Effect of a Lactobacillus Species on Incidence of Diarrhea in Calves and Change of the Microflora Associated with Growth

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To evaluate the effect of Lactobacillus plantarum strain Hokkaido, which was isolated from a kind of Japanese pickle, on the incidence of diarrhea in calves and on the intestinal microflora, we performed feeding tests with a milk replacer containing Lactobacillus sp. In Experiment 1, thirty two male Holstein calves were divided into two groups, a control (C) group and LPH group. L. plantarum strain HOKKAIDO was orally administered to the LPH group for 35 days. The diarrhea score and the number of calves with watery or soft stool were significantly (p<0.05) smaller in the LPH group than in the C group. In Experiment 2, ten male Holstein calves were divided into three groups: a control group, LPH group and BOV group. BovactinTM was administered to the BOV group and the experimental protocol followed that of Experiment 1. No significant difference was observed in the incidence of diarrhea among the three test groups. However, when the data of Experiments 1 and 2 were pooled, the incidence of diarrhea in the LPH group was significantly (p<0.05) lower than that of the control group. These results indicate that L. plantarum strain Hokkaido reduces the incidence of diarrhea in calves. Analysis of the microflora and measurement of the stool type of the fecal samples that were collected 0, 15 and 28 days after the start of administration were performed using a T-RFLP method and visual analysis, respectively. The clustering of the T-RFLP profiles indicated that when the significance of the distributions of the samples among the clusters was tested, a significant difference (p<0.01) was observed only among the sampling-date groups. The average value of the pairwise Pearson r within each sampling-date group indicated that T-RFLP profiles varied considerably among the calves on day 0 and day 15, while the profiles of day 28 closely resembled each other. From these results, we infer that the intestinal microflora of calves are less settled in the early days of life, and this might partially explain the higher incidence of diarrhea in this period. Bacteria belonging to the class Clostridia were most predominant at all the sampling-date groups. The day 0 samples were characterized by a larger population of bifidobacteria and lactic acid bacteria (LAB). The day 15 samples were characterized by larger populations of LAB and the class Bacteroidia. The day 28 samples were characterized by a larger population of Bacteroides.

Key words: calf; diarrhea; microflora; probiotic; T-RFLP

INTRODUCTION

The dairy and livestock industries have recently been confronted with large problems including zoonoses such as bovine spongiform encephalopathy and avian influenza, and the spread of antibiotic-resistant bacteria through feeds containing antibiotics. In this context, the healthcare of livestock has become an important issue. Animals are hosts to many kinds of bacteria, and a large number of bacteria colonize in the intestinal tract, forming a community known as the intestinal microflora. The microflora affects the health of the host through their metabolites and immunomodulatory activities, while the microflora are affected by diet, medicine, stress and so on. In recent years, probiotics (7) or prebiotics (22) that improve the host microflora have received a lot of attention. In livestock, probiotic and prebiotic-supplemented feed have been evaluated and exploited for decreasing diarrheal disease, reducing odors, and improving growth (1, 12, 24, 29, 31). However, there seems to be no report describing the effect of probiotics on the microflora.

Conventionally, analysis of the intestinal microflora, which is necessary for evaluation of probiotics or
prebiotics, has been performed by the culture method. However, molecular tools targeting the 16S ribosomal RNA gene (6, 10, 14, 15, 27, 28, 35, 36) have recently been developed, since many species (70–80%) of environmental bacteria are difficult to culture or are unculturable. Of these molecular tools, terminal restriction fragment length polymorphism (T-RFLP) is known to have the advantages of reproducibility and higher throughput (20), therefore, it has been frequently used in the studies of individual differences, diversity and dynamics of microflora (9, 11, 21). We have developed a modified method of T-RFLP that makes it possible to presumptively identify phylogenetic bacterial groups from the size of terminal restriction fragments (T-RFs), and another method to confirm the bacterial groups by cloning and sequencing of T-RFs (16, 17).

*Lactobacillus plantarum* has been isolated from various kinds of fermented foods or silage (3, 19, 27, 28). Nagata et al. (18) reported the suppression of human allergic reaction by an oral intake of *L. plantarum* isolated from a pickled scallion. In addition, Jonganurakkun et al. (13) published data suggesting *L. plantarum* isolated from a pickled Japanese radish stimulated immune activities and allergenic inhibition.

We isolated a strain of *L. plantarum* named strain HOKKAIDO (abbreviated as strain Hokkaido) from a pickled celery and demonstrated that this strain inhibited the adhesion of *E. coli* O-157 to Caco-2 cells, a human intestinal epithelial cell line; initiated the production of IL-12, a Th1-inducible cytokine, from human primary cultured dendritic cells or mouse spleen cells (unpublished data); and could tolerate human gastric and intestinal juices to reach the bowels in a living state (19).

The incidence of diarrhea is high in the calf, especially in the first four weeks of life and seems to be closely associated with other major diseases and mortality. The causes of diarrhea are considered to be bacterial or viral infection due to immunocompetence or intestinal microflora being immature and various stresses like mother-infant separation, transportation, marketing and dietary change (29, 34).

The objective of the current study was to evaluate the effect of the administration of the strain HOKKAIDO on the incidence of diarrhea and analyze the change of the microflora during its administration using our T-RFLP method and an improved method for cloning and sequencing of T-RFs.

**MATERIALS AND METHODS**

*Bacterial strain and culture*

*Lactobacillus plantarum* strain HOKKAIDO was isolated from Japanese pickles produced in Hokkaido and identified by microscopic, physiological and biochemical tests, utilization tests of carbohydrates, 16S ribosomal RNA gene sequencing together with a *recA*-targetting multiplex PCR (30). This strain is deposited at the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Japan (Deposit no. FEMR-P-19645).

The bacterial cells were cultured in GYP medium (1% glucose, 1% yeast extract, 0.5% polypeptide, 0.2% Na-acetate · 3H2O, 20 ppm MgSO4 · 7H2O, 1 ppm MnSO4 · 4H2O, 1 ppm FeSO4 · 7H2O, 1 ppm NaCl, pH 6.8) at 37°C for 16 hr, concentrated by centrifugation and freeze-dried with 10% skim milk.

**Experiment 1 (Exp 1)**

Thirty two male Holstein calves (aged from 21 to 55 days old, average age of 33.0 days old, mean body weight 62.5 kg) that were purchased from a livestock market were divided into two groups based on their appearance, weight and condition to avoid group bias. The control group (C group) were given 2 l of a milk replacer, which was produced by dissolving 500 g of chow (42% skimmed milk, 30% condensed whey proteins, 25% fats and 3% vitamin-mineral mix) in 4 l of warm water, two times a day. The second group (LPH group) were similarly given the milk replacer with addition of approximately 1 x 10⁹ CFU of the strain HOKKAIDO. In addition to the milk replacer, chow was continuously available throughout the test period. The calves were housed in 2 rooms (16 animals/room) and ingested the milk replacers for 35 days (Oct.–Nov. 2005). Measurements of body weights were performed on days 0, 7, 21 and 35, and observation of the fecal condition and measurement of the consumption of chow were performed every day. The stool properties, whose evaluation was visually performed, were scored as 2, 1 and 0 for watery, soft and normal stools, respectively.

