J Appl Genet 51(3), 2010, pp. 395–402

Original article

Bacterial diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis

P. R. Pandya[2], K. M. Singh[1], S. Parnerkar [2], A. K. Tripathi[1], H. H. Mehta[1], D. N. Rank[3], R. K. Kothari[4], C. G. Joshi[1]

[1] Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry (CVSAH), Anand Agricultural University (AAU), Anand, Gujarat, India
[2] Animal Nutrition Research Station, CVSAH, AAU, Anand, Gujarat, India
[3] Department of Animal Genetics & Breeding, CVSAH, AAU, Anand, Gujarat, India
[4] Department of Microbiology, Christ College, Rajkot, Gujarat, India

Abstract. Bacterial communities in buffalo rumen were characterized using a culture-independent approach for a pooled sample of rumen fluid from 3 adult Surti buffaloes. Buffalo rumen is likely to include species of various bacterial phyla, so 16S rDNA sequences were amplified and cloned from the sample. A total of 191 clones were sequenced and similarities to known 16S rDNA sequences were examined. About 62.82% sequences (120 clones) had >90% similarity to the 16S rDNA database sequences. Furthermore, about 34.03% of the sequences (65 clones) were 85–89% similar to 16S rDNA database sequences. For the remaining 3.14%, the similarity was lower than 85%. Phylogenetic analyses were also used to infer the makeup of bacterial communities in the rumen of Surti buffalo. As a result, we distinguished 42 operational taxonomic units (OTUs) based on unique 16S r DNA sequences: 19 OTUs affiliated to an unidentified group (45.23% of total OTUs), 11 OTUs of the phylum Firmicutes, also known as the low G+C group (26.19%), 7 OTUs of the *Cytophaga-Flexibacter-Bacteroides* phylum (16.66%), 4 OTUs of Spirochaetes (9.52%), and 1 OTU of Actinobacteria (2.38%). These include 10 single-clone OTUs, so Good's coverage (94.76%) of 16S rRNA libraries indicated that sequences identified in the libraries represent the majority of bacterial diversity present in rumen.

Keywords: *Bubalus bubalis,* molecular diversity, rumen bacteria, Surti buffalo, 16S rDNA, 16S rRNA.

Introduction

Livestock production in India is subsidiary to plant production. In tropical countries, the ruminants are fed on lignocellulosic agricultural by-products (like cereal straw, tree foliage, etc.) and cakes of oil seeds. Ruminants digest such plant materials by virtue of the extensive microbial community, including bacteria, fungi, and protozoa (Miron et al. 2001), which are found in the rumen and provide the host with nutrients, predominantly in the form of volatile fatty acids and microbial protein. According to Woese et al. (1990), all microbes present in the rumen ecosystem can be classified into 3 domains: Bacteria, Archaea (methanogens), and Eucarya (protozoa and fungi). Historically, most of the knowledge of the microbial composition of rumen fluid has been derived using traditional methods, such as the roll-tube technique (Hungate 1969) or most probable number (MPN) estimates (Dehority et al. 1989). The rumen bacteria have been shown by traditional procedures to belong to some 22 predominant species (Krause and Russell 1996). However, recent observations made it clear that prior estimation represents only a small portion of the total diverse microbial community that colonises the rumen (Krause et al. 2003). DNA-based technologies (16s RNA/ 18s RNA gene) have been used extensively to study rumen microbes

