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# Effects of Supplementing Propionibacteria in Lactation Dairy Diets on Ruminant Fermentation in Continuous Cultures

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**EFFECTS OF SUPPLEMENTING *PROPIONIBACTERIA* IN  
LACTATION DAIRY DIETS ON RUMINAL FERMENTATION  
IN CONTINUOUS CULTURES**

by

**Karmella Ann Dolecheck**

**Thesis submitted in partial fulfillment  
of the requirements for the degree**

of

**HONORS IN UNIVERSITY STUDIES  
WITH DEPARTMENTAL HONORS**

in

**Animal, Dairy, and Veterinary Sciences  
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## Abstract

The aim of the present study was to assess characteristics of in vitro ruminal fermentation when mixed cultures were offered lactation dairy diets supplemented with the direct-fed microorganism, *Propionibacterium* P63 in continuous cultures. The design of the experiment was a  $2 \times 2$  factorial with 4 replications. Diets based on corn silage and alfalfa hay as the forage sources were formulated; high forage (HF) or low forage (LF) diet with a forage-to-concentrate ratio of 60:40 or 40:60 (DM basis), respectively, was combined without or with P63 to form 4 treatments: HF without P63, HF with P63, LF without P63, and LF with P63. Approximately 700 mL of the strained ruminal fluid obtained from 2 lactating dairy cows was inoculated into each of 4 fermentors with a continuous dual-flow system. The cultures were allowed 6 d of adaptation to the treatments followed by 3 d of sampling and data collection. Feed totaling 40.0 g of DM was added to each fermentor daily in equal portions delivered at 0800 and 2000 h. The P63 treatments received  $7 \times 10^8$  cfu of P63/fermentor/feeding. Supplementing P63 decreased culture pH ( $P = 0.05$ ) in the LF diet, but not in the HF diet. Feeding the LF diet increased total VFA concentration compared with the HF diet ( $P = 0.01$ ), and supplementing P63 increased total VFA concentration regardless of level of forage in the diet ( $P < 0.01$ ). Molar concentrations and proportions of acetate and propionate did not differ in response to supplementing P63 in the HF and the LF diet. However, molar concentration and proportion of butyrate increased due to P63 supplementation ( $P < 0.05$ ) only in the LF diet, resulting in interactions between level of forage and P63 supplementation ( $P < 0.08$ ). Overall results in this in vitro study indicate that P63 supplementation enhanced ruminal fermentation by increasing VFA production, but its impacts on continuous culture fermentation differed between the HF and the LF diet.

**Keywords:** continuous cultures, lactation dairy diets, *Propionibacteria*, ruminal fermentation

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## Introduction

The widespread use of antibiotic feed additives by the North American cattle industry to maximize animal performance and production efficiency has prompted an interest in possible alternatives, such as bacterial direct-fed microbials (**DFM**; Yoon and Stern, 1995). Direct-fed microbials, also referred to as probiotics, are live, naturally occurring bacterial supplements. The mode of action of DFM depends largely on the type and dose of the microorganism fed. In a literature summary, an improvement of 2.5% in feed efficiency was reported in feedlot cattle supplemented with lactate-utilizing bacteria, lactate-producing bacteria, or both (Krehbiel et al., 2003). On the other hands, DFM supplementation in dairy rations has become a generally accepted practice with the following stated benefits: increased ruminal digestion, dry matter intake (**DMI**), milk production, and reduced body temperature (Piva et al., 1993; Higginbotham et al., 1994; McGilliard and Stallings, 1998).

Certain bacterial DFM are believed to improve ruminal function. Feeding of lactate-producing DFM (*Lactobacillus* and *Enterococcus* strains) alone or in combination with lactate-utilizing bacteria (*Propionibacterium*) have shown some indications of reduced ruminal acidosis (Ghorbani et al., 2002; Elam et al., 2003; Nocek et al., 2006). The concept behind elevation of ruminal pH with a lactic acid producing DFM is that production of a ‘tonic’ concentration of lactic acid may stimulate and sustain an active population of lactic acid utilizers in the rumen (Nocek et al., 2006). Therefore, there is evidence of reduced ruminal acidosis with feeding of *E. faecium* or *Lactobacillus* sp., alone or in combination with *Propionibacterium* (Ghorbani et al., 2002; Nocek et al., 2006; Oetzel et al., 2007).