**Experiment 2 (Exp 2)**

Ten male Holstein calves (aged from 8 to 27 days old, average age of 15.8 days old, mean body weight 53.6 kg) that were purchased from a livestock market were divided into three groups based on their appearance, weight and condition to avoid group bias. Four calves were allocated to the control group (C group). Three calves each were allocated to the second and third test groups, respectively. The second group (LPH group) received a daily dose of milk replacer containing approximately 2 x 10⁹ CFU of the strain HOKKAIDO, and the third group (BOV group) received a daily dose of milk replacer containing 20 g of
Bovactin™ (MIYARISAN Pharmaceutical Co., Ltd., Tokyo, Japan), consisting approximately $1 \times 10^7$, approximately $1 \times 10^6$ and approximately $1 \times 10^5$ CFU/g of Lactobacillus plantarum strain 220, Streptococcus faecium and Clostridium butyricum strain Miyari, respectively. Bovactin™ was selected from commercial materials containing viable bacteria which claim to regulate the functions of the intestines, because it contains L. plantarum. The calves were individually housed in calf-hutches for 35 days (Jun.–Jul. 2006) and were given 1 l of milk replacer 4 times a day during the period. The other conditions were the same as the design for Exp 1. The sampling of the feces for microflora analysis was performed on days 0, 15 and 28.

Fecal DNA extraction, PCR and T-RFLP analysis

Fecal DNA extraction and T-RFLP analysis were performed using a previously described method (16) with some modifications. Briefly, the fecal samples (approximately 0.1 g) were suspended in a solution containing 4 M guanidine thiocyanate, 100 mM Tris-HCl (pH 9.0) and 40 mM EDTA, and then beaten in the presence of zirconium beads using a bead beater (Micro Smash™ MS-100, Tomy Medico Ltd., Tokyo, Japan). Thereafter, DNA was extracted from the bead-treated suspension using a mixture of phenol:chloroform:isoamylalcohol (25:24:1) and purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd., Buckinghamshire, UK).

The PCR was performed using the fecal DNA and primers (0.1 μM each) of Hex-labeled 516f (5’-TGCCAGCAAGCCGGTGTA-3’; E. coli positions 516 to 532) and 1510r (5’-GGTTACCTTGTTACGACTT-3’; E. coli positions 1510 to 1492). The mixture containing the resulting 16S rDNA amplicons and 4 U of BslI (New England BioLabs Japan, Tokyo, Japan), in which internal standard DNAs were not contained, was incubated for 1 hr at 55°C and the BslI-digest was fractionated using an automated sequence analyzer (ABI PRISM 310 Genetic Analyzer, Life Technologies Japan Ltd., Tokyo, Japan) in GeneScan mode (the injection time was 20 sec and the run-time was 40 min).

T-RF cloning and sequencing

T-RF cloning and sequencing were performed using a previously described method (16) with some modifications, resulting in a higher efficiency. The PCR was performed as described above except that 0.4 μM of each of biotin-labeled NotI-516f (5’-TAGAGCCGGCCGCTGCCAGCAGCCGGGTA-3’) and 1510r was used as the primer set, and that an equal mixture of the fecal DNAs from 10 calves on the same sampling date was used as the template. The PCR products (100 μl) were purified using S-400HR MicroSpin Columns (GE Healthcare), mixed with 100 μl of MPG streptavidin (Takara Bio Inc., Otsu, Japan), suspended in 2 × B & W buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2.0 M NaCl), then incubated at a room temperature for 15 min. After washing twice with 100 μl of T10E0.1 (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA), the DNA-bead conjugate (100 μl) was treated for 3 hr with 50 U of BssII at 55°C. The DNA in the conjugate (20 μl) was blunted-ended using T4 DNA polymerase (Toyobo Co., Ltd. Osaka, Japan), washed three times with T10E0.1 and suspended in 50 μl of T10E0.1. Five microliters of the DNA solution were incubated with 1 pmole (1 μl) of EcoRI-NotI-BamHI Adaptor (Takara Bio) in 12 μl of ligation mixture (DNA Ligation Kit <Mighty Mix>, Takara Bio, Inc.) at 16°C for 30 min. After washing three times followed by suspension, the PCR was performed using 50 μl of the reaction mixture containing 5 μl of adaptor-ligated DNA solution, 2.5 U of Taq polymerase (Promega corporation, Madison, WI), 1 × PCR buffer (Promega), each dNTP at a concentration of 200 μM, 2.5 mM MgCl2, and each primer at a concentration of 0.4 μM. The primers used were 516f and NotI-BamHI primer (5’-CGGCGGGCCGCGGATCC-3’). The amplification program was as follows: preheating at 94°C for 1 min, followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, extension at 72°C for 1 min and finally, a terminal extension at 72°C for 7 min. The amplified products were subjected to electrophoresis through 2% agarose gel (Nippon Gene Co., LTD, Tokyo, Japan), followed by ethidium bromide staining. The DNA fragments were cut out from the gel, purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), cloned into E. coli TOP10 using the TOPO TA Cloning Kit for Sequencing (Life Technologies Japan Ltd.). Insert DNA fragments (approximately 100 to 1,000 bp long) were recovered with the colony direct PCR method using T3 and T7 primers. Each DNA product was purified using the MultiScreen FB filter plate (Millipore Corporation, Billerica, MA) and sequenced using a BigDye Terminator Cycle Sequencing Kit (Life Technologies Japan Ltd.). Homology searches of the obtained sequences were performed with FASTA programs at the web site of the DNA Data Bank of Japan (DDBJ).

Statistical analysis

Statistical analysis of the data were performed using the Wilcoxon t-test, Friedman’s test or the Chi square test with...
Variation among individuals and alteration with growth of fecal microflora

To investigate the correlation between the stool types and intestinal microflora, the fecal samples in Exp 2 were collected before and, 15 and 28 days after the start of the ingestion of the milk replacers and their characters are summarized in Table 4. From these samples, DNA was extracted and used in the T-RFLP analysis. The pair-wise Pearson correlation coefficient (Pearson \( r \)) was calculated using the resulting T-RFLP data (see Appendix 1); then, the samples were clustered on the basis of the Pearson \( r \) by UPGMA, resulting in three clusters: I, II and III (Fig. 1). As shown in Table 5, when the significance of the distributions of the samples among the clusters was tested, no significant differences were observed among the feeding groups and the stool-type groups. On the other hand, a significant difference (\( p<0.01 \)) was observed among the sampling-date groups; the day 28 samples were clearly concentrated in Cluster I as shown in Fig. 1. To make clear the difference within the sampling-date group, the T-RFLP profiles were presented for the respective groups (Fig. 2, Appendix 1), and the average of pair-wise Pearson \( r \) within each group was calculated; it was 0.58 for the day 0 samples, and 0.40 and 0.89 for the day 15 and 28 samples, respectively. These values indicate that the day 0 and 15 samples had considerable variation in the T-RFLP profile among animals, while the day 28 samples closely resembled each other.

