Introduction

More than 400 different species colonize the human and animal gastrointestinal (GI) tract. This microbial community has been designated the microbiota and its metabolism has a significant impact on gut environmental conditions. Previous studies have suggested that some species of the microbiota are more beneficial than others and that the population of these beneficial microorganisms can be increased by special dietary components known as prebiotics, including fibers and oligosaccharides. Recently, many plant- and bacteria-derived materials have proved to have prebiotic potential. However, the different effects of individual prebiotics on microbiota have not yet been clarified.

The recent development of stable isotope probing (SIP) of nucleic acid (SIP-DNA and SIP-RNA) technology enables enrichment and detection of microbial groups involved in the metabolism of $^{13}$C- or $^{15}$N-labeled compounds from a microbial community (Radajewski, 2000). SIP-DNA and -RNA techniques are based on the hypothesis that microorganisms which ferment stable isotope-labeled compounds more efficiently than non-fermenters, also incorporate $^{13}$C into their DNA or RNA, respectively. This can be detected using stable isotope probing technologies.

The aim of this study was the development of an efficient method to identify the prebiotics-assimilating bacteria in gut microbiota using DNA-stable isotope probing (DNA-SIP) technology. For efficient probing of microbiota with stable isotopes, a small-scale repeated batch culture using a low-carbon-source-containing medium was developed. Fecal samples from cattle were inoculated and $[\text{U-}^{13}\text{C}]$-fructose was applied to the culture after 24 h stabilization. Organic acid production, pH value of the period and the total diversity of microorganisms of the culture were successfully maintained during the chasing period. DNA samples were extracted from the culture and were subjected to isopycnic centrifugation and fractionation in order to separate fructose fermenters from non-fermenters. T-RFLP (Terminal Restriction Fragment Length Polymorphism) and the modified T-RFLP of each fraction suggested that $\text{Streptococcus bovis}$ was the most dominant fructose fermenter in this culture. In addition, we improved the modified T-RFLP method and successfully identified $\text{Lactobacillus vitulinus}$ and $\text{Megasphaella eldenii}$ as minor fructose-fermenters and several species of $\text{Clostridium}$ cluster IV as non-fermenters. From these results we concluded that the methods shown here provide a means for assessing the importance of individual prebiotics on gut microbiota.

Key Words—fructose; microbiota of cattle feces; modified T-RFLP; phylogenetic identification; stable isotope probing
nucleotides more efficiently. The labeled nucleotides are separated by isopycnic density gradient centrifugation, and the diversity of 16S rRNA genes of the active microorganisms are then analyzed using various fingerprinting techniques such as T-RFLP (Friedrich, 2006; Hayashi et al., 2002a), DGGE (Heilig et al., 2002; Satokari et al., 2001), and clone library (Hayashi et al., 2002b). Among these methods, T-RFLP is the most suitable method for the high-throughput analysis of a microbial community. It is simple, reproducible, and semi-quantitative. In addition, in silico analysis of the Ribosomal Database (RDP, (Marsh et al., 2000)) provides annotation of individual T-RF. For these reasons, many researchers have applied T-RFLP to the metagenomic and SIP studies of bacterial communities in various environments including soil (Lueders et al., 2004b, c; Pumphrey and Madsen, 2008; Rangel-Castro et al., 2005), activated sludge (Hatamoto et al., 2007; Manefield et al., 2002), and gut (Egert et al., 2007; Kovatcheva-Datchary et al., 2008).

However, the combination of nucleotide-SIP technology and T-RFLP analysis has several limitations that apply to the study of prebiotics. First, there has been no suitable in vitro culturing system for nucleotide-SIP experiments. Several fine-tuned culture systems which are suitable for stable cultivation of the dominant group of gut microbiota in vitro have been developed (Macfarlane et al., 1998; Mäkivuookko et al., 2006; Minekus et al., 1999; Molly et al., 1993). Because those systems are large and continuous culturing systems, a large amount of stable isotope is necessary for the sufficient labeling of microbiota in these cultures. However, most prebiotics have a high molecular weight compared to compounds such as phenol, benzene, and methane, which were used in the study of non-gut environments. Because it is difficult and expensive to obtain a large amount of labeled prebiotics, small-scale in vitro fermentation techniques are essential. Second, annotation of each T-RF to the in silico database is not always sufficient for identification. The sizes of the T-RFs did not correspond exactly to the fragment sizes in the 16S rDNA database (Kaplan and Kitts, 2003) and same-size T-RFs can be generated from 16S rRNA genes of multiple species. Thus, it is necessary to test several combinations of enzymes and primers in order to obtain a number of T-RFLP profiles from one sample for precise identifications. Construction of a clone library is an alternative, but it is a time-consuming undertaking.

