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Full Length Research Paper

Comparison of bacterial diversity in large intestine of Xiangxi yellow cattle (*Bos taurus*) associated with different diet: Fresh *Miscanthus sinensis* and mixed forage

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Ruminants harbour a complicate ecosystem consisting mostly of microorganisms which have the ability to digest the fibrous cell walls of plant materials from host's food. To discover the novel enzyme from ruminant's digestive tract for exploration of the new biofuel plant-*Miscanthus sinensis*, we comparatively analyzed the distribution of microbiome in the large intestine of two groups of local cattle fed with either *M. sinensis* [*M. sinensis* (+) group] or mixed forage [*M. sinensis* (-) group]. Two libraries of 16S rRNA gene from intestinal microbiome of two groups of local cattle were constructed respectively, and subjected to restriction fragment length polymorphism (RFLP) and sequence analysis. After analyzing the sequences of 16S rRNA genes, our results indicated that the intestinal bacteria of *M. sinensis* (+) group were composed of *Firmicutes* (48.89%), *Bacteroidetes* (6.67%), rumen bacteria (10%) and uncultured bacteria (34.44%), and the intestinal bacteria of *M. sinensis* (-) group were composed of *Firmicutes* (52.87%), *Bacteroidetes* (1.27%), rumen bacteria (9.55%), uncultured rumen bacteria (4.46%) and uncultured bacteria (31.21%). As expected, we found that five species of potential cellulolytic becteria can only be detected in the library of *M. sinensis* (+). Besides, through phylogenetic tree analysis, we found that the ratio of uncultured genus which were lack of recognized sequences in *M. sinensis* (+) library and *M. sinensis* (-) library were 55.96 and 51.56%, respectively, indicating that the cattle fed on *M. sinensis* will produce more novel uncultured genus which probably have specific metabolic effect on decomposition of *M. sinensis*.

Key words: Cattle, bacterial diversity, large intestine, *Miscanthus sinensis*, 16S rRNA gene library, phylogenetic analysis.

INTRODUCTION

Cattle is a functionally important ruminant animal that was able to digest plant materials with high fiber concentration, as in its gastrointestinal tract there was a complex community of fibrolytic aerophilic and anaerobic microbes (Pandya et al., 2010). Most of the previous studies for investigating the microbial community in digestive system of ruminants usually focused on rumen, the largest and most important stomach compartment (Tajima et al., 1999, 2000; Pitta et al., 2010). However, the large intestine, especially the caecum and colon, has also received a lot of attention since it is also an important organ for nutrition supply and absorption of ruminant. Some studies suggested that the large intestine provide an active fermentation condition similar to the reticulo-rumen (Schwarz, 2001). Previous studies indicated

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that the large intestine of cattle is an important organ for digesting glucose and soluble α-glucoside (Huntington et al., 2006; Noziere et al., 2005; Warner et al., 1972). In addition, cellulose and hemicellulose have also been reported to be largely digested in the large intestine of cattle, as the digestion of cellulose accounted for 18% to 27% of total digested cellulose, and the hemicellulose digested in the large intestine accounted for 30 to 40% of the total hemicellulose digestion (Hoover, 1978; Warner et al., 1972). It is reasonable that the large intestine has a strong digestive ability for cellulose and hemicellulose, as cecal bacteria in large intestine produce some special cellulolytic enzymes that could hydrolyze pentosans and hemicellulose (Bailey and MacRea, 1970). Thus, to identify novel fibrolytic enzymes with strong digestive ability for cellulose from the large intesteine's microorganism of cattle would be feasible and significant.

Miscanthus sinensis, as an abundant source of biomass for biopower or biofules (Ge et al., 2011; Paul et al., 2011), has been of great interest to a lot of European countries. Retroactively, in 1992, 'European Miscanthus Network' has been established (Vega et al., 1997). A kind of Chinese *M. sinensis*, called Chinese silvergrass, has been found to be extensively distributed in China. With high cellulose content and high yield, Chinese *M. sinensis* would be a gold plant for providing energy by biotransformation or chemical conversion or combination of both approaches (Shumny et al., 2011). Due to the complexity of lignocellulose's frame, this plant is very difficult to be degraded (Vega et al., 1997). However, the ruminant animal such as cattle would be able to digest *M. sinensis* as the result of numerous cellulolytic microorganisms habiting in its digestive tract (Maas and Glass, 1991; Ogura, 2011), which could be a potential source for identifying bio-enzyme. In consideration of this, we proposed that *M. sinensis*-fed cattle would have an increased population of cellulolytic microorganisms in rumen and large intesteine which provide much higher possibility for us to identify novel fibrolytic enzymes.

