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SELECTION OF *PROPIONIBACTERIUM* STRAINS CAPABLE OF UTILIZING LACTIC ACID FROM *IN VITRO* MODELS

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ABSTRACT

Forty-four strains representing four species of *Propionibacterium* were screened for lactic acid utilization to examine their potential for use in a direct-fed microbial to prevent lactic acidosis in feedlot cattle. Strains were tested for utilization of lactic acid and growth in a nutrient broth supplemented with 80 mM lactic acid at two different pH values - one representing the pH of an acidic rumen (5.0) and the other that of a forage-fed ruminant (7.0). No differences in growth and lactic acid utilization were detected among strains at pH 7.0. Data from pH 5.0 experiments showed one strain of *P. freudenreichii* (#2) utilized up to 78.59 mM of lactic acid, which was significantly more compared to other strains. Compared with strains of *P. acidipropionici, P. jensenii and P. thoenii, P. freudenreichii* strains reached significantly higher cell densities and utilized more lactic acid at pH 5.0. Rumen fluid simulation models were used to examine the ability of fourteen selected propionibacteria strains to survive and utilize lactic acid produced by native ruminal microorganisms. Eleven of the fourteen propionibacteria strains tested utilized lactic acid in the rumen model. Gompertz non-linear curve fitting equation was used to determine which strains significantly increased the lag time for lactic acid accumulation and suppressed the rate of H+ concentration.

(Key words: *Propionibacterium,* rumen, lactic acid utilization,)

INTRODUCTION

Feeding of grains such as corn, to cattle improves the rate and efficiency of gain, improves the tenderness and flavor of the meat by increasing the intramuscular fat deposits (marbling) and increases milk production in dairy cows. However, feeding grains and other rapidly fermented carbohydrates to ruminants has increased the incidence of metabolic disorders such as lactic acidosis.

Over consumption of readily fermented carbohydrates leads to an accumulation of ruminal lactic acid; its accumulation perturbs the normal ruminal flora (Dunlop, 1972; Slyter 1976; Elam, 1976). Lactic acid producing microorganisms, namely Streptococcus bovis and *Lactobacillus* species that produce lactate, presumably are primarily responsible for the decline in rumen pH (Allison *et al.*, 1975; Dunlop, 1972; Hungate *et al.*, 1952; Mann, 1970).

Treatments that inhibit the growth of *S. bovis* can help to prevent lactic acidosis. Antibiotics such as lasalocid, monensin, thiopeptin, and virginiamycin have a narrow spectrum of activity against gram-positive organisms and generally help to prevent the decrease in pH seen with cattle and sheep engorged with readily fermented carbohydrates (Nagaraja *et al.*, 1982; Tung and Kung, 1993; Muir *et al.*, 1981). The sensitivity of *S. bovis* to thiopeptin and monensin has been demonstrated using *in vitro* experiments (Muir and Barreto, 1979; Tung and Kung, 1993).

Inoculation of the rumen with lactic acid-utilizing organisms is a logical alternative prophylactic for ruminal acidosis due to current consumer perceptions of antibiotic residues in the food supply. Using *in vitro* fermentation with a mixed population of ruminal microorganisms, lactic acid accumulation was significantly reduced by inoculating with *Megasphaera elsdenii* (Kung and Hession, 1995). *M. elsdenii* inoculation of beef cattle at the time of experimentally inducing acidosis resulted in ruminal pH values of 5.51 compared to control values of 4.65. However, inoculating cattle with the same bacteria 8 h prior to inducing acidosis resulted in no significant difference between treated and control ruminal pH values (Greening, *et al.*, 1991). These data suggest that the *M. elsdenii* introduced do not have the ability to become sufficiently established to significantly inhibit pH reductions in ruminal acidosis.

Propionibacterium are normal inhabitants of the rumen and account for approximately 1.4% of the total microbial population (Oshio *et al.*, 1987), therefore may provide an additional biological approach to help prevent the accumulation of lactic acid in the rumen. A denitrifying strain of *Propionibacterium* was shown to establish an active population when introduced into the rumen of beef cattle (Swartzlander, 1994). This data suggest that the ruminal inoculation of propionibacteria can affect the formation of undesirable endproducts such as lactic acid.

