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| Title | Effects of difructose anhydride III (DFA III) administration on rat intestinal microbiota. |
| Author(s) | Minamida, Kimiko; Shiga, Kazuki; Sujaya I., Nengah et al. |
| Citation | Journal of Bioscience and Bioengineering, 99(3), 230-236<br>https://doi.org/10.1263/jbb.99.230 |
| Issue Date | 2005-03 |
| Doc URL | https://hdl.handle.net/2115/14822 |
| Type | journal article |
| File Information | JB&B99-3.pdf |

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# The Effects of Difructose Anhydride III (DFA III) Administration on Rat Intestinal Microbiota

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Received 6 September 2004/Accepted 3 December 2004

Running title: CHANGES IN RAT INTESTINAL MICROBIOTA

[**Key words**: difructose anhydride III (DFA III), denaturing gradient gel electrophoresis (DGGE), *Ruminococcus* sp.]

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## ABSTRACT

The effects of difructose anhydride III (di-D-fructofuranose-1,2': 2,3'-dianhydride; DFA III) administration (3% DFA III for 4 weeks) on rat intestinal microbiota were examined using denaturing gradient gel electrophoresis (DGGE). According to DGGE profiles, the number of bacteria related to *Bacteroides acidofaciens* and uncultured bacteria within the *Clostridium lituseburense* group decreased, while that of bacteria related to *Bacteroides vulgatus*, *Bacteroides uniformis* and *Ruminococcus productus* increased in DFA III-fed rat cecum. In the cecal contents of DFA III-fed rats, a lowering of pH and an increase in short chain fatty acids (SCFAs), especially acetic acid, were observed. The DFA III-assimilating bacterium, *Ruminococcus* sp. M-1, was isolated from the cecal contents of DFA III-fed rats. The strain had 98% similarity with *R. productus* ATCC 27340$^{T}$ (L76595), and mainly produced acetic acid. These results confirmed that the bacteria harmful to host health were not increased by DFA III administration. Moreover, DFA III stimulated the growth of *Ruminococcus* sp. M-1 producing acetic acid, which may alter the intestinal microbiota towards a healthier composition. It is expected that DFA III would be a new candidate as a prebiotic.

## INTRODUCTION

Di-D-fructofuranose-1,2': 2,3'-dianhydride (DFA III) is found in chicory tubers but in small amounts. Large-scale production of DFA III from inulin was achieved using inulase II from *Arthrobacter* sp. H65-7 (1). DFA III is a non-digestible oligosaccharide having half the sweetness of sucrose (2, 3). It was reported that DFA III enhances Ca absorption *in vivo* (4-6) and *in vitro* experiments (7-9). It has recently been clarified that DFA III directly affects the epithelial tissue and activates the passage of tight junctions which are located on the luminal side of adjacent epithelial cells (8). Low calcium content among certain populations has recently become a serious health problem. Thus, DFA III is a premier food supplement candidate and is now being developed as a functional food.

When DFA III was given to rats, studies using conventional culture techniques showed that the number of lecithinase-negative clostridia increased in the rat cecum (2). Recent publications showed that only 15% of intestinal microbiota could be recovered using culture-dependent techniques (10, 11) and the application of a culture-independent approach can give a more realistic view. Prior to this study, we investigated the effect of DFA III administration (9 g/d DFA III for 4 weeks) on human intestinal microbiota

using denaturing gradient gel electrophoresis (DGGE). Our results showed that no difference in DGGE profiles after DFA III administration was observed in healthy subjects (12). Moreover, DFA III was not assimilated by the type strains of typical intestinal microorganisms: bifidobacteria, lactobacilli and *Bacteroides* spp. (2). However, when rats were fed 2-3% DFA III, a lowering of pH and an increase in short chain fatty acids (SCFAs) were observed in the rat cecum (2-6). This indicated that DFA III-assimilating bacteria existed in the rat intestine and the intestinal microbiota of the rat was changed by DFA III ingestion. Thus, we investigated the effects of DFA III administration on rat intestinal microbiota by DGGE.