Received: August 4, 2009. Revised: March 21, 2010. Accepted: June 2, 2010.
Correspondence: P. R. Pandya, Ass. Research Scientist (ANRS), College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, 388 001 Gujarat, India; e-mail: kmsingh18@gmail.com

both qualitatively (Sylvester et al. 2004) and quantitatively (Shin et al. 2004). These techniques have been used to construct a library of 16S rDNA clones of rumen microbes and to demonstrate considerable diversity of rumen bacteria. Molecular research on microbial ecology of animals provides a broad perspective of application in the field of animal sciences (An et al. 2005). India possesses more than 50% of the world buffalo population, which produces more than 60% milk in India (Kumar et al. 2007). Surti is a popular breed of buffalo found in central Gujarat state. Surti buffaloes are of medium size and docile temperament, with body weight of 350-375 kg at maturity. The breed is distinguished by a fairy broad and long head with a convex shape at the top between horns. The present study was aimed to examine the diversity of rumen bacteria in Surti buffalo offered a diet of green fodder Napier bajra (*Pennisetum purpureum*), mature pasture grass (*Dichanthium annulatum*), and compound concentrate mixture. The molecular techniques now available were used to construct a library of 16S rDNA clones of rumen bacteria, and a phylogenetic tree for the clones isolated.

Materials and methods

Animals, diet and collection of rumen fluid

The experiment was carried out on 3 adult Surti buffaloes, at approximately 3 years of age and with a mean live weight of 201±18 kg. They were reared at the Department of Animal Nutrition, College of Veterinary Science and Animal Husbandry, Anand. The permission of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was obtained prior to initiation of the study. All the animals were maintained under a uniform feeding regime (ICAR, 1998) for at least 21 days. The diet comprised green fodder Napier bajra 21 (*Pennisetum purpureum*), mature pasture grass (*Dicanthium annulatum*), and concentrate mixture (20% crude protein, 65% total digestible nutrients). The animals were offered 10 kg green, ad-lib dry grass, and 2.5 kg of concentrate mixture daily. Approximately 500 ml of rumen fluid was collected *via* a stomach tube located in the middle part of the rumen and connected to a vacuum pump at 0, 2, 4, or 6 h after feeding (Khampa et al. 2006). About 100 ml of rumen fluid was passed through 4 layers of cheese cloth to remove particulate matter. Remaining rumen fluid was stored at –80°C for further study. Total DNA (0, 2, 4, 6 h × 3 animals) was extracted separately by using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA) and we finally pooled all the DNA samples. The total DNA mixture (pooled) was used as a template in PCR to amplify 16S rRNA.

PCR amplification of the 16S rRNA gene

The PCR primers used to amplify 16S rDNA fragments were bacteria-specific primers (Lane 1991): a forward primer F27 (5'-AGAGTTTGATCM TGGCTCAG-3') and a reverse primer R1492 (5'-ATAGGYTACCTTGTTACGACT-3'). Subsequently, 16S rDNA was amplified by PCR using the metagenomic DNA and Master mix (Fermentas, UK). A total of 25 µL of reaction mixture consisted of 10 pmol of each primer, 30 ng of template DNA, and 12.5 µL of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) 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 50°C and 2 min of elongation at 72°C, with a final extension at 72°C for 10 min for the first set. The anticipated product of approximately 1.4 kb was separated by agarose gel electrophoresis and cleaned by using a QIAquick DNA Gel Extraction Kit (QIAGEN, CA) in accordance with the directions of the manufacturer.

Cloning and sequencing

The purified PCR products were cloned in InstaTA cloning kit (Fermentas, UK) according to the instructions of the manufacturer. The recombinant plasmids were next extracted by the Mini-prep Plasmid Extraction Kit (QIAGEN, CA). Sequencing was performed for all the clones in the library with an ABI Prism 310 Genetic Analyser (Applied Biosystems Inc., CA) using BigDye Terminator (version 3.1) in the Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India.

Sequence analyses and phylogenetic tree constructing

All reference sequences were obtained from the GenBank/EMBL/DDBJ/RDP (Benson et al. 2007). Sequences (~650 bp) from the current study were analysed by the CHECK_CHIMERA program (Maidak et al. 2001) to remove any chimeric rDNA clone. The similarity searches for se-

