ORIGINAL ARTICLE

Seasonal differences in rumen bacterial flora of wild Hokkaido sika deer and partial characterization of an unknown bacterial group possibly involved in fiber digestion in winter

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ABSTRACT

Rumen digesta was obtained from wild Hokkaido sika deer to compare bacterial flora between summer and winter. Bacterial flora was characterized with molecular-based approaches and enrichment cultivation. *Bacteroidetes* was shown as a major phylum followed by *Firmicutes*, with similar proportions in both seasons. However, two phylogenetically unique groups in *Bacteroidetes* were found in each season: unknown group A in winter and unknown group B in summer. The ruminal abundance of unknown group A was the highest followed by *Ruminococcus flavefaciens* in winter. Moreover, the abundance of these two was higher in winter than in summer. In contrast, the abundance of unknown group B was higher in summer than in winter. In addition, this group showed the highest abundance in summer among the bacteria quantified. Unknown group A was successfully enriched by cultivating with oak bark and sterilized rumen fluid, particularly that from deer. Bacteria of this group were distributed in association with the solid rather than the liquid rumen fraction, and were detected as small cocci. Accordingly, unknown group A is assumed to be involved in degradation of fibrous materials. These results suggest that wild Hokkaido sika deer develop a rumen bacterial flora in response to changes in dietary conditions.

Key words: ecology, fiber digestion, quantitation, rumen bacteria, sika deer

Ruuning head: Rumen bacteria of Hokkaido sika deer

1 INTRODUCTION

Wild ruminant animals are excellent models for the functional surveillance of rumen microbes (Kobayashi, 2006), since these animals are expected to harbor specific rumen microbes in response to the dietary conditions to which they are exposed. Changes of rumen microbiota likely depend on the area in which the wild animals inhabit. In particular, harsh dietary conditions might select for rumen microbiota to digest and ferment fibrous materials as a main dietary ingredient in order to maximize nutrient extraction. This is because the rumen is the main digestive organ for degrading fibrous foods by microbes and also acts as a reservoir of ingested foods (Itabashi 1998).

Hokkaido, the northernmost island of Japan, is home to more than a million head of wild sika deer (Hokkaido sika deer, *Cervus nippon yesoensis*), many of which live in snowy areas with limited access to food. We have elucidated seasonal changes of body size, ingesta, body fat reserve, and rumen fermentation products of sika deer in the Shiretoko Peninsula of Hokkaido, characterized by severe winter conditions (long duration of low temperatures with heavy snowfall), leading to a precarious nutritional status (Ichimura et al. 2004). Despite this, the population of sika deer in this area has increased since the 1980s (Okada 2000). One of the reasons for their survival under such harsh dietary conditions could be the change in rumen microbes as an adaptation to poor dietary conditions in winter. This was partially characterized by morphological classification of rumen bacteria in relation to ingesta by Ichimura et al. (2004), who found that more Gram-negative cocci and fewer Gram-negative curved rods were found in winter. To confirm bacterial adaptation to a winter diet, functional characterization of the bacteria is necessary.

Similar attempts have been made for other wild or semi-wild ruminants including reindeer (Orpin et al. 1985), fallow deer, axis deer, white-tailed deer (Dehority et al. 1999), red deer and hill sheep (Hobson et al. 1975). More recently, deep sequencing of reindeer rumen microbiota has detected abundant unknown bacteria, whose function requires investigation (Mackenzie et al. 2015). In fact, such possibly functional but unknown bacteria represent an attractive research target to gain insight

into the rumen metabolism of wild animals as well as the future development of additives including pro- and pre-biotics and other industrial materials (Kobayashi 2006). Rumen microbiota of sika deer in the above area might be an ideal source for such purposes and could provide insight for future application.

We aimed to partially characterize the rumen bacteria of Hokkaido sika deer in the present study by qualitative, quantitative and visualization approaches as well as discuss their ecology and function with special reference to the winter diet condition.

2 MATERIALS AND METHODS

2.1 Animals and samples

Twenty rumen samples were chosen from a total of 32 wild Hokkaido sika deer that had been characterized with respect to food habit and rumen fermentation products (Ichimura et al. 2004), based on body weight (average body weight of 65.9 kg in summer and 62.4 kg in winter). Summer samples (July and August) were from 4 males and 6 females, while winter samples (March) were from 5 males and 5 females. The winter sample was defined as the sample collected at the end of a long winter, in which the deer had been exposed to snow-covered land with limited access to food (Ichimura et al. 2004). All deer were more than 1 year of age. These deer were culled for research purposes or for nuisance control in the Shari district on the Shiretoko Peninsula of Hokkaido, Japan (Figure 1). According to climate data for the sampling period of this area (Utoro, Shari), the average temperatures were 17°C in July and August, 2001 and -1°C with 125 cm of snow accumulation in March, 2002. The sampling area was dominated by mixed forest with *Sasa* spp as undergrowth, and forbs were seen along the woodland paths. More details of the vegetation can be found in Ichimura et al. (2004).

2.2 Characterization of rumen bacterial flora

Total DNA was extracted and purified as described previously (Koike et al. 2007; Shinkai & Kobaayashi 2007). Briefly, rumen digesta was subjected to glass bead beating, and extracted DNA was treated with proteinase K and RNase, then precipitated and diluted for use in the clone library analysis and quantitative PCR (qPCR).

