influenced ($P<0.05$) by addition with Bl, Cb or yeast culture, with highest IVOMD observed when Bl or Cb was combined with 60 mg yeast culture. Total volatile fatty acids were affected by Bl and yeast culture ($P<0.01$), but not by Cb ($P>0.05$). There were significant interaction effects on pH, acetate to propionate ratio and ammonia-N between yeast culture and Bl or Cb. From the above results, it is indicated that Bl and Cb may be more effective as feed additives when combined with yeast culture than when offered separately.

KEY WORDS: *Bacillus licheniformis*, *Clostridium butyricum*, *in vitro* digestibility, yeast culture, rumen fermentation parameters

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INTRODUCTION

Numerous studies have been conducted in an attempt to increase ruminant productivity by manipulating the rumen environment and to increase digestibility and nutrient utilization by the animals. One approach that has recently been widely investigated is the application of live microbial preparations, in order to promote digestion and intestinal hygiene (Gourinier-Chateau et al., 1994), enhance animal performance and reduce usage of antibiotics (Jouany and Morgavi, 2007; Guedes et al., 2008; Wallace et al., 2008). It is indicated that microbial additives may benefit ruminant nutrition in terms of liveweight gain and milk production by a magnitude of 7 to 8% (Wallace, 1994).

Yeast cells promote growth of rumen bacteria, and cellulolytic and lactate-utilizing bacteria can be preferentially stimulated (Chaucheyras-Durand et al., 2008). Yeast cells are thought to stimulate bacterial growth through removal of oxygen that occurs in ruminal fluid at various times during the feeding cycle and in that way prevents the toxicity to the ruminal anaerobes (Chaucheyras-Durand et al., 2008). Yeast culture may also provide stimulatory factors for cellulolytic bacteria, such as B vitamins or branched-chain fatty acids (Weidmeier et al., 1987). On lysis, the yeast cells also provide protoplasm, which is a source of nutrition for the rumen microbes (Arambel and Kent, 1990).

Bacillus licheniformis (Bl), on the other hand, is a facultative aerobic and saprophytic bacterium with the ability to produce α-amylase and protease enzymes. A thermostable α-amylase extracted from Bl has been shown to increase starch digestion especially in ruminants fed on diets high in grains with low digestion rates, e.g., sorghum (Rojo et al., 2005). The products of starch digestion would be utilized by non-amylolytic bacteria including cellulose-digesting microbes in a cross-feeding mechanism (Tricarico et al., 2008). A study by Qiao et al. (2010) in lactating Holstein cows showed that although Bl had no significant influence on feed intake and body weight, there were significant increases in rumen microbial protein synthesis which led to significantly higher nutrient digestibility, decreased ammonia nitrogen (N) and increased total volatile fatty acids (VFA) and acetate concentrations in the rumen. Milk yield and milk protein were also significantly increased by Bl (Qiao et al., 2010). Another study by Kritas et al. (2006) in ewes treated with Bl combined with *B. subtilis* showed that milk yield, milk fat and protein were significantly improved compared to the controls.

Clostridium butyricum (Cb) is a strictly anaerobic spore-forming bacterium capable of promoting feed digestibility through its ability to produce amylase, vitamin B and vitamin K (Song and Wu, 2006). Cross-feeding mechanisms are a general feature of the ruminal microbial ecosystem and those microorganisms that utilize hydrolysis products from other species will contribute to ruminal

fermentation (Koike and Kobayashi, 2009) and improve feed digestibility.

The mode of action of direct-fed microbials (DFMs) differs according to their composition, but more research is required to define the mechanism of their action (Chaucheyras-Durand et al., 2008). Animal responses to DFM addition have been highly variable, apparently influenced by the composition of the diet and much remains to be elucidated about the dose- and diet-dependence of DFM effects (Chaucheyras-Durand et al., 2008). In China, various studies have been conducted to determine the effect of DFMs, such as Bl and Cb on porcine performance. Scientists are now searching for experimental evidence to support beliefs on beneficial effects associated with these DFMs in ruminants.

In case none of these DFM survives in the rumen, then their lysis would provide protoplasm, which is a source of nutrients for the rumen microbes (Arambel and Kent, 1990). It is therefore hypothesized that Bl and Cb alone may not directly affect fibre digestibility, but may indirectly stimulate cellulolytic microbes in the rumen through cross-feeding mechanisms. Fibre digestibility may be improved by combining Bl or Cb with yeast culture through synergistic effects. Information on the effect of Bl and Cb on ruminants is scarce; therefore, the objective of this study is to select a suitable dose of Bl and Cb and their combination with yeast culture for feeding ruminants by evaluating their effects on rumen fermentation characteristics and *in vitro* organic matter digestibility (IVOMD).

