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

Jing Liu · Yi-Yi Pu · Qian Xie · Jia-Kun Wang · Jian-Xin Liu

Received: 6 March 2014/Accepted: 22 June 2014/Published online: 2 September 2014 Springer Science+Business Media New York 2014

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*ie e a genus. The growth of the Tee a genus was remarkably supported by the inclusion of pectin,

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.

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 microecosystem.

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

J. Liu · Y.-Y. Pu · Q. Xie · J.-K. Wang (⊠) · J.-X. Liu (⊠) MoE Key Laboratory of Molecular Animal Nutrition, Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China e-mail: jiakunwang@zju.edu.cn

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		mer 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 i e e a group	F	GGCAGCAGCTAAGAATATTCC	575	88	Bekele et al. [3]	
	R	CCGTCAATTCCTTTGAGTTT			Watanabe et al. [34]	
Tie e a bi a ii (B25)	F	AGTCGAGCGGTAAGATTG	421	97	Tajima et al. [32]	
	R	CAAAGCGTTTCTCTCACT			Tajima et al. [31]	
Fibi bac ei Acci ge e (S85)	F	GTTCGGAATTACTGGGCGTAAA	121	96	Denman and McSweeney [6]	
	R	CGCCTGCCCTGAACTATC			Denman and McSweeney [6]	
Rr i c cer albr (8)	F	CCCTAAAAGCAGTCTTAGTTCG	176	93	Koike and Kobayashi [16]	
	R	CCTCCTTGCGGTTAGAACA			Koike and Kobayashi [16]	
R i c cc i e efacie (Y1)	F	CGAACGGAGATAATTTGAGTTTACTTAGG	132	92	Denman and McSweeney [6]	
5	R	CGGTCTCTGTATGTTATGAGGTATTACC			Denman and McSweeney [6]	
Pie ella 🏞 i ic la	F	GAAAGTCGGATTAATGCTCTATGTTG	74	99	Stevenson and Weimer [29]	
(ATCC19189)	R	CATCCTATAGCGGTAAACCTTTGG			Stevenson and Weimer [29]	

The culture stains used are indicated in brackets

respective pure-cultured strains including *P.* fr *i ic la* ATCC19189, *T. b*; *a ii* B25, *Fib*; *bac e*; *frci ge e* S85, *R*, *i c cc*, *alb*, 8, and *R. frci ge e* S85, *R*, *i c cc*, *alb*, 8, and *R. frci 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. alb*, 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 μ l of 2 × SYBR Premix Ex Taq, 0.4 μ l of 50 × ROX Reference Dye, 10 ng of the template DNA, and 0.2 μ M of each primer in a total volume of 20 μ l. 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,

Item [#]	Treatments*							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

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

 $^{\rm a}$ 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 diand 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 amplied using the primers GC-338F and 533R. The treatments are indicated at the *f he la e* (*CS* corn stover, *AH* alfalfa hay, *Pe* pectin, *S* corn starch, *CSP* corn stover and pectin, *CSS* corn stover and corn starch). The h = h e e e f 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* (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

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 Tre e a may be extensively involved in pectin digestion. It has been reported that T. *i* leckii ferments only pectin and lacks the ability to utilize starch [25]. Although both pectin and starch support the growth of T. acchai hit, 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. acchai hit, 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 *Tie* 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 Tee e a in alfalfa-fed ruminants compared to those fed stover, grass, or concentrate, and the highest proportions of Tee e a

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 Tee e a, whereas B2 and B3 were dominated with species of Pee ella, 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 Tie e a have been described: *T. bi* a *ii* [28], *T. acchai* hit. [23], and *T. i leckii* [25]. A phylogenetic study conducted by Bekele et al. [3] suggested the existence of distinct

Table 3 Identi cation of PCR-DGGE bands

Band	Clone ID	Species (GenBank accession no.)	% Similarity
B1	1 3	<i>Tie e a ici</i> strain 14V28 (NR_042942)	92
	4 7	Tie e a bi 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 *Tie e a* members (strains 606, 709 and 710) with outstanding feature of pectinolytic activity. Pectin is decomposed by Tee e a strains via trans-elimination mechanism, yielding a mixture of saturated and unsaturated degradation products. Similar to T. acchae hit, 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 $T_{ee} = e a$ involved in the rumen pectin digestion.

