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Abstract: The objectives of this study were to determine whether *Propionibacterium* affects in-vitro VFA production and/or increases the digestibility of fiber and starch. A 4 x 4 x 4 factorial arrangement was used. The main factors were feed source, length of fermentation, and level of added *Propionibacterium*. Fermentations were conducted in batch cultures with *Propionibacterium* added to the flasks just prior to the addition of rumen inoculum. The total VFA, acetate, and propionate concentrations increased 9, 5, and 18%, respectively with the addition of *Propionibacterium*. *Propionibacterium* decreased dry-matter digestibility by 2% and fiber digestibility by 14%. A second study was conducted to examine the effect of pre-hydration and the carrier of the *Propionibacterium* on in-vitro fermentation. All levels of *Propionibacterium* were examined again. The acetate and propionate concentrations decreased by 5% when *Propionibacterium* was autoclaved; however, autoclaving did not increase dry-matter or fiber digestibility when compared to *Propionibacterium* alone. *Propionibacterium* increased the total VFA, acetate, and propionate concentrations, and therefore may enhance the performance of ruminant livestock.

Key Words: *Propionibacterium*, Volatile Fatty Acids, Neutral Detergent Fiber, Starch, Digestibility

Propionibacterium Strain P5'ın In vitro Uçucu Yağ Asitleri Üretimi, Ham Sellüloz ve Nişasta Sindirimi Üzerine Etkileri

Özet: Bu çalışmanın amacı *Propionibacterium*'un in vitro uçucu yağ asitleri üretimini değiştirme, ve/veya ham sellüloz ile nişasta sindirimini arttırma etkinliğini saptamaktır. Araştırmada uygulanan faktörler, yem kaynağı, fermentasyon süresi ve fermentasyon ortamına katılan *Propionibacterium* düzeyi olup, denemede 4x4x4 faktöriyel deneme deseni kullanılmşıtır. *Propionibacterium*, fermentasyon tüplerine rumen kültürü (rumen sıvısı) katılmadan hemen önce katılmış ve tüpler daha sonra toplu halde su banyosuna yerleştirilerek fermentasyon işlemi gerçekleştirilmiştir. *Propionibacterium*'un kullanılmasıyla toplam uçucu yağ asitleri, asetik asit ve propiyonik asit yoğunlukları sırasıyla %9, %5 ve %18 oranında artmıştır. *Propionibacterium* kuru madde sindirimini %2, ham sellüloz sindirimini ise %14 oranında düşürmüştür.

Ön-sulandırmanın ve *Propionibacterium*'un taşıyıcısı olarak kullanılan maddenin in vitro rumen fermentasyonuna etkilerini saptamak amacıyla ikinci bir araştırma düzenlenmiştir. Bu çalışmadada *Propionibacterium*'un daha önce kullanılan oranlarının tümü tekrar incelemeye alınmıştır. Denemede otoklavdan geçirilmiş *Propionibacterium*'un, otoklavdan geçirilmemiş *Propionibacterium*'a göre asetik ve propiyonik asit yoğunluklarını %5 oranında azalttığı halde, kuru madde veya ham sellüloz sindirimini artırmadığı saptanmıştır. *Propionibacterium*'un toplam uçucu yağ asitleri, asetik asit ve propiyonik asit yoğunluklarını artırdığı ve bu nedenle geviş getiren hayvanların performansını geliştirebileceği söylenebilir.

Anahtar Sözcükler: *Propionibacterium*, Uçucu Yağ Asitleri, Nötr Deterjan Lif, Nişasta, Sindirilebilirlik

Introduction

The genus *Propionibacterium* (PB) contains a diverse and versatile number of organisms. They are Gram-positive, non-motile, non-spore-forming facultative anaerobes (1, 2), which are found naturally in the rumen (3) at levels of 10^5 to 10^6 CFU/ml of rumen contents (C. Hibberd, personal communication). The main characteristic of this genus is the production of large amounts of propionic and acetic acids as metabolic end products. *Propionibacterium* strains are used mostly in the cheese industry as dairy starter cultures (4, 5, 6), and in the ensiling process of high moisture corn (7, 8). Recently, researchers at Oklahoma State University (OSU)

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isolated and tested a strain of PB that effectively reduced rumen and blood levels of nitrite in cattle consuming high-nitrate pearl millet hay (9). After several cattle and sheep feeding trials at OSU, the researchers concluded that a viable population of this strain could be established and maintained in the rumen. It was also found that PB stimulated dry-matter intake (DMI) when compared to control groups, regardless of the nitrate content of the consumed forages.