MATERIAL AND METHODS

Material

Two DFMs, *Clostridium butyricum* (Cb) and *Bacillus licheniformis* (Bl), were prepared at the Zhejiang Academy of Agricultural Sciences Institute of Microbiology. The preparations were in powder form consisting of the bacteria and their respective carrier fermentation media. Colony forming units (cfu) of Cb and Bl were 3.8×10^8 and 1.0×10^{11} per gram, respectively. Fermentation medium for Cb consisted of 20 g glucose and 3.7 g maize steep flour per litre at pH 7.5. Ingredients for Bl fermentation medium included 15 g maize starch, 90 g defatted soyabean powder and 6 g maize steep liquor per litre. Yeast culture in powder form was manufactured by Diamond V Mills, Inc. (Cedar Rapids, Iowa, USA), and included yeast (*Saccharomyces cerevisiae*) and the media on which the yeast grew consisting of ground yellow maize, hominy feed, maize gluten feed, wheat middlings, rye middlings, diastatic malt and maize syrup and cane molasses. Chemical composition (g/kg DM) of yeast culture was as follows: crude protein (CP) 120, ether extract 30, and crude fibre 65.

Grass hay and maize powder were used as substrate for gas test. The CP and ash content were 49 and 201 g/kg DM for hay, and 69 and 135 g/kg DM for maize powder, respectively.

Experimental design

A two-way factorial design was used to investigate the effects of four doses of Cb or Bl and their combinations with four doses of yeast culture on rumen fermentation characteristics *in vitro*. The doses were 0.5, 1, 5 and 10 mg per 200 mg substrate for Cb and Bl, and 18, 27, 36 and 60 mg for yeast culture, respectively. These doses were calculated based on values recommended by manufacturers for application in animal feeding.

In vitro gas test

The *in vitro* gas method based on syringes (Menke and Steingass, 1988) was used to record gas value. Inoculum for the *in vitro* fermentation was obtained from three rumen-fistulated sheep fed twice daily on a basal diet of Chinese wildrye (*Leymus chinensis*) grass hay supplemented with 400 g per day of a concentrate mixture ingredients (g/kg): maize 650, wheat bran 150, bean residue 160, calcium dihydrogen phosphate 30 and sodium chloride 10. Rumen fluid was collected before the morning feeding and strained through four layers of gauze into a pre-warmed and insulated bottle. All laboratory handling of rumen fluid was carried out under a continuous flow of carbon dioxide (CO_2).

Substrate (grass hay and maize powder at a ratio of 50:50, w/w) and the appropriate DFM doses were incubated in buffered medium using 100-ml glass syringes as described by Menke and Steingass (1988). *In vitro* incubations were conducted in two consecutive runs, each involving triplicates. Three blank syringes containing 30 ml of medium only were included in each assay. The syringes were placed in a water bath (39^{o}C) with a shaking bed. The gas volume was recorded after 2, 4, 6, 9, 12, 24, 36, 48 and 72 h incubation.

Sampling and chemical analyses

For determination of pH, VFA and ammonia-N, samples of syringe contents were collected after 24 h of incubation. The pH measurements were taken using a portable pH-meter immediately after sampling. Samples intended for VFA and ammonia-N determination were placed in ice as quickly as possible after collection to avoid volatilization of the VFAs and to slow down microbial activities. Samples for ammonia-N determination were centrifuged at 3 500 rpm for 10 min and the

supernatants frozen at -20°C prior to analysis. Samples for VFA determination were mixed with 20% ortho-phosphoric acid at a 4:1 ratio and centrifuged at 20 000 *g* for 10 min and the supernatants frozen at -20°C before analysis.

Ammonia-N and VFA were determined using the methods described by Hu et al. (2005). Briefly, The VFA was determined with a gas chromatograph (GC-2010, Shimadzu) equipped with a Flame Ionization Detector (FID) and a capillary column (HP-INNOWAX, 1909N-133). The temperature of detector and column was 220°C and 170°C, respectively. Nitrogen was used as a carrier, total flow and column flow was 63.8 ml/min, respectively. Concentration of ammonia-N was determined by a spectrometer (Model 723, Phenix) using colorimetry (Searle, 1984) with ammonium chloride solution as a standard.

