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Pectin Induces an In Vitro Rumen Microbial Population Shift Attributed to the Pectinolytic *Treponema* Group

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Abstract Pectin is a non- ber carbohydrate (NFC) that exists in forages, but it is not clear how pectin exerts its effect on populations of either known microbial species or uncultured ruminal bacteria. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and real-time PCR analysis were used in the present study to investigate the effects of pectin on microbial communities in an in vitro rumen fermentation system. The fermentations were conducted using forage (corn stover or alfalfa), an NFC source (pectin or corn starch), or their combination as the substrates. Addition of pectin increased acetate ($P < 0.05$), whereas inclusion of starch increased butyrate production ($P < 0.05$). The pectate lyase activity was higher with alfalfa than with corn straw, or with pectin than with corn starch ($P < 0.05$), while the amylase activity was higher in corn starch-included treatments than the others ($P < 0.05$). The cluster analysis of the bacterial 16S rRNA gene showed that the DGGE banding patterns differed signi - cantly between the treatments and led to the identi cation of three groups that were highly associated with the NFC sources. The speci c bands associated with pectin-rich treatments were identi ed to be dominated by members of the *T e e a* genus. The growth of the *T e e a* genus was remarkably supported by the inclusion of pectin, highlighting their speci c ability to degrade pectin. The results from the present study expand our knowledge of the microbial populations associated with pectin digestion, which may not only facilitate future research on utilization of pectin in feeds, but also improve our understanding of pectin digestion with respect to the rumen micro-ecosystem.

Introduction

A shortage of available carbohydrates can primarily limit the microbial activity and nitrogen utilization in the rumen [33]. Zhu et al. [41] observed that dairy cows fed alfalfa hay, as a primary forage source, exhibited a higher rumen microbial protein yield than those fed corn stover, and this difference was attributed to the higher non- ber carbohydrate (NFC) content in alfalfa hay. The typical NFC content (% of dry matter) in alfalfa hay ranges from 23.2 to 31.5 %, corresponding primarily to a pectin content of 10.5 14.2 % [21], but total NFC in corn stover is only 5.3 % [22]. Both pectin and starch are the primary types of NFCs that exist in feeds, but their digestion and fermentation characteristics differ markedly [11] primarily due to their speci c effects on ruminal bacteria [20].

There appear to be limited studies that focused on the effects of pectin on microbial populations. Through in vitro fermentations with different carbohydrate sources (pectin vs. starch), Poulsen et al. [26] found that the addition of pectin resulted in a different microbial community compared with that obtained with starch treatment, and these differences may be attributed to a selective enhancement of groups of pectin-utilizing bacteria. However, the exact groups or species of pectin-utilizing bacteria remain

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unknown due to the limitations of the methodology (terminal-restriction fragment length polymorphism analysis) used by these researchers.

The technique of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) followed by sequencing analysis is widely used as a method to compare the ruminal bacterial structures obtained with different diets and/or speci c groups of bacteria associated with a particular function in the rumen [5, 13, 15]. Quantitative PCR has proven to be a

Table 1 Pure cultures and primers used in this study

Target		Primer sequences	Product size (bp)	PCR ef ciency (%)	References
Total bacteria	F	CGGCAACGAGCGCAACCC	141	91	Denman and McSweeney [6]
	R	CCATTGTAGCACGTGTGTAGCC			Denman and McSweeney [6]
T e e a group	F	GGCAGCAGCTAAGAATATTCC	575	88	Bekele et al. [3]
	R	CCGTCAATTCCTTTGAGTTT			Watanabe et al. [34]
T e e a b a ii (B25)	F	AGTCGAGCGGTAAGATTG	421	97	Tajima et al. [32]
	R	CAAAGCGTTTCTCTCACT			Tajima et al. [31]
Fib bac e cci ge e (S85)	F	GTTCGGAATTACTGGGCGTAAA	121	96	Denman and McSweeney [6]
	R	CGCCTGCCCCTGAACTATC			Denman and McSweeney [6]
R i c cc a b (8)	F	CCCTAAAAGCAGTCTTAGTTCG	176	93	Koike and Kobayashi [16]
	R	CCTCCTTGCGGTTAGAACA			Koike and Kobayashi [16]
R i c cc avefacie (Y1)	F	CGAACGGAGATAATTTGAGTTTACTTAGG	132	92	Denman and McSweeney [6]
	R	CGGTCTCTGTATGTTATGAGGTATTACC			Denman and McSweeney [6]
P ev e a i ic a (ATCC19189)	F	GAAAGTCGGATTAATGCTCTATGTTG	74	99	Stevenson and Weimer [29]
	R	CATCCTATAGCGGTAAACCTTTGG			Stevenson and Weimer [29]