However, it should be noted that the CS treatment also supported a relatively signi cant growth of the Teee a group that is similar to that found using pectin alone as the substrate. Moreover, the proportion of the Teee 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 *Tie 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 Tie e a [24], have often been observed during the study or isolation of cellulolytic bacteria [30].

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

Target species	Treatments*							SEM	P value
	Bk	CS	AH	CSP	CSS	Pe	St		
<i>Tie e a</i> 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
Tie eabiaii	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
Fibs bac estacci 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
Rr i c ctr albr	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 ct i efacie	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
Pie ella 🛧 i ic la	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

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)

^{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. alb*. are able to degrade pectin through secreted pectate lyases [4]. Gradel and Dehority [10] also demonstrated that the cellulolytic strain *R. cefacie* B34b and *R. alb*. 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. \uparrow , *i ic la* is well-known ruminal amylolytic bacteria [30] and have also been reported as important rumen pectinolytic bacteria [10]. The population of *P.* \uparrow , *i ic la* 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.* \uparrow , *i ic la* 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* ie *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 i ic la 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* ie e a in vivo, and the isolation of new species of *Tie e a* 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.

References

- 1. Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. J Mol Biol 215(3):403 410
- Ariza P, Bach A, Stern MD et al (2001) Effects of carbohydrates from citrus pulp and hominy feed on microbial fermentation in continuous culture. J Anim Sci 79(10):2713 2718
- Bekele AZ, Koike S, Kobayashi Y (2011) Phylogenetic diversity and dietary association of rumen Treponema revealed using group-speci c 16S rRNA gene-based analysis. FEMS Microbiol Lett 316(1):51 60
- 4. Cai SC, Li JB, Hu FZ et al (2010) Cettal il iter tr i ic la a newly described rumen bacterium that possesses redundant brolytic-protein-encoding genes and degrades lignocellulose with multiple carbohydrate-borne brolytic enzymes. Appl Environ Microbiol 76:3818 3824
- Chen Y, Penner GB, Li M et al (2011) Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. Appl Environ Microbiol 77(16):5770 5781
- 6. Denman SE, McSweeney CS (2006) Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol Ecol 58(3):572 582
- Dice LR (1945) Measures of the Amount of Ecologic Association Between Species. Ecology 26(3):297 302
- Ďuskova D, Marounek M (2001) Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium Lachnospira multiparus. Lett App Microbiol 33(2):159 163
- Fondevila M, Dehority BA (1994) Degradation and utilization of forage hemicellulose by rumen bacteria, singly in coculture or added sequentially. J Appl Bacteriol 77(5):541 548
- Gradel CM, Dehority BA (1972) Fermentation of isolated pectin and pectin from intact forages by pure cultures of rumen bacteria. Appl Microbiol 23(2):332 340
- Hall MB, Larson CC, Wilcox CJ (2010) Carbohydrate source and protein degradability alter lactation, ruminal, and blood measures. J Dairy Sci 93(1):311 322