Our final goal is to identify novel high efficient fibrolytic enzyme from cellulolytic microorganisms for exploitation of the new biofuel plant-*M. sinensis*. To start this project, it is of significance for us to first understand the complexity and diversity of microorganisms in the digestive system of cattle which were fed on *M. sinensis,* which would not only increase our chances to identify novel fibrolytic enzyme, but also provide more knowledge for understanding *M. sinensis*-fed cattle's physiology and nutrition. Although many investigations have been carried out to study microbial diversity in rumen of cattle (Bekele et al., 2010, 2011; Chen et al., 2011), and our investigation on microbial diversity in rumen of *M. sinensis*-fed cattle are also ongoing, the phylogenetic diversity in the large intestine of cattle, especially for *M. sinensis*-fed cattle, has rarely been studied. In the present study, we extracted the total microorganism's DNA from large intestines of *M. sinensis*-fed Xiangxi Yellow Cattle (*Bos taurs*) as well as mixed forage-fed cattle respectively, and then followed by polymerase chain reaction (PCR), cloning, and sequencing of 16S rRNA genes. We found that the distribution of large intestinal microbiome varied with the diets. Five species of potential cellulolytic becteria can only be detected in the library *M. sinensis* (+) group. Moreover, we also found that a more significant part of unidentified sequences showed in phylogenetic tree analysis of *M. sinensis* (+) library, indicating that the cattle fed on *M. sinensis* can produce more novel uncultured genus which probably have specific metabolic effect on decomposition of *M. sinensis*, and these sequences data were previously unidentified.

MATERIALS AND METHODS

Animal diet, rumen content collection

Six male cattle sampled were all from the same colony maintained in Liu Yang (Hunan provice, eastern region of China), belonging to Xiangxi Yellow Cattle (*Bos taurs*), the most widely distributed cattle in Hunan Province. They were separated into two groups. One group including three cattle were always fed on common feedstuff, including vinasse, bean dregs, dry straw (3:3:1), while the other group including three cattle were only given fresh *M. sinensis* diet from 2.5 years old for 18 months. All of the cattle in this experiment were the same age and their weight were 100±3 kg.

Each diet was fed for 18 months and large intestinal samples were derived from each group of animals. Briefly, the whole contents in large guts of each group of cattle were extracted by hand with sterile gloves and were thoroughly mixed, then these pooled samples were separated in small aliquots (50±1 g) and were frozen by liquid nitrogen. Samples were stored at -80°C until DNA extraction.

Isolation and purification of genomic DNA

The genomic DNA was extracted from the large gut content of both treatments using the modified method as below. Briefly, 10 g sample was suspended completely in 30 mL PBS (pH 7.4) by bead-beating for 20-30 min, then centrifuged at 600×g for 10 min at 4°C. The supernatant was collected and centrifuged again at 8000×g for 10 min to obtain the cell pellet. 6.3 mL DNA extraction buffer [1500 mM NaCl, 100 mM Tris-HCl (pH 8.0), 100 mM PBS, 100 mM EDTA, 2% CTAB], 2% (w/v) PVPP, 50 µL lysozyme (100 µg ml^{-1}) were added to the pellet, then incubated at 37°C for 30 min. Furthermore, the mixture was added with proteinase K (50 µg ml^{-1}) and incubated at 37°C for 30 min followed by added with 0.7 mL 20% SDS and incubated at 65°C for another 90 min. After that, the whole mixture was centrifuged at 8000×g for 15 min and the supernatant was transferred to a clean tube. The liquid was then purified twice with chloroform: isoamyl alcohol (24:1). Two volumes of ethanol were used to precipitate DNA.

Primers and PCR conditions

The PCR reaction was conducted using the 27F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse primer (5'-GGTTACCTTGTTACGACTT-3') (Pandya et al., 2010). Ex Taq polymerase (TaKaRa, Dalian, China) was used for PCR thermocycling. Each 50 µL PCR reaction contained: 30 ng of purified bacterial genomic DNA, 200 µmol of dNTP mixture, 5 µL of

10×PCR Buffer, 10 pmol of each primer. PCR amplification was performed using the following program: denaturing at 95°C for 5 min, followed by 30 cycles of 30 s of denaturing at 95°C, 30 s of annealing at 58°C and 2 min of elongation at 72°C, with a final extension at 72°C for 10 min.

16S rRNA gene library construction

The purified PCR products were cloned into pMD-18 T vecter (TaKaRa, Dalian, China), and recombinant plasmids were transformed into competent *Escherichia coli* DH5α cells. Positive clones grew after incubation overnight at 37°C. The 16S rRNA gene library from Chinese silvergrass group was named as *M. sinensis* (+) library (S) and another 16S rRNA gene library from mixed forage group was *M. sinensis* (-) library (D).

Restriction fragment length polymorphism (RFLP) screening of 16S rRNA gene clones

Inserted rRNA genes from recombinant clones were reamplified by PCR (common primers-M13). Aliquots of crude reamplified rRNA gene PCR products were digested twice with 0.5U of Mspl and Afal (TaKaRa, Dalian, China) in a final volume of 15 µL, for 3 h at 37°C. The operational taxonmic units (OTUs) was initially classified based on the RFLP patterns.

Sequencing and phylogenetic analysis

Positive clones were sequenced by company (BGI, Beijing Genomics Institute, China). The sequences were automatically aligned using CLUSTAL X, and phylogenetic trees were constructed by neighbor-joining distance matrix methods with the programs in the software package Mega, version 4.0. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. Good's coverage was calculated as [1-(*n*/*N*)]×100%, where *n* is the number of single-clone OTUs and *N* is the library size, that is, the total number of clones for the analyzed sample (Schloss and Handelsman, 2005).

