Curr Microbiol (2015) 70:67–74
DOI 10.1007/s00284-014-0672-y

Electronic supplementary material The online version of this article (doi:10.1007/s00284-014-0672-y) contains supplementary material, which is available to authorized users.

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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 specific groups of bacteria associated with a particular function in the rumen [5, 13, 15]. Quantitative PCR has proven to be a powerful method for the rapid and sensitive monitoring of microbial populations in the complex ruminal environment [29, 31]. Thus, based on our hypothesis of the existence of specific groups or species related to pectin digestion, the objective of this study was to evaluate the effects of pectin on microbial populations in in vitro fermentations using the PCR-DGGE and quantitative PCR methods. Corn starch was used as an additional substrate for comparative purposes.

Materials and Methods

In Vitro Fermentations with Specific Substrates

The in vitro fermentations were conducted as described previously [18]. Briefly, fermentations were set up for seven treatments with three replicates each. Each fermentation was conducted in 90 ml of buffer medium [32] in 180-ml serum bottles. The following treatments were used: (1) blank control (Bk) with no substrate, (2) 150 mg of pectin (Pe, from citrus peel, Fluka, Sigma Aldrich, Denmark), (3) 150 mg of corn starch (St, Aladdin Reagent Company, Shanghai, China), (4) 600 mg of alfalfa hay (AH), (5) 600 mg of corn stover (CS), (6) CS and 150 mg of pectin (CSP), and (7) CS and 150 mg of corn starch (CSS). The medium preparation and rumen fluid collection were conducted according to Liu et al. [18]. Each bottle was injected with 10 ml of the rumen fluid as the inoculum and placed in an incubator at 39 °C with shaking for 24 h. At 3, 6, 9, 12, and 24 h of incubation, gas production was measured according to Zhang et al. [40].

Sampling and Chemical Analysis

At the end of the incubation, the fermentation fluid was sampled for DNA extraction to determine pH, volatile fatty acids (VFA), and enzyme activities. The pH and VFA were determined using the methods described by Hu et al. [12]. After the incubation terminated, the contents in the bottle were completely rinsed out and filtered through filter bags. The bags were put into an oven at 55 °C for 48 h to determine dry matter digestibility (DMD). The pectate lyase activity was measured according to Yuan et al. [39], by measuring the increase in absorbance at 235 nm. One activity unit (U) of pectate lyase was defined as the lyase that produced 1 mmol of unsaturated galacturonide from 100 ml of fermentation fluid at 39 °C per minute. The amylase activity was determined using starch–iodine method [37], and one activity unit was defined as the amylase that hydrolyzed 10 mg starch from 100 ml of fermentation fluid at 39 °C in 30 min.

DNA Extraction

The DNA was extracted using the RBB+C method [38] and then quantified using the Qubit dsDNA HS assay kit (Invitrogen, USA) with a Qubit 2.0 fluorometer (Invitrogen, USA). The extracted DNA was diluted to 50 or 10 ng/μl for PCR-DGGE or real-time PCR analysis, respectively.

PCR-DGGE and Sequence Analysis

The V3 region of the 16S rRNA gene was amplified with the bacterial primer set GC-338f (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG) and 533r (TTACCGCGGCTGCTGGCAC). The PCR was performed in a volume of 50 μl containing 10 μl of 5 × GoTaq Reaction Buffer, 1.25 U of GoTaq DNA Polymerase (Promega, Madison, WI, USA), 0.2 mM dNTP, 0.2 μM of each primer, and 50 ng of the DNA template. The PCR program consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and a final elongation for 7 min at 72 °C.

The DGGE analysis was running with the DCode apparatus (Bio-Rad, USA) at 60 °C and 85 V for 16 h, using a 6–12 % polyacrilamide gel with a 40–60 % denaturation gradient. Gel bands were visualized through silver staining [15] and analyzed using the Quantity One® software (Bio-Rad, USA). The similarity of the DGGE profiles was calculated using Dice coefficient index [7]. A dendrogram was then constructed using the unweighted pair group method with the arithmetic averages clustering algorithm. The DGGE bands of interest were isolated and re-amplified using the same primers without GC clamp. Following cloning and sequencing, the similarities of bands were further queried using the BLAST program [1].