In the present study, we focused on the metabolism of fructose, which generates a number of prebiotics, including fructo-oligo-saccharides (FOS) and inulin, and developed a small scale in vitro cultivation method suitable for nucleotide-SIP analysis. [U-13C]-fructose was added to this culture and both fructose-fermenters and fructose-non-fermenters were successfully enriched based on isopycnic density-gradient centrifugation. In addition, we succeeded in directly identifying the phylogenetic species of these fermenters and non-fermenters using a modified T-RFLP analysis based on adaptor-ligation, 2nd PCR, and polyacrylamide gel electrophoresis.

Materials and Methods

Media and microbial source. Fresh cattle feces were collected from adult Holstein grown on silage and grass at the Jiyuu-Gakuen Nasu farm in Fukushima Prefecture, Japan. After samples were collected, they were immediately frozen at −80°C until use as seed inocula for culture.

Ninety-nine percent-labeled [U-13C]-fructose was purchased from ISOTEC (Miamisburg, OH).

Repeated batch culture and stable isotope probing.

Eighty-milliliter batch cultures in 125 ml vials were inoculated with 10-fold dilutions of homogenized intestine microbiota derived from cattle feces. All handling was performed anaerobically under N2 gas. Incubations were performed at pH 6.6 and 37°C. Forty milliliter samples were removed every 24 h and replaced with the same amount of fresh media. Two types of media were used. Medium A was prepared according to a previous report (Minekus et al., 1999). Medium B was same as medium A except that the starch concentration was one-tenth that of medium A. The experimental design of the stable isotope probing analysis is illustrated in Fig. 1. Twenty milliliters of fecal sample were inoculated to each vial and after 24 h stabilization, [U-13C]-fructose was added to final concentrations of 0.05%. Forty milliliters of fecal sample was collected after 0 h (control), 8 h, 24 h, 32 h, and 48 h cultivation. The samples were centrifuged at 12,000 × g (20 min), and the pellet and supernatant were subjected to DNA extraction and chemical analysis, respectively.

Chemical analysis. Acetate, butyrate, citrate, formate, isobutyrate, isovalerate, lactate, malate, propionate, pyruvate, and succinate were measured by a
Fig. 1. Stable isotope labeling procedure and extraction of isotopically-labeled DNA.

(A) Experimental procedure for stable isotope labeling in repeated batch culture. The hatched triangle indicates the time of seeding. Filled triangles indicate times of media exchange and addition of [U-13C]-fructose to a final concentration of 0.05%. Open triangles indicate sampling times. (B) CsCl density gradient centrifugation of DNA from fecal samples cultivated in [U-13C]-fructose-containing media. A mixture of chromosomal DNA extracted from Ruminococcus productus JCM1471T, Bifidobacterium longum JCM1217T, Collinsella aerofaciens JCM10188T, Bacteroides vulgatus JCM5826T, and Clostridium perfringens JCM1290T cultured with [U-13C]-glucose or non-labeled DNA, respectively.

DNA extraction and isopycnic centrifugation. Post-centrifugation (12,000 × g) fecal sample pellets were suspended in 2 ml of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, pH 9.0) and were washed three times in this buffer. DNA samples were extracted using the FastDNA SPIN Kit for soil (Qiogene, Carlsbad, CA) according to the manufacturer’s instructions.

A density gradient was prepared by mixing CsCl and TE (pH 8.0) with an average of refractive index value of 1.395 nD, and supplemented with 80 µl of a 10 mg/ml ethidium bromide to 1 ml of CsCl/TE buffer. The mixture was added to 5.5 ml 5PA seal tubes (HI-500, Tokyo, Japan) equipped with a Shimadzu SPR-H column, 250 × 7.8 mm (Shimadzu), held at 45°C. A flow rate of 0.5 ml/min.