The purpose of this study was 1) to identify those propionibacteria strains capable of reducing sub-acute levels (80 mM/L) of lactic acid at pH values similar to the acidic rumen (pH 5.0), and 2) to determine the ability of selected strains to inhibit the accumulation of lactic acid and subsequent pH reduction of a rumen simulation model supplemented with glucose.

MATERIALS AND METHODS

Bacterial strains. *Propionibacterium* cultures used in this study were maintained at -75 °C in a sodium lactate broth (NLB) supplemented with 10% glycerol (Hofherr and Glatz, 1983).

Culture conditions. Strains were activated by placing a portion of the frozen suspension in 10 ml of NLB and incubated at 32 °C for 36-48 hours. Strains were sub-cultured by transferring a 1% volume of the culture at mid-log growth to fresh NLB. Cultures were transferred a minimum of three times before being tested. The purity of tested strains was monitored by regularly streaking cultures onto a sodium lactate agar (NLA).

***In vitro* acidified and neutralized broth medium**. Primary strain selection involved testing the growth and lactic acid utilization of cultures in a basal broth media. The acidified medium was prepared by including 80 mM L(+) lactic acid in a basal broth containing 1% yeast extract, 1% tryptone, dipotassium phosphate and distilled water. The pH of the broth medium was raised to pH 5.0 using 5.0 M NaOH. Following filter sterilization (Gelman Sciences, Ann Arbor, Michigan), the medium was dispensed at a volume of 10 ml into sterile screw cap test tubes. Neutralized broth medium was prepared the same as acidified media except that the pH of broth was raised to 7.0 with 5.0 M NaOH prior to filter sterilization.

Rumen fluid simulation medium. Ruminal fluid was collected via ruminal cannula 2 h post feeding from a cross-bred beef heifer fed a high roughage diet. The ruminal fluid was strained through four layers of cheesecloth and transported to the laboratory in an insulated container. Test ruminal fluid media contained 250 ml of strained ruminal fluid, 62.5 ml McDougall's buffer

(McDougall, 1948), and 1.5% dextrose. The added dextrose served as a readily fermented carbohydrate to simulate conditions found in the rumen of animals following grain engorgement. Strained ruminal fluid, buffer, and dextrose were dispensed into sterilize 500 ml bottles and allowed to equilibrate in a water bath at 39 °C for approximately 15 minutes prior to inoculation. Initial pH of the rumen fluid model ranged from 6.6 to 6.9 depending on date of collection

High Pressure Liquid Chromatography. Samples were prepared for HPLC analysis by aseptically removing 1.0 ml from the test medium at the appropriate sampling times. Samples were placed in a 1.5 ml microcentrifuge tube and the cells were pelleted by centrifugation (10 minutes, at 12,500 rpm). A sample of the supernatant fluid (0.5 ml) was transferred to a clean tube and acidified with an equal volume of 0.01 M sulfuric acid solution to stop fermentation. These samples were stored at -20 C until analysis was performed. For analysis, frozen tubes were allowed to thaw at room temperature and filtered through 0.2 um filters directly into 2 ml HPLC autosampler vials and capped.

Samples were analyzed using a Hewlett Packard 1090 HPLC system equipped with a diode-array detector (Hewlett Packard, Atlanta, Georgia). The sample was injected into 0.005 M H_2SO_4 mobile phase heated to 65°C and separated using a BioRad HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, California). The peaks were detected with a diode array detector at 210 nm. Other wavelengths were recorded and examined for peak purity, but 210 nm was the optimum setting for determining peak height with minimum background noise. Peak areas were used to determine compound concentrations by comparison with external standards. Peak purity was monitored by UV scanning techniques as an aid in identifying abnormal wavelength patterns present in a single peak

***In vitro* broth medium experimental procedures**. For each propionibacteria strain, duplicate tubes were inoculated with a 1% inoculum of a 48 h culture for each propionibacteria strain. Tubes were incubated under static conditions at 32 °C for 48 h. Growth was determined by measuring increases in optical density at 0 h and ever 8 hours beginning with 16 h using a Milton Roy Spectronic 601 spectrophotometer (Milton Roy, Rochester, New York) set to a wavelength of 600 nm. Samples (1 ml) were aseptically removed from each tube at the time optical density readings were taken and prepared for organic analysis by HPLC.