Real-Time PCR Quantification of the 16S rRNA Gene of Target Ruminal Bacterial Species

Plasmids containing a species-specific amplicon were used as the standard for the estimation of the 16 s rRNA gene copy numbers of the respective target species. Standards for quantifying *Prevotella ruminicola*, *Treponema bryantii*, and three cellulolytic bacteria were prepared from

Table 1 Pure cultures and primers used in this study

Target	Primer sequences		Product size (bp)	PCR efficiency (%)	References
Total bacteria	F	CGGCAACGAGCGCAACCC	141	91	Denman and McSweeney [6]
	R	CCATTGTAGCACGTGTGTAGCC			Denman and McSweeney [6]
Treponema group	F	GGCAGCAGCTAAGAATATTCC	575	88	Bekele et al. [3]
	R	CCGTCAATTCCTTTGAGTTT			Watanabe et al. [34]
Treponema bryantii (B25)	F	AGTCGAGCGGTAAGATTG	421	97	Tajima et al. [32]
	R	CAAAGCGTTTCTCTCACT			Tajima et al. [31]
Fibrobacter succinogenes (S85)	F	GTTCGGAATTACTGGGCGTAAA	121	96	Denman and McSweeney [6]
	R	CGCCTGCCCCTGAACTATC			Denman and McSweeney [6]
Ruminococcus albus (8)	F	CCCTAAAAGCAGTCTTAGTTCG	176	93	Koike and Kobayashi [16]
	R	CCTCCTTGCGGTTAGAACA			Koike and Kobayashi [16]
Ruminococcus flavefaciens (Y1)	F	CGAACGGAGATAATTTGAGTTTACTTAGG	132	92	Denman and McSweeney [6]
	R	CGGTCTCTGTATGTTATGAGGTATTACC			Denman and McSweeney [6]
Prevotella ruminicola (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. ruminicola* ATCC19189, *T. bryantii* B25, *Fibrobacter succinogenes* S85, *Ruminococcus albus* 8, and *R. flavefaciens* Y1. Whereas the standard for *Treponema* group was obtained by cloning the amplicon amplified from the genomic DNA of *T. bryantii* B25 using *Treponema* group-specific primers, the standard for total bacteria was generated from the amplicon amplified using the bacterial universal primers (Table 1) with the genomic DNA of *R. albus* 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 amplification 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 five-fold dilution series of the respective plasmid DNA standard was run with the samples, which were run in triplicate. The amplification efficiencies 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 significance 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 significantly increased acetate production ($P < 0.05$), whereas inclusion of starch significantly increased butyrate production ($P < 0.05$, Table 2). Propionate production was not significantly 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 profile 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 significantly different between CSP and CSS or Pe and St ($P > 0.05$, Supplementary Table S1).

The pectate lyase activity was significantly 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 specific 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, *St* corn starch

pectate lyase activity increased significantly ($P < 0.05$). 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 significantly higher with CSS and St than the other treatments ($P < 0.05$).

Comparison of Bacterial DGGE Profiles and Sequence Analysis of Specific Bands

The bacterial DGGE profiles 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 specific 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 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

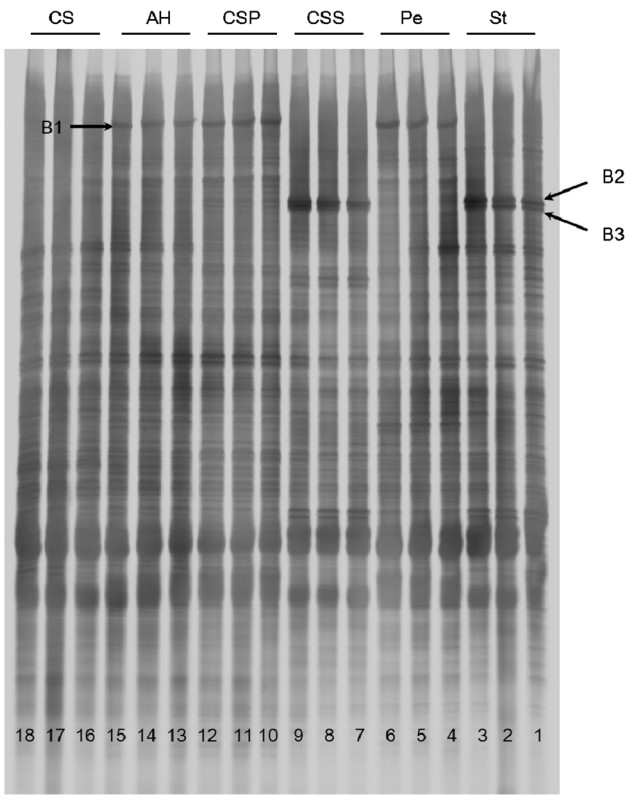

Fig. 1 PCR-DGGE fingerprints of bacterial 16S rRNA gene fragments from the DNA obtained from the rumen fluid from in vitro fermentations with specific substrates. The fragments were amplified using the primers GC-338F and 533R. The treatments are indicated at the *top of the lanes* (*CS* corn stover, *AH* alfalfa hay, *Pe* pectin, *St* corn starch, *CSP* corn stover and pectin, *CSS* corn stover and corn starch). The *numbered lanes* 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 *arrows* (B, band)