quences were carried out by BLAST (BLAST/Blast.cgi, Madden et al. 1996) and alignment was done using CLUSTAL W (Thompson et al. 1994). Ambiguously and incorrectly aligned positions were aligned manually. The distance matrix was calculated using the DNADIST program included in PHYLIP (Felsenstein 1985) and used to assign sequences in various operational taxonomic units (OTUs) or phylotypes by DOTUR (Schloss and Handelsman 2005) and a total of 42 OTUs were distinguished, based on unique 16S r DNA sequences. Good's coverage was calculated as $[1 - (n/N)] \times 100$, where n is the number of single-clone OTUs and N is the library size, i.e. the total number of sequences (clones) for the analysed sample (Schloss and Handelsman 2005). Phylogenetic tree was constructed by the neighbour joining method using MEGA 4.0 software (Tamura et al. 2007). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies (Felsenstein 1985).

Nomenclature and nucleotide sequence accession numbers

The prefix KM was used to denote the OTUs identified. Nucleotide sequences are deposited in the GenBank database under the accession numbers EU348296-EU348106.

Results

Sequence similarity

A total of 191 16S rDNA clones of partial length were isolated from the rumen liquor of the buffaloes. All the clones were subjected to sequence analysis, followed by homology search using databases: the GenBank and the Ribosomal Database Project (RDP) database. In our library, about 62.82% clones (120 clones) had ≥90% similarity to 16S rDNA data sequences from those databases. Furthermore, about 34.03% (65 clones) of the sequences were 85-89% similar to 16S rDNA data sequences, while for the remaining 3.14 % (6 clones) the similarity was less than 85% (Table 1).

Phylogenetic analysis

The collection of cloned 16S rRNA gene sequences of bacteria encompassed several major bacterial lineages. Although the similarity for most of the sequences with those of known rumen bacteria was too low to identify the sequence as representing a particular species, a phylogenetic tree was constructed to investigate their taxonomic affiliation. All 191 clone sequences were included in a phylogenetic analysis and were grouped into 42 operational taxonomy units (OTUs) affiliated to an unidentified group (19 OTUs, 45.23%), the phylum Firmicutes, also known as the low G+C group (11 OTUs, 26.19%), the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum, (7 OTUs, 16.66%), Spirochaetes (4 OTUs, 9.52%) and Actinobacteria (1 OTU, 2.38%) (Table 2). Thus bacterial sequences obtained from rumen formed tightly-clustered, deeply-diverging groups affiliated to the different bacterial phyla as well unclassified groups. The total includes 10 single-clone OTUs, so Good's coverage (94.76%) of 16S rRNA libraries indicated that the sequences identified in libraries represent the majority of bacterial diversity present in rumen.

Within the phylum Firmicutes, 4 OTUs showed similarity to sequences of species. These correspond to *Succiniclasticum ruminis* (KM22, 6 clones), *Acetovibrio cellulolyticus* (KM27, 4 clones), *Streptococcus* sp. (KM21, 2 clones), and *Ruminococcus callidus* (KM26, 6 clones). The remaining 7 OTUs belonged to independent clusters, which are associated with the phylum Firmicutes. In the CFB phylum, 1 OTU was similar to *Prevotella ruminicola* (KM3, 16 clones) and another 6 OTUs constituted separate clusters that were remotely related to the *Bacteroides fragilis* group. Another 4 OTUs belonged to *Treponema* spp. (KM41, KM17, KM42, and KM12, 13 clones, Table 2).

Discussion

None of the clones in our library were identified below the genus level, because the similarity values of our sequences were too low to assign them to particular taxa. Although there are no exact 16S rDNA similarity limits for defining specific taxa, such as genera and species, species definition in general requires sequence having greater than 98% similarity (Vandamme et al. 1996). Thus if a sequence has a greater than 98% similarity to a 16S rDNA of a known bacterium, it is considered to be a member of that species. In the present study, we examined rumen bacterial composition by PCR-based analysis of bacterial 16S rDNA sequences. This culture-independent method offers possibility of characterizing microbial ecosystems, independent isolation, maintenance, and propagation of bacteria under laboratory condi-

Table 1. Similarity values of operational taxonomic units (OTUs) based on 16S rDNA sequences of 191 clones retrieved from the rumen fluid of Indian Surti buffalo.