The culture stains used are indicated in brackets

respective pure-cultured strains including *P. i ic a* ATCC19189, *T. b a ii* B25, *Fib bac e cci ge e* S85, *R i c cc a b* 8, and *R. avefacie* Y1. Whereas the standard for *T e e a* group was obtained by cloning the amplicon ampli ed from the genomic DNA of *T. b a ii* B25 using *T e e a* group-speci c primers, the standard for total bacteria was generated from the amplicon ampli ed using the bacterial universal primers (Table 1) with the genomic DNA of *R. a b* 8 as a template. The respective plasmid DNA standard was prepared according to Koike et al. [17].

The real-time PCR assays were performed using a 7500 Real-Time PCR System (Applied Biosystems, USA) with the SYBR Premix Ex Taq (TaKaRa Bio, Dalian, China). The PCR mixture solution contained 10 µl of 2 × SYBR Premix Ex Taq, 0.4 µl of 50 × ROX Reference Dye, 10 ng of the template DNA, and 0.2 µM of each primer in a total volume of 20 µl. The ampli cation procedure consisted of one cycle of 95 °C for 30 s for the initial denaturation and 40 cycles of 95 °C for 5 s and annealing/extension at 60 °C for 34 s. A ve-fold dilution series of the respective plasmid DNA standard was run with the samples, which were run in triplicate. The ampli cation ef ciencies and the relative abundance of each target species were calculated according to Liu et al. [18].

Statistical Analysis

The statistical analyses were performed using the SAS software [27] with one-way ANOVA, and the mean separation was conducted using Tukey's studentized range test. The level of signi cance was set to 0.05.

Results and Discussion

In Vitro Fermentation Parameters and Enzymes Activities in the Fermentation Fluid

Fermentation of pectin by known pectinolytic bacteria strains yielded acetate as major end products, whereas they produced more butyrate, formate or lactate when they grow on glucose [8, 19]. When comparing CSP with CSS or Pe with St, addition of pectin signi cantly increased acetate production ($P < 0.05$), whereas inclusion of starch significantly increased butyrate production ($P < 0.05$, Table 2). Propionate production was not signi cantly different between CSP and CSS or Pe and St ($P > 0.05$). Our results agree with those of Ariza et al. [2], who found pectin-rich citrus pulp diet resulting in a greater acetate/propionate ratio compared with starch-rich hominy feed diet under continuous culture fermentation system. Marounek et al. [20] also demonstrated that mixed cultures of rumen microorganisms generated a metabolite pro le that is high in acetate and low in butyrate with pectin fermentation. Not surprisingly, the AH had higher DMD than CS ($P < 0.05$). Incorporation of either pectin or starch with CS resulted in higher DMD than CS alone ($P < 0.05$), with no difference between CSP and CSS ($P > 0.05$). The pectin treatment induced higher gas production in early time point than starch, whereas after 24 h, total gas production was not signi cantly different between CSP and CSS or Pe and St ($P > 0.05$, Supplementary Table S1).

The pectate lyase activity was signi cantly higher with AH than with CS, or with Pe than with St (Table 2, $P < 0.05$). When CS was supplemented with pure pectin,

Table 2 Effects of speci c substrates on fermentation parameters and enzyme activities at 24 h of in vitro incubation