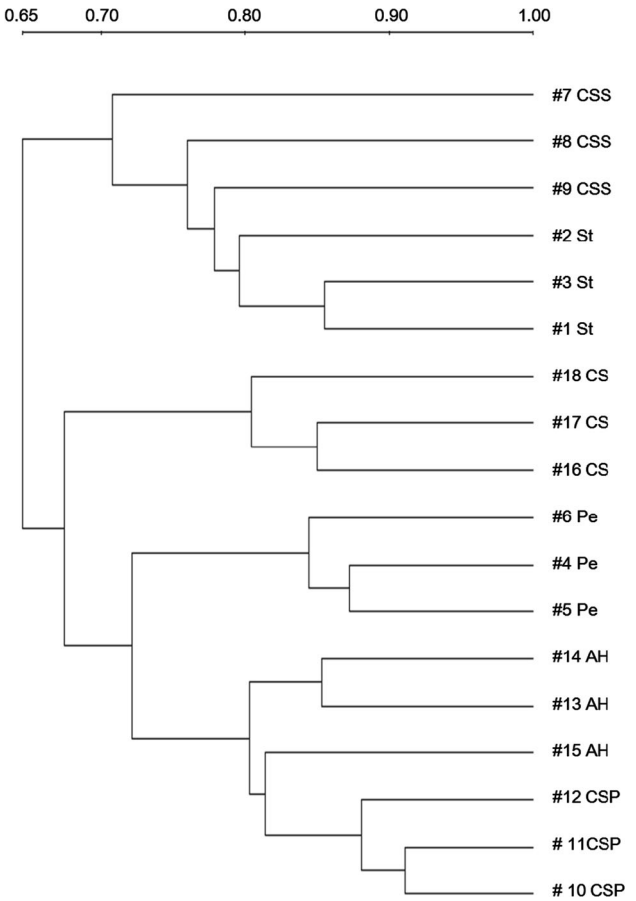

Fig. 2 PCR-DGGE-derived UPGMA dendrogram showing the effects of specific substrates on ruminal bacterial communities in an in vitro fermentation system (*CS* corn stover, *AH* alfalfa hay, *Pe* pectin, *St* corn starch, *CSP* corn stover and pectin, *CSS* corn stover and corn starch). The *scale* is related to the % similarity

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 identified 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 *Treponema,* whereas B2 and B3 were dominated with species of *Prevotella*, 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 identified bands. These may originate from species that are related to pectin and starch metabolism or may be a result of the amplification of a heteroduplex of the 16S rRNA gene [5].

To date, only three species of the genus *Treponema* have been described: *T. bryantii* [28], *T. saccharophilum* [23], and *T. zioleckii* [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 confirmed the findings reported by Bekele et al. [3] and suggest that various members of *Treponema* may be extensively involved in pectin digestion. It has been reported that *T. zioleckii* ferments only pectin and lacks the ability to utilize starch [25]. Although both pectin and starch support the growth of *T. saccharophilum* 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 specific pectinolytic bacterium and may play an important role in pectin digestion. One clone (No. 15) that showed 99 % similarity with *T. saccharophilum* was recovered from B1, further confirming 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 *Treponema* group in the total rumen bacteria were as high as 25.7 and 24.9 % for AH and CSP, respectively. Bekele et al. [3] and Liu et al. [18] observed a higher population size of *Treponema* in alfalfa-fed ruminants compared to those fed stover, grass, or concentrate, and the highest proportions of *Treponema* were 1.05 and 3.05 % in the rumen of sheep and dairy cows, respectively. The values observed in our in vitro study were approximately 10-fold higher than the results of the above-mentioned studies. A possible explanation for this difference may be that the in vitro environment was not able to support the survival of all of the ruminal bacteria, whereas the members of *Treponema* were able to quickly adapt to the in vitro conditions and thus became the dominant group. In addition, starch as the sole substrate supported the poorest growth of *Treponema*, suggesting that the members of this group are unable to utilize starch. In contrast, an increase in the proportion of *Treponema* was always observed ($P < 0.05$) in comparisons of Pe with St and of CSP with CSS, suggesting that pectin stimulates the growth of *Treponema* members. This finding is consistent with the results obtained using PCR-DGGE techniques, confirming the hypothesis that various members of *Treponema* are specifically involved in pectin digestion. Similar to the results reported by Liu et al. [18], who found that the population of *T. saccharophilum* is significantly higher with alfalfa as the substrate compared with corn stover ($P < 0.05$), the *Treponema* group also showed the highest growth in the AH treatment, likely due to the rich pectin content found in alfalfa hay.