Calculation and statistical analysis

Cumulative gas production (GP, ml) at time *t* was fitted to a modified exponential model (Dhanoa, 1988) with the exclusion of the intercept (Sallam, 2005):

$$GP = B(1\text{-}\exp^{-c(t-L)}),$$

where: *B* - the GP from the insoluble fraction (ml), *c* - the GP rate constant for the insoluble fraction (*B*), *t* - incubation time (h), and *L* - lag time (h).

Equation developed by Menke and Steingass (1988) was used to estimate the IVOMD from the GP:

$$IVOMD\ (\%) = 14.88 + 0.8893\ GP + 0.0448\ CP$$

where: GP - 24 h net gas production (ml/200 mg DM); CP - crude protein (g per kg DM).

Data were analysed by the GLM procedure of SAS (1999). A two-way factorial analysis of variance was performed and comparisons of means among treatments were made using the least significant difference procedure when significant F-test for the main effect was found. Significant differences were accepted if $P<0.05$.

RESULTS

The 24 h gas volume was significantly increased ($P<0.05$) with inclusion of Bl, Cb and yeast culture (Tables 1 and 2). Significant interaction ($P<0.05$) was observed on 24 h gas volume between Bl and yeast culture (Table 1), but not ($P>0.05$) between Cb and yeast culture (Table 2). The *B* value was influenced ($P<0.05$) by Bl, Cb and yeast culture (Tables 1 and 2). Similar to the 24 h gas

Table 1. Effect of *Bacillus licheniformis* (Bl) and yeast culture (Yc) on gas production parameters and *in vitro* organic matter digestibility (IVOMD)

Items[1]	Bl, mg	Yeast culture, mg					SEM	P[2]		
		0	18	27	36	60		Yc	Bl	Yc×Bl
GP, ml	0	42.7	41.1	45.7	44.5	49.3	1.10	***	***	***
	0.5	38.3	41.5	47.3	52.3	54.6				
	1	39.5	44.0	47.1	49.9	54.1				
	5	40.9	46.2	47.3	53.9	55.9				
	10	45.0	47.3	49.0	53.8	56.2				
B, ml	0	53.0	51.0	56.0	55.5	57.8	1.15	***	***	*
	0.5	50.9	49.2	56.1	60.7	64.7				
	1	50.1	52.4	56.6	58.9	63.0				
	5	52.2	54.0	56.8	63.2	66.2				
	10	54.7	55.2	59.3	63.5	66.9				
c, h^{-1}	0	7.02	6.80	6.93	6.88	8.12	0.423	***	NS	NS
	0.5	5.88	7.66	7.72	8.54	7.64				
	1	6.27	7.67	7.42	8.05	8.22				
	5	6.13	8.12	7.50	8.27	8.22				
	10	7.18	8.30	7.43	7.97	7.73				
Lag time, h	0	-0.68	-0.48	-0.50	-0.68	-0.28	0.245	***	**	NS
	0.5	-0.85	-0.36	-0.08	0.14	-0.56				
	1	-0.93	0.10	-0.18	0.23	-0.32				
	5	-1.23	0.13	0.07	0.40	0.17				
	10	-0.33	0.50	0.13	0.27	-0.15				
IVOMD, %	0	0.67	0.65	0.69	0.68	0.72	0.010	***	***	***
	0.5	0.63	0.65	0.71	0.75	0.77				
	1	0.64	0.68	0.70	0.73	0.77				
	5	0.65	0.70	0.71	0.76	0.78				
	10	0.68	0.71	0.72	0.76	0.79				

[1] GP - gas production at 24 h incubation; *B* - the GP from the insoluble fraction (ml); *c* - the GP rate constant for the insoluble fraction (*B*)

[2] Yc - yeast culture effect; Bl - *Bacillus licheniformis* effect; Yc×Bl - interaction effect between Yc and B; * P<0.05, ** P<0.01; *** P<0.001; NS - not significant

value, there was significant (P<0.05) interaction effect between Bl and yeast culture on *B* value (Table 1). The *B* value was significantly (P<0.05) higher when 60 mg of yeast culture was added to the fermentation medium, and when Bl or Cb was used in combinations with yeast culture, with the highest *B* values in Bl or Cb combining with 60 mg yeast culture. The *c* value was not influenced by Bl or Cb alone, but increased (P<0.05) with inclusion of yeast culture (Tables 1 and 2). Bl and yeast culture significantly influenced (P<0.05) lag time of GP (Table 1), but generally the lag time was numerically small.