### Table 1. Comparison of average weights of test groups by day

| Day of measurement | Average weight (kg) of each test group |
|--------------------|--------------------------------------|
|                    | C          | LPH         | BOV         |
| 0 Exp 1            | 62.2 (7.8) | 62.7 (6.7)  | -           |
| 7 Exp 2            | 51.8 (9.4) | 53.7 (3.8)  | 55.3 (4.0)  |
| 21 Exp 1           | 74.3 (10.1)| 77.5 (7.4)  | -           |
| 35 Exp 1           | 89.8 (12.1)| 92.4 (8.4)  | -           |
| 21 Exp 2           | 76.3 (11.8)| 79.3 (17.4) | 76.3 (5.9)  |

Figures in parentheses represent standard deviations.
**Probiotic Effect of Lactobacillus in Calf and Analysis of Microflora**

BP (T-RF clones were named p3-, p9- and p14- for days 0, 15 and 28, respectively), 400–500 bp (T-RF clones were named p4-, p10- and p15- for days 0, 15 and 28, respectively), 300–400 bp (T-RF clones were named p5-, p11- and p16- for days 0, 15 and 28, respectively) or 100–200 bp (T-RF clones were named p6- for day 0) in length, were generated by *Bsl*I-digestion of the 16S rDNA-amplicon mixtures derived from the 0-, 15-, or 28-day samples. We consider these results support the accuracy of the assumptive groupings of the microflora (Appendix 2). The microflora analysis of the calf feces, irrespective of the feeding group, suggested the following: (i) bifidobacteria were the relatively predominant intestinal bacteria in calves at the earlier days of life; (ii) the class *Bacteriodia* became more predominant with the growth of a calf; and (iii) the class *Clostridia* was most predominant in calf microflora.

**Discussion**

From the point of view of food safety, it is considered that probiotics should be more widely used as a tool for health control in the livestock industry. We isolated a *Lactobacillus* sp., *Lactobacillus plantarum* strain Hokkaido, from well-fermented Japanese pickles and have data which shows that the strain Hokkaido inhibited the adhesion of harmful pathogens to intestinal epithelial...
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cells and activated the cellular immunity (unpublished data). Thus we evaluated this strain as a diarrhea suppressant in calves and at the same time analyzed the change of the intestinal microflora during its

Table 3. Total number of calves with watery, soft or normal stool in the 35-day experiments

| Feeding groups | Stool types |        |       |       |
|----------------|-------------|--------|-------|-------|
|                | Watery | Soft | Normal |
| Exp 1*1        | C       | 13    | 29    | 518   |
| LPH            | 5      | 13    | 542   |
| Exp 2*2        | C       | 14    | 36    | 90    |
| BOV            | 9      | 29    | 67    |
| LPH            | 8      | 23    | 74    |
| Exp 1 & 2*3    | C       | 27    | 65    | 608   |
| LPH            | 13    | 36    | 616   |

*1 A significant difference (p=0.014) was indicated by the Chi square test
*2 No significant difference (p=0.910) was indicated by the Chi square test
*3 A significant difference (p=0.002) was indicated by the Chi square test

Table 4. Characteristics of stool samples used in T-RFLP analysis

| Stool samples | Feeding groups | No. of individuals | Date of sample collection (days) | Stool types |
|---------------|----------------|--------------------|----------------------------------|-------------|
| C1-0-N        | C              | 1                  | 0                                | Normal      |
| C2-0-D        | C              | 2                  | Watery                           |
| B3-0-D        | BOV            | 3                  | Watery                           |
| L4-0-S        | LPH            | 4                  | Soft                             |
| B5-0-S        | BOV            | 5                  | Soft                             |
| L6-0-N        | LPH            | 6                  | Normal                           |
| C8-0-S        | C              | 8                  | Soft                             |
| B10-0-S       | BOV            | 10                 | Soft                             |
| L11-0-S       | LPH            | 11                 | Soft                             |
| C12-0-S       | C              | 12                 | Soft                             |
| C1-15-N       | C              | 1                  | 15                               | Normal      |
| C2-15-N       | C              | 2                  | Normal                           |
| B3-15-N       | BOV            | 3                  | Watery                           |
| L4-15-N       | LPH            | 4                  | Normal                           |
| B5-15-N       | BOV            | 5                  | Normal                           |
| L6-15-N       | LPH            | 6                  | Normal                           |
| C8-15-S       | C              | 8                  | Soft                             |
| B10-15-N      | BOV            | 10                 | Normal                           |
| L11-15-N      | LPH            | 11                 | Normal                           |
| C12-15-N      | C              | 12                 | Normal                           |
| C1-28-N       | C              | 1                  | 28                               | Normal      |
| C2-28-N       | C              | 2                  | Normal                           |
| B3-28-N       | BOV            | 3                  | Normal                           |
| L4-28-N       | LPH            | 4                  | Normal                           |
| B5-28-D       | BOV            | 5                  | Watery                           |
| L6-28-S       | LPH            | 6                  | Soft                             |
| C8-28-N       | C              | 8                  | Normal                           |
| B10-28-N      | BOV            | 10                 | Normal                           |
| L11-28-N      | LPH            | 11                 | Normal                           |
| C12-28-N      | C              | 12                 | Normal                           |

The ages of calves from which the samples of C1-0-N, C2-0-D, B3-0-D, L4-0-S, B5-0-S, L6-0-N, C8-0-S, B10-0-S, L11-0-S and C12-0-S were derived, were 21, 15, 16, 27, 22, 10, 8, 9 and 15 days old, respectively.

Table 5. Distribution of samples among clusters from clustering of T-RFLP profiles

| Groups | Frequency (no. of samples) |
|--------|---------------------------|
| Clusters | I | II | III |
| Date of sample collection (days)*1 | 0 | 1 | 3 | 6 | 15 | 2 | 4 | 4 | 28 | 10 | 0 | 0 |
| Stool-types*2 | Watery | 1 | 0 | 2 | Soft | 1 | 2 | 5 | Normal | 11 | 5 | 3 |
| Feeding groups*3 | C | 5 | 4 | 3 | BOV | 4 | 0 | 5 | LPH | 4 | 3 | 2 |

*1 A significant difference (p=0.005) was indicated by the Chi square test
*2 No significant difference (p=0.322) was indicated by the Chi square test
*3 No significant difference (p=0.651) was indicated by the Chi square test

Fig. 1. Clustering diagram of T-RFLP profiles from fecal samples on the basis of the Pearson r by UPGMA. For sample names, refer to Table 4.
administration.