*Propionibacteria* naturally inhabit the rumen and can alter volatile fatty acids (**VFA**) and ammonia production by influencing the bacterial composition in the rumen (Daivis, 2006).

Increasing the number of *Propionibacteria* in the rumen may increase ruminal concentrations of propionate and plasma concentration of glucose and insulin. The need for glucose and other energy-providing substrates increases dramatically, as rate of growth or production increases rapidly in growing or high producing ruminants. If these needs are not met, animal health and growth performance can be compromised. Ruminally derived propionate is the major precursor for gluconeogenesis in rapidly growing ruminants, and increasing ruminal propionate can also increase energetic efficiency via reduced fermentation losses (Rogers and Davis, 1982) and reduced heat increment (Orskov and Allen, 1966). Parrott et al. (2001) assessed 44 strains representing 4 species of *Propionibacterium* for lactic acid utilization to examine their potential for use as a DFM to prevent ruminal acidosis in cattle consuming large amounts of highly fermentable carbohydrate. Among the 44 strains, *Propionibacterium* 63 (**P63**) increased the lag time for lactic acid accumulation and suppressed the rate of H<sup>+</sup> concentration, suggesting that P63 may be able to utilize ruminal lactic acid, thus preventing ruminal pH decline in cattle consuming high concentrate rations (Parrott et al., 2001). Inclusion of P63 in the diet of feedlot steers for 10 d did not influence average daily gain (**ADG**) or feed efficiency (Swinney-Floyd et al., 1999). Feeding P63 to heifers on a high concentrate diet for 126 d did not influence ADG, DMI, or feed efficiency (Huck et al., 2000). When heifers were fed a high concentrate diet with P63 for 28 d, then *Lactobacillus acidophilus* for 120 d, ADG was improved without an effect on feed efficiency (Huck et al., 2000). On the other hands, heifers fed a high concentrate diet with the *L. acidophilus* for 28 d, then P63 for 120 d, had increased ADG and improved feed efficiency by 5.0% and 5.1%, respectively (Huck et al., 2000). However, there has been lack of data available to explain how ruminal microbiota respond to supplementing P63 in lactation dairy diets containing different forage-to-concentrate ratios. We hypothesized that ruminal

fermentation and microbial community composition would have different patterns in response to supplementing P63 in high forage (**HF**) or low forage (**LF**) diet. The aims of the present study were to assess characteristics of ruminal fermentation and microbial community composition when ruminally mixed cultures were administered with P63 in HF or LF diet.

## **Materials and Methods**

### **Experimental Design, Diets, and Treatments**

The design of the experiment was  $2 \times 2$  factorial with 4 replicated runs ( $n = 4$ ). Diets based on corn silage and alfalfa hay as forage sources were formulated to maintain different forage-to-concentrate ratios (60:40 vs. 40:60, DM basis). The concentrate contained beet pulp, steam flaked corn, dried distillers grains with solubles, safflower seed, and soybean meal. Four dietary treatments were tested: HF without P63 (**HF–P63**), HF with P63 (**HF+P63**), LF without P63 (**LF–P63**), and LF with P63 (**LF+P63**). Before use in the fermentors, the diets were dried at 55°C for 48 h and ground through a 4.0 mm screen. An independent run was composed of 4 fermentors that were inoculated simultaneously with ruminal contents obtained from the same cows.