Propionibacterium may exert some type of physiological effect that stimulates appetite. This strain of PB may possibly alter rumen fermentation sufficiently to increase intake, perhaps by shifting fermentation end products, increasing feed digestibility, or increasing the clearance of undigested feed from the rumen. According to the types of feeds offered in the OSU studies, it is most probable that PB increased fiber digestibility, which resulted in greater DMI. Although greater digestibility may be caused by direct fermentation of fiber by the PB, it is more likely due to an enhanced rumen environment for the growth of other fiber-digesting microorganisms.

This study was designed to investigate the physiological basis for the increased intake response noted in ruminants inoculated with PB strain P5. The objectives were to determine the ability of various levels of PB to alter the end products of in-vitro fermentation, specifically volatile fatty acids (VFA), and to enhance the fermentation of dry matter (DM), fiber and starch in diverse feed sources.

Materials and Methods

Trial 1

A 4 x 4 x 4 factorial arrangement was used in Trial 1. There were 4 feeds: alfalfa silage, fescue hay, wheat straw, and corn grain; 4 incubation times: 6, 18, 36, and 120 h for fiber and 3, 6, 12, and 24 h for starch; and 4 levels of PB: 0, 1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/ml of the final in-vitro solution.

The 4 feed samples, alfalfa silage, fescue hay, wheat straw and corn grain, were obtained from Southern Illinois University Farm, dried at 55ºC for 48 h, and then ground through a Wiley mill fitted with a 1-mm screen (Arthur H. Thomas, Philadelphia, PA). In-vitro fermentations were conducted in batch cultures and were based on a system described by Goering and Van Soest (10), consisting of a warm water bath, fermentation flasks with feed samples, the addition of growth/buffer media and reducing solution (sodium sulfide and cysteine), and continuous flushing of each flask with CO_2. Three separate dried preparations of PB strain P5 (1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/g; one for each level of PB studied) were provided by a commercial source (Far-Mor Biochem, Inc., Milwaukee, WI). Aqueous PB solutions were prepared by mixing PB with distilled water, and added into in-vitro flasks just prior to the addition of rumen inoculum. Ruminal digesta were obtained from the ventral rumen of a lactating dairy cow receiving a high-quality diet which consisted of alfalfa silage, corn silage, ground corn grain, soybean meal, and mineral and vitamin premix. Ruminal digesta were collected 1 to 2 h after feeding. The digesta were blended under CO_2, strained through four layers of cheesecloth and glass wool, and 10 ml of rumen fluid was then injected into each flask, thus starting incubation.

The feed samples were analyzed for neutral detergent fiber (NDF) according to modified procedures of Van Soest and Robertson (11). At the end of each incubation period, the fermentation was arrested by reducing the solution pH via addition of 1 ml of 6 N H_2SO_4 to each flask. Then 20 ml of solution was siphoned from each flask to be analyzed for NDF, and this was centrifuged at 30,000 x g for 30 min in order to remove undigested feed particles. About 15 ml of supernatant was collected and kept frozen (-20ºC) for later VFA analysis, and the remaining solution and particles were returned to the flask. The remaining contents of the fermentation were analyzed for NDF immediately (11). The flask contents were washed into a 600 ml Berzelius beaker with 100 ml of neutral detergent solution, and 1 ml of heat stable amylase (4% (v/v) Termamyl; Termamyl 120L; Novo Nordisk Bioindustrials, Inc., Danbury, CT) was added. This was then refluxed for 1 h. The beaker contents were washed into tared Gooch crucibles, which were placed on a filtering manifold, washed twice with hot distilled water, then washed twice again with acetone, and finally sucked dry. The crucibles were dried in a forced-air oven at 105ºC for 8 h. The true DM digestibility was calculated as the difference in the NDF residue following incubation (100 - %NDF residue after incubation), on the assumption that all neutral detergent solubles had been completely digested. The digestibility of NDF was calculated as the percentage of the total NDF in each sample. Supernatants were analyzed for VFA content using a Hewlett Packard 5890A (Hewlett Packard Co.,