RESULTS

Bacterial community structure in the large intestine of *M. sinensis* (+) group and *M. sinensis* (-) group

To analyze the differences of bacterial community structure in the large intestine between *M. sinensis* (+) group (*M. sinensis*-fed cattle) and *M. sinensis* (-) group (mixed forage-fed cattle), two 16S rRNA gene libraries were constructed. A total of 109 16S rDNA clones, grouped into 41 OTUs, from *M. sinensis* (+) library and 128 16S rDNA clones, typed into 43 OTUs, from *M. sinensis* (-) library were analyzed. The OTUs based on RFLP and the accession numbers of the closest relative sequences were categorized in Tables 1 and 2, respectively. As a result, in *M. sinensis* (+) library, about 58.72% clones (64 clones) had <97% similarity and 31.28% clones (45 clones) had ≥97% similarity to 16S rRNA gene data sequences from GeneBank by BLAST. All the sequences consisted of *Firmicutes* (48.89%), *Bacteroidetes* (6.67%), rumen bacteria (10%) and uncultured bacteria (34.44%) (Table 3), including a diverse array of bacterial species: *Clostridium*, *Acetivibrio*, *Butyrivibrio*, *Eubacterium*, *Sporobacter*, *Oscillospira*, *Lachnospira*, *Ruminococcus*, *Anaerostipes*, *Marinilabilia*. Moreover, eight OTUs (S-2, S-5, S-18, S-20; S-33; S-12, S-29; S-14) could only be grouped at the family level (*Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae* or *Clostridiaceae*); two OTUs (S-16, S-22) could only be identified at the order level (*Clostridiales*); and an unique OUT (S-9) could only be labeled at phylum level (*Bacteroidetes*); others could only be classified to bacteria. Among all of the analyzed sequences, the most abundant OTU was S-37, which comprised 9.17% of the analyzed clones, belonging to uncultured bacterium (Table 1).

However, in *M. sinensis* (-) library, about 48.44% clones (62 clones) had <97% similarity and 51.56% clones (66 clones) had ≥97% similarity to the known 16S rRNA gene sequences of NCBI database (www.ncbi.nlm.nih.gov/blast). These corresponded to *Firmicutes* (52.87%), *Bacteroidetes* (1.27%), rumen bacteria (9.55%), uncultured rumen bacteria (4.46%) and uncultured bacteria (31.21%) (Table 3), including *Christensenella*, *Eubacterium*, *Acetomicrobium*, *Clostridium*, *Butyricicoccus*, *Oscillospira*, *Acetivibrio*, *Sporobacter*, *Oscillibacter*, *Lachnospira*, *Ruminococcus*, *Hydrogenoanaero bacterium*. And seven OTUs (D-2, D-22; D-18; D-10, D-13, D-14, D-16) could only be ascribed at the family level (*Lachnospiraceae*, *Clostridiaceae* or *Ruminococcaceae*); five OTUs (D-5) could only be identified at the order level (*Clostridiales*). Besides, D-22 was showed as the most abundant OTU, including 9 clones (7.03%) of all, which was considered as *Lachnospiraceae bacterium* (Table 2).

Phylogenetic analysis of *M. sinensis* (+) group and *M. sinensis* (-) group

The phylogenetic tree was constructed to further investigate bacteria's taxonomic affiliation. BLAST searching tool had been used to find the neighbors of these sequences, and 2-3 the nearest relative sequences were chosen to construct the phylogenetic tree together with the representative sequenced clones of each OTUs.

In the library of the *M. sinensis* (+), all 109 sequences underwent in a phylogenetic analysis and could be classified into 41 OTUs. As shown in Table 1, almost 1/3 of the representive clones (13 OTUs) were close to uncultured bacteria. Furthermore, within the phylum Firmicutes, *Lachnospiraceae bacterium* (S-2, S-5, S-18, S-20) was the prevalent bacterial genera, whose proportion was 14.45% in the whole library, followed by uncultured *Ruminococcaceae* (5.56%), while the abundance of *Bacteroidetese* was 6.67%. The total library of the *M. sinensis* (+) contained 9 single-clone OTUs, and the Good's coverage of this library was

Table 1. Similarity values of operational taxonomic units (OTUs) based on RFLP of 16S rRNA gene sequences derived from *M. sinensis* (+) library.