### **Operation of Continuous Cultures**

Whole ruminal contents were taken from 2 ruminally fistulated lactating cows fed a TMR diet. The rumen contents were collected prior to morning feeding (730 h). Grab samples of ruminal contents were taken from various locations within the rumen and transported to the laboratory in sealed, preheated containers. At the laboratory, the contents were strained through polyester screens (PeCAP, pore size 355  $\mu\text{m}$ ; B & SH Thompson, Ville Mont-Royal, QC) and then composited. Approximately 700 mL of contents were then placed in each of the 4 fermentors. A

dual-flow continuous-culture system based on Teather and Sauer (1988) was used, consisting of 1-L gas-tight fermentor vessels (Prism Research glass Inc., Research Triangle Park, NC). Natural stratification of contents in a manner similar to the rumen was accomplished with the use of a glass “T” that served as the fermentor overflow. The entrance to the outlet was located near the bottom of the suspended particle phase, allowing differential liquid and solid turnover rates. The slow rate at which the culture contents were stirred and the presence and maintenance of the rumen mat in the cultures allowed for protozoa to be maintained. A constant flow of CO<sub>2</sub> was maintained at a rate of 20 mL/min to allow for anaerobic fermentation conditions. Artificial saliva (Slyvter et al., 1966) was constantly delivered to each fermentor at a rate of 10.0%/h. The temperature of the fermentors was maintained at 40°C by a circulating water bath. Each independent run lasted 9 days including 6 days of adaptation and 3 days of data and sample collection. Fermentors receiving the LF diet were adapted from HF to LF by combinations of HF:LF of 70:30 on d 1, 50:50 on d 2, and 30:70 on d 3. By d 4, all fermentors received the full experimental diet. Each fermentor received 40 g/d (DM basis) of the experimental diet as 2 equal portions added to the diet at 0800 and 2000 h. The P63 treatments received  $7 \times 10^8$  colony forming units of P63/fermentor/feeding starting on d 2. Diets were manually fed to the fermentor through a feed port on the fermentor vessel. Data and samples were taken on d 7 to 9.

### **Sampling and Chemical Analysis**

Culture pH was recorded through a pH electrode connected to a pH meter (model 63, Jenco Instruments Inc., San Diego, CA) every hour for 12 h on d 8 and d 9. Methane samples were taken from the gas of each fermentor at 0, 3, 6, 9, and 12 h after the morning feeding using a 10- $\mu$ L gastight syringe (Hamilton Co., Reno, NV) and analyzed for CH<sub>4</sub> with a GLC (model CP-3900, Varian, Walnut Creek, CA). Immediately after CH<sub>4</sub> testing, 5 mL of ruminal fluid was



taken at 3, 6, and 9 h and directly stored at -40°C until later analysis for VFA could be completed. Ruminal VFA were separated and quantified using a GLC (model 6890 series II, Hewlett Packard Co.) with a capillary column (30 m × 0.32 mm i.d., 1-μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA) and flame-ionization detection. On the final day of each run (d 10), the complete fermentor contents were collected and blended using a waring blender (Waring Products Division, New Hartford, CT) for 1 min. A total of 15 mL of blended ruminal fluid was collected and freeze-dried (FreeZone 12 L; Labconco Co., Kansas City, MO) for FA analysis. The samples were methylated (Kramer et al., 1997) and analyzed for long chain FA by GLC (model CP-3380, Varian).

### **Statistical Analysis**

The Proc Mixed procedures of SAS were used to analyze the data in this study (SAS Institute, 2007). Data for VFA and ruminal FA profiles were analyzed using the following model:  $Y_{ijkl} = \mu + R_i(F_j) + F_k + P_l + (FP)_{kl} + e_{ijkl}$ , where  $Y_{ijkl}$  = individual response variable measured;  $\mu$  = overall mean;  $R_i(F_j)$  = random effect of fermentor  $j$  within independent run  $i$ ;  $F_k$  = fixed effect of forage level  $k$  (HF vs. LF;  $k = 1$  to  $2$ );  $P_l$  = fixed effect of P63 (without vs. with P63;  $l = 1$  to  $2$ );  $(FP)_{kl}$  = interaction between forage level  $k$  and P63  $l$ ; and  $e_{ijkl}$  = residual error. Degrees of freedom in the denominator were estimated using the Kenward-Roger option. Differences were considered significant at  $P < 0.05$ , and trends were discussed at  $0.05 < P < 0.10$ . Results are reported as least squares means.