OTU	No. of clones	Nearest valid relative (GenBank accession no.)	Sequence identity (%)
KM1	5	URB (DQ394664)	92
KM2	13	URB (EF436391)	96
KM3	16	*Prevotella aff. ruminicola* Tc2-24 (AJ009933)	93
KM4	2	URB (EU845097)	89
KM5	24	URB (EU381899)	90
KM6	19	*Butyrivibrio fibrisolvens* isolate L8 (AY699274)	86
KM7	2	URB (EU844704)	87
KM8	6	*Selenomonas ruminantium* strain 65 (EF112197)	91
KM9	5	URB (AB270078)	90
KM10	2	URB (AB034150.)	86
KM11	5	uncultured equine intestinal *Eubacterium* sp. (AJ408101)	88
KM12	6	*Treponema* sp. (AF023049)	86
KM13	9	URB (EU381619)	90
KM14	7	URB (AB270141)	93
KM15	5	uncultured member of Lachnospiraceae (AB218344)	91
KM16	3	URB (AB185560)	87
KM17	3	URB (EU844824)	89
KM18	1	URB (AB034150)	84
KM19	3	URB (EF686622)	94
KM20	4	uncultured Firmicutes clone NI_217 (FJ650689)	91
KM21	2	uncultured Firmicutes bacterium (CU925746)	83
KM22	6	*Succiniclasticum ruminis* strain DSM 9236 (NR_026205)	90
KM23	2	uncultured Firmicutes bacterium (CU922655)	83
KM24	2	URB (EU259464))	95
KM25	5	uncultured *Acetivibrio* sp (EU703284)	87
KM26	6	*Butyrivibrio fibrisolvens* isolate L8 (AY699274)	87
KM27	4	URB (EF686520)	93
KM28	2	*Prevotella ruminicola* strain TC2-28 (AF218619)	87
KM29	3	rumen bacterium YS2 (AF544207)	89
KM30	1	URB (EU259402)	96
KM31	3	uncultured member of Ruminococcaceae (EU794145)	87
KM32	2	URB (EU842702)	93
KM33	2	URB (EF686519)	93
KM34	1	not available	
KM35	1	*Ruminococcus gnavus* strain A2 (EU139255)	86
KM36	1	uncultured *Prevotella* sp. (AM420039)	96
KM37	1	URB (EU719249)	87
KM38	1	URB (EF686612)	93
KM39	1	URB (AB244116)	90
KM40	1	URB (FJ983064)	85
KM41	1	*Treponema bryantii* (M57737)	87
KM42	3	URB (FJ028789)	92

URB = uncultured rumen bacteria

tions. However, PCR-based methodologies are subject to certain limitations (Acinas et al. 2005). As discussed by Wintzingerode et al. (1997), care should be taken in experimental procedures and in interpretation of the results. Thus our findings reveal that rumen of the Surti buffaloes contained a diverse array of bacterial species. More than 11 OTUs (26.19%) belonged to the known phylum Firmicutes, 7 OTUs (16.66%) to the *Bacteroides fragilis* group, 4 OTUs (9.52%) to Spirochaetes, and 1 OTU (2.38%) to the Actinobacteria. About 19 OTUs (45.23%) belonged to unidentified groups (Table 2). The proportions of bacterial communities in Surti buffalo rumen are similar to those reported previously in other rumen ecology studies. In a metagenome analysis by pyrosequencing of rumen sample of Angus-Simmental cross steers (*Bos taurus*) fed a diet of grass-legume

Table 2. Analysis of diversity of 16S rDNA phylotypes retrieved from the rumen of Indian Surti buffalo.