Palo Alto, CA) gas chromatograph, as described by Erwin et al. (12). The chromatograph was fitted with a 1/8" x 6" stainless steel column packed with 10% SP-1200/1%H_3PO_4 on 80/100 chromosorb W-AW (Supelco Inc., Houston, TX). The column, injector and detector temperatures were 135, 160 and 160ºC, respectively. The starch content of the feed samples and in-vitro digested samples was determined with a procedure developed by MacRae and Armstrong (13), as modified by Dado and Beek (14). The feed samples were autoclaved with 25 ml distilled water at 130ºC for 1h, and then incubated with 50 ml of 0.2 M sodium acetate buffer (pH 4.5) containing 200 mg of amyloglucosidase (#A7255, Sigma Chemical, St. Louis, MO) at 55ºC for 24 h. The incubated samples were filtered through Whatman #1 filter paper into a 100-ml volumetric flask, and diluted to volume with distilled water. The glucose content was measured by colorimetric assay via glucose oxidase (Diagnostic Kit #510-A, Sigma Chemical, St. Louis, MO). The starch content was found to be 90% that of the total glucose and was recorded as a percentage of DM. The starch content of the in-vitro incubated samples was determined as previously described with some minor changes. These changes were as follows: 25 ml of buffer containing 200 mg of enzyme was used; the samples were transferred to 100-ml volumetric flasks and diluted to volume with distilled water; a 15 ml aliquot was centrifuged at 2000 x g for 10 min and filtered; and 10 ml was transferred to a 100-ml volumetric flask and diluted to volume with distilled water. The starch content was determined via glucose as previously described; however, for longer in-vitro incubation times, larger volumes of samples were used in the color assay. The addition of starch via PB was accounted for when calculating the starch digestibility. All the measurements were conducted in duplicate. Some additional replicates were run when duplicates were not in agreement.

An analysis of variance (15) was conducted to determine the significance of the main factors and interactions. The full model used included the main effects of PB, feed source and time, and all two-way interactions between these. Orthogonal contrasts were used to compare levels of PB.

Trial 2

In Trial 2, pre-hydration was conducted to determine whether additional hydration time would result in a more viable PB culture before addition to the fermentation. The effect of the PB carrier was also examined. There were 4 levels of PB: 0, 1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/ml of the final in-vitro solution; 3 levels of autoclaved PB: 1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/ml of the final in-vitro solution; 3 levels of hydrated PB: 1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/ml of the final in-vitro solution; 2 different feeds: alfalfa silage and fescue hay; and 3 different incubation times: 6, 18, and 120 h. Aqueous solutions of PB were autoclaved at 130ºC for 1h. The fermentation procedures and measurements were the same as those described in Trial 1, but with the omission of the starch digestibility measurement. An analysis of variance (15) was conducted to determine the significance of the main factors and interactions. The full model used included the main effects of PB, feed source, time, and all two-way interactions between these. Contrasts were used to compare no PB to the average of all levels of PB without autoclaving or hydration (same as Trial 1), the average of all levels of PB with the average of all levels of autoclaved PB, and the average of all levels of PB with the average of all levels of hydrated PB.

Results

Trial 1

The starch and NDF contents of the feed sources and the starch content of the PB carrier are presented in Table 1. The starch content of the feed sources varied from 0.83 to 65.0% of DM, and alfalfa silage had the lowest starch content. The neutral detergent fiber content was highest in wheat straw. The starch contents of the PB carriers were 10.8, 8.9 and 26.1% of DM for 1 x 10^3, 1 x 10^6, and 1 x 10^9 CFU/g PB, respectively.

Table 1. Starch and neutral detergent fiber (NDF) concentration in feed sources and *Propionibacterium* carrier.

Items	Starch	NDF
	(% of DM)	
Feed sources		
Alfalfa silage	0.83	47.9
Fescue hay	3.40	71.4
Wheat straw	1.85	82.0
Corn grain	65.0	9.4
Carrier of *Propionibacterium*		
1 x 10^3 CFU/g	10.8	...
1 x 10^6 CFU/g	8.9	...
1 x 10^9 CFU/g	26.1	...

The total and individual VFA concentrations are summarized in Table 2. Compared to the control, total VFA production was 9% higher for all levels of PB ($P < .01$). The interaction between PB and time was not significant ($P > .05$), but the interaction between PB and feed source was statistically significant ($P < .01$). Except in the case of corn grain ($P > .05$), PB increased total VFA production ($P < .05$) across all feed sources. In general, addition of PB increased the concentrations of acetate, propionate, butyrate, and valerate ($P < .01$); however, it did not affect the production of isobutyrate ($P = .14$), and decreased isovalerate concentrations ($P < .01$). Interactions between PB and time were significant ($P < .05$) for acetate, isobutyrate, and isovalerate, and interactions between PB and feed source were significant ($P < .05$) for all VFA except for valerate. Addition of PB decreased the A:P ratio by 14% ($P < .01$).