Clone library analysis was performed as follows. Bacterial DNA from each rumen digesta was amplified by PCR with the primers 530F (5'-GTGCCAGCMGCCGCGG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3'). The reaction mixture for library construction consisted of 5.0 µl of 10 x Ex-Taq buffer, 5.0 µl of 2 mM dNTP, 2.5 µl of each primer (10 pmol/µl), 33.0 µl of water, 1.0 µl of rTaq polymerase (TOYOBO, Osaka, Japan), and 1.0 µl of template DNA (10 ng/µl). PCR conditions were as follows: initial denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, and extension at 72°C for 1.0 min. Final extension was performed at 72°C for 9 min. The PCR product was purified by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) after performing agarose gel electrophoresis, then ligated with pGEM-T Easy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM109 (Nippon Gene, Tokyo, Japan). White colonies developed on ampicillin- and Xgal-containing Luria-Bertani plates were employed for plasmid isolation using a QIAprep Spin Miniprep Kit (Qiagen), and the purified plasmid DNA was subjected to sequence analysis (Thermo Sequenase Cycle Sequencing Kit, and DSQ 2000L DNA Sequencer, Shimadzu, Kyoto, Japan). The obtained nucleotide sequences were compared with known sequences in the GenBank database using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain similarity values. The Clustal X program (http://www.clustal.org/） for multiple sequence alignment and phylogenetic analysis was used. A neighbor-joining tree with a Kimura-2 correction was created (Collins et al., 1994). Two libraries were compared by LIBSHUFF (Singleton et al. 2001). The sequences obtained were deposited in the DDBJ nucleotide sequence database under the accession numbers AB214694 through AB214833.

Bacterial communities were quantitatively characterized by real-time PCR assay with species/group-specific primers for the representative groups, which included total bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Prevotella ruminicola*, *Prevotella bryantii*, *Treponema bryantii*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Eubacterium ruminantium*, unknown group A, and unknown group B (see below for these unknown groups), using a LightCycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). All primers except those for the unknown groups were as described by Muyzer et al. (1993), Tajima et al. (2001), Koike & Kobayashi (2001), and Koike et al. (2007). Primers for the unknown groups detected in the 16S rDNA library but unique for the sika deer rumen investigated (*see* Results for details) were newly designed. PCR reactions and related information are listed in Table 1. For quantification of unknown groups A and B, primer specificity was assessed by sequencing clones in a mini-library constructed using the designed primers. Other validation in qPCR for unknown groups, such as amplification efficiency and quantification range, was performed as described by Koike et al. (2007).

Fluorescence in situ hybridization (FISH) was carried out essentially as described in our previous study (Shinkai & Kobayashi 2007). Then, a group-specific probe for unknown group A (5'-CTTCCGACGTTGAGCGCC -3': 631-648 of 16S rRNA target site) was newly designed. The specificity of this probe was checked with the Probe Match tool of RDP II (http://rdp.cme.msu.edu/index.jsp) and the BLAST search tool. The 5’ end of the oligonucleotide probe was labeled with Cy3 (Hokkaido System Science, Sapporo, Japan). Samples from the unknown group A enrichment study (see below) were used for FISH analysis. The established FISH procedure for unknown group A bacteria is as follows. Fixation was performed with 3% paraformaldehyde for 3 h at 4°C. Sequential dehydration was carried out in 50, 80, 96 and 100% ethanol (3 min each). Hybridization was performed with 10% formamide at 46°C for 1.5 h. The slide was rinsed in a washing buffer for 15 min at 48°C. To reduce the auto-fluorescence of feed particles,

toluidine blue O (Division Chroma) was added to the slide sample. Then, the sample was rinsed, air-dried and incubated in 99.5% ethanol for an appropriate period of time to remove the dye from the bacterial cells but not from the feed particles. Then, the samples were immediately washed with distilled water. Total bacteria were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI) contained in Vectashield H-1200 (Vector Laboratories, Inc., Burlingame, CA). For microscopic observation of bacteria and their fluorescence signals, a microscope (BX51, Olympus, Tokyo, Japan) with a universal reflected-light illuminator (BX-URA2, Olympus) and cooled CCD camera (Cool Snap, Roper Scientific Photometrics, Champaign, IL) was used. Images were processed with Adobe Photoshop version 6.0 (Adobe Systems Co., Ltd, Tokyo, Japan).

2.3 Enrichment of unknown bacteria

Bacteria belonging to unknown group A were enriched by incubating diluted (1000 fold) rumen digesta with an anaerobic medium of Bryant and Burkey (1953), to which one of various carbohydrate sources such as Sasa leaf, elm bark, oak bark, rice straw hay, Orchardgrass hay, alfalfa hay, soybean husk, lablab bean husk, filter paper or Avicel was added (5 mg/mL). *Sasa* leaf and the two types of bark are representative diets for deer in winter (Ichimura et al. 2004), while the two types of bean husk are known to stimulate fibrolytic rumen bacteria (Ngwe et al. 2012; Fuma et al. 2012). Avicel was obtained from Asahi Kasei Corp. (Tokyo, Japan). All of the above carbohydrate sources were dried (60°C for 48 h) and ground into 1 mm particles. As another medium component, sterilized and centrifuged rumen fluid (Ogimoto & Imai 1981) from 2 sheep on a hay diet or 2 deer (in winter of the present study) was added at 30% level for a habitat simulation. The inoculant was diluted (1000 fold) in rumen digesta and incubated at 38°C for 120 h with the above medium in a Hungate tube with a butyl rubber stopper and plastic cap after flushing the head space with CO_2 gas (n=3). After incubation, the degree of the enrichment of unknown group A bacteria was assessed by quantifying unknown group A with group-specific qPCR.