Taxon	No. of operational taxonomic units (OTUs) and % of total OTUs	No. of clones
Firmicutes (low G+C group)	11 OTUs (26.19%)	41
a. *Succiniclasticum ruminis*	1 OTU (KM22)	6
b. *Streptococcus* sp.	1 OTU (KM21)	2
c. *Acetivibrio cellulolyticus*	1 OTU (KM27)	4
d. *Ruminococcus callidus*	1 OTU (KM26)	6
e. Unclassified	7 OTUs	23
CFB phylum	7 OTUs (16.66%)	27
a. *Prevotella ruminicola*	1 OTU (KM3)	16
b.Unclassified	6 OTUs	11
Spirochaetes	4 OTUs (9.52%)	13
Treponema spp.	(KM41, KM17, KM42, KM12)	
Actinobacteria	01 OTU (2.38%)	1
Actinomyces bovis	(KM18)	
Unidentified bacteria	19 OTUs (45.23%)	109
Total	42 OTUs	191

CFB = *Cytophaga-Flexibacter-Bacteroides*

hay (Brulc et al. 2009), 62% of sequences were identified as the Firmicutes and 21% of sequences as the *Bacteroides fragilis* group. Tajima et al. (1999) reported that 52.4% of clones identified in the rumen liquor of Holstein cow (*Bos taurus*) fed a diet of hay belonged to the Firmicutes, and 38.1% to the CFB phylum. Edwards et al. (2004), summarizing the published data for rumen bacteria, reported that on average 54% of rumen bacteria were members of the Firmicutes and 40% were from the CFB phylum. Yuhei et al. (2005) found that 81.3% clones represented the Firmicutes, while 14.4% clones belonged to the Bacteroides *fragilis* group and Actinobacteria and Proteobacteria in fecal microbiota of cattle. Deng et al. (2007) assigned 57.1% of clones to the phylum Firmicutes, 42.2% of clones to the CFB phylum, and one clone (0.7%) to Spirochaetes in gayals (*Bos gaurus frontalis*, syn. *B. frontalis*) fed a diet composed of fresh bamboo leaves and twigs (*Sinarundinaria* sp.), with 50.5±3.16 % dry matter, 10.2± 0.40 % crude protein, 38.8±1.17 % crude fiber. Dowd et al. (2008), using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), evaluated ubiquitous bacteria from the cattle faeces, which included *Clostridium*, *Bacteroides*, *Porpyhyromonas*, *Ruminococcus*, *Alistipes*, *Lachnospiraceae*, *Prevotella*, *Lachnospira*, *Enterococcus*, *Oscillospira*, *Cytophaga*, *Anaerotruncus*, and *Acidaminococcus* spp. Regarding cellulose degrader, *Fibrobacter*-like bacteria may not have been detected in the Surti buffaloes in the present study. The reason can be due to a small population, adherence to feed, specificity of the primers, or experimental PCR biases. The other classic fibrolytic bacteria, such as *Ruminococcus bromii* and *Butyrivibrio fibrosolvens* (Hungate 1969; Srinivas and Krishnamoorthy 2005; Khampa et al. 2006) were recovered in 3 OTUs. One OTU (KM26, 6 clones) identified in the present study was related to the known fibrolytic bacterium *Ruminococcus cadillus*. The acid-utilizing bacterium *Succiniclasticum ruminis* (KM22, 6 clones), sugar-utilizing bacterium *Streptococcus* sp. (KM21, 2 clones), and *Acetovibrio cellulolyticus* (KM27, 4 clones) were also recovered from the rumen of Surti buffalo (Table 2). These results support the results of Koike et al. (2003) for a domestic rumen sample and co-culture sample. Another 7 OTUs of the phylum Firmicutes were clustered independently and their confirmation requires further investigation.