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Fingerprinting analysis of bacterial 16S rRNA gene in gradient fractions. Bacterial 16S rRNA gene was amplified from 1 µl of each fraction using 35F (5’-CTG GCTCAGGATGAACG-3’, Hayashi et al., 2004) and 1492R (5’-GGTACCTTGTTACGACTT-3’) primers. The forward primer 35F was labeled with Beckman Dye 3 at the 5’-end. PCR was carried out in a total volume of 50 µl with 50 ng Template DNA, 0.2 µM of each primer, 0.2 mM (each) dNTP, 1 × standard PCR buffer (Perkin-Elmer Corp., Norwalk, CT), 0.1% BSA (TaKaRa Bio, Inc., Otsu, Japan), and 1.25 U AmpliTaq Gold DNA Polymerase (Perkin-Elmer Corp.). PCR cycling consisted of an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The fluorescently labeled PCR products were purified using Microcon YM-100 (Millipore, Bedford, MA) and eluted in a final volume of 30 µl of distilled water. An aliquot (7 µl) of the purified PCR products was digested with 20 U of Hhal (TaKaRa Bio, Inc.) at 37°C for 10 h. The lengths of the fluorescently-labeled terminal restriction fragments were determined for each sample using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Operation taxonomy units (OTU) of T-RFs were determined in the range of 60 to 600 bp, which was the range of the size marker. Putative microorganisms were identified based on the RDP 9 database (http://rdp.cme.msu.edu/).

Direct identification of T-RF by modified T-RFLP. Modified T-RFLP was conducted as shown in Fig. 2. The first PCR was conducted with 35F and 1492R primers and the amplified fragments were digested with Hhal (TaKaRa Bio, Inc.) as described above. The double-stranded Hhal-adapter was prepared by annealing the single-stranded oligonucleotides ad1_Hhal (5’-GAGCATCTGACGAGTAA-3’) and ad2_Hhal (5’-CCATGCTTCGATCGTCCG-3’). The Hhal digested fragments were diluted 10-fold and were ligated with 10 pmol of Hhal adapter using a ligation kit (TaKaRa Bio, Inc.), at 16°C for 1 h. An aliquot (0.5 µl) of this ligation mixture was subjected to the second PCR with 35F and pHhal (5’-CCATGCCTCAGATGCTC
CGC-3') primers. Twenty microliters of the PCR mixtures were subjected to electrophoresis on a 13% (w/v) polyacrylamide gel at 90 V for 16 h at 8°C with a 100 bp ladder as the size standard (TaKaRa Bio, Inc.). The gel was stained with VistraGreen nucleic acid gel stain (GE Healthcare, Buckinghamshire, UK) for 15 min and the resulting image was acquired using a FluorImager™ 595 (Molecular Dynamics, Sunnyvale, CA). The band of interest was carefully excised and the DNA was eluted into 30 µl water by performing a freeze and thaw cycle three times. The eluted DNA was subjected to PCR using 35F and p\textit{Hha}I primers. To separate co-migrating T-RFs within the same band, p\textit{Hha}I_N (5'CCATGCACAGATGCTCCGCN-3', N: A, T, G, or C) were used as reverse primers instead of p\textit{Hha}I.

PCR products were subcloned with a TOPO TA cloning kit (Invitrogen, Ltd., Paisley, UK) for sequencing. Microorganisms associated with each T-RF were identified by annotating the sequence to the 16S rRNA gene database of the DNA data bank of Japan (DDBJ) using BLASTN. The results were aligned using Clustal W. Phylogenetic inferences were performed based on Jukes Cantor distance determination and Neighbor-Joining dendrogram construction with MEGA 3.1 (Kumar et al., 2004).

Quantitative real-time PCR. Quantitative PCR was conducted as reported (Nishida et al., 2008). Amplifications of the 16S rRNA gene were performed with the SYBR Premix Ex Taq™ (TaKaRa Bio, Inc.) containing 0.2 µM of each primer and 0.01% BSA using LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). For quantification of Streptococcus bovis, StrBov01_F (5'CTAATACCGCATAACAGCA-3') and StrBov01_R (5'-AGAAACTTCCTATCTCTAGG-3') (Stevenson and Weimar, 2007) were used as primers with the condition of 5 s at 95°C followed by 50 cycles of 95°C for 15s, 57°C for 20 s, and 72°C for 15 s. For quantification of Megasphaera elsdenii, MegEls2F (5'-AGATGGGCAACAGCTGGGA-3') and MegEls2R (5'-CGAAAGCTCGAAGGCCT-3') were used as primers (Tajima et al., 2001) with the condition of 5 s at 95°C followed by 45 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s. For quantification of Clostridium cluster IV, sg-Clept-F (5'-GCACAAGCAGTGAGTG-3') and sg-Clept-R (5'-CTTCCTCGTTTTGTCAA-3') were used as primers (Matsuki et al., 2004) with the condition of 5 s at 95°C followed by 45 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 30 s. S. bovis ATCC33317T, M. elsdenii JCM1772T, and Faecalibacterium prausnitzii ATCC 27766T, were used as standard strains of S. bovis, M. elsdenii, and Clostridium cluster IV, respectively. Standard curves were constructed from the dilution series of chromosomal DNA extracted from a dilution series of the standard strains.