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## MATERIALS AND METHODS

**Animals and diets** Male Sprague-Dawley rats (4-week-old, weighing about 100 g; Clea Japan, Tokyo) were housed individually in stainless steel cages in a room with controlled temperature (22 ± 2°C), relative humidity of 40-60%, and lighting (lights on from 8:00-20:00). Rats were freely provided with tap water and a basal diet shown in Table 1 for an acclimatization period of 5 d, and then were divided into two groups ($n$ = 9) based on body weight. The rats in one group were fed with the test diet

containing DFA III (DFA III diet, 30 g DFA III/kg diet; Nippon Beet Sugar Mfg., Obihiro) (Table 1). The rats in the other group were fed with the test diet without DFA III (control diet). Both test diets were prepared according to an AIN-93G formulation (13). All rats were fed with the assigned test diets and deionized water for 4 weeks. Body weight and food intake were measured every day. At the end of the experiment, the rats were killed after application of sodium pentobarbital anesthesia (50 mg/kg body weight; Nembutal, Abbott Laboratories, North Chicago, IL, USA). The cecum was removed with its contents, and the contents were quickly collected and weighed. A portion of these contents for DNA extraction was placed in eppendorf tubes, and another portion for isolation of DFA III-assimilating bacteria was quickly placed in test tubes containing a diluted solution for anaerobic bacteria (4.5 g of $KH_2PO_4$, 6.0 g of $Na_2HPO_4$, 0.5 g of Tween 80, 0.5 g of L-cysteine hydrochloride and 1.0 g of agar per liter) (14). The remaining cecal contents were immediately frozen with liquid nitrogen and stored at -40°C for subsequent analyses.

The study design was approved by Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University's guidelines for the care and use of laboratory animals throughout this study.

**DGGE analysis** DNA extraction for DGGE was performed as described previously (12). Then, two PCR amplications (for bacteria and *Bacteroides* spp.) were done as described previously (12) with some modifications. For bacteria, the first-round PCR was done using universal primers targeting the V3 regions of the bacterial 16S rDNA (15) and the second-round PCR was done using 1 µl of the first-round PCR products as a template and using the same primers as in the first-round PCR. The second-round PCR was attempted to intensify the band patterns obtained in the first-round PCR. For *Bacteroides* spp., the first-round PCR was done using *Bacteroides*-specific primers (16) and the second-round PCR was done using 1 µl of the first-round PCR products as a template and bacterial universal primers targeting the V3 of the bacterial 16S rDNA.

The second-round PCR products (50-µl PCR reaction mixtures) were concentrated by ethanol precipitation and dissolved in 10 µl of autoclaved water. Prior to DGGE, samples were heated at 95°C for 5 min and 65°C for 60 min, and then left at 37°C overnight. The DGGE was performed on the DCode$^{TM}$ system (Bio-Rad Laboratories, Hercules, CA, USA) at 65 V, 60°C in 1×TAE for 14 h on 10% polyacrylamide gels containing a 35% to 60% gradient of urea-formamide, where 100% was defined as 7M urea and 40% (v/v) formamide (15). The gel was stained with

SYBR® Green I (Cambrex Bio Science Rockland, Rockland, ME, USA) for 50 min. The stained gel bands were excised with a razor blade and were washed once using autoclaved water, and then were stored at -20°C until use. Then, sequencing of DGGE fragments and analysis of the sequence data were done as described previously (12). Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession nos. AB125220 to AB125230.

**Analyses of pH and SCFAs in cecal contents** The cecal contents were diluted with 4 volumes of deionized water and homogenized using a Teflon homogenizer. The pH of these homogenates was measured with a semiconducting electrode (ISFET pH sensor 0010-15C; Horiba, Kyoto) to determine the pH of cecal contents. Pools of SCFAs (acetic, propionic, and butyric acids) in the homogenate of cecal contents were measured after sample preparation by the procedure described previously (17) using HPLC (LC-10ADvp; Shimadzu Seisakusyo, Kyoto) with two Shim-pack SCR-102H columns (8 mm i.d.×30 cm long; Shimadzu Seisakusyo) and an electroconductibility detector (CDD-6A; Shimadzu Seisakusyo).

**Isolation of DFAIII-assimilating bacteria** The isolation medium for DFA

III-assimilating bacteria contained 52.5 g of GAM without dextrose (Nissui Pharmaceutical, Tokyo) supplemented with 10 g of DFA III, 13.5 g of agar and 20 ml of 0.2% Bromocresol purple (BCP) solution per liter of deionized water. The pH of the medium was adjusted to 7.1 and then it was sterilized at 115°C for 15 min. After sterilization, 10 mg of hemin and 5 mg of vitamin K were added into 1 *l* of medium. The medium was stored in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) until use.

Cecal samples were diluted by the sterilized physiological saline, and inoculated onto plates of the isolation medium in an anaerobic chamber. After cultivation at 37°C for 2 d, colonies with yellow zones were picked up as DFA III-assimilating strains. After single-colony isolation, the cell morphology of the isolates by microscopy and sugar assimilation tests was examined.