Item[#]	Treatments*						SEM	*P* value
	CS	AH	CSP	CSS	Pe	St		
Total VFA (mmol)	23.6^{c}	30.1^{b}	33.3^{a}	32.2^{ab}	19.6^{d}	17.9^{d}	0.5	<0.01
Acetate	17.2^{c}	21.9^{b}	25.3^{a}	22.5^{b}	16.1^{c}	12.5^{d}	0.4	<0.01
Propionate	4.0^{b}	5.6^{a}	5.5^{a}	5.8^{a}	1.8^{c}	1.8^{c}	0.1	<0.01
Butyrate	2.4^{c}	2.5^{c}	2.6^{c}	3.9^{a}	1.7^{d}	3.6^{b}	0.0	<0.01
Acetate/propionate	4.3^{cd}	3.9^{d}	4.6^{c}	3.9^{d}	9.0^{a}	6.9^{b}	0.1	<0.01
DMD (%)	47.3^{d}	66.6^{b}	61.5^{c}	62.5^{c}	100^{a}	100^{a}	0.6	<0.01
Amylase	2.7^{b}	4.3^{b}	4.7^{b}	11.7^{a}	2.4^{b}	11.5^{a}	1.0	<0.01
Pectate lyase	0.85^{c}	1.81^{b}	2.58^{a}	0.43^{cd}	0.80^{c}	0.01^{d}	0.12	<0.01

[a d] Means with different letters with a row differ ($P < 0.05$)

[#] *VFA* volatile fatty acids, *DMD* dry matter digestibility

* *CS* corn stover, *AH* alfalfa hay, *CSP* corn stover and pectin, *CSS* corn stover and corn starch, *Pe* pectin, *S* corn starch

pectate lyase activity increased signi cantly ($P < 0.05$). Pectinolytic enzymes, predominant as pectin lyases, were produced by pectinolytic species and released into the rumen environment, degrading pectin to unsaturated di- and trigalacturonides [35] which are further metabolized intracellularly to generate a high yield of acetate [14]. Thus, higher pectin lyases associated with AH, CSP, and Pe suggested the increments of pectinolytic bacteria population under these treatments. As expected, the amylase activities were signi cantly higher with CSS and St than the other treatments ($P < 0.05$).

Comparison of Bacterial DGGE Pro les and Sequence Analysis of Speci c Bands

The bacterial DGGE pro les were clearly divided into three clusters (Fig. 1): CSS and St in group 1; CS in group 2; and Pe, AH, and CSP in group 3. The members of each group exhibited similarities greater than 71, 80, and 72 % to each other, respectively. The cluster analysis indicated that starch supplementation (CSS and St) apparently diverted the patterns of the DGGE bands to form an outgroup with 65 % similarity with groups 2 and 3 (Fig. 2). The CS cluster (i.e., group 2) exhibited approximately 67.5 % similarity with group 3. However, the combination of CS with pectin as the substrate (CSP) resulted in the band patterns being closely grouped with those of AH. Based on the comparison of the microbial responses to CSP and CSS using the microbial structure of AH as the standard, the results indicate, from a microbial ecological aspect, that pectin but not starch is one of the important nutritional differences that exist between CS and AH.

As shown in Fig. 1, one of the speci c bands (B1) located at the top of the gel was found only in the AH- and pectin-added treatments, and the other two bands (B2 and B3) were associated with starch treatments. Because the