2.4 Calculations and statistical analysis

Microbial data by qPCR were expressed in log-transformed value for 16S rDNA gene copies per gram of rumen digesta and the relative proportion of each species or group in total bacteria. Values in each season (n=10) were averaged and compared by Student's t test, since no sex differences were detected for these data. In the enrichment study for unknown group A, data from 3 tubes were averaged. A significant difference was declared at $P < 0.05$ and a trend was defined at $P < 0.10$.

3 RESULTS

Cloned sequences in the present study covered ca. 890 bp of the bacterial 16S rRNA gene and partly allowed the comparative characterization of the bacterial community structure between summer and winter deer rumen samples. A total of 140 clones (71 and 69 from summer and winter samples, respectively) were used for further analysis to partially characterize the bacteria inhabiting the rumen of wild deer in the experimental area.

The bacterial community structure is shown as a phylogenetic tree in Figure 2. The bacterial classification did not differ greatly between summer and winter samples, and indicated that the majority of bacteria was represented by *Bacteroidetes* followed by *Firmicutes*. LIBSHUFF analysis revealed no significant difference in bacterial community between the two seasons. However, two clusters consisting of uncultured bacteria belonging to *Bacteroidetes* were distributed in a season-specific manner, i.e., one cluster originated from winter samples only, while the other was from summer samples only. These two clusters were designated as unknown groups A and B, respectively, in which the nearest known relative was *Prevotella ruminicola* having < 90% in sequence similarity. Meanwhile, these unknown groups shared ≥ 97% similarity in sequence with many uncultured bacteria. Then, qPCR assays for these unknown groups were successfully established with 1.8678 and 1.9026 in amplification efficiency and 10^3-10^8 copies/g in quantifiable ranges, respectively.

The results for bacterial quantification by qPCR are presented in Figure 3. *R. flavefaciens* and unknown group A showed a higher abundance (both in absolute number and relative proportion) in winter than in summer, while unknown group B had a lower abundance in winter than in summer. Of 11 species and groups detected, unknown group A exhibited the highest abundance followed by *R. flavefaciens* and *S. bovis* in winter. Meanwhile, unknown group B showed the highest abundance followed by *S. bovis* in summer. *Eubacterium ruminantium* was not in the quantifiable range for both the summer and winter samples.

The degree of enrichment of unknown group A depended on the carbon source. Japanese oak bark showed the best growth stimulation of unknown group A bacteria among the 10 different carbon sources tested. That is, in comparison with no incubation, incubation with oak bark resulted in increased growth of unknown group A bacteria (1033 fold), while the other carbon sources produced lower growth (2-51 fold) even though they stimulated the growth of other bacteria. Further enrichment was attempted by adding sterilized rumen fluid as a habitat-simulating agent. The addition of sheep rumen fluid increased not only unknown group A but also other bacteria; thus, the degree of enrichment was low. However, the addition of deer rumen fluid produced higher growth stimulation for unknown group A in comparison with other bacteria, resulting in 2.9-4.1 fold enrichment (Table 2). The established FISH protocol allowed successful visualization of unknown group A bacteria in the enrichment culture, as shown in Figure 4. Unknown group A bacteria were observed as clustered cocci among the other bacteria. The size of unknown group A bacteria was estimated as approximately 1-2 μm. qPCR analysis of this enriched culture showed a higher abundance of unknown group A in the solid than the liquid fraction (19.4×10^8 vs. 3.0×10^8 copies of 16S rRNA gene / g of culture).

4 DISCUSSION

In the previous report, we emphasized the seasonal difference in ingested foods of wild deer in the Shiretoko Peninsula of Hokkaido. In particular, the deer were exposed to poor dietary conditions, in

which fibrous materials such as bark were the main food source (Ichimura et al. 2004). In the present study, we confirmed the adaptation of rumen bacteria to such a harsh diet in the late winter; fibrolytic *R. flavefaciens* increased in both absolute and relative abundance in winter, together with the increase of the so far uncharacterized unknown group A bacteria (Figure 3). According to Gram-staining and morphological classification in the previous report, Gram-negative cocci increased in winter compared to summer, even though total bacterial counts did not differ (Ichimura et al. 2004). As *R. flavefaciens*, a Gram-positive coccus, often stains as a Gram-negative bacterium, the previous result might support the increase of *R. flavefaciens* in the present report. The reported increase of acetate proportion during winter (Ichimura et al. 2004) appears reasonable, as *R. flavefaciens* produces acetate as a major metabolite (Ogimoto & Imai, 1981). In regards to adaptive ability to a woody diet in the winter, *R. flavefaciens* might be superior to *F. succinogenes* in terms of attachment to and growth rate on fibrous materials (Roger et al. 1990; Latham et al. 1978). *F. succinogenes* is reported to show decreased attachment to cellulose under high pH and low temperature conditions (Miron et al. 2001). Therefore, the rumen environment of the present deer in winter may not be suitable for *F. succinogenes* to exert its superior fibrolytic actions as generally accepted (Kobayashi et al. 2008). It was reported that protozoa were represented by *Entodinium* spp. (> 99%) and no seasonal differences were observed (Ichimura et al. 2004), which strongly suggests that protozoa do not significantly contribute to the rumen fermentation and nutrition of the deer investigated. Meanwhile, the function of fungi in deer rumen remains uncharacterized and requires future investigation.