Besides, Paster and Canale-Parola [23] suggested that the anaerobic degradation of pectin by *T. saccharophilum* was undertaken via the Entner-Doudoroff pathway, generating

Table 3 Identification of PCR-DGGE bands

Band	Clone ID	Species (GenBank accession no.)	% Similarity
B1	1–3	*Treponema porcinum* strain 14V28 (NR_042942)	92
	4–7	*Treponema bryantii* strain RUS-1 (NR_104781)	95
	8	*Treponema bryantii* strain RUS-1 (NR_104781)	90
	9	*Treponema bryantii* strain RUS-1 (NR_104781)	93
	10	*Treponema succinifaciens* DSM 2489 (NR_074755)	92
	11	*Treponema succinifaciens* DSM 2489 (NR_074755)	91
	12	*Treponema primitia* ZAS-2 (NR_074169)	89
	13	*Treponema primitia* ZAS-2 (NR_074169)	85
	14	*Treponema saccharophilum* strain ATCC 43261 (NR_044745)	99
	15	*Treponema saccharophilum* strain ATCC 43261 (NR_044745)	86
	16	*Treponema zuelzerae* strain DSM 1903 (NR_104797)	88
	17	*Treponema denticola* ATCC 35405 (NR_074582)	91
	18	*Ruminococcus gnavus* (NR_036800)	98
	19	*Lutaonella thermophilus* (NR_044451)	91
	20	*Corynebacterium ureicelerivorans* (NR_042558)	99
	21	*Prevotella denticola* F0289 (NR_102922)	95
	22	*Prevotella maculosa* strain W1609 (NR_044270)	97
B2	23	*Prevotella maculosa* strain W1609 (NR_044270)	95
	24	*Prevotella maculosa* strain W1609 (NR_044270)	96
	25,26	*Ruminobacter amylophilus* strain H18 (NR_026450)	93
	27	*Prevotella baroniae* (NR_043224)	96
	28,29	*Prevotella dentalis* DSM 3688 (NR_102481)	97
	30	*Prevotella dentalis* DSM 3688 (NR_102481)	96
B3	31	*Prevotella bryantii* B14 (NR_028866)	91
	32	*Prevotella bryantii* B14 (NR_028866)	93
	33,34	*Prevotella denticola* F0289 (NR_102922)	94
	35	*Prevotella maculosa* strain W1609 (NR_044270)	97
	36	*Prevotella maculosa* strain W1609 (NR_044270)	96
	37	*Clostridium populeti* strain 743A (NR_026103)	99
	38	*Eubacterium ruminantium* (NR_024661)	96

acetate as a major end product of pectin fermentation. Using two small pectinolytic spirochetes (strains 692 and 791) isolated from the rumen, Ziołecki 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 Ziołecki [36] described three isolated large rumen *Treponema* members (strains 606, 709 and 710) with outstanding feature of pectinolytic activity. Pectin is decomposed by *Treponema* strains via trans-elimination mechanism, yielding a mixture of saturated and unsaturated degradation products. Similar to *T. saccharophilum*, 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 *Treponema* involved in the rumen pectin digestion.

However, it should be noted that the CS treatment also supported a relatively significant growth of the *Treponema* group that is similar to that found using pectin alone as the substrate. Moreover, the proportion of the *Treponema* 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 *Treponema* in terms of substrate utilization: one group is highly specialized in pectin utilization, and the other group is involved in fiber metabolism. Rumen spirochaetes, which are predominantly *Treponema* [24], have often been observed during the study or isolation of cellulolytic bacteria [30].

T. bryantii is likely a representative of the *Treponema* group that does not favor pectin utilization but is involved in fiber digestion. In the present study, the highest

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

Target species	Treatments*							SEM	*P* value
	Bk	CS	AH	CSP	CSS	Pe	St		
Treponema 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
Treponema bryantii	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
Fibrobacter succinogenes	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
Ruminococcus albus	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
Ruminococcus flavefaciens	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
Prevotella ruminicola	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 significantly different ($P < 0.05$)

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

population of *T. bryantii* 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. bryantii*, Stanton and Canale-Parola [28] showed a beneficial interaction of *T. bryantii* with the cellulolytic bacterium *F. succinogenes*. *F. succinogenes* and *R. albus* are able to degrade pectin through secreted pectate lyases [4]. Gradel and Dehority [10] also demonstrated that the cellulolytic strain *R. flavefaciens* B34b and *R. albus* 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. ruminicola is well-known ruminal amylolytic bacteria [30] and have also been reported as important rumen pectinolytic bacteria [10]. The population of *P. ruminicola* was significantly 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. ruminicola* can efficiently utilize pectin when this species is co-cultured with a cellulolytic species [9].

The present study provides the first exploration of the specific relationship of the *Treponema* 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. ruminicola* 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 *Treponema* in vivo, and the isolation of new species of *Treponema* is required to fully explore the ecological mechanism underlying the digestion of pectin by these species.

Acknowledgments This research was supported by the Grants from the National Basic Research Program of China Ministry of Science and Technology (Grant No. 2011CB100801). The authors are thankful to Dr. Chris McSweeney at CSIRO Livestock Industries, Australia, for donating the pure rumen bacterial strains.

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