Addition of Bl, Cb and yeast culture significantly influenced IVOMD (P<0.05) (Tables 1 and 2). Significant (P<0.05) interaction effect on IVOMD was observed between Bl and yeast culture (Table 1), but not between Cb and yeast culture (P>0.05), as shown in Table 2. The IVOMD was significantly (P<0.05) improved when 60 mg of yeast culture was added to the fermentation medium. Improved IVOMD (P<0.05) was also observed in the combinations of Bl or Cb with yeast culture, with the highest (P<0.05) IVOMD coefficients observed in Bl or Cb with yeast culture at the 60 mg level (Tables 1 and 2).

Table 2. Effect of *Clostridium butyricum* (Cb) and yeast culture (Yc) on gas production parameters and *in vitro* organic matter digestibility (IVOMD)

Items[1]	Cb, mg	Yeast culture, mg					SEM	P[2]		
		0	18	27	36	60		Yc	Cb	Yc×Cb
GP, ml	0	42.7	41.1	45.7	44.5	49.3	1.05	***	***	NS
	0.5	40.9	44.8	48.9	49.1	54.4				
	1	42.5	43.9	46.8	48.6	54.9				
	5	41.4	43.2	48.5	48.3	54.4				
	10	45.1	44.4	50.7	51.4	56.7				
B, ml	0	53.0	51.0	56.0	55.5	57.8	1.38	***	**	NS
	0.5	47.6	54.2	58.9	57.7	63.8				
	1	52.0	53.1	55.7	60.3	64.9				
	5	51.6	54.2	58.7	58.6	62.8				
	10	54.5	53.9	61.9	59.8	66.0				
c, h^{-1}	0	7.02	6.80	6.93	6.88	8.12	0.387	***	NS	NS
	0.5	8.40	7.15	7.28	7.93	8.12				
	1	7.27	7.30	7.68	6.87	7.82				
	5	6.53	6.58	7.34	7.32	8.48				
	10	7.33	7.08	7.00	8.30	8.38				
Lag time, h	0	-0.68	-0.48	-0.50	-0.68	-0.28	0.337	NS	NS	NS
	0.5	-0.05	-0.75	-0.44	-0.01	-0.17				
	1	0.05	-0.13	-0.02	-0.10	-0.30				
	5	-0.85	-0.88	-0.46	0.14	0.18				
	10	-0.60	-0.52	-0.50	0.30	0.33				
IVOMD, %	0	0.67	0.65	0.69	0.68	0.72	0.009	***	***	NS
	0.5	0.65	0.68	0.72	0.72	0.77				
	1	0.66	0.68	0.70	0.72	0.77				
	5	0.65	0.67	0.71	0.71	0.77				
	10	0.69	0.68	0.74	0.74	0.79				

[1] as inTable 1

[2] Yc - yeast culture effect; Cb - *Clostridium butyricum* effect; Yc×Cb - interaction effect between Yc and Cb; **P<0.01; ***P<0.001; NS - not significant

The pH value was significantly influenced (P<0.05) by Bl and yeast culture (Table 3), but not by Cb (P>0.05) (Table 4). There were significant interaction effects on pH between yeast culture and Bl (Table 3) or Cb (Table 4). At lower doses pH was significantly higher (P<0.05) than controls and at higher doses pH was significantly lower than controls (P<0.05). Ammonia-N concentration was significantly influenced (P<0.05) by Bl and yeast culture, but not by Cb (P>0.05) (Tables 3 and 4). Significant interaction effect on ammonia-N was observed between yeast culture and Bl (Table 3) or Cb (Table 4).

Table 3. Effect of *Bacillus licheniformis* (Bl) and yeast culture (Yc) on *in vitro* fermentation characteristics