OTU	Length (bp)	No. of clones	Sequence identity (%)	Nearest valid relative (GeneBank accession no.)
S1	1495	3	94	UB (EU466462)
S2	1462	2	97	*Lachnospiraceae bacterium* (AF550610)
S3	1491	3	90	*Acetivibrio sp.* (AB596889)
S4	1493	1	92	*T.aceticus* (Z49863)
S5	1486	5	98	*Lachnospiraceae bacterium* (HM099641)
S6	1497	3	94	UB (FJ848445)
S7	1486	2	97	UB (FJ825506)
S8	1492	3	95	*Eubacterium siraeum* (EU266550)
S9	1496	2	91	*Bacteroidetes bacterium* (GU409274)
S10	1481	2	92	UB (EU469073)
S11	1496	2	93	UB (EU774692)
S12	1487	2	97	*Rikenellaceae bacterium* (EU774692)
S13	1475	3	95	UB (DQ799972)
S14	1491	2	91	*Clostridiaceae bacterium* (AB298755)
S15	1495	5	90	Rumen bacterium (AB239481)
S16	1492	2	94	*Clostridiales bacterium* (AB477432)
S17	1495	3	93	*Clostridium sp.* (AB596885)
S18	1479	5	92	*Lachnospiraceae bacterium* (DQ789118)
S19	1464	1	95	*Oscillospira guilliermondii* (AB040497)
S20	1473	4	93	*Lactobacillales bacterium* (AY581272)
S21	1476	2	95	Rumen bacterium (GU324401)
S22	1492	2	90	*Clostridiales bacterium* (HQ452854.1)
S23	1501	1	94	*Paenibacillus campinasensis* (EU169230.1)
S24	1503	4	89	Rumen bacterium (GU324364)
S25	1493	2	97	UB (FJ951884)
S26	1492	1	89	*Lachnospira pectinoschiza* (AY169414)
S27	1496	1	92	Butyrate-producing bacterium (AY305315)
S28	1497	3	97	UB(FJ848398.1)
S29	1489	2	92	*Rikenellaceae bacterium* (AB298736)
S30	1472	1	95	*B.crossotus* (X89981)
S31	1487	2	97	*Ruminococcus flavefaciens* (AF104841)
S32	1478	3	98	UB (EU466127)
S33	1398	2	96	Uncultured Ruminococcaceae (EU466127)
S34	1478	2	97	UB(FJ681013)
S35	1477	4	97	Uncultured Ruminococcaceae (EU794113)
S36	1472	3	100	UB (GQ451212)
S37	1496	10	99	UB (FJ825523)
S38	1483	1	94	*Marinilabilia salmonicolor* (AB517713.1)
S39	1494	1	96	*Anaerostipes caccae* (AB243986)
S40	1492	2	90	*Lactobacillales bacterium* (EU728724)
S41	1476	5	98	UB (EU475425)

UB=uncultured bacteria, URB=uncultured rumen bacteria, S: *Miscanthus sinensis* (+).

91.74%. That indicated the sequences identified in *M. sinensis* (+) library represent the majority of bacterial diversity present in the large gut. Through phylogenetic analysis of these OTUs, we found there was a relative big part of clade having no recognized sequences. It was named clade II, including 24 OTUs, 61 clones (Figure 1).

In *M. sinensis* (-) library, as the data summarized in Table 2, 11 OTUs, nearly 25.6% clones were grouped to uncultured bacteria. Moreover, among the phylum Firmicutes, uncultured *Ruminococcaceae* (D-10, D-13, D-

Table 2. Similarity values of operational taxonomic units (OTUs) based on RFLP of 16S rRNA gene sequences derived from *M. sinensis* (-) library.

OTU	Length (bp)	No. of clones	Sequence identity (%)	Nearest valid relative (GeneBank accession no.)
D1	1496	5	96	UB (EU464240)
D2	1477	3	95	*Lachnospiraceae bacterium* (AF550610)
D3	1476	2	96	UB (FJ848449)
D4	1460	2	94	URB (GU304334)
D5	1495	2	94	*Clostridiales bacterium* (DQ168656)
D6	1485	2	97	UB (EU774974)
D7	1483	2	99	UB (GQ448460)
D8	1471	2	95	Rumen bacterium (AB239481)
D9	1493	6	98	UB (EU776277)
D10	1475	3	99	Uncultured Ruminococcaceae bacterium (HQ132395)
D11	1472	4	97	UB (EU466343)
D12	1483	4	98	*Acetivibrio sp.* (AB596889)
D13	1477	6	97	Uncultured Ruminococcaceae bacterium (EU794228)
D14	1472	2	98	Uncultured Ruminococcaceae bacterium (EU794227)
D15	1477	2	97	UB (EU465566)
D16	1476	2	97	Uncultured Ruminococcaceae bacterium (EU794158)
D17	1486	2	90	*Hydrogenoanaero bacterium* (EU170433)
D18	1475	6	89	*Clostridiaceae bacterium* (AB298755)
D19	1490	3	90	*T.aceticus* (Z49863)
D20	1468	2	96	UB (EU464238)
D21	1476	4	97	UB (FJ680663)
D22	1484	9	95	*Lachnospiraceae bacterium* (DQ789118)
D23	1477	1	90	Bacterium (DQ286651)
D24	1479	2	91	*Lactobacillales bacterium* (AY581272)
D25	1502	2	90	*Eubacterium oxidoreducens* (FR733672)
D26	1471	2	95	*Ruminococcus sp.* (EU815223)
D27	1483	5	100	*Clostridium sp.* (AB093546)
D28	1472	5	96	Rumen bacterium (GU324408)
D29	1473	2	94	*Christensenella minuta* (AB490809)
D30	1494	1	93	*Lachnospira pectinoschiza* (AY699282)
D31	1496	2	90	*Acetomicrobium faecale* (FR749983)
D32	1476	6	98	UB (FJ848445)
D33	1476	1	94	*Oscillibacter sp.* (HM626173)
D34	1492	1	97	*Oscillospira guilliermondii* (AB040498)
D35	1476	4	97	*Eubacterium siraeum* (L34625)
D36	1477	5	99	UB (EU773761)
D37	1475	3	99	*Rumen bacterium* (GU324404)
D38	1491	1	98	*Butyricicoccus pullicaecorum* (EU410376)
D39	1494	2	96	Uncultured Ruminococcaceae bacterium (EU794237)
D40	1468	3	94	URB (GU304068)
D41	1507	1	99	*Lactococcus lactis subsp.* (EU337112)
D42	1491	1	94	*Blautia schinkii* (X94964)
D43	1473	3	98	UB (FJ848445)