Results

Optimization of culture condition for DNA-SIP analysis

To develop a small-scale culture which maintains the diversity of microbiota, we used a repeated batch culture with small anaerobic vials and optimized cultivation media to maintain the gut microbiota. Organic acid production and pH were measured to assess the robustness of the culture.

Using medium A, the previously reported medium (Minekus et al., 1999), the pH was found to drop from 6.6 to 4.2 within one day (Fig. 3A). Half of the medium...
was changed and the pH was adjusted daily to 6.3 with NaOH. However, the pH was found to drop to 5.3, 5.9, and 6.0 on day 2, 3, and 4, respectively. Chemical analysis suggested that lactate was dominantly produced up to 12 mM on the first 2 days (Fig. 3B), and was then converted to propionate, butyrate, and acetate. T-RFLP analysis suggested that the number of dominant species (more than 1% of total) in the culture decreased from 17 to 2 after 24 h cultivation. The most abundant OTU was annotated to *Lactobacillus* sp., which comprised 35% of the total bacteria on day 1 and 70 to 80% on day 2 to day 4 (*HhaI*-OTU [256 bp], data not shown). These results suggested that this culture was not stable and was not suitable for the nucleotide-SIP experiment. It was likely that overgrowth of lactate-forming bacteria prevented the growth of the other species under this condition.

In order to keep greater biological diversity of the culture, several media were tested. Finally, we found that limiting-carbohydrate-concentration-media was suitable for this purpose. In medium B, which contained one-tenth of the initial carbohydrate concentration of medium A, the pH of the culture was stably maintained and did not drop under 6.5 during the cultivation period (Fig. 3C). No lactate but large amounts of acetate, propionate, and butyrate were detected in the culture using medium B (Fig. 3D). In addition, a variety of OTUs were detected in this culture by T-RFLP analysis. The dominant genera were *Streptococcus* (18 to 25%), *Lactobacillus* (2 to 8%), *Clostridium* (13 to...
20%), *Ruminococcus* (5 to 10%), *Bacteroides* (7 to 25%), and *Fusobacterium* (3 to 14%).

**Enrichment and detection of 13C-fructose fermenting bacteria**

We conducted [U-13C]-fructose feeding experiments according to the schedule illustrated in Fig. 1A. Neither the concentration of organic acid nor pH in the culture was changed significantly by the additions of 13C-fructose (Fig. 3E and 3F). DNA extracted from the fecal samples collected at 0, 8, 24, 32, and 48 h was subjected to CsCl density gradient centrifugation. The fluorometric analysis showed that the non-labeled DNA (0 h) ranged between control 12C-DNA and completely labeled 13C-DNA (Fig. 1B). This is likely due to the variable GC-content of DNA as previously reported (Lueders et al., 2004a). The density shift of the isotopically-labeled DNA began within 8 h and continued with greater incorporation of 13C during the cultivation period (Fig. 1B).

DNA from 0, 8, 24, and 32 h cultivation samples was fractionated and was subjected to T-RFLP analysis. The T-RFLP profiles of the 8 and 24 h samples are shown in Fig. 4. The results indicated that the *Hha*OTU[574 bp] and *MspI*-OTU[549 bp] peak increased significantly in fraction 1. In silico annotation suggested that *Lactococcus garvieae* and several *Streptococcus* species were possible candidates for *Hha*OTU[574bp] and that *Lactococcus garvieae*, several species of *Streptococcus*, and *Clostridia* were candidates for *MspI*-OTU[549 bp].

In addition, several peaks including *Hha*OTU[180 bp], *Hha*OTU[195 bp], *Hha*OTU[218 bp], and *Hha*OTU[375 bp] disappeared in the high density fraction. It is likely that these OTUs were generated from the DNA of 13C-fructose non-fermenter in this culture.

**Direct identification of 13C-fructose fermenting bacteria by modified T-RFLP**

Though two enzymes were employed for T-RFLP analysis of the fructose-fermenting bacteria, we were not able to annotate them at either the species or genus level. In order to identify the fructose-fermenters, we attempted to clone the *Hha*OTU[574 bp] using a modified T-RFLP analysis.