Rumen model experimental procedures. Duplicate bottles were inoculated with the appropriate propionibacteria strain to be tested at a level of 1×10^7 cfu/ml. Bottles were flushed with CO_2, capped, and incubated at 39 °C for 48 h. Every 6 h during the 48 h incubation period, samples were collected and analyzed for pH, lactic acid and volatile fatty acid (VFA) concentrations. Additional samples were collected at 16 h and 48 h for use in microbiological analysis. Lactic acid and VFA samples were prepared by aseptically collecting a 1 ml sample in a 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation (10 minutes at 12,500 x g). One-half ml of supernatant was mixed with an equal volume of 10 mM H_2SO_4 and filtered through a 0.2 um membrane filter.

Microbiological analysis consisted of plating serial dilutions (10^{-3}, 10^{-4} and 10^{-5}) of the *in vitro* rumen fluid medium on a propionibacteria selective-differential medium (PSA).

Differences in pH and lactic acid concentration between inoculated and uninoculated controls at each sampling time were calculated and regressed against incubation time up to 24 h in order to select the best lactic acid utilizing strains. Strains for which a change over time in lactate or pH was detected (an $R > 0.50$ against sampling time) were compared using Duncan's Multiple Range procedures (SAS, 1985). Additionally, Gompertz equation was used to analyze the sigmoidal curves for pH decrease and lactic acid concentration increase (Zwietering *et al*. 1990).

RESULTS

Broth medium supplemented with 80 mM lactic acid, pH 7.0 Five strains had utilized over 40 mM lactic acid by 24 h. These strains were from *P. acidipropionici* (P90), *P freudenreichii* (P104, P49, P99), and *P. thoenii* (P85) species. By 48 h, no significant differences were observed for lactic acid utilization among strains. Means for lactic acid utilization at pH 7 are shown in Figure 1. Comparison of the mean lactic acid utilization for each species at 24 hours indicated *P. freudenreichii* strains utilized the highest concentrations with a mean of 40 mM while *P. jensenii* strains utilized the lowest concentration with a mean of 17 mM. However, all species had similar lactic acid utilization values by 48 h.