In-vitro true DM, NDF and starch digestibility are summarized in Table 3. Compared to the control, the overall mean for DM and NDF digestibility of PB was significantly lower ($P < .01$). Addition of PB reduced DM and NDF digestibility by 2 and 14%, respectively. This reduction was consistent across all times and feed sources, except for a few data points for grass hay and wheat straw at longer fermentation times. The interaction between PB and time was not significant for true DM ($P = .95$) and NDF digestibility ($P = .89$). In addition, there was no interaction between PB and feed source for true DM ($P = .06$) and NDF digestibility ($P = .20$). Because of the low starch content of each feed (Table 1) (less than 4% of the DM except for corn grain), the effects of PB on starch digestibility are not clear. Compared to the control, a significant increase (11%) in the overall mean for starch digestibility was observed ($P = .01$) with PB. However, with the exception of alfalfa silage, PB had no effect on starch digestibility. Alfalfa silage was sensitive to the addition of starch and analytical errors may have occurred because of the very low starch content.

Trial 2

The total and individual VFA concentrations are summarized in Table 4. Compared to the control, PB again caused an increase in the total VFA concentration ($P < .01$) as in Trial 1. Autoclaving PB caused a decrease in the total VFA concentration when compared to PB alone ($P = .02$) (86.1 vs 81.8 mM). Hydration tended to increase the total VFA concentration (86.1 vs. 88.3 mM), however, the difference was not significant ($P = .20$). The acetate, propionate, and butyrate concentrations were 12, 29, and 15% higher, respectively, for all levels of PB when compared to the control ($P < .01$). Autoclaving PB caused a significant 5% decrease in the acetate ($P < .01$) and propionate ($P = .04$) concentrations when compared to PB alone. All the VFA concentrations were numerically higher for hydrated PB than autoclaved

Table 2. Influence of *Propionibacterium* (PB) on concentration of volatile fatty acids (VFA) averaged across all feed sources and times (Trial 1).

Measurement	*Propionibacterium*[1]				SEM	Contrast		
	0	10^3	10^6	10^9		CP[2]	L[3]	Q[4]
	(mM)					(P > F)		
Total VFA	100.1	108.4	110.3	107.4	0.80	<.01	.41	.03
Acetate (A)	56.9	59.3	60.7	59.0	0.50	<.01	.70	.03
Propionate (P)	24.1	28.7	28.9	27.6	0.30	<.01	<.01	.04
Butyrate	12.8	14.0	14.3	13.9	0.20	<.01	.59	.09
Valerate	2.52	2.80	2.77	2.88	0.08	<.01	.50	.43
Isobutyrate	1.44	1.34	1.36	1.54	0.01	.14	<.01	<.01
Isovalerate	2.44	2.22	2.29	2.53	0.03	<.01	<.01	.01
A:P	2.58	2.19	2.22	2.23	0.03	<.01	.36	.72

[1]The level of PB: 0, $1x10^3$, 1x106, $1x10^9$ CFU/ml.
[2]Control versus PB.
[3]Linear effect of PB.
[4]Quadratic effect of PB.

Table 3. Influence of *Propionibacterium* (PB) on in-vitro digestibility of nutrients averaged across all feed sources and times (Trial 1).

Measurement	*Propionibacterium*[1]				SEM	Contrast		
	0	10^3	10^6	10^9		CP[2]	L[3]	Q[4]
	(% of DM)					(P > F)		
TDMD[5]	63.0	61.2	61.9	61.3	0.40	<.01	.88	.18
NDFD[6]	33.2	28.2	29.0	28.7	0.90	<.01	.67	.63
Starch	42.5	42.9	39.7	59.1	1.60	.01	<.01	<.01

[1]The level of PB: 0, 1x10^3, 1x106, 1x10^9 CFU/ml.
[2]Control versus PB.
[3]Linear effect of PB.
[4]Quadratic effect of PB.
[5]True dry matter digestibility.
[6]Neutral detergent fiber digestibility.

Table 4. Influence of *Propionibacterium* (PB) on in-vitro digestibility and concentration of volatile fatty acids (VFA) averaged across all feed sources and times (Trial 2).