We identified the presence of unknown groups A and B in the deer rumen that were uniquely detected in winter and summer, respectively (Figure 2). Unknown group B bacteria showed decreased abundance in winter (Figure 3). In contrast, unknown group A showed increased ruminal abundance in winter (Figure 3), and was thus targeted for further ecologic characterization. In fact, unknown group A can be considered to be a key group for the ability of deer to utilize a highly fibrous diet in winter. Based on BLAST analysis, 315 sequences having ≥ 97% identity to the representative clone

SWD59, assigned to unknown group A, were retrieved. These sequences were from the rumen of a wide variety of wild and domestic ruminants including deer (this study), musk oxen, reindeer, yaks, camels, buffaloes, goat, sheep, cattle, and zebu cattle, and even from the abomasal mucosa of cattle and feces of gorillas and orangutans (Table 3). Thus, unknown group A bacteria are thought to be widely distributed irrespective of animal species, diets, and geographic boundaries. Henderson et al. (2015) described the world-wide distribution of unclassified *Bacteroidetes* to which the unknown group A proposed in the present study is assigned. Furthermore, ecological adaptation and substrate versatility is suggested in uncultured *Bacteroidetes* based on the presence of polysaccharide utilization loci (Mackenzie et al. 2015). This phylum also includes unknown group A. Our previous study dealt with fiber-associated rumen bacteria, one of which (Orchardgrass-associated bacterium clone no.5, assigned to the tentatively proposed U1 group) (Koike et al. 2003) was assigned to unknown group A in the present study. The isolation and physiological analysis of unknown group A bacteria is expected to assist in the functional characterization of this potentially important member.

As the first step to improve isolation success, enrichment of unknown A bacteria was attempted by using various carbon sources. Bean husk from soybean and lablab bean is known to selectively stimulate the growth of *Fibrobacter* and *Ruminococcus* (Ngwe et al. 2012; Fuma et al. 2012). However, oak bark rather than bean husk was successful for the enrichment of unknown group A, especially when supplemented with the rumen fluid from deer rather than sheep (Table 2). This is due largely to the adaptation of rumen bacteria to the winter diet (mainly wooden bark) and the possible presence of growth factor(s) in the deer rumen fluid. In fact, oak bark is reported to be one of the favorite winter food sources for deer of this area (Ichimura et al. 2004).

Due to quite limited functional information of unknown group A bacteria, it is not easy to predict their contribution to animal nutrition. However, according to wide distribution of this group among various herbivores including domestic cattle (Table 3), the bacteria play a certain role in plant digestion. Based on successful enrichment with oak bark (Table 2), unknown group A bacteria may be involved in hemicellulose digestion because oak inner bark contains high level of hemicellulose

(Kojima et al. 2006). The hemicellulose to cellulose ratio in oak bark (0.83) (Kojima et al. 2006) is higher than those in easily degradable bean husk (0.15 - 0.36) that stimulates the growth of representative rumen cellulolytics (Fuma et al. 2012). This indirectly suggest that unknown group A bacteria may prefer more complexed fiber rich in hemicellulose rather than pure cellulose that *Fibrobacter* prefers. However, this possibility is to be confirmed by further detailed study.

FISH successfully visualized unknown group A bacteria in the enrichment culture (Figure 4), which indicates that they are metabolically active in the culture supplemented with oak bark as the sole carbon source. This may suggest that unknown group A is directly or indirectly involved in the digestion of bark, a highly fibrous food. In fact, unknown group A bacteria was associated with the solid rather than the liquid fraction in the enriched culture, indicating their involvement in fiber digestion. Using the roll tube method with rumen fluid-glucose-cellobiose agar medium (Ogimoto & Imai 1981) and enriched culture as the inoculum, we picked >600 colonies and screened unknown group A bacteria through PCR detection. However, none of those were PCR-positive. Although reasons for the unsuccessful isolation are not clear, an agar medium may not be suitable for growth of the target bacteria. Further hypotheses and experimentation will be necessary to characterize this group of bacteria. In the meantime, the omics approach to targeting this group may provide new insights into the complex mechanism of fiber digestion as well as the seasonal adaptation of rumen flora to the poor-quality winter diet of deer.

Deer in the research area are thought to be capable of more effectively utilizing the less nutritious fibrous foods available in winter (Takahashi & Kaji 2001) by developing a fibrolytic bacterial population, including *R. flavefaciens* and possibly unknown group A bacteria. This may be key to supplying the energy needed for the survival of deer during the 5-month snowy season. The rumen bacterial flora of Svalbard reindeer was the first indication of such dietary adaptation in wild ruminants (Orpin et al. 1985). Subsequent detailed analysis revealed that > 90% of rumen bacteria in these reindeer represented novel strains (Sundset et al. 2007). A similar finding was reported for Hokkaido native horses grazed on woodland pastures year-round (Yamano et al. 2008). Thus, research on the gut

microbiota of wild or semi-wild herbivores exposed to harsh nutritional conditions might provide a greater understanding of the nutritional strategies of host animals.

5 CONCLUSION

Wild deer in Shiretoko Peninsula of Hokkaido can extract nutrients from a highly fibrous diet and survive during the lengthy winter by the adaptation of rumen bacteria. The observed increase of fibrolytic *R. flavefaciens* in winter represents one such adaptation. Another could be the development of unknown group A bacteria belonging to unclassified *Bacteroidetes*. Further functional characterization of this group in the rumen will shed light on this important biological process.