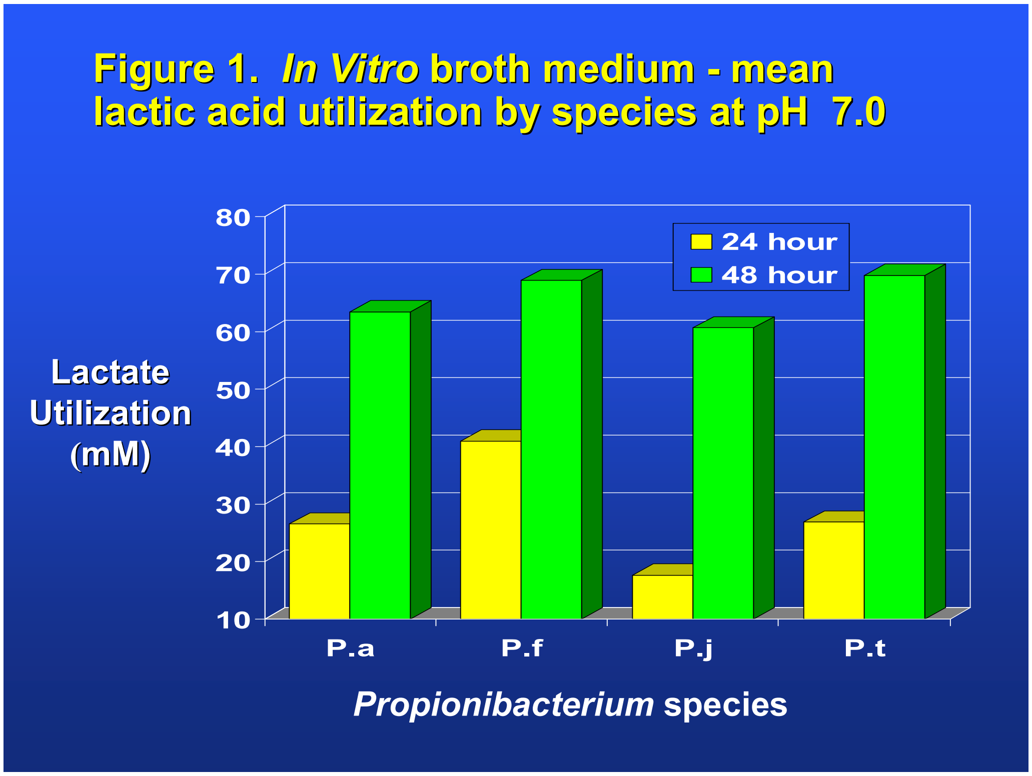
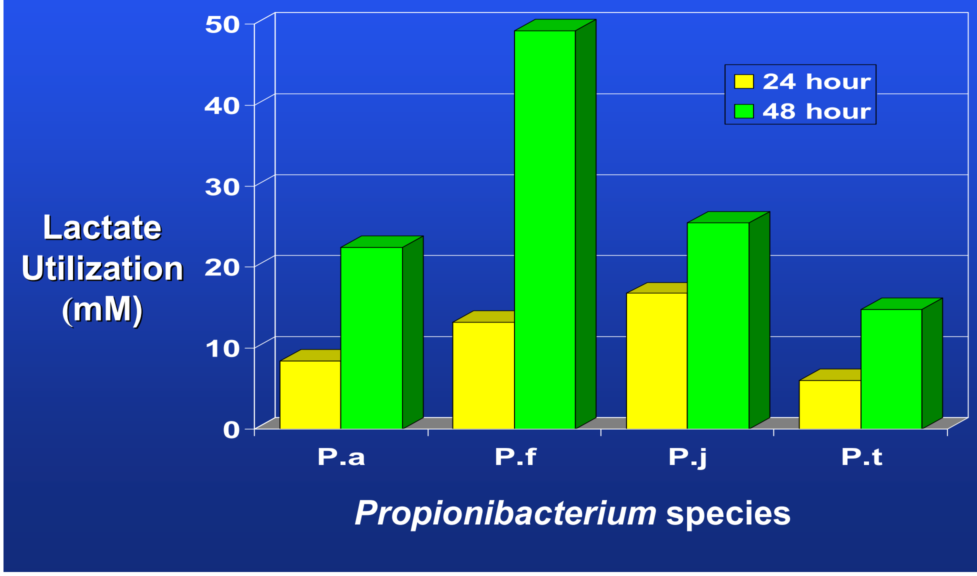


Figure 1. *In Vitro* broth medium - mean lactic acid utilization by species at pH 7.0

Data for 24 and 48 h optical density values for each species grown at pH 7.0 showed that only thirteen strains failed to reach an optical density of 1.0 by 24 h. Of those thirteen, six strains were classified as *P. jensenii*, suggesting that this species tended to have longer lag times. By 48 h, most strains had optical density readings above 2.0. The means of species optical densities suggests only minimal differences after 24 or 48 h of incubation.