- Hu W-L, Liu J-X, Ye J-A et al (2005) Effect of tea saponin on rumen fermentation in vitro. Anim Feed Sci Technol 120(3): 333 339
- Huws SA, Lee MRF, Muetzel SM et al (2010) Forage type and sh oil cause shifts in rumen bacterial diversity. FEMS Microbiol Lett 73(2):396 407
- Kasperowicz A (1993) Comparison of utilization of pectins from various sources by pure cultures of pectinolytic rumen bacteria and mixed cultures of rumen microorganisms. Acta Microbiol Polonica 43(1):47 56
- 15. Kocherginskaya SA, Aminov RI, White BA (2001) Analysis of the Rumen Bacterial Diversity under two Different Diet Conditions using Denaturing Gradient Gel Electrophoresis, Random Sequencing, and Statistical Ecology Approaches. Anaerobe 7(3):119 134
- Koike S, Kobayashi Y (2001) Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus avefaciens. FEMS Microbiol Lett 204(2):361 366
- Koike S, Yabuki H, Kobayashi Y (2007) Validation and application of real-time polymerase chain reaction assays for representative rumen bacteria. Anim Sci J 78(2):135 141
- Liu J, Wang J-K, Zhu W et al (2013) Monitoring the rumen pectinolytic bacteria Treponema saccharophilum using real-time PCR. FEMS Microbiol Ecol 87(3):576 585
- Marounek M, Ďuskova D (1999) Metabolism of pectin in rumen bacteria Butyrivibrio brisolvens and Prevotella ruminicola. Lett Appl Microbiol 29(6):429 433
- 20. Marounek M, Bartos S, Brezina P (1985) Factors In uencing the Production of Volatile Fatty Acids from Hemicellulose, Pectin and Starch by Mixed Culture of Rumen Microorganisms. Zeitschrift fur Tierphysiologie Tierernahrung und Futtermittelkunde 53(1 5):50 58
- Mertens DR (2002) Nutritional implications of ber and carbohydrate characteristics of corn silage and alfalfa hay. California Animal Nutrition Conf Fresno, CA:94 107
- Mullen CA, Boateng AA, Goldberg NM et al (2010) Bio-oil and bio-char production from corn cobs and stover by fast pyrolysis. Biomass Bioenergy 34(1):67 74
- Paster BJ, Canale-Parola E (1985) Treponema saccharophilum sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl Environ Microbiol 50(2):212 219
- 24. Paster BJ, Dewhirst FE, Weisburg WG et al (1991) Phylogenetic analysis of the spirochetes. J Bacteriol 173(19):6101 6109
- Piknova M, Guczynska W, Miltko R et al (2008) Treponema zioleckii sp. nov., a novel fructan-utilizing species of rumen treponemes. FEMS Microbiol Lett 289(2):166 172
- 26. Poulsen M, Jensen BB, Engberg RM (2012) The effect of pectin, corn and wheat starch, inulin and pH on in vitro production of methane, short chain fatty acids and on the microbial community composition in rumen uid. Anaerobe 18(1):83 90
- SAS Institute (2000) SAS User's Guide: Statistics. Version 8.01. SAS Inst. Inc., Cary, NC

- Stanton TB, Canale-Parola E (1980) Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch Microbiol 127:145 156
- 29. Stevenson DM, Weimer PJ (2007) Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quanti cation real-time PCR. Appl Microbiol Biotechnol 75(1):165 174
- Stewart CS, Flint HJ, Bryant MP (1997) The rumen bacteria. In: Hobson PN, Stewart CS (eds) The rumen microbial ecosystem, 2nd edn. Blackie Academic & Professional, London, pp 10 72
- Tajima K, Aminov RI, Nagamine T et al (2001) Diet-dependent shifts in the bacterial population of the rumen revealed with realtime PCR. Appl Environ Microbiol 67(6):2766 2774
- 32. Theodorou MK, Williamsa BA, Dhanoaa MS et al (1994) A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Technol 48(3–4):185–197
- Van Soest PJ (1995) What constitutes alfalfa quality: New considerations. In: Proceedings of the 25th Natl Alfalfa Symposium, Liverpool, NY:1 15
- 34. Watanabe K, Kodama Y, Harayama S (2001) Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community ngerprinting. J Microbiol Methods 44(3):253 262
- Wojciechowicz M (1970) Partial characterization of pectinolytic enzymes of Bacteroides ruminicola isolated from the rumen of a sheep. Acta Microbiol Polonica Series A 3(1):45 56
- Wojciechowicz M, Zio ecki A (1979) Pectinolytic enzymes of large rumen treponemes. Appl Environ Microbiol 37(1):136–142
- Xiao Z, Storms R, Tsang A (2006) A quantitative starch iodine method for measuring alpha-amylase and glucoamylase activities. Anal Biochem 351(1):146–148
- Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36(5):808 812
- Yuan P, Meng K, Wang Y et al (2012) Abundance and genetic diversity of microbial polygalacturonase and pectate lyase in the sheep rumen ecosystem. PLoS ONE 7(7):e40940
- 40. Zhang C, Guo Y, Yuan Z et al (2008) Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial ora *i i i* Anim Feed Sci Technol 146(3):259 269
- 41. Zhu W, Fu Y, Wang B et al (2013) Effects of dietary forage sources on rumen microbial protein synthesis and milk performance in early lactating dairy cows. J Dairy Sci 96:1727 1734
- Zio ecki A (1979) Isolation and characterization of large treponemes from the bovine rumen. Appl Environ Microbiol 37(1):131 135
- Zio ecki A, Wojciechowicz M (1980) Small pectinolytic spirochetes from the rumen. Appl Environ Microbiol 39(4):919 922