## **Results and Discussion**

### **Experimental Diets**

Table 1 includes diet ingredients and chemical composition. Corn silage was used as the main forage in both the HF and the LF diet. The amount of steam-flaked corn included in the LF diet was twice that included in the HF diet to maintain a lower forage-to-concentrate ratio. Therefore, the CP concentration was similar between the HF and LF diet (16.4%), while NDF concentration was higher in the HF diet.

### **Ruminal Fermentation Characteristics**

Unexpectedly, we observed very little difference in culture pH as a result of different forage-to-concentrate ratios between the HF and the LF diet (Table 2). We would expect to observe a pH decrease in the LF diet due to lactic acid production upon digestion of the starches found in the concentrates. Additionally, supplementing P63 decreased culture pH ( $P = 0.05$ ) in the LF diet, but not in the HF diet (Figure 1). Adding P63 decreased ( $P = 0.05$ ) LF culture pH on average from 5.83 to 5.63. The decrease in culture pH in the LF diet is consistent with our VFA data, which shows an increase in VFA production in both the HF and the LF diet with the addition of P63 ( $P < 0.01$ ). However, we would therefore have also expected culture pH with the HF diet to fall with greater VFA production which was not the case. This may be partially due to the fact that increased fiber content, like that seen in the HF diet, helped to decrease large fluctuations in culture pH. Still, we must consider that the lack of pH response in the HF diet with regards to increased VFA concentration could be an effect of the supplemented P63.

The pattern of daily pH fluctuation in the culture, including following feeding, was similar throughout all treatments, with the highest pH values observed just prior to feeding and the lowest being observed between 3 and 4 hours after feeding as shown in Figure 1. The HF curves, regardless of P63 addition, were comparatively flatter than the LF curves.

Total VFA concentration increased ( $P = 0.04$ ) in the LF diet compared to the HF diet, while supplementing P63 increased total VFA concentration regardless of level of forage in the diet ( $P = 0.01$ , Table 2). The greater VFA production seen in the LF diet compared to the HF diet in continuous cultures was expected from past literature review (Yang et al., 2001; Eun et al., 2004). Therefore, discussion of forage-to-concentration ratio effect was limited since the focus of this study was on the understanding of P63 supplementations effect on ruminal fermentation and microbial community composition.

As expected, we observed an increase in propionate concentration in the LF diet when compared with the HF diet. This is due to greater starch digestion in the LF diet which is directly connected to propionate production. Contrary to our prediction, molar concentration of acetate and propionate did not differ in response to P63 supplementation in either the HF or LF diet. We would have expected that increasing the number of *Propionibacteria* in the rumen would increase ruminal concentrations of propionate due to their mode of action. In both the LF and HF diet, molar concentration of butyrate increased due to P63 supplementation ( $P < 0.01$ ). There was also a noticeable interaction between level of forage and P63 supplementation on isovalerate concentration ( $P = 0.01$ ). This increased VFA production has the ability to positively affect rumen health through microbial growth. The lower A:P ( $P < 0.01$ ) and (A+B):P ( $P < 0.01$ ) ratios observed in the LF diet were expected due to greater propionate production from the digestion of the starches in the LF diet.

### **Implications**

Overall, the increase in VFA production with P63 supplementation in both the HF and the LF diet is a positive effect, enhancing in vitro ruminal fermentation. Greater VFA concentration

increases energy availability for the use of various tissues, which can then support an increase in milk production. However, the effect of P63 supplementation on culture pH and specific VFA concentrations between the HF and the LF diets was inconsistent with suspected mode of action for P63. Further research is needed to investigate the results of various dose levels of P63 used in different forage-to-concentrate ratios in diets.