UB=uncultured bacteria, URB=uncultured rumen bacteria, D: *Miscanthus sinensis* (-).

14, D-16) accounted for 15% of the total sequences, *Lachnospiraceae bacterium* (D-2, D-22) accounted for 10.19%, only one OTU D-222 affiliated to the CFB phylum, which had relatively remote similarity with *Acetomicrobium faecale.* Besides, the Good's coverage of this library was 95.32% owing to 6 single-clone OTUs

Table 3. Estimated composition of species of bacteria identified from the two libraries.

S/No		Proportion (%)	
	Taxon	***M. sinensis* (+)S**	***M. sinensis* (-) D**
firmicutes		48.89(21OTUs)	52.87(24OTUs)
1	*Lachnospiraceae bacterium*	14.45(4OTUs)	10.19 (2OTUs)
2	Uncultured Ruminococcaceae bacterium	5.56(1OTU)	15(4OTUs)
3	*Ruminococcus flavefaciens*	2.22(1OTU)	0
4	*Ruminococcus* sp.	0	1.27(1OTU)
5	*Clostridiaceae bacterium*	2.22(1OTU)	5.1(1OTU)
6	*Clostridium* sp.	3.33(1OTU)	5.1(1OTU)
7	*Clostridiales bacterium*	3.33(2OTUs)	2.55(2OTUs)
8	*Lactobacillales bacterium*	2.22(1OTU)	1.27(1OTU)
9	*Lachnospira pectinoschiza*	1.11(1OTU)	0.64(1OTU)
10	*Hydrogenoanaero bacterium*	0	1.27(1OTU)
11	*Eubacterium oxidoreducens*	0	1.27(1OTU)
12	*Eubacterium siraeum*	3.33(1OTU)	3.82(1OTU)
13	*Oscillospira guilliermondii*	1.11(1OTU)	1.27(1OTU)
14	*Oscillibacter* sp.	0	0.64(1OTU)
15	Butyrate-producing bacterium	1.11(1OTU)	0
16	*B.crossotus*	1.11(1OTU)	0
17	*Blautia schinkii*	0	0.64(1OTU)
18	*Butyricicoccus pullicaecorum*	0	0.64(1OTU)
19	*Paenibacillus campinasensis*	1.11(1OTU)	0
20	*Anaerostipes caccae*	1.11(1OTU)	0
21	*Acetivibrio* sp.	3.33(2OTUs)	3.18(1OTU)
22	*T. aceticus*	1.11(1OTU)	2.55(1OTU)
23	*Lactococcus lactis* subsp.	0	0.64(1OTU)
24	*Christensenella minuta*	0	1.27(1OTU)
CFB phylum (Bacteroidetes)		6.67(5OTUs)	1.27(1OTU)
1	Uncultured Bacteroidales	2.22(1OTU)	0
2	Unclassified Bacteroidetes bacterium	1.11(1OTU)	0
3	*Rikenellaceae bacterium*	2.22(2OTUs)	0
4	*Marinilabilia salmonicolor*	1.11(1OTU)	0
5	*Acetomicrobium faecale*	0	1.27(1OTU)
URB		0	4.46(2OTUs)
rumen bacteria		10(2OTUs)	9.55(3OTUs)
unidentified bacteria		0	0.64(1OTU)
UB		34.44(13OTUs)	31.21(11OTUs)

UB=uncultured bacteria, URB=uncultured rumen bacteria, S: *Miscanthus sinensis* (+), D: *Miscanthus sinensis* (-).

in this library. This value also means the chosen clones was representative. Through the phylogenetic analysis, we also could find clade II, including 25 OTUs, 66 clones (Figure 2).