Timmerman et al. (29) reported that 4 experiments with veal calves were conducted to assess the influence on growth and health indicators of a multispecies probiotic (MSPB) containing different probiotic species of human origin and a calf-specific probiotic (CSPB) containing 6 *Lactobacillus* strains, which were originally isolated from calf feces. They pooled the data for the 4 experiments with respect to the treatments by the two kinds of probiotics and calculated a general health score that was calculated using a defined formula, in which the incidence of diarrhea and therapeutic treatments for digestive, respiratory, or other diseases were weighted differently. The general health score was significantly (*p*<0.05) increased compared with the control. Moreover, the CSPB treatment significantly (*p*<0.05) reduced the incidence of diarrhea in one of the 4 experiments and the different results among the experiments were likely due to combinations of the origins of the calves, experimental conditions, and management systems which would have influenced the susceptibility to probiotics of the calves. In our experiments, the group administered lactobacilli (the LPH group) showed a significantly lower incidence of diarrhea than the control group in Exp 1. On the other hand, no differences were observed among the three feeding groups in Exp 2: the control, BOV and LPH groups. We consider that the conflicting results of the two experiments might be ascribable to the smaller number of animals in Exp 2 and the different physical conditions of the animals used in the two experiments. In fact, the diarrhea score in Exp 2 was several-fold higher than that in Exp 1. Other possible reasons may exist; however, when the data for the two experiments were pooled and the control and LPH groups were compared, the incidence of diarrhea in the latter group was significantly (*p*<0.05) lower than that of the control group during the
were observed in the T-RFLP profile, compared with that no characteristic changes of the intestinal microflora microbiological agent was administered to the calves, but have largely affected the microflora.

In our experiments, 10^9 CFU/animal/day of microbiological agent was administered to the calves, which was calculated by a pair-wise comparison of all the obtained T-RFLP profiles and the profiles were clustered on the basis of Pearson r. No significant differences were observed with respect to the distribution of the samples from not only the feeding groups but also the stool-type groups among Clusters I, II and III which were derived from the clustering of the T-RFLP profiles, while a significant difference was observed with respect to the distribution of samples by sampling-date groups. In our experiments, 10^9 CFU/animal/day of microbiological agent was administered to the calves, but no characteristic changes of the intestinal microflora were observed in the T-RFLP profile, compared with that of the control calves. Considering that there is 10^12 CFU/g of feces in the large intestine (2), the administration of the above-mentioned amount of microbes should not have largely affected the microflora.

Judging from the values of pair-wise Pearson r of the T-RFLP profiles within each sampling-date group, the intestinal microflora of the calf seems to be variable in the early days of its life, becoming more stable as it grows. On the other hand, the incidence of diarrhea appears to reduce as a calf grows (Table 2). It is likely that younger calves have lower tolerance to infection because of the instability of the microflora, and a higher incidence of diarrhea as a result. Probiotics may play some role in suppressing the injurious effects derived from the instability of microflora colonization, encouraging immunostimulation, and inhibiting epithelial and mucosal adherence and epithelial invasion by harmful pathogens such as enterotoxigenic Escherichia coli (2). To elucidate the mechanism of diarrhea suppression by probiotics, further investigations are necessary.

Some investigations of the diversity of LAB (3) or bifidobacteria (8, 28) and of microflora (5, 21, 32, 33) have been reported for cattle. Busconi et al. (3) demonstrated by colony isolation, clustering of AFLP banding patterns and 16S rDNA sequencing, that the most representative genera of LAB in the calf intestinal tract were Lactobacillus (54% of total) and Streptococcus (32% of total), while the most frequent species was L. mucosae (51% of the Lactobacillus spp.) which was characterized by high in vitro mucus-binding activity. We detected sequences of Lactobacillus and Streptococcus in a 3:1 ratio in the day 0 fecal sample and in a 1:1 ratio in the case of the day 15 fecal sample (Table 7). We also detected a sequence closely related to L. mucosae as 30% of all LAB related sequences in the day 0 sample. In addition, we detected sequences closely related to L. gasseri, L. johnsonii, L. salivarius, L. viridans, L. reuteri, Streptococcus bovis, S. agalactiae and Enterococcus cecorum in the day 0 or 15 samples (Appendix 2); L. salivarius, L. reuteri, S. bovis were also detected by Busconi et al. (3). LAB related sequence was not detected in the day 28 sample. Vlková et al. (28) has described that in the calf, bifidobacteria constituted a minor group 3 days after birth, increasing rapidly after 7 days and then decreasing slowly during the next 7 weeks of life, which seems to support our conclusion that bifidobacteria are the relatively predominant intestinal bacteria in the calf in the early days of its life. Ozutsumi et al. (21) reported the analysis of fecal microflora in three castrated Holstein cattle using the random cloning method targeting the 16S ribosomal RNA gene. They analyzed 284 sequences which were affiliated with Firmicutes (including clostridia, 81.3%), Bacteroidetes (14.4%), Actinobacteria (2.5%) and Proteobacteria (1.4%) and detected a number of unidentified bacteria. Dowd et al. (5) reported the analysis of the microbiome using the

| OTU | Phylogenetic bacterial groups |
|-----|-----------------------------|
| 106 | Clostridium subcluster XIVa   |
| 110 | Clostridium cluster IX, Megamonas |
| 124 | Bifidobacterium              |
| 168 | Clostridium cluster IV       |
| 317 | Prevotella                   |
| 332 | Lactobacillales              |
| 338 | Clostridium cluster XI       |
| 370 | Bacteroides, Clostridium cluster IV |
| 423 | Clostridium cluster XVIII    |
| 469 | Bacteroides                  |
| 494 | Clostridium subcluster XIVa  |
| 505 | Clostridium subcluster XIVa  |
| 517 | Clostridium subcluster XIVa  |
| 520 | Lactobacillales              |
| 643 | NA                          |
| 650 | Clostridium cluster XVIII    |
| 657 | Lactobacillales              |
| 749 | Clostridium cluster IV       |
| 754 | Clostridium subcluster XIVa  |
| 853 | Bacteroides                  |
| 919 | Clostridium clusters XI, subcluster XIVa |
| 940 | Clostridium subcluster XIVa, Enterobacteriales |
| 955 | Clostridium subcluster XIVa  |
| 968 | NA                          |
| 990 | Clostridium subcluster XIVa  |