Items[1]	Bl, mg	Yeast culture, mg					SEM	P-value[2]		
		0	18	27	36	60		Yc	Bl	Yc×Bl
GP, ml	0	44.9	45.8	47.3	48.2	53.2	1.85	***	**	*
	0.5	42.4	43.8	43.8	47.5	52.0				
	1	45.3	50.0	47.9	49.9	45.9				
	5	49.2	45.8	49.4	50.2	54.2				
	10	47.3	46.8	49.5	47.2	62.0				
pH	0	6.44	6.68	6.78	6.65	6.34	0.022	***	***	***
	0.5	6.74	6.52	6.51	6.43	6.36				
	1	6.80	6.74	6.51	6.42	6.37				
	5	6.69	6.78	6.49	6.38	6.41				
	10	6.82	6.51	6.48	6.35	6.29				
Ammonia-N, mg/dl	0	19.4	15.7	17.2	16.0	15.8	1.48	*	*	**
	0.5	18.3	15.3	15.8	16.2	17.8				
	1	16.0	19.5	13.7	18.2	23.0				
	5	14.6	18.4	14.5	22.7	19.5				
	10	17.0	14.6	14.2	15.4	14.9				
Total VFA, µmol/ml	0	27.3	35.8	34.1	34.3	43.9	1.97	***	**	NS
	0.5	33.7	28.3	32.5	33.1	36.1				
	1	31.5	36.2	33.0	36.9	39.2				
	5	33.9	40.0	37.4	37.3	39.2				
	10	32.2	31.0	36.2	34.7	40.8				
Ac/Pr ratio	0	3.89	3.57	3.88	3.40	3.43	0.162	***	NS	*
	0.5	3.75	4.03	3.85	3.47	3.64				
	1	4.02	3.76	3.45	3.62	3.82				
	5	3.50	4.17	3.59	3.32	3.55				
	10	4.29	3.97	3.48	3.54	3.52				

[1] GP - gas production at 24 h incubation; VFA - volatile fatty acids; Ac/Pr ratio - ratio of acetate to propionate

[2] Yc - yeast culture effect; Bl - *Bacillus licheniformis* effect; Yc×Bl - interaction effect between Yc and Bl; * P<0.05; **P<0.01; ***P<0.001; NS - not significant

Total VFA concentration was significantly influenced (P<0.05) by Bl and yeast culture but not by Cb (P>0.05) (Tables 3 and 4). Significantly higher (P<0.05) total VFA concentrations were observed in treated syringes compared to controls. No interaction effects on total VFAs were observed between yeast culture and Bl (Table 3) or Cb (Table 4). Acetate to propionate ratio was significantly influenced (P<0.05) by Cb and yeast culture, but not by Bl (P>0.05) (Tables 3 and 4). Acetate to propionate ratio significantly decreased (P<0.05) with inclusion of yeast culture in the combinations with Bl (Table 3). There was significant interaction effect on acetate to propionate ratio between yeast culture and Bl (Table 3) or Cb (Table 4).

Table 4. Effect of *Clostridium butyricum* (Cb) and yeast culture (Yc) on *in vitro* fermentation characteristics

Item[1]	Cb, mg	Yeast culture, mg					SEM	P-value[2]		
		0	18	27	36	60		Yc	Cb	Yc×Cb
Gas, ml	0	44.9	45.8	47.3	48.2	53.2	2.11	***	NS	NS
	0.5	44.2	37.5	47.3	45.4	50.0				
	1	45.6	45.2	45.9	51.4	53.5				
	5	43.8	47.2	43.7	52.0	51.7				
	10	45.5	50.7	46.5	47.7	48.7				
pH	0	6.44	6.68	6.78	6.65	6.34	0.022	***	NS	***
	0.5	6.69	6.70	6.50	6.48	6.38				
	1	6.81	6.71	6.54	6.40	6.36				
	5	6.69	6.74	6.53	6.40	6.39				
	10	6.82	6.75	6.50	6.33	6.33				
Ammonia-N, mg/dl	0	19.4	15.7	17.2	16.0	15.8	1.77	*	NS	*
	0.5	17.6	18.6	16.4	18.0	17.7				
	1	16.5	20.2	16.9	16.1	22.3				
	5	17.5	20.3	14.5	18.8	23.0				
	10	17.3	23.6	13.2	17.1	13.5				
Total VFA, µmol/ml	0	27.3	35.8	34.1	34.3	43.9	2.32	***	NS	NS
	0.5	30.4	36.4	33.0	35.9	36.0				
	1	28.6	35.0	33.8	40.5	37.5				
	5	38.0	38.2	34.3	38.5	35.6				
	10	34.2	41.4	33.2	41.5	40.3				
Ac/Pr ratio	0	3.89	3.57	3.88	3.40	3.43	0.140	*	**	*
	0.5	3.61	3.87	3.88	3.57	3.67				
	1	4.19	3.87	3.68	3.94	4.07				
	5	3.56	3.90	3.85	3.52	3.63				
	10	4.10	3.65	3.78	3.53	3.39				

[1] as in Table 3

[2] Yc - yeast culture effect; Cb - *Clostridium butyricum* effect; Yc×Cb - interaction effect between Yc and Cb; * P<0.05; **P<0.01; ***P<0.001; NS - not significant