DISCUSSION

In the present study, we for the first time comparatively analyzed the bacterial diversity in large intestine of two

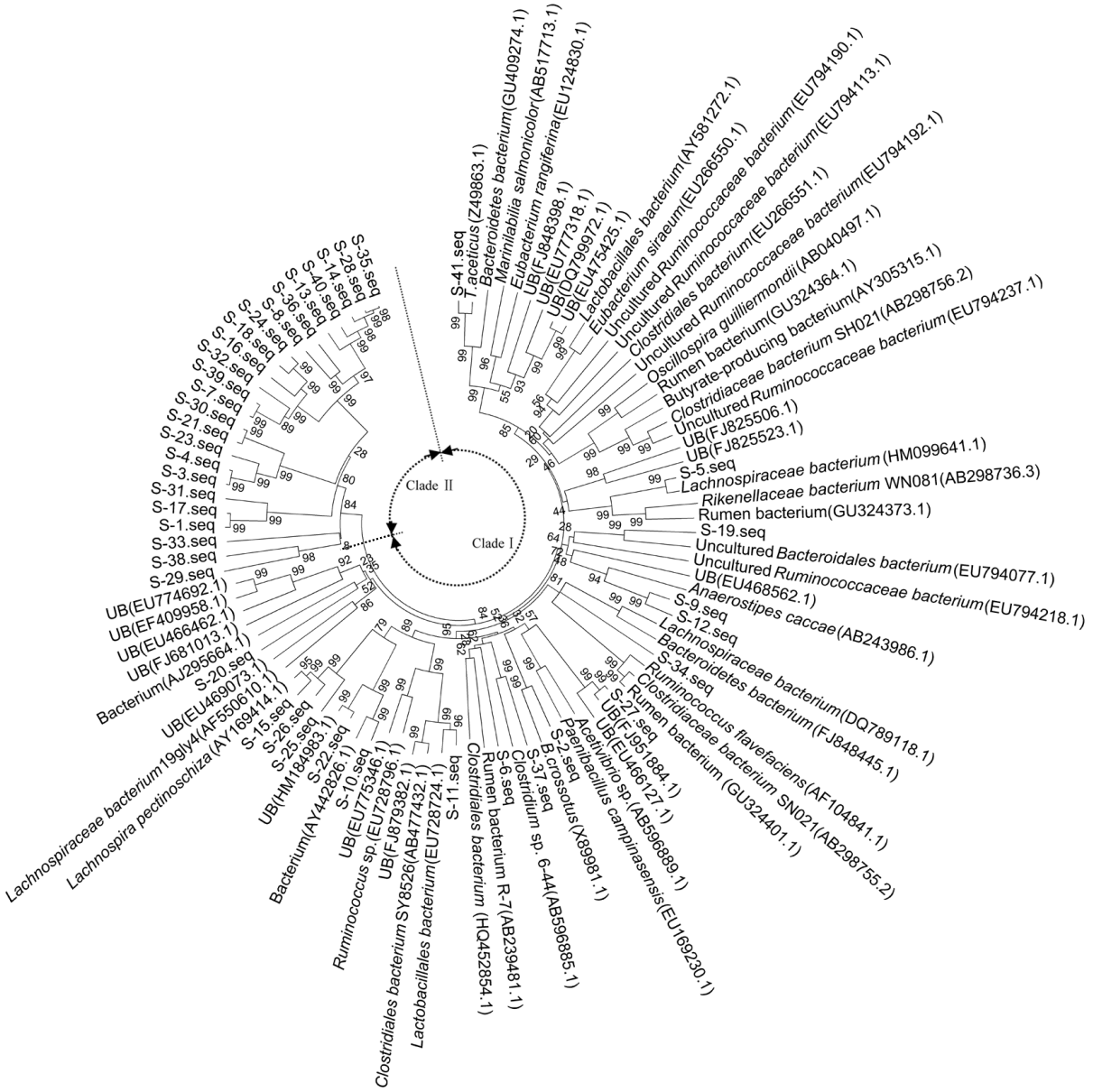

Figure 1. Phylogenetic tree derived from 16S rRNA sequence data recovered from *Bos taurus* (*M. sinensis* (+)) large intestine. The tree was constructed depending on neighbour-joining analysis of a distance matrix obtained from a multiple sequence alignment. Bootstrap value was expressed as a percentage of 1000 trees.

groups of cattle raised with/without *M. sinensis*. Important experimental findings from the present work were summarized below. Sequence analysis of 16S rRNA gene clone libraries constructed using specific PCR primers have been frequently used to analyze diversity of microorganism community (Tajima et al., 1999, 2000, 2007). Based on this method, in this study we found that there are changed diversity profiles and previously rarely detected OTUs in the bacterial community of *M. sinensis* (+) group compared with that of *M. sinensis* (-) group. It was worthy of noting that compared with species in *M. sinensis* (-) library, a few species such as *Ruminococcus flavefaciens*, Butyrate-producing bacterium, *Paenibacillus campinasensis*, *Anaerostipes caccae* could only been identified in *M. sinensis* (+) library. *R. flavefaciens* was a cellulolytic ruminal bacterium that could utilize cellobiose but not glucose as a substrate for growth (Helaszek and White, 1991), and played an important role in the digestion of hemicellulose and cellulose in plant cell walls. It has been reported that they inherented in the pony and donkey cecum (Julliand et al., 1999). Butyrate-producing bacterium was essential for digestion of plant polysaccharides (Paggi and Fay, 2004), and was found in digestive tract of human (Barcenilla et al., 2000).