**Table 1.** Ingredient and chemical composition of the diets provided to the continuous cultures

Item	Diet	
	High forage	Low forage
Ingredient, % DM		
Alfalfa hay	17.5	13.1
Corn silage	52.7	39.6
Corn grain, steam flaked	10.8	21.7
Beet pulp	3.50	5.17
Soybean meal, 48%	8.39	10.5
Safflower seed	1.71	1.92
Corn dried distillers grains with solubles	4.33	6.63
Vitamins and mineral premix <sup>1</sup>	1.14	1.28
Chemical composition, % DM		
DM, %	56.3	64.1
OM	91.6	94.6
CP	16.4	16.4
NDF	35.3	25.7
ADF	21.8	18.3
Starch	20.8	28.6

<sup>1</sup>Formulated to contain (per kg DM): 71.3 g of P (from monosodium phosphate), 68.9 g of K (from potassium sulfate), 94.6 mg of Se (from sodium selenate), 6.56 g of Cu (from copper

sulfate), 25.8 g of Zn (from zinc sulfate), 4,131.3 kIU of Vitamin A, 515.4 kIU of Vitamin D, 5,728.8 IU of vitamin E, and 19.6 mg of Rumensin (Elanco Animal Health, Greenfield, IN).

**Table 2.** Culture pH, VFA profiles, and methane production by supplementing P63 in the high or low forage diet in continuous cultures (n = 4)

Item	Treatment <sup>1</sup>				SEM	Significance of effect <sup>2</sup>		
	HF		LF			F	P	F × P
	-P63	+P63	-P63	+P63				
Culture pH <sup>3</sup>	5.80	5.81	5.83	5.63	0.064	0.35	0.23	0.15
Total VFA, mM	63.4	69.3	68.3	71.8	2.03	0.04	0.01	0.45
Individual VFA, mM								
Acetate (A)	31.9	33.3	31.8	33.1	1.32	0.88	0.20	0.93
Propionate (P)	17.8	19.9	22.8	22.7	0.860	< 0.01	0.26	0.20
Butyrate (B)	7.25	8.37	7.24	8.95	0.378	0.36	< 0.01	0.35
Valerate	4.00	5.35	4.66	5.15	0.260	0.39	0.01	0.13
Isobutyrate	0.326	0.210	0.291	0.359	0.091	0.44	0.74	0.22
Isovalerate	0.785 <sup>a</sup>	0.340 <sup>b</sup>	0.323	0.250	0.057	< 0.01	< 0.01	0.01
A:P	1.80	1.68	1.38	1.47	0.075	< 0.01	0.76	0.08
(A + B):P	2.22	2.10	1.70 <sup>a</sup>	1.88 <sup>b</sup>	0.102	< 0.01	0.64	0.05
Individual VFA, mol/100 mol								
Acetate	50.2	48.3	46.6	46.1	0.776	0.01	0.15	0.37
Propionate	28.2	28.9	33.8 <sup>a</sup>	31.5 <sup>b</sup>	1.00	0.01	0.24	0.04
Butyrate	11.6	12.1	10.6	12.5	0.530	0.43	0.01	0.09
Valerate	6.38	7.76	6.78	7.22	0.399	0.85	0.04	0.24
Isobutyrate	0.522	0.304	0.430	0.489	0.126	0.59	0.36	0.13

Isovalerate	1.25 <sup>a</sup>	0.495 <sup>b</sup>	0.469	0.351	0.076	< 0.01	< 0.01	< 0.01
Methane, mmol/d	5.46	5.37	4.16	2.68	0.713	0.06	0.44	0.48