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REFERENCES

Bryant MP. Burkey LA. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *Journal of Dairy Science* **36**, 205–217.

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandezgarayzabal J, Carcia P, Cai J, Hippe H, Farrow JAE. 1994. The phylogeny of the genus Clostridium: proposal of five new genera and

eleven new species combinations. *International Journal of Systematic Bacteriology* **44**, 812–826

Dehority BA, Demarais S, Osborn DA. 1999. Rumen ciliates of white-tailed deer, axis deer, sika deer, fallow deer from Texas. *The Journal of Eukaryotic Microbiology* **46**, 125-131.

Fuma R, Oyaizu S, Nukui Y, Ngwe T, Shinkai T, Koike S, Kobayashi Y. 2012. Use of bean husk as an easily digestible fiber source for activating the fibrolytic rumen bacterium Fibrobacter succinogenes and rice straw digestion. *Animal Science Journal*, **83**, 43-49.

Henderson G, Cox F, Ganesh S, Jonker A, Young W; Global Rumen Census Collaborators, Janssen PH. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Scientific Report **5**, 14567.

Hobson PN, Mann SO, Summers R. 1975. Rumen micro-organisms in red deer, hill sheep and reindeer in the Scottish highlands. *Proceeding of the Royal Society of Edinburgh (B)* **75**, 171-180.

Ichimura Y, Yamano H, Takano T, Koike S, Kobayashi Y, Tanaka K, Ozaki N, Suzuki M, Okada H, Yamanaka M. 2004. Rumen microbes and fermentation of wild sika deer on the Shiretoko peninsula of Hokkaido island, Japan. *Ecological Research* **19**, 389-395.

Itabashi H. 1998. Digestion in the rumen. In: Nutritional Physiology of the Ruminant. (ed. Y.Obara) pp.87-112. Nosangyoson Bunka Kyokai, Tokyo.

Kobayashi Y. 2006. Inclusion of novel bacteria in rumen microbiology: needs for basic and applied science. *Animal Science Journal* **77**, 375–385.

Kobayashi Y, Shinkai T, Koike S. 2008. Ecological and physiological characterization shows that *Fibrobacter succinogenes* is important in rumen fiber digestion. *Folia Microbiologica* **53**, 195–200.

Koike S, Kobayashi Y. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiology Letters* **204**, 361–366.

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

Koike S, Yabuki H, Kobayashi Y. 2007. Validation and application of real-time polymerase chain reaction assays for representative rumen bacteria. *Animal Science Journal* **78**, 135–141.

Kojima Y, Yasui Y, Orihashi K, Terazawa M, Kamoda S, Kasahara H, Takahashi Y. 2006. Relationship between bark preferences of *Cervusn nippon yesoensis* and inner bark components of small-diameter tree trunks. *Journal of the Japanese Forestry Society*, **88**, 337-341.

Latham MJ, Brooker BE, Pettipher GL, Harris PJ. 1978. Adhesion of *Bacteroides succinogenes* in pure culture and in the presence of *Ruminococcus flavefaciens* to cell walls in leaves of perennial ryegrass (*Lolium perenne*). *Applied and Environmental Microbiology* **35**, 1166-1173.

Mackenzie AK, Naas AE, Kracun SK, Schückel J, Fangel JU, Agger JW, Willats WG, Eijsink VG, Pope PB. 2015. A polysaccharide utilization locus from an uncultured *Bacteroidetes* phylotype suggests ecological adaptation and substrate versatility. *Applied and Environmental Microbiology* **81**, 187-195.

Miron J, Ben-Ghedalia D, Morrison M. 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. *Journal of Dairy Science* **84**, 1294-1309.

Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695–700.

Ngwe T, Nukui Y, Oyaizu S, Takamoto G, Koike S, Ueda K, Nakatsuji H, Kondo S, Kobayashi Y. 2012. Bean husks as a supplemental fiber for ruminants: potential use for activation of fibrolytic rumen bacteria to improve main forage digestion. *Animal Science Journal*, **83**, 43-49.

Ogimoto K, Imai S. 1981. *Atlas of Rumen Microbiology*. Japan Scientific Societies Press, Tokyo, Japan.

Okada H. 2000. Hokkaido sika deer in Shiretoko. In: Mammals in Shiretoko (ed. Shari Munusipal Shiretoko Meusium) pp.12-26. Hokkaido Shinbun, Sapporo.

Orpin CG, Mathisen SD, Greenwood Y, Blix AS. 1985. Seasonal changes in the ruminal micloflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). *Applied and Environmental Microbiology* **50**, 144-151.

Roger V, Fonty G, Komisarczuk-Bondy S, Gouet P. 1990. Effect of physiochemical factors on the adhesion to cellulose avicel of the ruminal bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*. *Applied and Environmental Microbiology* **56**, 3081-3087.

Shinkai T, Kobayashi Y. 2007. Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence *in situ* hybridization and real-time PCR. *Applied and Environmental Microbiology* **73**, 1646–1652.

Singleton DR, Furlong MA, Rathbun SL, Whitman WB. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Applied and Environmental Microbiology* **67**, 4374–4376.

Sundset MA, Praesteng KE, Cann IK, Mathiesen SD, Mackie RI.2007. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. *Microbial Ecology* **54**, 424-438.

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Applied and Environmental Microbiology* **67**, 2766-2774.