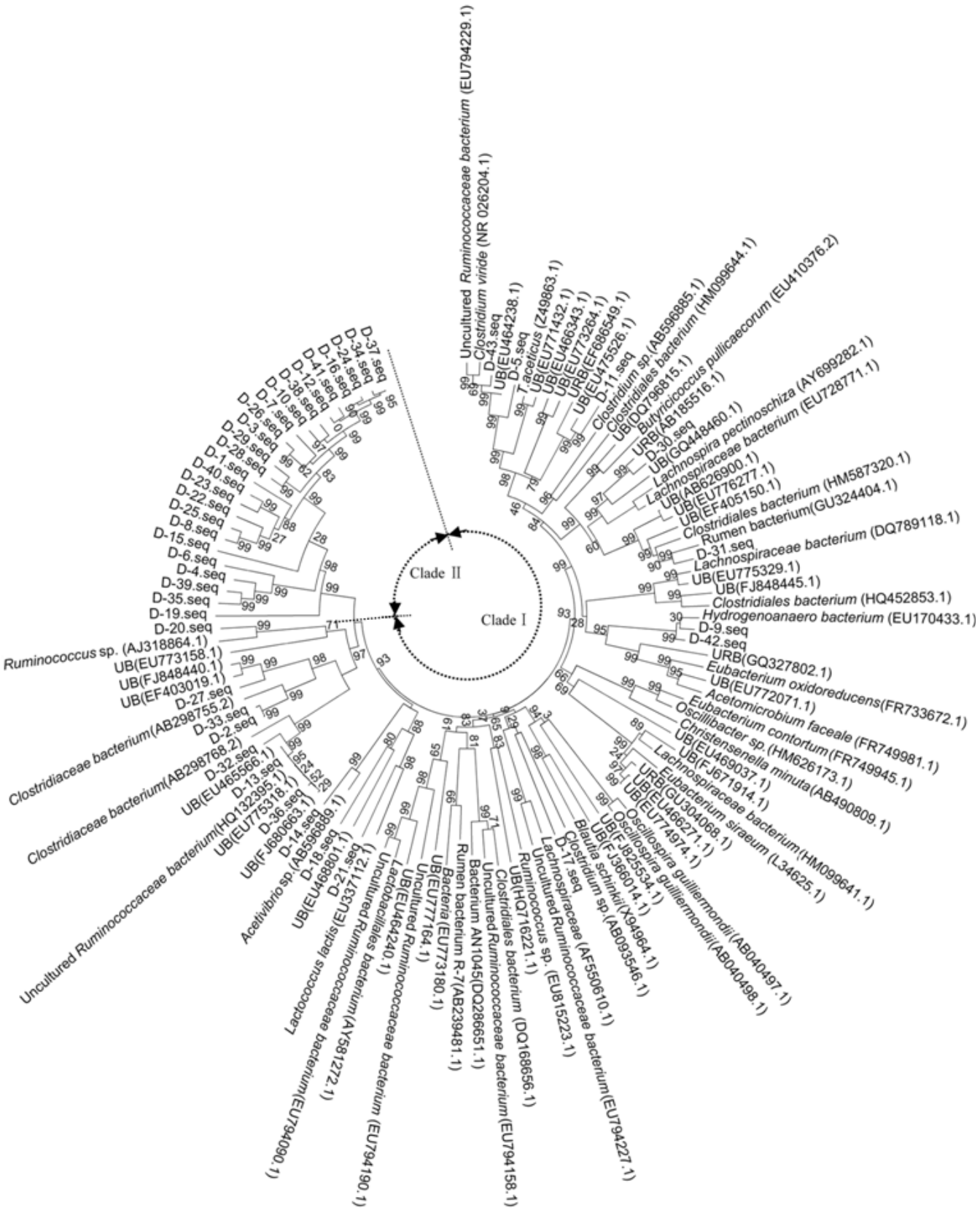

Figure 2. Phylogenetic tree derived from 16S rRNA sequence data recoverd from Bos taurus (*M. sinensis* (-)) large intesine. The tree was constructed depending on neighbour-joining analysis of a distance matrix obtained from a multiple sequence alignment. Bootstrap value was expressed as a percentage of 1000 trees.

Moreover, previous study indicated high-fiber intake increased the amounts of Butyrate-producing bacterium (Mrazek et al., 2006). Additionally, *Paenibacillus campinasensis* has drawn much attention recently. *P. campinasensis* BL11, which produces multiple extracellular polysaccharide-degrading enzymes, including one xylanase, three cellulases, one pectinase and one cyclodextrin glucanotransferase, has a strong potential for plant material degradation and bioresource utilization (Ko et al., 2007). *Anaerostipes caccae* has been identified as a new saccharolytic, acetate-utilising, butyrate-producing bacterium (Schwiertz et al., 2002). As these species of *Firmicutes* were crucial for the host in decomposition of plant-derived material, our result thus implied that the intake of *M. sinensis* was possibly responsible for increasing fibrolytic or cellulolytic bacteria.