Broth medium supplemented with 80 mM lactic acid, pH 5.0 . The levels of lactic acid utilization at 24 and 48 h of incubation are listed in Table 1. Means for lactic acid utilization at pH 5 of each species are shown in Figure 2. Twenty-five of the forty-four strains tested utilized 8 mM (10%) or more by 24 h. Seven of the twenty-five strains utilized over 20.0 mM (25%) of the available lactic acid *P. thoenii* strains had the lowest utilization (6 mM) while *P. jensenii* strains utilized the most lactic acid (16 mM) at 24 h. By 48 h of incubation, twenty-four strains had utilized over 20 mM (25%), with eight strains utilizing more than 40 mM (50%). The utilization of lactic acid ranged from 1.10 mM to 78.59 mM. Two *P. freudenreichii* strains utilized 76.90 mM and 78.59 mM respectively, which was significantly more lactic acid compared to other strains. Two strains utilized less lactic acid at 48 h when compared to 24 h. This may be due to a shift in lactic acid production by the strains or in experimental error since all other strains had utilized more lactic acid at 48 h compared to 24 h. Six of the eight most active lactic acid utilizers were classified as *P. freudenreichii.* Means of lactic acid utilization for each species (Figure 1) indicate *P. freudenreichii* strains utilized the most (49 mM). while *P. thoenii* strains utilized the least (14 mM).

Figure 2. ***In- vitro broth medium- mean lactic acid utilization by species of Propionibacterium*** **at pH 5.0.**



Optical density values for each species at 24 and 48 h of incubation showed that after 24 h only two strains had optical density values over 0.30. All species had similar lag time as suggested by similar strain means at 24 h. By 48 h, *P. freudenreichii* strains had reached a higher maximum optical density when compared to other species.

The variation for both optical density and lactic acid utilization within species was lower for strains grown in pH 7.0 broth when compared to pH 5.0 at both 24 and 48 h. Most all strains had increased amounts of lactic acid utilization and higher optical densities when grown at pH 7.0. Most of the top ranking strains were not greatly affected by the decreased pH condition. However many strains were inhibited by the lower pH level and had decreased lactic acid utilization at pH 5.0 by as much as 88%.

Rumen simulation. High variability was observed among strains tested in the rumen simulation model. Strain performance across and within experiments was quite variable. This may have been due to variation in the rumen fluid collected on different days from the donor animal; since similar fluctuations were noted in control tubes.

The rate of change in pH and lactic acid concentration was determined by regressing the difference between inoculated and control rumen fluid incubations against time. Only when the regression coefficient for rate of change in pH and lactate was greater than 0.50 for an inoculated flask was the data included in the statistical analysis (Table 2). Compared with other strains, strain P42 had the highest rate of pH increase (0.0377 units/h), but was not statistically ($P<0.05$) different from P63, P54, P25 and P41. Strain P42 also had the highest rate of lactic acid utilization (1.61 mM/h) compared to others, but was not statistically ($P<0.05$) different from P63, P54, P25, P41, P111, P81 and P104. Since linear regression analysis did not adjust for differences in lag times, other non-linear methods were employed

Ruminal fluid simulation data was analyzed using the Gompertz non-linear equation technique (Table 3). Values up to 24 h were used in the analysis since a decrease in lactic acid concentration was observed after 24 h in all controls. Flasks inoculated with strain P54 and P63 had significantly lower rates of hydrogen ion accumulation (Table 3). (Note- it was later determined via total genomic analysis that strains P54 and P63 were genetic equivalents meaning they are the same strain). When the rate of H+ increase of inoculated flasks was compared to the control (0.00018), strain P63 had significantly different values. Strain P63 also had a significant impact on the lactic acid production lag time. Strain P63 increased the lag time of lactic acid accumulation, thereby slowing the accumulation of acid. On the other hand, some strains decreased the lag time of inoculated samples thus resulting in faster lactic acid accumulation.
Strain Survival. The viable plate counts of strains at 16 h and 48 h of incubation in the rumen simulation model showed that nine strains maintained a population of at least 1.0×10^4 cfu/ml for 48 hours. Six of the nine strains exceeded 1.0×10^5 cfu/ml; strain P63 had the highest rates of survival at 1.0×10^6 cfu/ml.

DISCUSSION

Slyter and Rumsey (1991) reported L-lactate levels increase to concentrations as high as 90 mM in the rumen of beef cattle 24 h after the diet was changed from 90% forage to 100% concentrate. In this study, experiments were performed to test the ability of propionibacteria strains to grow and utilize lactic acid when grown in conditions similar to those found in the acidotic rumen.