Takahashi H, Kaji K. 2001. Fallen leaves and unpalatable plants as alternative foods for sika deer under food limitation. *Ecological Research* **16**, 257-262.

Yamano H, Koike S, Kobayashi Y, Hata H. 2008. Phylogenetic analysis of hindgut microbiota in Hokkaido native horses compared to light horses. *Animal Science Journal*, **79**, 234-242.

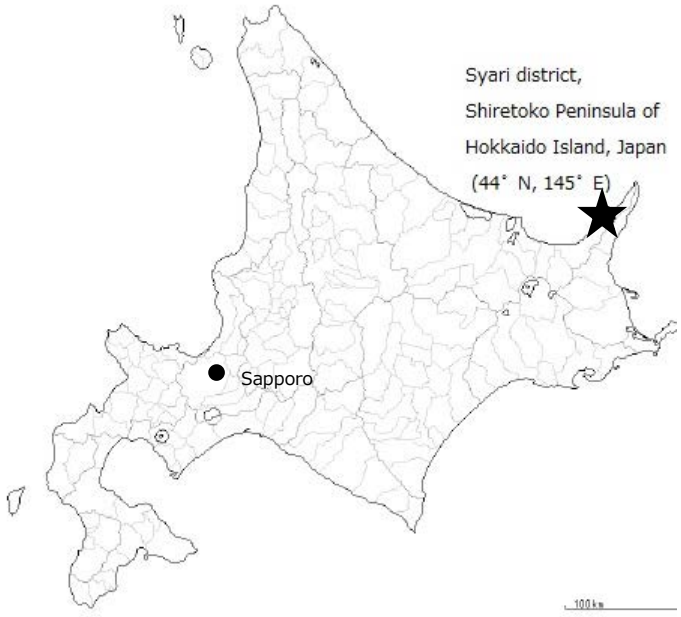


Figure 1 Sampling site and map of Hokkaido Island, Japan. Hokkaido sika deer (*Cervus Nippon yesoensis*) investigated were shot and slaughtered in July and August (summer) and in March (late winter).