Measurement	*Propionibacterium*[1]				Autoclaved[2]			Hydrated[3]			SEM	Contrast		
	0	10^3	10^6	10^9	10^3	10^6	10^9	10^3	10^6	10^9		CP[4]	PA[5]	PH[6]
					(mM)							(P > F)		
Total VFA	75.1	89.1	87.1	82.1	80.3	82.6	82.4	89.8	89.4	85.7	1.90	<.01	.02	.20
Acetate (A)	45.2	51.9	51.0	48.9	46.8	48.4	49.0	52.0	51.8	50.7	0.90	<.01	<.01	.26
Propionate (P)	16.3	22.4	21.6	19.2	20.2	20.8	19.1	22.7	22.5	20.2	0.60	<.01	.04	.15
Butyrate	7.4	8.8	8.7	8.0	8.1	8.2	8.2	9.0	9.1	8.4	0.20	<.01	.06	.11
Valerate	2.80	2.62	2.57	2.35	2.31	2.20	2.50	2.58	2.71	2.49	0.21	.26	.33	.65
Isobutyrate	1.17	1.14	1.15	1.28	1.04	1.03	1.26	1.19	1.16	1.33	0.05	.72	.08	.39
Isovalerate	2.24	2.22	2.14	2.35	1.95	1.96	2.37	2.23	2.22	2.57	0.08	.97	.07	.15
A:P	2.78	2.34	2.38	2.54	2.33	2.35	2.56	2.31	2.31	2.50	0.03	<.01	.83	.11
					(% of DM)									
TDMD[7]	50.9	49.4	48.8	49.7	49.1	49.7	49.1	49.7	49.4	49.4	0.20	<.01	.98	.37
NDFD[8]	18.5	16.1	14.8	16.6	15.3	16.6	15.4	16.4	15.8	16.1	0.50	<.01	.90	.50

[1]The level of PB: 0, 1x10^3, 1x106, 1x10^9 CFU/ml.
[2]Autoclaved PB.
[3]Hydrated PB.
[4]Control versus PB.
[5]PB versus autoclaved PB.
[6]PB versus hydrated PB.
[7]True dry matter digestibility.
[8]Neutral detergent fiber digestibility.

PB, but none were significant. All these effects are summarized in Figure 1.

In-vitro true DM and NDF digestibility are summarized in Table 4. Compared to the control, the difference in the overall mean for the true DM and NDF digestibility of PB was again significant (P < .01). Neither autoclaving nor hydration of PB changed the digestibility of true DM and NDF when compared to PB alone across feed sources and time (P > .25).

Discussion

A significant decrease in the total VFA, acetate and propionate concentrations for autoclaved PB when compared to PB alone suggests that PB was viable in the mixed fermentation culture and was the possible cause, at least partially, for increases in the total VFA, acetate, and propionate concentrations. These results are consistent with the main characteristic of PB, which is the production of large amounts of propionic and acetic acids

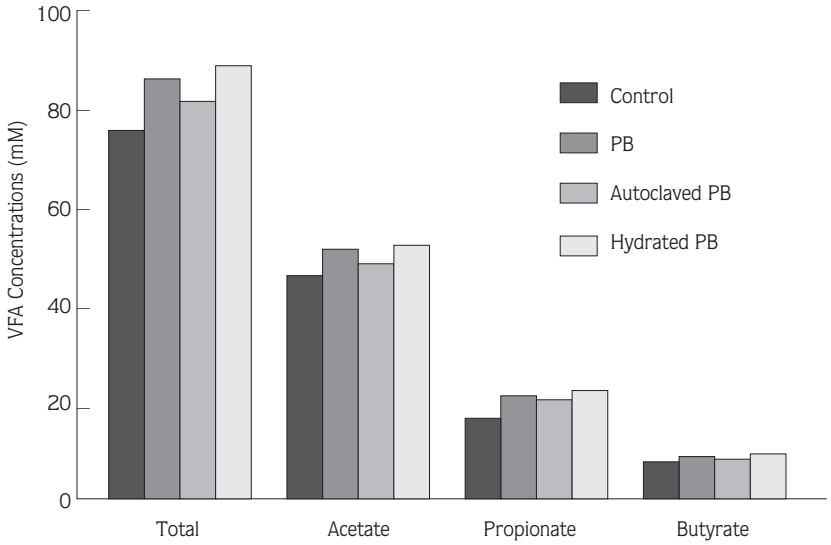

Figure 1. Influence of *Propionibacterium* (PB) on concentration of volatile fatty acids (VFA) averaged across all feed sources and times.