The differences observed in growth and lactic acid utilization at pH 5.0 indicated some species were better able to function at the lower pH. *P. freudenreichii* strains were clearly the better species for growth and lactic acid utilization under low pH conditions. Values for growth and lactic acid utilization at pH 7.0 were consistent with those reported in literature. Crow (1986) examined the substrate preference of *P. freudenreichii* strains grown in a complex media supplemented with 176 mM of DL-lactic acid at pH 6.5 and found that L-(+) lactic acid was reduced by 73 mM (83%) at 24 h.

Maximum values for growth and lactic acid utilization were generally much lower at pH 5.0 than at pH 7.0. However, many strains had reduced growth and lactic acid utilization when the pH was reduced to 5.0.

The inability of certain strains within a species group to tolerate pH and ruminal fluid typical of an acidotic rumen confirms the importance of studying and selecting organisms under environmental conditions in which they will be used. While *P. freudenreichii* strains were determined to grow well and utilize more lactic acid at pH 5.0 in broth experiments, strains failed to function in the competitive environment of rumen simulation models. A lack of strain survival may account for the inadequate performance of strains of this species in rumen simulation models. That is why it was important to screen the propionibacteria strains selected from pure culture experiments in rumen simulation models. The rumen simulation models tested the strains ability to utilize lactic acid produced by native ruminal microorganisms, compete for nutrients in a complex microbial system and survive increased osmotic pressure and microbial predation.

Many strains could not be isolated from the rumen simulation models following 48 h of incubation. One assumption is that strains not detected were not able to survive in the rumen based media, however other possibilities do exist. Those strains not recovered may have been impossible to enumerate from the highly diverse community of ruminal organisms using the selective-differential medium employed. In addition, rumen models were maintained as batch culture systems with no outflow of endproducts. Thus endproduct accumulation which may injure cells, rendering them non-culturable on the selective-differential media. Transferring an amount of contents after 24 h of incubation to fresh rumen fluid media may help reduce this problem as suggested by Theodorou, *et al.*, (1987).

The ruminal fluid model used for this experiment was much less effective as a screening tool for selecting lactic acid utilizing strains after 24 h of incubation. In control flasks even when no propionibacteria strains were added, concentrations of lactic acid concentrations decreased and pH increased after 24 h of incubation. As a result, only the first 24 h of the incubation was considered in statistical analysis. Kung and Hession (1995) observed similar reductions following 24 h and attributed this to the accumulation of metabolic endproducts.

A non-linear curve fitting technique was employed to detected differences in pH and lactic acid lag periods between treated and control flasks. Gompertz equation was successful in predicting a non-linear curve for 24 h incubation data. Observed values were located on the predicted curve more than 95% of the time. Gompertz analysis revealed that strain P63 significantly increased the lag time of lactic acid accumulation and suppressed rate of H+ accumulation.

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Table 1. Lactate utilization of *Propionibacterium* strains grown in 80 mM broth at pH 5