The classification of the class Clostridia is based on Collins et al. (3). NA, not applicable.
TABLE 7. Classification of sequences harbored on T-RF clones

| Phylogenetic bacterial groups | Number and percent of T-RF clones that harbor sequences homologous to that of the indicated bacterial group with respect to fecal samples that were collected on the indicated days after administration |
|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                             | 0 day                   | 15 days                  | 28 days                  |
|                             | No.  %                   | No.  %                   | No.  %                   |
| Gram-positive bacteria      |                         |                          |                          |
| *Firmicutes*                | (54) (75.0)              | (41) (73.2)              | (40) (69.0)              |
| *Clostridia*                | (35) (48.6)              | (28) (50)                | (34) (58.6)              |
| *Clostridium* rRNA cluster IV | 2 (2.8)                 | 2 (3.6)                 | 7 (12.1)                 |
| *Clostridium* rRNA cluster IX | 6 (8.3)                 | 0 (0.0)                 | 0 (0.0)                  |
| *Clostridium* rRNA cluster XI | 1 (1.4)                 | 1 (1.8)                 | 2 (3.4)                  |
| *Clostridium* rRNA subcluster XIVa | 14 (19.4)               | 15 (26.8)               | 12 (20.7)                |
| *Clostridium* rRNA cluster XVI | 5 (6.9)                 | 2 (3.6)                 | 2 (3.4)                  |
| Others                      | 7 (9.7)                 | 7 (12.5)                | 11 (19.0)                |
| *Bacilli*                   | (15) (20.8)              | (10) (17.9)             | (0) (0.0)                |
| *Enterococciaceae*          | 2 (2.8)                 | 0 (0.0)                 | 0 (0.0)                  |
| *Lactobacilliaceae*         | 10 (13.9)               | 4 (7.1)                 | 0 (0.0)                  |
| *Streptococciaceae*         | 3 (4.2)                 | 4 (7.1)                 | 0 (0.0)                  |
| Unknown                     | 3 (4.2)                 | 4 (7.1)                 | 6 (10.3)                 |
| *Actinobacteria*            | (8) (11.1)              | (0) (0.0)               | (0) (0.0)                |
| *Bifidobacteriaceae*        | 8 (11.1)                | 0 (0.0)                 | 0 (0.0)                  |
| Gram-negative bacteria      |                         |                          |                          |
| *Bacteroidetes*             | (7) (9.7)               | (16) (28.6)             | (16) (27.6)             |
| *Bacteroidia*               | (6) (8.3)               | (9) (16.1)              | (7) (12.1)               |
| *Bacteroidaceae*            | 1 (1.4)                 | 2 (3.6)                 | 1 (1.7)                  |
| *Prevotellaceae*            | 5 (6.9)                 | 6 (10.7)                | 5 (8.6)                  |
| Others                      | 0 (0.0)                 | 1 (1.8)                 | 1 (1.7)                  |
| Unknown                     | 1 (1.4)                 | 7 (3.6)                 | 9 (15.5)                 |
| *Proteobacteria*            | (3) (4.2)               | (1) (1.8)               | (2) (3.4)               |
| *Gammaproteobacteria*       | (2) (2.7)               | (1) (1.8)               | (2) (3.4)               |
| *Enterobactriaceae*         | 2 (2.8)                 | 1 (1.8)                 | 0 (0.0)                  |
| Others                      | 0 (0.0)                 | 0 (0.0)                 | 2 (3.4)                  |
| Unknown                     | 1 (1.4)                 | 0 (0.0)                 | 0 (0.0)                  |
| Unknown                     | 1 (1.4)                 | 0 (0.0)                 | 0 (0.0)                  |
| Total clones                | 72                      | 56                      | 58                      |

This table was prepared on the basis of Appendix 2 without distinction of the feeding groups, because no statistical difference of T-RFLP profile was observed among the groups.

The classification of the class *Clostridia* is based on Collins et al. (4). The sequences whose homology with the most closely related species were less than 90% are listed under “Unknown”.

feces of 20 adult lactating Holstein dairy cattle in 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing. They obtained 46,865 sequences in total and found 274 different species corresponding to 142 separate genera, in which *Clostridium, Bacteroides, Porphyromonas, Ruminococcus, Alistipes, Lachnospiraceae, Prevotella, Lachnospira, Bacteroidales, Akkermansia, and Enterococcus* spp. were predominant. At the level of phylum, *Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria* occupied on average 48.4, 45.4, 2.3 and 0% of the population across all cows, respectively. Taken together, the results of these reports are basically consistent with our present results, indicating that our methods for T-RFLP and the cloning and sequencing of T-RF are a useful set of tools for the analysis of microflora.

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### Appendix 1. Data of T-RFLP analysis in Exp 2

| Sample names (days) | 110 | 124 | 168 | 317 | 332 | 338 | 370 | 469 | 494 | 505 | 517 | 643 | 650 | 749 | 754 | 853 | 919 | 940 | 956 | 989 | 990 | X |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| C1-0-N 21           | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C2-0-D 15           | 2.5 | 4.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| B3-0-D 15           | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| L4-0-S 16           | 0.0 | 0.0 | 0.0 | 8.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| B5-0-S 27           | 1.1 | 17.0 | 3.4 | 2.9 | 0.0 | 4.9 | 5.6 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 23.8 | 0.0 | 2.4 | 0.0 | 12.4 | 29.9 | 0.0 | 0.0 | 14.1 | 0.0 |
| L6-0-N 22           | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 3.2 | 1.0 | 2.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| C8-0-S 10           | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| B10-0-S 8           | 0.0 | 5.3 | 0.0 | 0.0 | 11.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.6 | 33.9 | 0.0 | 2.7 | 0.0 | 8.0 |
| L11-0-S 9           | 9.4 | 1.9 | 0.0 | 0.0 | 16.2 | 0.0 | 13.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.3 | 10.5 | 0.0 | 12.3 |
| C12-0-S 15          | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 12.6 | 2.0 | 0.9 | 0.0 | 5.7 | 25.4 | 13.0 | 8.1 | 22.0 | 0.0 |