as metabolic end-products (1, 16, 17). Because autoclaving did not alter VFA molar percentages when compared to PB alone, viable PB appears to universally increase all VFA concentrations. De Visser et al. (18) studied the influence of maturity of grass silage and flaked corn starch on the production and metabolism of VFA in dairy cows, and found that adding 2 kg of rumen degradable starch to the diet did not affect molar percentages of VFA when compared to the control, which contained no rumen degradable starch. However, increasing the amount of rumen degradable starch in the diet to 4 kg slightly decreased only the molar percentage of acetic acid when compared to the control (66 vs 65.25 mol/100 mol, $P < .01$). It was concluded that rumen degradable starch had minor effects on the molar percentages of VFA in the rumen fluid. Another study (19) found that increasing the amount of high moisture corn in the dairy cow ration from 24% to 40% (dry-matter basis) did not change total VFA (142 vs 144 mM, $P = .8$, respectively); however, the molar percentage of acetic acid decreased (68.9 vs 66.4 mol/100 mol, $P < .014$, respectively), and that of propionic acid increased (20.3 vs 22.2 mol/100 mol, $P < .048$, respectively). In that study, the amount of starch added to the diet was much higher than in the present study. In the present study, the amount of starch added to the fermentation via PB carrier may be significant since it accounted for up to 2.8% of the DM in the VFA assay.

The efficiency of VFA production increased with viable PB. The concentration of VFA per unit of DM digestibility increased from 1.66 mM/% (averaged total VFA production, 81.77 mM, divided by averaged DM digestibility, 49.3% from autoclaved PB) for the carrier alone to 1.75 mM/% (averaged total VFA production, 86.10 mM, divided by averaged DM digestibility, 49.3% from PB without treatment) with viable PB. Because VFA are the major source of energy for ruminants (20), the use of PB in vivo may enhance the performance of ruminant animals even though fiber digestibility may be lower.

The addition of PB resulted in lower true DM and NDF digestibilities when compared to the control. A decrease in NDF digestion may have occurred because of increased VFA concentrations, particularly acetate, which may inhibit acetate producing bacteria, thus slowing the rate of fiber digestion (21). Autoclaving and hydration did not change digestibility when compared to PB alone across both feed sources and times. These data suggest that the resulting decrease in digestibility with the addition of PB was primarily due to the carrier, which is perhaps most likely because of the starch content in the PB carrier. Grigsby et al. (22) and Piennar et al. (23) both reported that DM and NDF digestion decreased in the rumen with increasing levels of corn. In another study (24), a moderate depression in pH to approximately 6.0 as a result of starch resulted in a small decrease in fiber digestion, but the numbers of fibrolytic organisms were not significantly affected. It was reported that further

decreases in pH to 5.5 or 5.0 resulted in depressed growth rates and a significant decrease in fibrolytic microbes, such that fiber digestion was almost completely inhibited. Sarwar et al. (25) found that when cereal grains were fed at increasing levels (over 50% of the diet), acid production from the fermentation of non-structural carbohydrates overcame the buffer capacity in the rumen, proportionately reducing rumen fluid pH, fiber digestion and, perhaps, DMI. In the present study, the amount of starch added to the fermentation via the PB carrier may be significant since it accounted for up to 2.8% of the DM in the fiber digestibility assay. Unfortunately, pH was not measured in the present study, but minimal changes in in-vitro pH would be expected because of buffers in the media. In another study, incremental amounts of starch (0, 40, 60, or 80% of the DM) were added in-vitro and increases in discrete lag prior to fiber digestion were observed with a pH maintained at 6.8 (26). The results of this study are in agreement with those of El-Shazly et al. (27), who reported that microorganisms preferentially used starch before fiber. In more recent work, the addition of starch was also found to influence the rate and extent of fiber digestion, especially when pH is low (28). In the present study, the entire depression in NDF digestibility may not be attributable to starch addition alone since far less starch was added than in other studies. In addition to starch, the PB may contain other substances that may have harmful effects on cellulolytic bacteria within the rumen, particularly when these microorganisms are not given appropriate time to adapt to the presence of these new compounds. In vivo, adapted ruminants should not have any problem with fiber digestibility due to added PB starch because of the small amount of starch present in this added form of PB. Other potential problems may also be readily accommodated in adapted animals.

Based on the findings of the present study, it can be concluded that viable PB was present in-vitro and increased total VFA, acetate and propionate concentrations. The addition of PB decreased the true DM and NDF digestibility of feeds in-vitro. Changes in feed digestibility may not be associated with the increase in feed intake observed in an earlier study. Further studies are recommended to examine PB addition in vivo to allow for rumen adaptation.

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