**Sugar assimilation tests** The base medium for sugar assimilation tests contained 26.25 g of GAM without dextrose and 20 ml of 0.2% BCP solution per liter of deionized water. Ten grams of DFA III or glucose (positive control) as sugar was added into 1 *l* of the base medium. No-sugar medium was used as a negative control. These media were adjusted to pH 7.1 and sterilized at 115°C for 15 min. After

sterilization, 10 mg of hemin and 5 mg of vitamin K were added into 1 *l* of medium. These media were stored in an anaerobic chamber until use. The isolates from glycerol stocks at -80°C were inoculated into 4 ml of GAM broth (Nissui Pharmaceutical) with hemin and vitamin K and were grown overnight at 37°C in an anaerobic chamber. Cells were collected by centrifugation at 3000 rpm for 10 min at 4°C, washed twice with sterilized physiological saline, and suspended in the same solution. Then, 10 µl of the suspension was inoculated into 4 ml of sugar assimilation test medium and incubated at 37°C for 24 h in an anaerobic chamber. After cultivation, the capability of sugar assimilation was determined by medium color: yellow was positive and purple was negative.

**Classification of DFA III-assimilating bacteria** For the identification of the isolates, 16S rDNA sequences were investigated. DNA extraction was performed by following the protocol of ISOPLANT II (Nippon Gene, Tokyo). The 16S rDNA gene of the isolates was amplified by PCR. The PCR products were purified using SUPREC$^{TM}$ PCR (Takara Bio, Otsu), and then were sequenced using the Big Dye Primer Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequences were automatically analyzed on a 3100 Genetic Analyzer (Applied

Biosystems). Homology searches were performed in the GenBank database with the BLAST search program. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession no. AB125231.

**Product of DFA III-assimilating bacteria** To examine the composition of SCFAs produced by the isolates, DFA III-assimilating bacteria after preculture were inoculated into 4 ml of GAM modified broth (26.25 g of GAM without dextrose and agar, 10 g of DFA III, 10 mg of hemin and 5 mg of vitamin K per liter of deionized water) as described above, and were incubated at 37°C for 4 d in an anaerobic chamber. Two hundred µl of 1M NaOH were added into the culture broth once a day in order to maintain a neutral pH. SCFAs in the culture broth were determined by the HPLC method outlined in the above section. In order to confirm the decrease in DFA III, TLC was carried out using a silica gel plate (Silica gel 60; Merck, Darmstadt, Germany) with a solvent system of 1-butanol-2 propanol-water-acetic acid (7: 5: 4: 2, v/v) (18). Spots were detected with a reagent containing *p*-anisaldehyde-$H_2SO_4$-ethanol (1: 1: 18, v/v) by heating.

**Statistical analysis** Student's *t* test was used to determine whether body weight

gain, food intake, the pH values and SCFA pools in the cecal contents were significantly different between the two dietary groups ($P < 0.05$).

## RESULTS AND DISCUSSION

**Changes in rat cecal conditions by DFA III administration** Body weight gain and food intake did not differ between the two dietary groups (data not shown). The pH of cecal contents in rats fed the DFA III diet (6.15 ± 0.115) was lower than that in rats fed with the control diet (7.44 ± 0.099, $P < 0.001$). Pools of total SCFAs (sum of acetic, propionic and butyric acids) in cecal contents were higher in the DFA III group (196 ± 30.6 μmol/cecal content) than in the control group (103 ± 13.3 μmol/cecal content, $P < 0.05$), and, in particular, the acetic acid pool was doubled upon DFA III administration with 150 ± 26.8 μmol/cecal content and 75.5 ± 8.66 μmol/cecal content for the DFA III group and control group, respectively. These results suggested that the increase in the acetic acid pool mainly contributes to the lowered cecal pH as a result of DFA III administration.

**The DGGE gel profiles** Figure 1 shows the DGGE profiles of rats consuming

control test diets (profile C) and 3% DFA III test diets for 4 weeks (profile D). The profiles C and D in Fig. 1 are representative of the two groups. Each band that was in the same position on the gel correlated with the same closest relative bacteria. Band no. 10 correlated with many close relatives but with low similarity.

Figures 1a and 1b show the results obtained using V3 region primers and the *Bacteroides*-specific primers, respectively. Band nos. 1, 2, 3, 4 and 5 in Figs. 1a and 1b represented *Bacteroides* spp. In previous analysis, bands related to *Bacteroides* spp. were not detected by V3 region primers (12), and at that time, the samples used were frozen because of long-distance transportation. In this study, samples were fresh and DNA extraction was performed immediately. Freezing the samples before DNA extraction may have damaged the *Bacteroides* spp. DNA.