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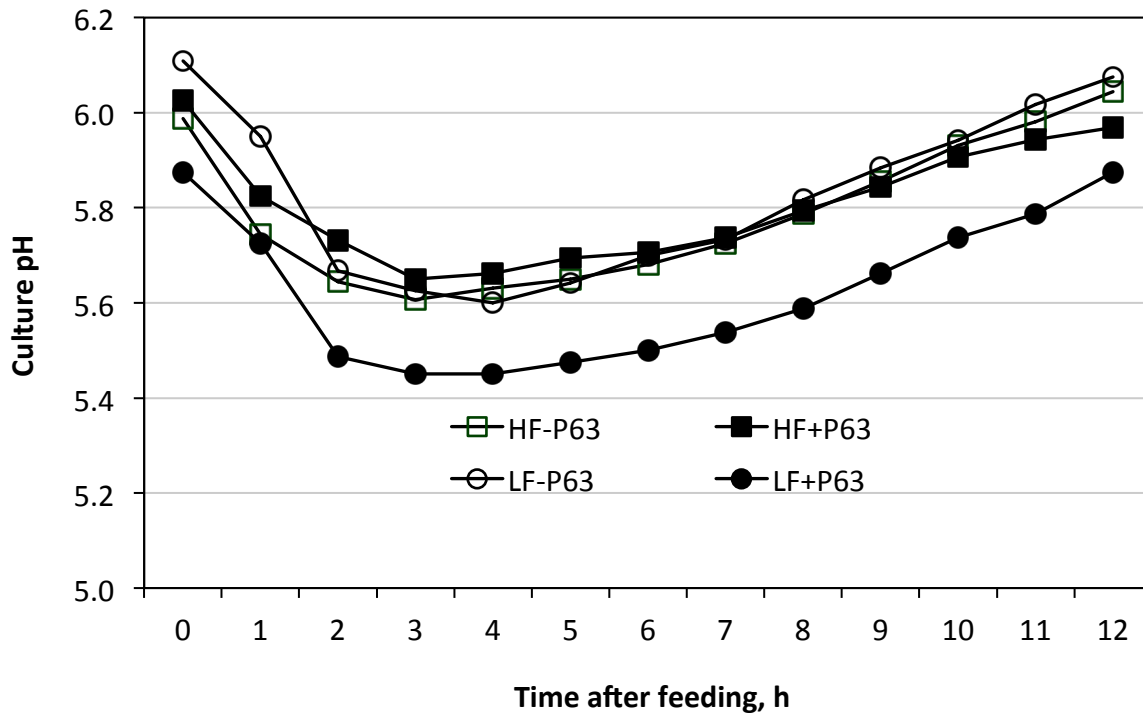
<sup>a,b</sup>Means in the same row within HF and LF subgroups with different superscripts differ based on single degree of freedom contrasts ( $P < 0.05$ ).

<sup>1</sup>-P63 = Without P63 supplementation; +63 = with P63 supplementation; HF = high-forage diet; LF = low-forage diet.

<sup>2</sup>F = Level of forage in the diet (high- vs. low-forage), P = P63 (without vs. with P63), and F × P = interaction between F and P.

<sup>3</sup>Decrease ( $P < 0.05$ ) by P63 in the LF diet.





**Figure 1.** Diurnal fluctuation of pH in continuous culture fermentors after feed addition as affected by forage level in the diet and P63 supplementation (n = 4). HF-P63 = high-forage diet without P63 supplementation; HF+63 = high-forage diet with P63 supplementation; LF-P63 = low-forage diet without P63 supplementation; and LF+P63 = low-forage diet with P63 supplementation. Least square mean for culture pH was 5.80, 5.81, 5.83, and 5.63 for HF-P63, HF+63, LF-P63, and LF+P63, respectively. Effect of level of forage in the diet (F; high- vs. low-forage), P63 (P; without vs. with P63), and F × P interaction was  $P = 0.35$ ,  $0.23$ , and  $0.15$  with SEM = 0.046, 0.044, and 0.059, respectively.

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### **Author's Biography**

Karmella Dolecheck was born in Lewiston, Idaho on January 25, 1990. At age 11 she moved to Twin Falls, Idaho where she graduated from Twin Falls High School as a Valedictorian in 2008. She attended Utah State University as a Presidential Scholar from 2008 to 2012 where she majored in Animal, Dairy, and Veterinary Sciences with an emphasis in Animal and Dairy Sciences and a minor in Agribusiness Management. While at school she was a member of the Utah State University Honors Program, the Utah State University Service Learning Scholars Program, served as a College of Agriculture Student Ambassador, and was honored as the College of Agriculture Scholar of the Year in the 2010-2011 school year. Following her undergraduate career she will be attending the University of Kentucky to obtain her Master's degree focusing on Dairy Reproductive Management.