DISCUSSION

The improved IVOMD and rate of gas production with inclusion of yeast culture observed in this study are in agreement with the findings of Kamel et al. (2004), where the organic matter degradation was improved and its degradation rate was accelerated significantly in response to *S. cerevisiae* supplementation in treated ruminants compared with controls. The effect of yeast culture on IVOMD may be explained by the suggestions of Chaucheyras-Durand et al. (2008) that yeast cells (*S. cerevisiae*) promote growth of rumen bacteria, particularly cellulolytic and lactate-utilizing bacteria. The significant influence of Bl on IVOMD is consistent with Rojo et al. (2005) where supplementation with a thermostable α-amylase from Bl increased OM digestibility in lambs. Information on the effect of Cb on IVOMD in ruminant is scarce; therefore, it is difficult to compare the results of this study with the findings by other researchers. Bacterial DFMs for ruminants contain various species of *Bifidobacterium,* lactate-producing *Enterococcus,* and *Bacillus*. Lactate-utilizing *Propionibacteria* may also be beneficial when fed to ruminants (Kung, 2001), as they are naturally found in large numbers in the rumen of animals fed forage and medium concentrate diets. All these organisms, however, appear to have little effect on ruminal fermentation and the suggested mode of action appears to be in the lower gut (Kung, 1998).

In the current study, the treatments which combined DFMs with a high dose of yeast culture proved to be the most beneficial in terms of nutrient digestibility (Tables 1 and 2). Due to the complexity of the ruminal ecosystem in terms of structure of microbial populations and activities, optimization of its function could be achieved by combinations of live yeast products and other additives to exert synergistic effects (Chaucheyras-Durand et al., 2008). Different combinations have been investigated, for example, yeast with probiotic bacteria such as *Enterococci*. Supplementation of *Enterococci* combined with yeast tended ($P<0.10$) to decrease OM digestibility (Beauchemin et al., 2003), but combination of *E. faecium* EF212 with *S. cerevisiae* did not have significant effect ($P>0.05$) on IVOMD (Yang et al., 2004). In another study, DM digestibility was improved, especially ruminal digestion of forage DM was increased in cows supplemented with a combination of *Enterococci* and yeast (Nocek and Kautz, 2006). Discrepancies between the results of the current study and those of Beauchemin et al. (2003) and Yang et al. (2004) may be explained by the difference in the type of DFMs, dose level, diet composition and mode of supplementation.

When Bl and yeast culture doses were low, pH was significantly higher ($P<0.05$) than controls, but at high doses pH was significantly lower ($P<0.05$) than controls (Tables 3 and 4). A study by Desnoyers et al. (2009) showed that yeast culture significantly increased rumen pH. According to Williams et al. (1991),

yeast culture elevated rumen pH by reducing the concentration of L-lactate in rumen fluid, probably due to yeast culture's stimulatory effect on lactate-utilizing microbes (Enjalbert et al., 1999). Significantly low pH at high doses in the current study is most likely the result of high lactate and VFA concentrations resulting from enhanced digestion. Ammonia-N results in the current study exhibited no distinct trend in relation to dose level. Most of the values, however, indicated a decline in ammonia-N concentration compared to controls. Relatively low ammonia-N is usually an indication that higher amounts of ammonia-N were incorporated into microbial cell protein (Moloney and Drennan, 1994).

Significantly elevated VFA concentrations ($P<0.05$) compared with controls observed in the current study are consistent with reports by Desnoyers et al. (2009). High concentrations of total VFA are the product of improved digestibility in treated syringes compared with controls. In the current study, yeast culture alone and in combination with Cb significantly decreased ($P<0.05$) acetate to propionate ratio. Similar observations were made by Enjalbert et al. (1999) where a decrease in acetate to propionate ratio associated with yeast culture supplementation was reported. The decrease in acetate to propionate ratio was caused by yeast culture increasing the molar proportion of propionate. There was only a minor variation in the effect of Cb on acetate to propionate ratio depending on whether yeast culture was at a higher or lower level, and *vice versa* in the current study.

CONCLUSIONS

Addition of *Bacillus licheniformis* (Bl), *Clostridium butyricum* (Cb) and yeast culture significantly influenced *in vitro* organic matter digestibility and rate of fermentation. Combination of yeast culture with Bl or Cb promoted the digestibility further, suggesting that Bl and Cb may be more effective as feed additives when combined with yeast culture.

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