Another interesting finding is that the ratio of Lachnospiraceae was significantly increased (4.26%) in the large intestine of *M. sinensis*-fed cattle compared with mixed forage-fed cattle (Table 1). Lachnospiraceae bacterium has been reported as a ubiquitous and important bacteria which could be often detected in the digestive tract of cattle (Dowd et al., 2008). Previous study implied that Lachnospiraceae bacterium could potentially involve in the degradation of cellulose, as Pope et al. (2010) have identified some genes that are homologous to genes present in the genomes of Lachnospiraceae and could express products with carboxymethylcellulase or xylanase activity. Our result also implied that the changes caused by *M. sinensis* might be due in part to variations in Lachnospiraceae. Further research is necessary to evaluate the potential role of Lachnospiraceae bacterium in the degradation of *M. sinensis*.

It is noteworthy that total CFB clones' number was much higher in the large intestine of *M. sinensis*–fed cattle compared to mixed diet-fed cattle (Table 3). Bacteroidetes has been demonstrated to be associated with hydrolysis of polysaccharides in intestine (Mariat et al., 2009; Martens et al., 2009), and high concentration of fiber diet might trigger the increasing trend of Bacteroides in the digestive tract of ruminant. For example, Yuhei et al. (2005) reported that only 14.4% clones belonged to the *Bacteroides fragilis* group in the fecal sample when cattle were fed with forage containing 41% corns, 23% wheat bran, 12% corn byproducts, 8% rapeseed meal, 7% soybean meal. While, Weidong et al. (2007) indicated that 42.2% clones could be assigned to the phylum CFB in gayals (*B. frontails*) when cattle were fed with a diet composed of fresh bamboo leaves and twigs. Pitta et al. (2010) also revealed that bermudagrass with a high fiber content could significantly increase the population of Bacteroides in the digestive tract of cattle. Consistent with previous findings, *M. sinensis* with high concentration of cellulose in our study could result in an increase of Bacteroides. The exact function of Bacteroides in the degradation of *M. sinensis* needs further investigation.

Moreover, from the taxonomic information generated by phylogenetic tree analysis, we found that the ratio of uncultured genus having no recognized relatives (clade II) in *M. sinensis* (+) library and *M. sinensis* (-) library were 55.96 and 51.56%, respectively, indicating that the cattle fed on *M. sinensis* will produce more novel uncultured genus which probably have specific metabolic effect on decomposition of *M. sinensis*. However, these bacteria needed to be further identified and their roles also needed to be deeply explored.

The diversity of micropopulation existing in digestive tract of ruminants has already been ascribed to breed, age, dietary composition, nutrient density and environmental conditions (Leng et al., 2012). Base on the finding of Dowd et al. (2008) they used 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) to study the diversity of cattle faeces, and identified Clostridium, Bacteroides, Porpyhyromonas, Ruminococcus, Alistipes, Lachnospiraceae, Prevotella, Lachnospira, Enterococcus, Oscillospira, Cytophaga, Anaerotruncus, and Acidaminococcus spp as the ubiquitous bacteria. However, in our study, only 6 out of 13 species mentioned above were detected: Clostridium, Bacteroides, Ruminococcus, Lachnospiraceae, Lachnospira, Oscillospira (Table 3). The reason for failing to detect other classification might be: Firstly, the size of our library was relatively small [*M. sinensis* (+) library and *M. sinensis* (-) library had 109 and 128 clones, respectively]. Secondly, experimental PCR biases might be unavoidable, like the specificity of the primers. It is been reported that DNA extracted from *Fibrobacter succinogenes* was amplified less efficiently by PCR in comparison to other gut bacteria (Reysenbach et al., 1992; Suzuki and Giovannoni, 1996). Thirdly, Xiangxi Yellow Cattle might form unique feature of their symbiotic microbe during the long-term evolution for better adaption to local environment.

In conclusion, our present study for the first time comparatively analyze the bacterial diversity in the large intestine of Xiangxi Yellow Cattle which were fed on *M. sinensis* or mixed forage. Our results indicated that the dietary transition from mixed forage to *M. sinensis* significantly altered the diversity of bacteria including *R. flavefaciens*, Butyrate-producing bacterium, *B. crossotus*, *P. campinasensis*, *A. caccae* in the phylum Firmicute and *Rikenellaceae* bacterium, *Marinilabilia salmonicolor* in the phylum Bacteroidete. In the large intestine of cattle, these species may play roles in the process of degradation of *M. sinensis*. Therefore, *M. sinensis* appeared to successfully increase the population of fibrolytic bacteria in the intestinal system of cattle. In this case, we would have a novel way to investigate the degradation of *M. sinensis* for replaceable energy. Undoubtedly, the information of symbiotic microbiota's diversity of cattle's large intestine adapting to *M. sinensis* diet will help us better understand the distribution of complex community

in cattle's digestion system, will benefit our discovery of bio-enzyme based on this system, and will provide the theoretical support for future work.

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