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Refer to Table 4 for sample names.
### Appendix 2. Results of cloning and sequencing of T-RFs

| Clone names | Size (bases) | Most closely related species | Accession no. | homology (%) | Phylum |
|-------------|--------------|------------------------------|---------------|--------------|--------|
| p1-08 | 1000 | *Clostridium thermosuccinogenes* | Y18180 | 86.7 | Firmicutes |
| p1-06 | 999 | *Lactobacillus reuteri* | CP000705 | 89.9 | Firmicutes |
| p1-13 | 974 | *Enterococcus coli* | CP000243 | 89.0 | Proteobacteria |
| p1-12 | 958 | *Ruminococcus gnavus* | X94967 | 94.0 | Firmicutes |
| p1-16 | 957 | *Coprooccus comes* | AJ270484 | 95.0 | Firmicutes |
| p1-04 | 955 | *Eubacterium formicigenes* | L34619 | 96.4 | Firmicutes |
| p1-03 | 939 | *Ruminococcus torques* | L76604 | 97.3 | Firmicutes |
| p4-13 | 939 | *Dorea formicigenes* | L34619 | 98.5 | Firmicutes |
| p1-13 | 938 | *Rosebacter faeicæs* | AY804150 | 93.7 | Firmicutes |
| p1-07 | 930 | *Eubacterium bifforme* | M59230 | 88.1 | Firmicutes |
| p1-05 | 926 | *Eubacterium bifforme* | M59230 | 97.2 | Firmicutes |
| p1-14 | 920 | *Enterococcus cocorum* | Y365054 | 99.9 | Firmicutes |
| p1-01 | 919 | *Clostridium glycyrhizinaŭicum* | AB233029 | 93.3 | Firmicutes |
| p1-02 | 918 | *Enterococcus cecorum* | AF061009 | 99.7 | Firmicutes |
| p2-01 | 755 | *Ruminococcus gnavus* | X94967 | 99.5 | Firmicutes |
| p2-04 | 755 | *Ruminococcus gnavus* | X94967 | 99.5 | Firmicutes |
| p2-06 | 755 | *Lactobacillus salivarius* | CP000233 | 95.6 | Firmicutes |
| p2-12 | 755 | *Lactobacillus mucosae* | DQ471799 | 92.8 | Firmicutes |
| p2-03 | 754 | *Ruminococcus lactaris* | L76602 | 95.2 | Firmicutes |
| p2-05 | 752 | *Subulagranum variabile* | AJ351869 | 93.2 | Firmicutes |
| p2-07 | 750 | *Mogibacterium neglectum* | AB037875 | 93.8 | Firmicutes |
| p2-10 | 749 | *Subulagranum variabile* | AJ351869 | 98.8 | Firmicutes |
| p2-08 | 748 | *Fusobacterium prausnitzii* | AJ413954 | 92.5 | Firmicutes |
| p4-12 | 571 | *Ruminococcus productus* | AY196512 | 92.4 | Firmicutes |
| p2-13 | 665 | *Lactobacillus salivarius* | DQ901733 | 99.2 | Firmicutes |
| p3-02 | 664 | *Lactobacillus gasseri* | CP000413 | 99.5 | Firmicutes |
| p3-10 | 664 | *Lactobacillus mucosae* | AF126738 | 99.7 | Firmicutes |
| p3-04 | 663 | *Lactobacillus mucosae* | AF126738 | 99.1 | Firmicutes |
| p3-12 | 663 | *Lactobacillus gasseri* | AY360337 | 99.5 | Firmicutes |
| p3-14 | 663 | *Lactobacillus gasseri* | AY360337 | 95.6 | Firmicutes |
| p3-09 | 662 | *Lactobacillus johnsonii* | AE017198 | 93.6 | Firmicutes |
| p3-13 | 662 | *Ruminococcus productus* | AB196512 | 94.6 | Firmicutes |
| p3-06 | 661 | *Streptococcus bovis* | AF104109 | 99.8 | Firmicutes |
| p3-05 | 655 | *Clostridium ramosum* | M2373 | 92.1 | Firmicutes |
| p3-01 | 654 | *Clostridium ramosum* | M2373 | 93.2 | Firmicutes |
| p3-07 | 654 | *Clostridium ramosum* | M2373 | 93.4 | Firmicutes |
| p3-08 | 654 | *Ruminococcus sp.* | AY960567 | 91.0 | Firmicutes |
| p3-11 | 654 | *Clostridium saccharogamia* | DQ100445 | 93.7 | Firmicutes |
| p4-03 | 521 | *Lactobacillus vitulinus* | AB210825 | 93.2 | Firmicutes |
| p4-07 | 514 | *Bacillus subtilis* | CP000034 | 96.7 | Firmicutes |
| p4-04 | 513 | *Faecalibacterium prausnitzii* | X85022 | 98.0 | Firmicutes |
| p4-06 | 490 | *Orbacterium nervosum* | AY323228 | 94.0 | Firmicutes |
| p4-09 | 490 | *Ruminococcus obeum* | AY169491 | 98.3 | Firmicutes |
| p2-02 | 488 | *Coprooccus comes* | EF031542 | 99.6 | Firmicutes |
| p4-10 | 488 | *Clostridium nucile* | AY169415 | 97.3 | Firmicutes |
| p4-02 | 485 | *Eubacterium bifforme* | M59230 | 97.3 | Firmicutes |
| p4-09 | 460 | *Spiroplasma chinense* | AY189126 | 87.1 | Tenericutes |
| p5-07 | 371 | *Megasenas hypermegalae* | AJ420107 | 97.3 | Bacteroidetes |
| p5-10 | 366 | *Prevotella capri* | AB084923 | 99.2 | Bacteroidetes |
| p5-11 | 366 | *Prevotella ruminicola* | AB219152 | 96.4 | Bacteroidetes |
| p5-05 | 365 | *Prevotella albensis* | AB011683 | 89.6 | Bacteroidetes |
| p5-12 | 337 | *Escherichia sp.* | DQ629916 | 96.6 | Proteobacteria |
| p5-06 | 335 | *Clostridium difficile* | AM180355 | 95.2 | Firmicutes |
| p5-04 | 329 | *Streptococcus bovis* | AF104109 | 99.7 | Firmicutes |
| p5-08 | 327 | *Streptococcus bovis* | AJ014090 | 100 | Firmicutes |
| p5-01 | 317 | *Prevotella capri* | AB064923 | 98.7 | Bacteroidetes |
| p5-03 | 316 | *Prevotella capri* | AB064923 | 99.0 | Bacteroidetes |
| p6-01 | 127 | *Bifidobacterium longum* | AE014295 | 97.6 | Actinobacteria |
| p6-02 | 127 | *Bifidobacterium longum* | AE014295 | 99.2 | Actinobacteria |
| p6-12 | 127 | *Bifidobacterium pseudacatenulatum* | AB125917 | 100 | Actinobacteria |
| p6-13 | 127 | *Bifidobacterium pseudacatenulatum* | AB125917 | 99.2 | Actinobacteria |
| p6-07 | 117 | *Veillonella ratti* | AF186071 | 97.3 | Firmicutes |
| p5-02 | 114 | *Bifidobacterium longum* | AE014295 | 99.1 | Actinobacteria |
| p6-03 | 114 | *Pectinatus frisingensis* | AF373027 | 97.3 | Firmicutes |
| p6-04 | 114 | *Mitsuokella jalaludini* | AF479674 | 100 | Firmicutes |
| p6-05 | 114 | *Pectinatus cerevisiophilus* | AF373026 | 97.3 | Firmicutes |
| p6-08 | 114 | *Bifidobacterium choerinum* | D86186 | 100 | Actinobacteria |
| p6-10 | 114 | *Pectinatus cerevisiophilus* | AF373026 | 97.3 | Firmicutes |
| p6-12 | 114 | *Bifidobacterium dentium* | EF140738 | 99.1 | Actinobacteria |
| Clone names | Size (bases) | Most closely related species | Accession no. | homology (%) | Phylum         |
|-------------|-------------|------------------------------|---------------|--------------|----------------|
| p7-06       | 998         | Ruminococcus obeum          | AY169419      | 98.1         | Firmicutes     |
| p7-12       | 997         | Ruminococcus obeum          | AY169419      | 90.7         | Firmicutes     |
| p7-01       | 995         | Eubacterium formigenerans   | L34619        | 87.3         | Firmicutes     |
| p7-04       | 984         | Prevotella multiformis      | AY207061      | 87.4         | Bacteroidetes  |
| p7-13       | 982         | Ruminococcus obeum          | AY169419      | 93.6         | Firmicutes     |
| p7-14       | 982         | Ruminococcus obeum          | AY169419      | 93.6         | Firmicutes     |
| p7-10       | 967         | Ruminococcus schinkii       | X94964        | 93.5         | Firmicutes     |
| p7-05       | 956         | Ruminococcus schinkii       | X94965        | 95.6         | Firmicutes     |