Amongst the bacteria comprising the CFB phylum, only one OTU (KM3, 16 clones) was closely related to *Prevotella runinicola*, which was identified previously as the most numerous of rumen bacteria (Stewart et al. 1997). The remaining 6 OTUs located in the CFB phylum were clustered loosely, and their identification requires further investigation. Overall, the present results were consistent with previous reports that CFB bacteria were less numerous in the rumen of wild animals (An et al. 2005), as compared to domestic cattle. Whitford et al. (1998) reported that 16S rDNA sequences similar to those of *Prevotella ruminicola* prevailed in isolated material from domestic cattle. Four OTUs (13 clones) were classified as Spirochaetes. A notable finding was the presence of *Actinomyces bovis* (Figure 1) grouped within the Actinobacteria. The 16S rRNAs from actinobacteria are commonly found in soils (Buckley and Schmidt 2003). Although Surti buf-

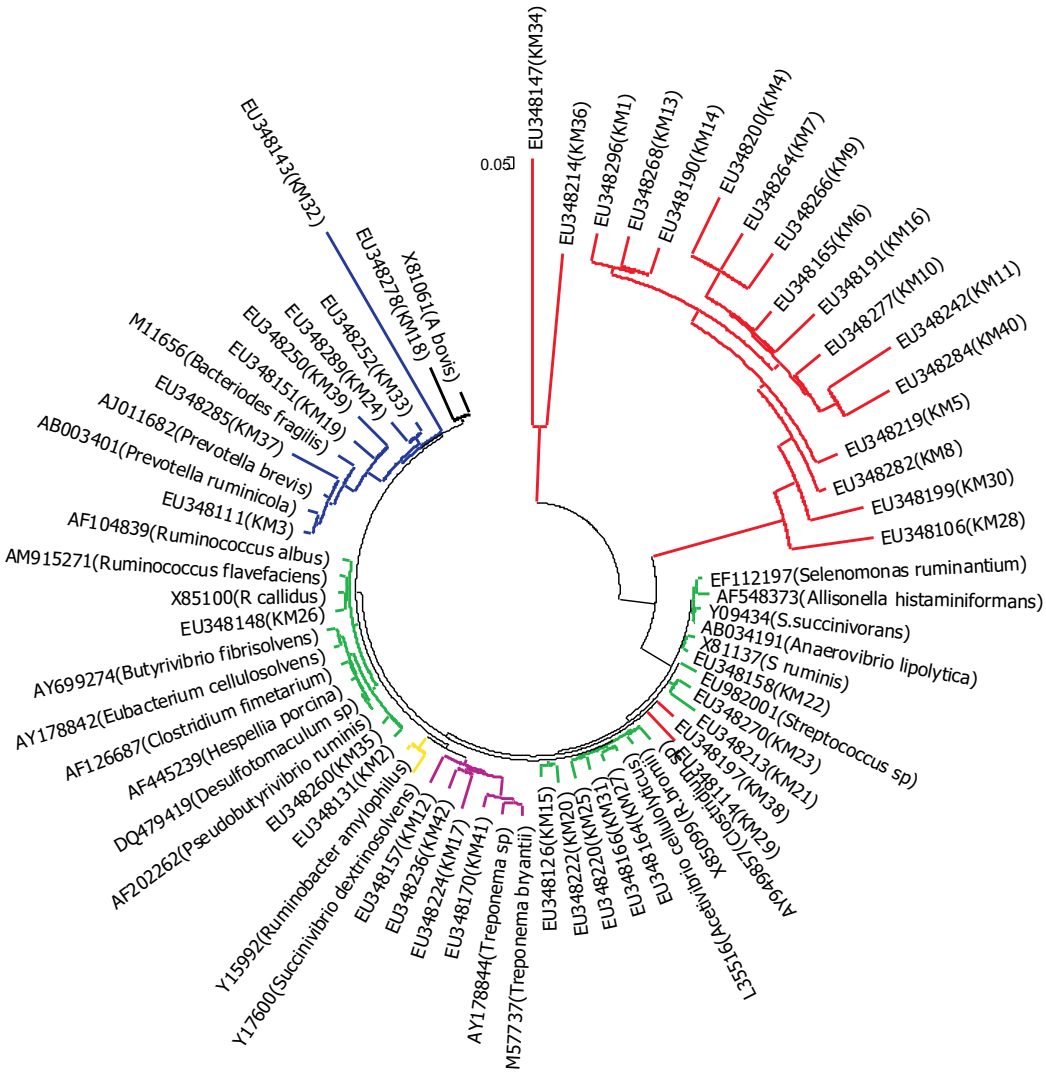

Figure 1. Phylogenetic relationships of partial 16S rDNA sequences of clones recovered from Surti buffalo rumen fluid samples. The unrooted tree was inferred by the neighbour joining method using the MEGA 4 software. The scale bar equals to an average of 5 nucleotide substitutions per 100 positions. Branch lines: green (Firmicutes), blue (CFB phylum), pink (Spirochaetes), yellow (Proteobacteria), black (Actinobacteria) and red (unidentified bacteria).

falo may accidentally eat soil through feed, it is unlikely that ingested actinobacteria, just passing through the gastrointestinal tract, would be detected in our analyses. *Actinomyces*-affiliated sequences have also been obtained from gastrointestinal tracts of diverse herbivores (Tajima et al. 1999) and humans (Eckburg et al. 2005). *A. bovis* were not reported in the clone libraries of bacterial 16S rDNA isolated from the gayals fed a diet of bamboo (*Sinarundinaria* sp.) leaves and twigs (Deng et al. 2007).

It is quite possible that the variations in the species of bacteria in the rumen are affected by diet. Latham et al. (1972) and Tajima et al. (2001) have shown that diet affects the composition of the microbial community of the rumen. However, differences between the present observations and those published previously are considered to be related, at least, with animal species. For example, *A. bovis* was retrieved from the rumen liquor of buffalo in the current study. However, this species of bacteria was not detected in bacterial 16S rDNA clone libraries of rumen fluid from gayals fed fresh bamboo leaves (Deng et al. 2007).