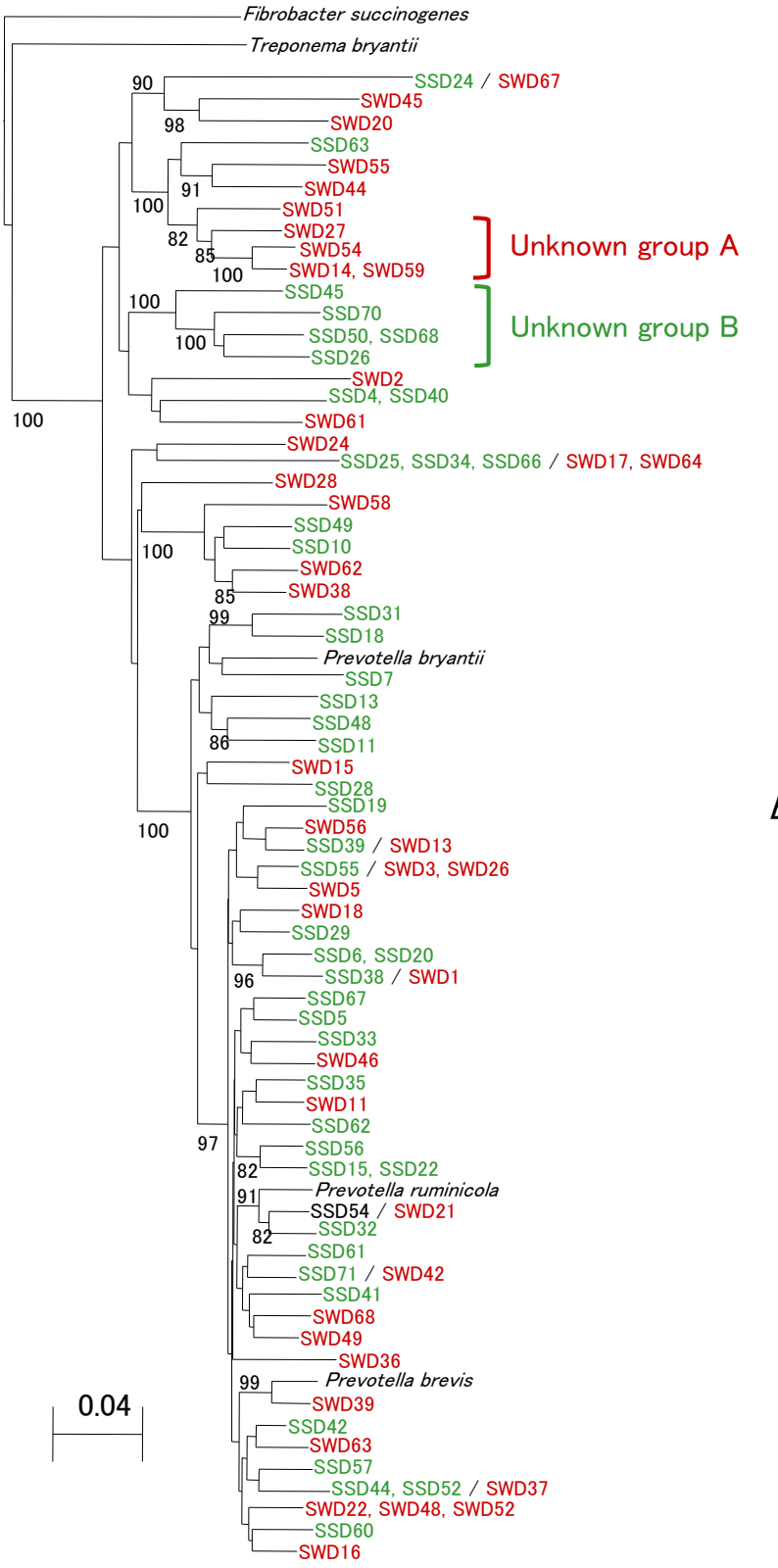
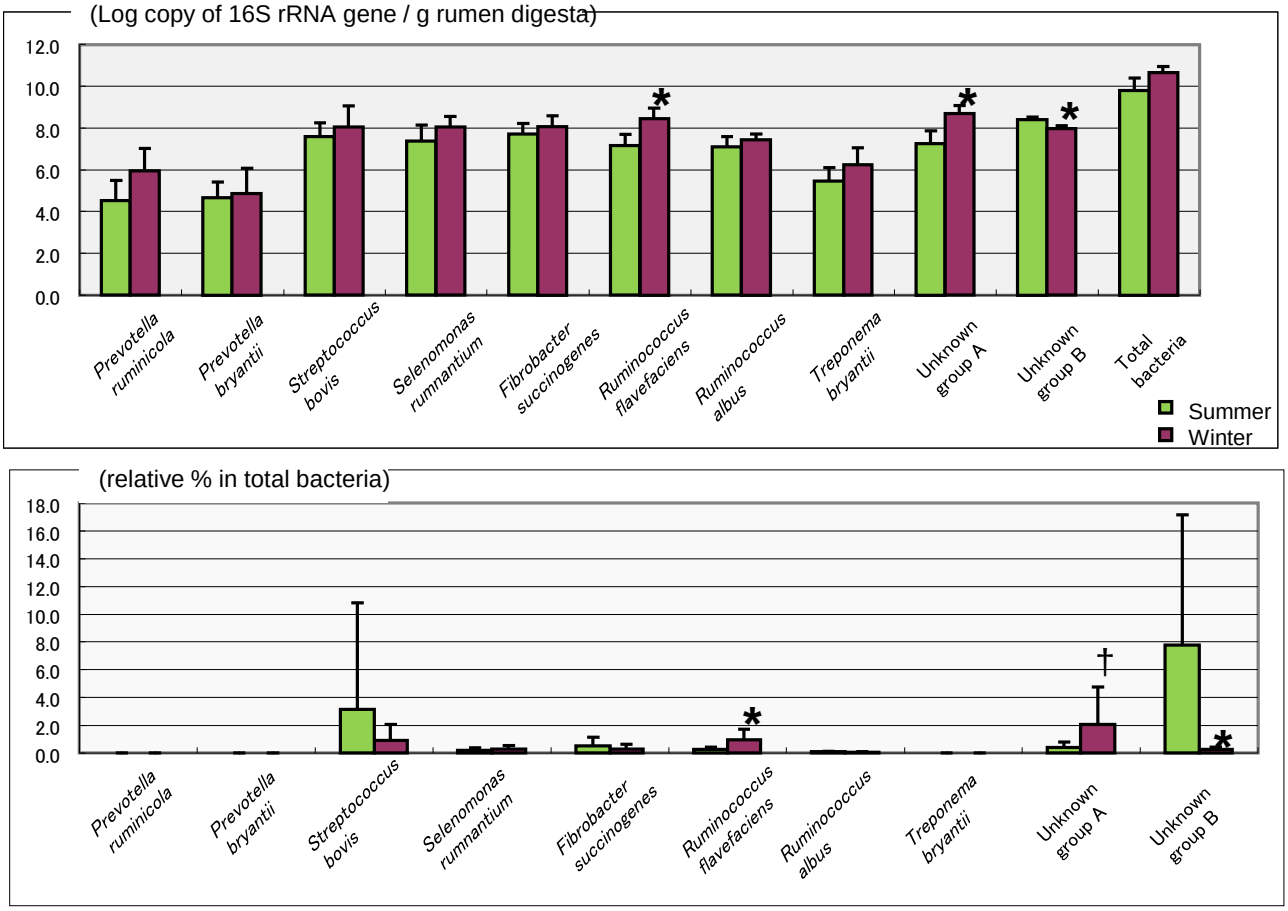


Figure 2 A part of phylogenetic tree of bacteria detected in the rumen of deer shot in summer (green) and winter (brown) of the Syari district, Shiretoko Peninsula, Hokkaido, Japan and season-specific clusters occupied by unknown bacteria named as unknown groups A (winter) and B (summer). Only bacteria affiliated to *Bacteroidetes* are shown. Numbers above each node are confidence levels (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.



*, $P < 0.05$, †, $P < 0.10$

Figure 3 Comparison between summer (green) and winter (brown) in rumen bacterial abundance of wild sika deer in the Syari district, Shiretoko Peninsula, Hokkaido, Japan. Each value represents average ± standard deviation (n=10).