| p7-09       | 940         | Clostridium leptum          | AJ262239      | 96.2         | Firmicutes     |
| p7-11       | 939         | Dorea formigenerans         | DQ279737      | 98.5         | Firmicutes     |
| p7-02       | 937         | Eubacterium formigenerans   | L34619        | 92.6         | Firmicutes     |
| p7-15       | 931         | Subdoligranulum variabilis  | AJ518869      | 93.7         | Firmicutes     |
| p7-08       | 926         | Clostridium orbiscindens    | Y18187        | 90.7         | Firmicutes     |
| p7-07       | 923         | Clostridium celercrecens    | AJ295659      | 93.8         | Firmicutes     |
| p8-04       | 956         | Eubacterium halii           | AY305318      | 95.5         | Firmicutes     |
| p8-06       | 955         | Eubacterium halii           | L34621        | 96.4         | Firmicutes     |
| p8-12       | 954         | Ruminococcus gnavus         | X94967        | 96.4         | Firmicutes     |
| p8-02       | 954         | Escherichia coli            | BA000007      | 98.8         | Proteobacteria |
| p8-09       | 954         | Ruminococcus obeum          | AY169419      | 97.9         | Firmicutes     |
| p8-05       | 953         | Papillibacter cinnamimovoran | AF167711      | 94.0         | Firmicutes     |
| p8-10       | 953         | Papillibacter cinnamimovoran | AF167711      | 93.9         | Firmicutes     |
| p8-11       | 949         | Subdoligranulum variabilis  | AJ518869      | 98.8         | Firmicutes     |
| p8-07       | 947         | Subdoligranulum variabilis  | AJ518869      | 99.2         | Firmicutes     |
| p8-08       | 947         | Subdoligranulum variabilis  | AJ518869      | 99.2         | Firmicutes     |
| p8-03       | 946         | Subdoligranulum variabilis  | AJ518869      | 97.2         | Firmicutes     |
| p9-05       | 963         | Lactobacillus johnsonii     | AE017198      | 93.9         | Firmicutes     |
| p9-09       | 963         | Lactobacillus johnsonii     | AE017198      | 99.7         | Firmicutes     |
| p9-10       | 963         | Lactobacillus reuteri       | AY735406      | 99.8         | Firmicutes     |
| p9-01       | 962         | Streptococcus bovis         | AF104109      | 100          | Firmicutes     |
| p9-08       | 962         | Streptococcus agalactiae    | CP000114      | 93.9         | Firmicutes     |
| p9-02       | 965         | Lactobacillus johnsonii     | AE017198      | 83.6         | Firmicutes     |
| p9-03       | 965         | Lactobacillus reuteri       | CP000705      | 82.7         | Firmicutes     |
| p9-07       | 965         | Lactobacillus vitulinus     | AB210825      | 91.3         | Firmicutes     |
| p9-06       | 964         | Eubacterium biiforme        | M59230        | 97.8         | Firmicutes     |
| p9-04       | 963         | Eubacterium halii           | L34621        | 89.7         | Firmicutes     |
| p10-08      | 490         | Clostridium nexile          | AY169415      | 97.9         | Firmicutes     |
| p10-04      | 487         | Eubacterium biiforme        | M59230        | 97.3         | Firmicutes     |
| p10-05      | 470         | Prevotella copri            | AB244772      | 98.9         | Bacteroidetes  |
| p10-14      | 469         | Prevotella ruminicola       | AB219152      | 98.9         | Bacteroidetes  |
| p10-03      | 467         | Prevotella copri            | AB244772      | 98.9         | Bacteroidetes  |
| p10-09      | 467         | Parabacteroides merdae      | AB238929      | 97.2         | Bacteroidetes  |
| p10-10      | 467         | Prolactibacter bellariavorns | AY918928      | 88.6         | Bacteroidetes  |
| p10-12      | 467         | Bacteroides thetaiotaomicron | AE015928      | 88.2         | Bacteroidetes  |
| p10-15      | 467         | Prolactibacter bellariavorns | AY918928      | 88.6         | Bacteroidetes  |
| p10-01      | 466         | Prevotella stercorea        | AB244774      | 98.1         | Bacteroidetes  |
| p10-07      | 466         | Sphingobacterium multivorum | AB020205      | 88.8         | Bacteroidetes  |
| p11-07      | 370         | Ruminococcus schinkii       | X94964        | 93.5         | Firmicutes     |
| p11-06      | 369         | Bacteroides capillosus      | AY136666      | 95.9         | Bacteroidetes  |
| p11-01      | 369         | Bacteroides coprophilus     | AB064923      | 96.7         | Bacteroidetes  |
| p11-03      | 366         | Bacteroides coprophilus     | AB260025      | 92.8         | Bacteroidetes  |
| p11-09      | 365         | Bacteroides intestinalis    | AB214328      | 86.0         | Bacteroidetes  |
| p11-13      | 355         | Bacteroides denticanum      | DQ156990      | 83.6         | Bacteroidetes  |
| p11-10      | 335         | Clostridium difficile       | AM180355      | 95.8         | Firmicutes     |
| p11-11      | 331         | Streptococcus bovis         | DQ394708      | 99.1         | Firmicutes     |
| p11-08      | 328         | Streptococcus bovis         | AF104109      | 100          | Firmicutes     |
| p11-12      | 318         | Prevotella copri            | AB244770      | 99.4         | Bacteroidetes  |
| Clone names | Size (bases) | Most closely related species                        | Accession no. | homology (%) | Phylum           |
|------------|-------------|-----------------------------------------------------|---------------|--------------|-----------------|
| p12-09     | 995         | Ruminococcus bromii                                | DQ882649      | 90.0         | Firmicutes       |
| p12-13     | 992         | Ruminococcus flavefaciens                          | AY445599      | 92.7         | Firmicutes       |
| p12-14     | 991         | Ruminococcus obeum                                 | AY169419      | 89.7         | Firmicutes       |
| p12-06     | 967         | Eubacterium formicigenicans                        | L34619        | 97.7         | Firmicutes       |
| p12-03     | 958         | Ruminococcus schinkii                              | X94965        | 93.8         | Firmicutes       |
| p12-05     | 957         | Clostridium indolis                                | AF028351      | 84.8         | Firmicutes       |
| p12-12     | 957         | Bacteroides capillosus                             | AY136666      | 92.8         | Bacteroides      |
| p12-04     | 955         | Clostridium leptum                                 | AF262239      | 95.2         | Firmicutes       |