The *Hha*-digested fragments were ligated to *Hha*

![Fig. 4. T-RFLP profiles of 16S rDNA obtained from fractions of [U-13C]-fructose feeding culture. Arrows and numbers indicate the sizes of T-RF in each fraction.](image-url)
adaptors and re-amplified with 35F and pHal primers. The re-amplified fragments were then subjected to electrophoresis. A major band of about 550 bp (Fig. 5, band 1) gradually increased in the higher density fractions, suggesting that this fragment was generated from Hhal-OTU[574 bp]. Band 1 was eluted from fructose-fermentors’ polyacrylamide gel and subjected to sub-cloning and sequencing. Six clones were sequenced and all of the clones comprised 571 bp fragments which were highly homologous to the Streptococcus bovis 16S rRNA gene (99 to 100% identity within the 550 bp region).

The quantity of band 2, which was not detected in the T-RFLP profiles, also increased in the higher density fraction. This band was likely to contain 16S rDNA of fructose-fermenters as well as band 1. In addition, the quantity of band 3 increased in the lower density fraction, meaning this band was likely to have been generated from non-fermenters (Fig. 5). To identify more fructose-fermenters and non-fermenters, these bands were also subcloned. Six clones of band 2 were sequenced and all encoded the 16S rRNA gene of Lactobacillus vitulinus (99 to 100% identity within a 430 bp region). Six clones of band 3 were sequenced. Band 3 included several homologous but not identical sequences. Phylogenic analysis using Clustal W suggested that all belonged to Clostridium cluster IV, which is known as a group of butyrate producer, in the gut and converts lactate to butyrate via the acrylic pathway (Fig. 6). Clostridium subterminale was the closest species for five of the clones (95 to 99% iden-

![Fig. 5. Polyacrylamide gel electrophoresis of T-RFs amplified using the pHal primer. Arrows indicate the bands subjected to sub-cloning and sequencing. The band pattern of fraction 6, which is not shown in this gel, was quite similar to that of fraction 5.](image)

![Fig. 6. Phylogenetic tree of 16S rDNA gene sequences cloned from band 3 in Fig. 5. Bootstrap values are shown at branch points. The scale bar represents genetic distance (10 substitutions per 100 nucleotides).](image)
tity within a 198 bp region) and Clostridium paraputrefaci- 
cum was the closest for one (93% identity within a 204 
bp region).

**Improvement of modified T-RFLP using 3’-extended primer**

Though *S. bovis* was the unique species identified 
from band 1 in Fig. 5, we speculated that band 1 might 
include several bands derived from the 16S rDNA gene 
of minor species. In order to identify these minor spe-
cies, primers pHhal_A, pHhal_T, pHhal_G, or pHhal_C, 
which comprise A, T, G, or C at the 3’-end of the pHhal 
primer, were used for a second PCR instead of pHhal. 
The amplified DNA samples were subjected to electro-
phoresis after Hhal digestion. The pHhal_T, pHhal_G, 
and pHhal_C primers were found to generate the 550 
bp fragment (Fig. 7), suggesting that fragments from 
more than three microorganisms were included in 
band 1. Eight clones from band 1A were sequenced 
and all of them encoded 16S rDNA of *S. bovis*. Six 
clones from band 1C and 1G were sequenced. Five 
clones from band 1C and all clones from 1G were 
found to encode 16S rDNA of *M. elsdenii* (99% identity 
within 560 bp region), suggesting that *M. elsdenii* also 
fermented fructose as well as *S. bovis*. One clone from 
band 1C encoded a fragment partially homologous to 
the 16S rDNA gene of *Streptococcus infantarius* (97% 
identity within a 378 bp region).

We also applied pHhal_N primers for further popula-
tion analysis of bands 2 and 3 in Fig. 5. As shown in 
Fig. 7, the pHhal_C primer was found to generate the 
bands 2C and 3C. The sequences of band 2C were 
identical to the 16S rRNA gene of *L. vitulinus* and 
*Clostridium* cluster IV, respectively.

**Quantitative PCR analysis**

In order to confirm that the species identified by the 
modified T-RFLP analysis were indeed enriched in 13C-
labeled fractions, quantitative PCR was performed. As 
shown in Fig. 8, the amount of 16S rDNA from *S. bovis* 
in fractions 2 and 3 were nearly 400 times higher than 
in fraction 8. The amount of 16S rDNA of *M. elsdenii* in 
fraction 2 was nearly 30 times higher than in fraction 8. 
On the other hand, the amount of 16S rDNA of *Clostrid-
ium* cluster IV was lower in the heavier fractions. These 
results strongly suggested that *S. bovis* and *M. els-
denii* utilized and assimilated 13C-fructose whereas 
*Clostridium* cluster IV did not.