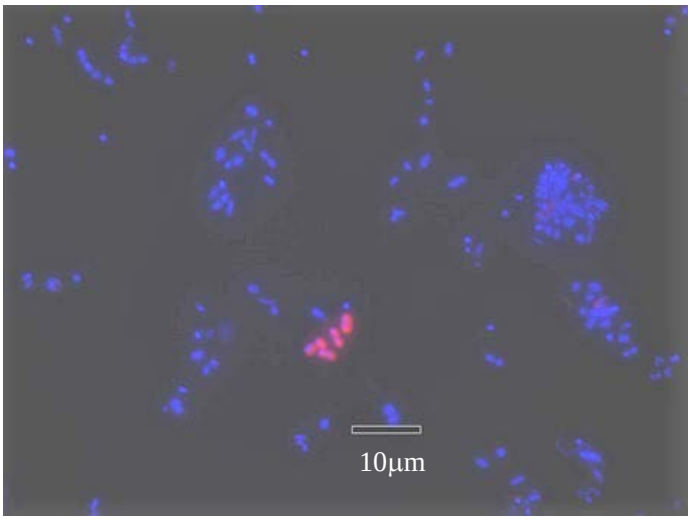


Figure 4 Detection of unknown group A bacteria in deer rumen digesta by fluorescent in situ hybridization
Whole cell hybridization was carried out using winter rumen digesta of deer and Cy3 labelled probe specific to unknown group A bacteria. Unknown group A bacteria are seen in clustered cocci (Red). Other bacteria are stained with 4',6-diamidino-2-phenylindole (Blue).

Table 1 Target bacterial species and groups and their PCR reactions for characterization of sika deer rumen

Target group	Annealing temp. (℃)	Extension time (sec)	Product size (bp)	References
Total bacteria	60	10	194	Muyzer et al. (1993)
Fibrobacter succinogenes	60	18	446	Tajima et al. (2001)
Ruminococcus flavefaciens	55	15	295	Koike and Kobayashi (2001)
Ruminococcus albus	55	10	175	Koike and Kobayashi (2001)
Prevotella ruminicola	53	20	485	Tajima et al. (2001)
Prevotella bryantii	68	22	540	Tajima et al. (2001)
Eubacterium ruminantium	57	30	671	Tajima et al. (2001)
Streptococcus bovis	57	35	869	Tajima et al. (2001)
Selenomonas ruminantium	57	22	513	Tajima et al. (2001)
Treponema bryantii	57	18	421	Tajima et al. (2001)
Unknown group A [1]	66	15	229	This study
Unknown group B [2]	64	10	150	This study

[1] Forward and reverse primers are 5'- GCCGGCGCTCAACGTCGGAA -3' and 5'- CCAGCGGTTAGCCATCATCG -3', respectively.
[2] Forward and reverse primers are 5'- CGGCGCAAGCTGTGAGGAAG -3' and 5'- GATCCGAACTGAGACGGGTT -3', respectively.

Table 2 Enrichment of bacteria belonging to unknown group A in a complex medium including powdered bark of Japanese oak and sterilized rumen fluid from sheep or deer as medium components.

Supplementation of rumen fluid	Unknown group A copy / tube	Fold change	Total bacteria copy / tube	Fold change	Degree of enrichment (%) (Unknown group A / total bacteria)
No Incubation	1.47×10^5	1	1.6×10^7	1	0.9
120h incubation with rumen of					
Sheep 1	1.54×10^5	1	6.51×10^9	407	0.002
Sheep 2	6.14×10^6	42	1.97×10^{11}	12,312	0.00003
Deer 1	6.39×10^9	43,469	2.22×10^{11}	13,875	2.9
Deer 2	1.67×10^{10}	113,605	4.08×10^{11}	25,500	4.1

Incubation (5mL) with powdered bark of Japanese oak (0.125 mg) and sterilized rumen fluid either from sheep or deer were carried out for 5d in triplicate and the degree of enrichment of unknown group A bacteria was monitored by quantifying unknown group A and total bacteria. Rumen fluid from two sheep on hay diet and two deer shot in winter in the experimental area were added at 30%.

Table 3 Sources of bacteria belonging to unknown group A * retrieved from GenBank

Source					No. of sequence
Digesta/Tissue	Common name	Scientific name	Country	Fed/Wild	(315 in total)
Rumen digesta	Hokkaido sika deer	*Cerevus nippon yesoensis*	Japan	Wild	4
Rumen digesta	Musk ox	*Ovibos moschatus*	USA	Fed	9
Rumen digesta	Reindeer	*Rangifer tarandus tarandus*	Norway	Fed	1
Rumen digesta	Yak	*Bos grunniens*	China	Fed	37
Rumen digesta	Golden takin	*Budorcas taxicolor bedfordi*	China	Fed (Zoo)	3
Rumen digesta	Swamp buffaloes	*Bubalus bubalis*	Thailand, Tiwan	Fed	16
Rumen digesta	Bactrian camel	*Camelus bactrianus*	China	Fed	2
Rumen digesta	Dromedary camel	*Camelus dromedarius*	Australia	Wild	4
Rumen digesta	Goat	*Capra aegagrus hircus*	China	Fed	1
Rumen digesta	Sheep	*Ovis aries*	Japan	Fed	31
Rumen digesta	Zebu cattle	*Bos indicus*	Thailand, Japan	Fed	13
Rumen digesta	Cattle	*Bos taurus*	USA, Canada, Japan, China	Fed	185
Abomasal mucosa	Cattle	*Bos taurus*	Austria	Fed	3
Feces	Rocky Hyrax	*Procavia capensis*	Canada	Fed (Zoo)	3
Feces	Western lowland gorilla	*Gorilla gorilla*	USA	Fed (Zoo)	2
Feces	Orangutans	*Pongo pygraeus abelii*	USA	Fed (Zoo)	1

* shraing ＞ 97% sequence identity of 16S rRNA gene