Species	**strain**	**24 h lactate utilization (mM)**	**48 h lactate utilization (mM)***
P. acidipropionici			
	90	$10.68^{cdefghi}$	58.70^{bc}
	111	29.97^{ab}	38.85^{ef}
	81	$9.44^{cdefghi}$	36.45^{fg}
	78	5.92^{efghi}	35.05^{fgh}
	52	8.29^{defghi}	26.21^{ghijk}
	53	21.47^{bcdef}	24.82^{hijkl}
	38	$14.91^{bcdefghi}$	23.68^{ijklm}
	2	7.88^{defghi}	17.44^{klmnop}
	35	8.33^{defghi}	15.21^{lmnopq}
	9	5.44^{efghi}	13.24^{mnopqr}
	42	4.72^{efghi}	12.45^{nopqr}
	50	3.66^{fghi}	10.27^{opqrs}
	5	7.57^{defghi}	9.29^{pqrs}
	3	0.74^{i}	8.74^{pqrs}
P. freudenreichii			
	99	26.36^{bc}	78.59^{a}
	49	$10.08^{cdefghi}$	76.90^{a}
	48	$11.21^{cdefghi}$	65.26^{b}
	104	$9.91^{cdefghi}$	61.68^{b}
	89	$19.15^{bcdefgh}$	51.22^{cd}
	31	22.60^{bcde}	48.68^{d}
	96	5.14^{efghi}	10.27^{opqrs}
	101	0.74^{i}	1.10^{s}
P. jensenii			
	88	$17.60^{bcdefghi}$	46.51^{de}
	41	$14.16^{bcdefghi}$	36.08^{fg}
	54	20.19^{bcdefg}	34.18^{fghi}
	106	43.49^{a}	30.14^{fghi}
	44	25.00^{bcd}	27.54^{ghijk}
	63	7.88^{defghi}	26.67^{ghijk}
	74	$10.51^{cdefghi}$	24.13^{ijklm}
	46	8.55^{defghi}	23.74^{ijklm}
	69	$10.96^{cdefghi}$	15.21^{lmnopq}
	86	$18.33^{bcdefghi}$	8.46^{pqrs}
	68	7.33^{defghi}	7.49^{pqrs}
P. thoenii			
	26	6.86^{efghi}	21.25^{jklmn}
	105	6.03^{efghi}	21.20^{jklmn}
	10	$10.51^{cdefghi}$	20.49^{jklmno}
	85	$9.51^{cdefghi}$	17.95^{klmnop}
	21	3.70^{fghi}	17.83^{klmnop}
	79	$11.01^{cdefghi}$	14.43^{lmnopq}
	20	1.55^{hi}	11.19^{nopqrs}
	4	2.72^{ghi}	5.53^{qrs}
	15	1.92^{hi}	3.19^{rs}

*Values with the same letter are not significantly different

Table 2. Impact of added *Propionibacterium* strains on rates of change in pH and lactate concentration of incubated rumen fluid models.

Strain	pH elevation, (Units/h)	Lactate decrease (mM/h)
42	.03770^{A}	1.61^{a}
63	.03627^{A}	1.30^{abc}
54	.02433^{Ab}	1.26^{abc}
25	.02380^{Ab}	1.12^{abc}
41	.02372^{Abc}	1.55^{ab}
111	.01691^{bcd}	1.05^{abc}
81	.01064^{bcd}	.71^{abcdef}
104	.00923^{bcde}	.88^{abcd}
89	.00785^{bcde}	.53^{bcdef}
88	.00590^{bcde}	.76^{abcde}
49	.00425^{cde}	.65^{abcdef}
48	.00366de	NA
99	.00051de	-.17^{def}
31	.00026de	-.22^{ef}
90	-.00917^{e}	-.32^{f}

Calculated by regressing the difference between inoculated and control fluid against incubation time.
Means in a column with the same superscript are not different (P<.05).

Table 3. Contrasts of maximum lactate accumulation and minimum pH of rumen models inoculated with various *Propionibacterium* strains.

Strain	Lactate production rate (mM/h)	H+ increase rate (x 10^{-5})	Time lag of lactate production (h)	Time lag of H+ increase(h)
P25	18.87	4.65	4.41+	4.20
P31	38.85	11.63	5.15	3.81
P41	23.31	11.15	4.65	3.29
P42	24.42	7.46	5.52	3.99
P48	38.85	1.45	5.45	3.27
P49	6.67	6.45	5.89	3.28
P54[a]	21.09	-1.45**	8.08**	3.56
63	9.99	2.18*	6.47+	2.68
P81	1.11	9.86	4.91	2.99
P88	14.43	11.49	5.76	2.87
P89	9.99	13.57	5.71	3.13
P90	14.43	5.18	5.00	3.28
P99	4.44	7.87	4.94	3.59
P104	-2.22	8.02	4.94	2.67
P111	14.43	5.17	4.97	5.74*
Control	38.85	17.99	5.45	4.72

* Values significantly different when compared to controls (P<.05)

\+ Values significantly different when compared to controls (P<.01)

** Values significantly different when compared to controls (p<.001)

[a] Analysis of total genomic DNA by pulse field gel electrophoresis showed P54 and P63 were genetic equivalents (same strain).