Conclusions

In spite of the possible deficiencies in analytical techniques, it is concluded that Surti buffalo has a diverse range of bacteria in the rumen. Unidentified sequences represent the majority of bacteria in the rumen of Surti. The sequences of cellulolytic bacterial groups (*Ruminococcus callidus*, *Acetovibrio cellulolyticus*, and *Streptococcus* sp.), proteolytic bacteria (*Prevotella ruminicola*), acid

utilizers (*Succiniclasticum ruminis*), and pectin utilizers (*Treponema* sp.) were also recovered in the present study. The present results are consistent with the observations of Deng et al. (2007), An et al. (2005) and Koike et al. (2003). Definitely, further studies should be undertaken to confirm and extend the present observations.

Acknowledgements. Financial support from the Department of Biotechnology, Government of India, New Delhi, is acknowledged with respect and gratitude.

REFERENCES

An D, Dong X, Dong Z, 2005. Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) estimated by 16S rDNA homology analyses. Anaerobe 11: 207–221.

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF, 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl Environ Microbiol 71: 8966–8969.

Buckley DH, Schmidt TM, 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ Microbiol 5: 441–452.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostelland J, Wheeler D, 2007. GenBank Nucleic Acids Res (35) D1–D25.

Brulc M, Antonopoulos D, Millera ME, Wilsona B, Anthony MK, et al. 2009. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Natl Acad Sci USA 6: 1948–1953.

Dehority BA, Tirabasso PA, Grifo AP, 1989. Most-probable-number procedures for enumerating ruminal bacteria, including the simultaneous estimation of total and cellulolytic numbers in one medium. Appl Environ Microbiol 55: 2789–2792.

Deng W, Wanapat M, Chen S, Ma J, Dongmei Xi, et al. 2007. Phylogenetic analysis of 16S rDNA sequences manifest rumen bacterial diversity in gayals (*Bos frontalis*) fed fresh bamboo leaves and twigs (*Sinarumdinaria*). Asian-Aust. J Animal Sci 7(20): 1057–1066.

Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, et al. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiology 8:125.

Edwards JE, McEwan NR, Travis AJ, Wallace RJ, 2004. 16S rDNA library-based analysis of ruminal bacterial diversity. Antonie Van Leeuwenhoek 86: 263–281.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. 2005. Diversity of the human intestinal microbial flora. Science 308: 1635–1638.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol 39: 783–791.