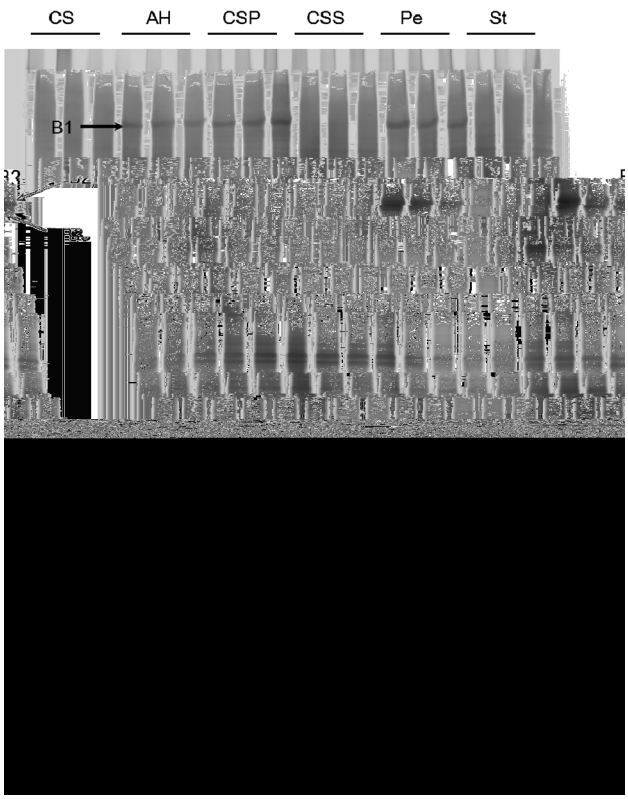

Fig. 1 PCR-DGGE ngerprints of bacterial 16S rRNA gene fragments from the DNA obtained from the rumen uid from in vitro fermentations with speci c substrates. The fragments were ampli ed using the primers GC-338F and 533R. The treatments are indicated at the f he a e (*CS* corn stover, *AH* alfalfa hay, *Pe* pectin, *S* corn starch, *CSP* corn stover and pectin, *CSS* corn stover and corn starch). The 2 be ed a e correspond to the treatment replicate number shown in Fig. 2. The distinct bands that appear to be only correlated to pectin or starch metabolism are indicated with a w (B, band)

distinct bands may represent the core species related to pectin or starch digestion, these were cloned and sequenced to characterize the taxonomic relationships. Because we

focused on species related to pectin digestion, more clones from B1 than B2 and B3 were selected to perform the sequencing analysis. Not surprisingly, the identi ed bands generated multiple reading sequences, and the taxonomic information of these sequences is shown in Table 3.

The bands that migrated to the same locations on the DGGE gel are likely to have the same identity at the genus level [5]. The sequences obtained from B1 were dominated by species of *T e e a*, whereas B2 and B3 were dominated with species of *P ev e a*, suggesting the important role of these species in pectin and starch digestion, respectively. Clones (18 22, 25 26, and 37 38) assigned to other genera were also recovered from the three identi ed bands. These may originate from species that are related to pectin and starch metabolism or may be a result of the ampli cation of a heteroduplex of the 16S rRNA gene [5].

To date, only three species of the genus *T e e a* have been described: *T. b a ii* [28], *T. accha hi* [23], and *T. i eckii* [25]. A phylogenetic study conducted by Bekele et al. [3] suggested the existence of distinct members of this group, the majority of which remains uncultured. Our sequencing results from B1 con rmed the ndings reported by Bekele et al. [3] and suggest that various members of *T e e a* may be extensively involved in pectin digestion. It has been reported that *T. i eckii* ferments only pectin and lacks the ability to utilize starch [25]. Although both pectin and starch support the growth of *T. accha hi* in a monoculture environment, Liu et al. [18] found that the addition of starch poorly supported the growth of the species in a mixed culture environment, suggesting that it is a speci c pectinolytic bacterium and may play an important role in pectin digestion. One clone (No. 15) that showed 99 % similarity with *T. accha hi* was recovered from B1, further con rming the implication of the previous study [18].

Relative Abundance of Typical Ruminal Bacteria

The relative abundances of the classical ruminal bacterial species are shown in Table 4. The relative proportions of the *T e e a* group in the total rumen bacteria were as high as 25.7 24.9 %-2441.70001(for)-444.4(AH-2441.9nts)-2442. population size of *T e e a* in alfalfa-fed ruminants compared to those fed stover, grass, or concentrate, and the highest proportions of *T e e a*

Table 3 Identi cation of PCR-DGGE bands

Band	Clone ID	Species (GenBank accession no.)	% Similarity
B1	1 3	*T e e a ci* strain 14V28 (NR_042942)	92
	4 7	*T e e a b a ii* strain RUS-1 (NR_104781)	95

acetate as a major end product of pectin fermentation. Using two small pectinolytic spirochetes (strains 692 and 791) isolated from the rumen, Zio ecki and Wojciechowicz [43] found that both strains growing fairly rapid on pectin but not on starch, and that strain 692 could only utilize pectin as an energy source. Wojciechowicz and Zio ecki [36] described three isolated large rumen *T e e a* members (strains 606, 709 and 710) with outstanding feature of pectinolytic activity. Pectin is decomposed by *T e e a* strains via trans-elimination mechanism, yielding a mixture of saturated and unsaturated degradation products. Similar to *T. accha hi*, all the above-mentioned strains produced acetate as a major end product of pectin fermentation with no propionate and butyrate produced [42, 43], consistent with the higher acetate production with Pe and CSP treatments (Table 2). Based on these pure culture-based studies and our molecular-based investigation, it is reasonable to speculate the existence of distinct members of rumen *T e e a* involved in the rumen pectin digestion.