| p12-15     | 938         | Eubacterium ruminantium                            | AB008552      | 93.5         | Firmicutes       |
| p12-01     | 936         | Papillibacter cinnaminoformans                     | AF167711      | 93.1         | Firmicutes       |
| p12-02     | 936         | Papillibacter cinnaminoformans                     | AF167711      | 93.4         | Firmicutes       |
| p12-07     | 936         | Papillibacter cinnaminoformans                     | AF167711      | 94.2         | Firmicutes       |
| p12-08     | 929         | Ruminococcus flavefaciens                          | AY445599      | 92.4         | Firmicutes       |
| p13-08     | 772         | Ruminococcus bromii                                | X85099        | 91.4         | Firmicutes       |
| p13-02     | 759         | Ruminococcus productus                             | AB196512      | 90.5         | Firmicutes       |
| p13-01     | 756         | Clostridium thermocellum                           | CP000568      | 86.2         | Firmicutes       |
| p14-11     | 754         | Succinivibrio dextrinosolvens                       | EF560776      | 91.7         | Proteobacteria   |
| p13-03     | 753         | Papillibacter cinnaminoformans                     | AF167711      | 93.0         | Firmicutes       |
| p13-04     | 753         | Faecalibacterium prausnitii                         | AJ413954      | 96.2         | Firmicutes       |
| p13-10     | 753         | Succinivibrio dextrinosolvens                       | Y17600        | 97.1         | Proteobacteria   |
| p13-05     | 748         | Eubacterium cylindroides                           | L34617        | 90.3         | Firmicutes       |
| p13-07     | 732         | Papillibacter cinnaminoformans                     | AF167711      | 93.9         | Firmicutes       |
| p13-13     | 732         | Papillibacter cinnaminoformans                     | AF167711      | 94.1         | Firmicutes       |
| p13-06     | 724         | Papillibacter cinnaminoformans                     | AF167711      | 93.9         | Firmicutes       |
| p14-12     | 671         | Ruminococcus productus                             | X94966        | 91.9         | Firmicutes       |
| p14-05     | 664         | Clostridium leptum                                 | AJ305238      | 85.8         | Firmicutes       |
| p14-09     | 664         | Ruminococcus bromii                                | X85099        | 89.1         | Firmicutes       |
| p14-08     | 663         | Butyribivibrio fibrisolvens                         | U77341        | 90.6         | Firmicutes       |
| p14-06     | 662         | Ruminococcus productus                             | AB196512      | 90.3         | Firmicutes       |
| p14-07     | 662         | Ruminococcus productus                             | AB196512      | 90.8         | Firmicutes       |
| p14-01     | 659         | Anaerococcus prevotii                              | AF542232      | 98.3         | Firmicutes       |
| p14-10     | 656         | Ruminococcus callidus                              | X85100        | 95.3         | Firmicutes       |
| p14-03     | 655         | Eubacterium cylindroides                           | L34617        | 91.7         | Firmicutes       |
| p15-08     | 490         | Ruminococcus schinkii                              | X94965        | 96.1         | Firmicutes       |
| p15-02     | 474         | Prolixibacter bellariaformans                      | AY918928      | 88.1         | Bacteroidetes    |
| p15-10     | 472         | Pseudoramibacter alactolyticus                     | AB036759      | 90.6         | Firmicutes       |
| p15-05     | 470         | Parabacteroides distasonis                         | CP000140      | 85.5         | Bacteroidetes    |
| p15-07     | 470         | Papillibacter cinnaminoformans                     | AF167711      | 90.8         | Firmicutes       |
| p15-14     | 469         | Oscillibacter valericigenes                        | AB238598      | 96.1         | Firmicutes       |
| p15-01     | 467         | Sphingobacterium multivorum                        | AB020205      | 88.6         | Bacteroidetes    |
| p15-12     | 467         | Alistipes massiliensis                             | AY547271      | 89.2         | Bacteroidetes    |
| p15-13     | 467         | Alistipes massiliensis                             | AY547271      | 90.5         | Bacteroidetes    |
| p15-06     | 466         | Pedobacter koreensis                               | DQ680836      | 84.9         | Bacteroidetes    |
| p15-15     | 462         | Pedobacter panaciterrae                            | EF195090      | 80.4         | Bacteroidetes    |
| p15-16     | 462         | Eubacterium hallii                                 | L34621        | 97.2         | Firmicutes       |
| p15-09     | 459         | Prolixibacter bellariaformans                      | AY918928      | 88.4         | Bacteroidetes    |
| p15-04     | 442         | Prevotella ruminicola                              | AF218619      | 91.1         | Bacteroidetes    |
| p16-10     | 370         | Anaerophaga thermohalophilia                       | AJ418048      | 82.8         | Bacteroidetes    |
| p16-07     | 368         | Clostridium xylanovorans                           | AF116920      | 87.7         | Firmicutes       |
| p16-01     | 367         | Sporobacter termitidis                             | Z49863        | 93.2         | Firmicutes       |
| p16-05     | 366         | Prevotella copri                                   | AB064923      | 97.0         | Bacteroidetes    |
| p16-09     | 366         | Prevotella ruminicola                              | AF218620      | 91.5         | Bacteroidetes    |
| p16-03     | 365         | Clostridium clostridiformes                        | M59089        | 84.4         | Firmicutes       |
| p16-02     | 364         | Prevotella copri                                   | AB064923      | 90.6         | Bacteroidetes    |
| p16-08     | 335         | Clostridium bififormans                            | AF320283      | 97.0         | Firmicutes       |
| p16-13     | 333         | Clostridium bififormans                            | AF604562      | 95.5         | Firmicutes       |
| p16-12     | 331         | Ruminococcus productus                             | AY937379      | 92.8         | Firmicutes       |