Hungate RE, 1969. A roll tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons DW, eds. Methods in microbiology, Vol. 3B. London and New York: Academic Press: 117–132.

ICAR, 1998. Nutrient requirements of livestock and poultry. Indian Council of Agricultural Research, New Delhi, India.

Khampa S, Wanapat M, Wachirapakorn C, Nontaso N, Wattiaux M, 2006. Effects of urea level and sodium DL-malate in concentrate containing high cassava chip on ruminal fermentation efficiency, microbial protein synthesis in lactating dairy cows raised under tropical conditions. Asian-Aust J Anim Sci 19: 837–844.

Koike S, Yoshitani S, Kobayashi Y, Tanaka K, 2003. Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. FEMS Microbiol Lett 229: 23-30.

Krause DO, Russell JB, 1996. How many ruminal bacteria are there? J Dairy Sci 79: 1467–1475.

Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, et al. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. FEMS Microbiol Rev 27: 663–693.

Kumar S, Nagarajan M, Sandhu J, Kumar N, Behl V, 2007. Phylogeography and domestication of Indian river buffalo. BMC Evol Biol 7:186.

Lane DJ, 1991. 16S/23S rRNA sequencing. In: Stackebrandt E and Goodfellow M, eds. Nucleic acid techniques in bacterial systematic. New York: John Wiley & Sons: 115–175.

Latham MJ, Storry JE, Sharpe ME, 1972. Effect of low roughage diets on the microflora and lipid metabolism in the rumen. Appl Microbiol 24: 871–877.

Madden TL, Tatusov RL, Zhan J, 1996. Application of network BLAST server. Meth Enzymol 266: 131-141.

Maidak BL, Cole JR, Lilburn TG, Jr CTP, Saxman PR, Farris RJ, et al. 2001. The RDP-II (Ribosomal Database Project). Nucl Acids Res 29: 173–174.

Miron J, BenGhedalia D, Morrison M, 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. J Dairy Sci 84: 1294–1309.

Schloss PD, Handelsman J, 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71: 1501–1506.

Shin EC, Cho KM, Lim WJ, Hong SY, An CL, et al. 2004. Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. J Appl Microbiol 97: 378-383.

Srinivas B, Krishnamurthy U, 2005. Influence of diet-induced changes in rumen microbial characteristics on gas production kinetics of straw substrates *in vitro*. Asian-Aust J Anim Sci 18: 990–996.

Stewart CS, Flint HJ, Bryant MP, 1997. The rumen bacteria. In: Hobson PN, Stewart CS, eds. The rumen microbial ecosystem. 2nd ed. New York: Chapman and Hall: 10–72.

Sylvester JT, Karnati SKR, Yu Z, Morrison M, Firkins JL, 2004. Development of an assay to quan-

tify rumen ciliate protozoal biomass in cows using real-time PCR. J Nutr 134: 3378–3384.

Tajima K, Aminov RI, Nagamine T, Ogata K, Nakamura M, et al. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol Ecol 29: 159–169.

Tajima K, Aminov RI, Nagamine T, Matsui H, et al. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl Environ Microbiol 67: 2766–2774.

Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596–1599.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 22: 4673–4680.

Vandamme P, Pot B, Gillis M, Vos De P, et al. 1996. Polyphasic Taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60: 407–438.

Whitford MF, Forster RJ, Beard CE, Gong J, Teather RM, 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe 4: 153–163.

Wintzingerode Von F, Goebel UB, Stackebrandt E, 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213–229.

Woese CR, Kandler O, Wheelis ML, 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proc Natl Acad Sci USA 87: 4576–4579.

Yuhei O, Hidenori H, Mitsuo S, Hisao I, Yoshimi B, 2005. Culture-independent analysis of fecal microbiota in cattle. Biosci Botechnol Bochem 69: 1793–1797.