However, it should be noted that the CS treatment also supported a relatively signi cant growth of the *T e e a* group that is similar to that found using pectin alone as the substrate. Moreover, the proportion of the *T e e a* group found in the treatment of CS plus Pe was equal to the proportion found with the CSP treatment, suggesting the existence of two groups of *T e e a* in terms of substrate utilization: one group is highly specialized in pectin utilization, and the other group is involved in ber metabolism. Rumen spirochaetes, which are predominantly *T e e a* [24], have often been observed during the study or isolation of cellulolytic bacteria [30].

T. b a ii is likely a representative of the *T e e a* group that does not favor pectin utilization but is involved in ber digestion. In the present study, the highest

Table 4 Effects of speci c substrates on the relative abundances of target gene copies for speci c species (% of total bacterial 16S rRNA gene)

Target species	Treatments*							SEM	*P* value
	Bk	CS	AH	CSP	CSS	Pe	St		
T e e a group	5.1^{cd}	14.7^{b}	25.7^{a}	24.9^{a}	10.9^{bcd}	11.8^{bc}	2.7^{d}	1.9	<0.01
T e e a b a ii	0.02^{c}	0.71^{a}	0.24^{b}	0.41^{b}	0.30^{b}	0.02^{c}	0.01^{c}	0.04	<0.01
Fib bac e cci ge e	1.0^{c}	16.8^{a}	5.3^{b}	10.2^{b}	8.2^{b}	0.3^{c}	0.3^{c}	0.9	<0.01
R i c cc a b	0.06^{b}	0.07^{b}	1.63^{a}	0.04^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.10	<0.01
R i c cc avefacie	0.008^{c}	0.024^{a}	0.018^{ab}	0.010^{bc}	0.012^{bc}	0.007^{c}	0.004^{c}	0.002	<0.01
P ev e a i ic a	0.20^{c}	0.54^{a}	0.51^{ab}	0.50^{ab}	0.22^{bc}	0.68^{a}	0.12^{c}	0.06	<0.01

$^{a\ d}$ Means with different letters within a row are signi cantly different ($P < 0.05$)

* *CS* corn stover, *AH* alfalfa hay, *Pe* pectin, *S* corn starch, *CSP* corn stover and pectin, *CSS* corn stover and corn starch, *Bk* blank control

population of *T. b a ii* was observed with the CS treatment ($P < 0.05$), followed by AH, CSP, and CSS, and neither pectin nor starch alone supported a high growth of this species. Although cellulose did not support the growth of *T. b a ii*, Stanton and Canale-Parola [28] showed a bene cial interaction of *T. b a ii* with the cellulolytic bacterium *F. cci ge e*. *F. cci ge e* and *R. a b* are able to degrade pectin through secreted pectate lyases [4]. Gradel and Dehority [10] also demonstrated that the cellulolytic strain *R. avefacie* B34b and *R. a b* 7 had partial ability to degrade pure pectin or pectin in alfalfa. However, the growths of cellulolytic species were not supported by pectin alone, probably because they had very limited ability of pectin utilization [10].

P. i ic a is well-known ruminal amylolytic bacteria [30] and have also been reported as important rumen pectinolytic bacteria [10]. The population of *P. i ic a* was signi cantly higher with the Pe compared with the St treatment ($P < 0.05$), and the addition of starch to CS even reduced the proportion of the species compared to that obtained with CS alone. It has been reported that *P. - i ic a* can ef ciently utilize pectin when this species is co-cultured with a cellulolytic species [9].

The present study provides the rst exploration of the speci c relationship of the *T e e a* group with pectin digestion in vitro using a molecular-based method. Although ruminal bacteria can grow on a relatively broad range of substrates under pure culture conditions, populations of *P. i ic a* were found to be stimulated by pectin rather than starch, suggesting that it may more likely function as pectinolytic bacteria in a mixed-culture environment. The results obtained in the present study may expand our understanding of pectin digestion from a ruminal ecological aspect. Further studies are needed to evaluate the role of *T e e a* in vivo, and the isolation of new species of *T e e a* is required to fully explore the ecological mechanism underlying the digestion of pectin by these species.

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