**Discussion**

Though nucleotide-SIP is a powerful tool to reveal a 
link between microbiota and diet, only two studies that 
have used this approach on gut microbiota have been 
published. Egert et. al. was the first to report the use of 
nucleotide-SIP on gut microbiota and revealed a glu-
cose-fermenter using an in vitro intestinal model. Us-
ing the same culturing system, Kovatcheva-Datchary 
et. al. studied the link between the microorganism di-
versity and starch-metabolism in the gut. However, 
Egert et. al. suggested that a high concentration of 
glucose and a starvation period were necessary for 
the identification of fermenting bacteria. In this study, 
we propose that the repeated batch culture containing 
a medium of low carbohydrate concentration is suit-
able for nucleotide-SIP experiments using minimal amounts of labeled microbiota. This small culture system was less stable than the continuous culture, but was stable enough to allow the survival of the most dominant bacteria over 3 to 4 days. The pH and organic acid concentration was also kept stable. The ratio of organic acid found in the repeated batch culture was similar to that in the GI tract (Belenguer et al., 2007). Further, the fructose fermentable bacteria were efficiently labeled within 8 h by the addition of 13C-fructose. The pH and organic acid concentrations were not significantly affected during this period. Based on these results, it is likely that this culturing system is a useful tool for rapid screening of bacteria using nucleotide-SIP.

We also applied an innovative analysis method for rapid and direct identification of fructose-fermenters and non-fermenters. The principle of the modified T-RFLP method was first proposed by Mengoni (Mengoni et al., 2002) and was later applied in two studies (Hyun-Kyung et al., 2008; Widmer et al., 2006). To our knowledge, this is the first report of this method on fecal microbiota. Previous studies of the modified T-RFLP have not compared the profiles to that of normal T-RFLP. Our results suggested that the profiles of these two methods were quite comparable (Figs. 4 and 5). The results of quantitative PCR strongly supported that the coupling of these two T-RFLP methods is likely to be a simple and reliable tool for screening and identification of diet-linked bacteria.

Using a primer extended by one base at the 3′-end, *M. elsdenii* was found to be a minor group of the fructose-fermenters (Fig. 7). It should be noted that *M. elsdenii* was detected in two sets of primer *pH*hal_G and *pH*hal_C. The sequence analysis suggested that the clones from band 1-C and 1-G comprised 581 bp, and 583 bp fragments, respectively. The only differences between these were that the clones of *pH*hal_G primer contained an additional GC at its 3′-end of the *Hhal*-digested fragments. It is likely that both OTUs were generated from the same 16S rDNA sequence, since in silico analysis suggested that the 16S rDNA gene of *M. elsdenii* (accession No. AY038996, U95027, DQ146765 and AY038994) comprised GGGCGCGC in this region and the two *Hhal* sites overlap.

*S. bovis* and *L. vitulinus*, which were the dominant fructose fermenter and lactate producer in our culture system, are the species often found in the GI tract of cattle (Russell and Dombrowski, 1980; Sharpe et al., 1973). Previous reports have demonstrated that *S. bovis* is one of the most acid-tolerant bacteria and that its overgrowth causes lactic acid acidosis in herbivores (Gill et al., 2000; Russell and Dombrowski, 1980). *S. bovis* has also been reported as a rapid glucose fermenter (Egert et al., 2007).

On the other hand, *M. elsdenii* has been known as an organic-acid-fermenter rather than a carbohydrate-fermenter in the gut. Many studies suggested that *M. elsdenii* species efficiently convert lactate to butyrate and propionate via the acrylate pathway (Counotte et al., 1981). However, several studies demonstrated that *M. elsdenii* is able to directly ferment fructose to produce propionate and butyrate (Forsberg, 1978; Marronek et al., 1989). Our results suggested that *M. elsdenii* played a role as the initial fermenter of fructose and presumably was responsible for the higher amount of production of propionate and acetate in the present culture.

Interestingly, several known polysaccharide degraders, such as species of genus *Prevotella* (*Hhal*-OTU[92 bp]), were not identified as fructose-fermenters in this study. *Prevotella* is known to ferment fructose as a
pure culture. This suggested the possibility that those species efficiently assimilated polysaccharides and oligosaccharides but not monosaccharide. A study using $^{13}$C-oligosaccharides would test this hypothesis, and this project is on-going in our lab.

References