The bands determined in DGGE were very few: seven bands (band nos. 1, 2, 4, 6, 8, 9, and 10) in control-fed rats and six bands (band nos. 3, 4, 5, 7, 8 and 9) in DFA III-fed rats. The DGGE banding patterns in control-fed rats were not any different among individuals. Those of DFA III-fed rats were also similar to each other. The reasons for this can probably be attributed to using specific pathogen-free (SPF) rats in this study and feeding them under the same environmental conditions. The phylogenetic tree of detected bands in Fig. 1 is shown in Fig. 2 and numbers of Fig.2 were consistent

with those of Fig. 1. The intestinal microbiota of experimental rats consisted of four groups: *Bacteroides* spp., *Clostridium* cluster IV (19), cluster XI and cluster XIVa. The phylogenetic tree showed that the major anaerobic bacteria in the intestine were detected by this method. However, indigenous facultative anaerobic bacteria such as lactobacilli were not detected although it was reported that they could be detected by DGGE analysis using V3 region primers (20, 21). The reasons for not detecting the lactobacilli in these experimental rats were perhaps because they were not dominant in the intestinal microbiota of the cecal contents and their numbers were below the detection limit of DGGE analysis.

**The changes in DGGE profile by DFA III administration** The DGGE profiles in Fig. 1 showed that the intensity of band no. 3 (*Bacteroides vulgatus* AB050111), no. 5 (*Bacteroides uniformis* AB050110) and no. 7 (*Ruminococcus schinkii* X94964 or *R. productus* L76595) increased by DFA III administration. *R. schinkii* was isolated from the rumen of sucking lambs (22) and has not been reported to be found in other body parts of animals except the rumen. *R. productus* was formerly named *Peptostreptococcus productus* (23) and is one of the dominant and indigenous members of the human and rat intestinal flora (23-25). Therefore, band no. 7 may be *R. productus*.

The results showed that DFA III administration stimulates the growth of dominant and indigenous bacteria in the rat intestine such as *Bacteroides* spp. and *R. productus*.

The DGGE profiles in Fig. 1 also showed that band nos. 1 and 2 (*B. acidofaciens* AB021157, AB021158), no. 6 (uncultured bacterium AF371814) and no. 10 disappeared following DFA III administration. The predominant *B. acidofaciens*, included in the *B. fragilis* group, was isolated from the cecum of mice (26). *B. acidofaciens* in mouse cecum was not stimulated by inulin (27), and this was also observed for DFA III. The phylogenetic tree (Fig. 2) showed that band no. 10 belongs to *Clostridium* cluster XI. This band had many close relatives but with low similarity, because the bacteria belonging to Cluster XI have the same sequences of the V3 regions of 16S rDNA.

**Identification of a bacterium showed band no. 10** As shown in above the section, band no.10 that disappeared following DFA III administration could not be identified using the sequences of V3 region. It has been known that the sequences of bacteria belong to *Clostridium* cluster XI vary in the V2 region of the bacterial 16S rDNA. Thus, specific primers for cluster XI; Clus11r-gc (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGA CCC GAA GGC CTT CAT CGC 3') and Clus11f (5' CTG TAC ACA CGG ATA ACA TAC CG 3') that amplified this

region were constructed and PCR was performed as described previously (12). The electrophorogram of PCR products is shown in Fig. 3a. PCR products from control-fed rat DNA were detected, while DFA III-fed rat DNA was not amplified. Then, the PCR products were separated by DGGE as described in the Materials and Method. The DGGE profile is shown in Fig. 3b. The DGGE band amplified by Clus11 primers was named band no. 11. It had 97% similarity with its closest relative, *Clostridium lituseburense* (M59107), which is not pathogenic to guinea pigs or mice (28). When the effects on both *C. histolyticum* and *C. lituseburense* groups (these may include a high proportion of pathogens) were investigated by oligofructose administration, the number of bacteria belonging to these groups decreased following short-chain oligofructose administration (29). In this study, the increase in the amount of SCFAs and lowering of the pH in the cecum may also suppress the growth of these bacteria. The phylogenetic analysis (Fig. 3c) showed that band no. 11 was positioned in Cluster XI as well as band no. 10.

**DFA III-assimilating bacteria** In order to find the cause of the increased amount of SCFAs, we tried to isolate DFA III-assimilating bacteria in cecal contents. The diluted cecal contents were inoculated onto the isolation media and incubated at

37°C for 2 d in an anaerobic chamber. Consequently, colonies with yellow zones were detected for the DFA III-fed rats and the count was $10^9$ cfu/g wet cecal contents. The isolates are strictly anaerobic gram-positive elliptical cocci, and occur in pairs or in short chains. They showed 98 % similarity in sequence data with *Ruminococcus productus* ATCC 27340$^T$ (L76595). One of them was isolated and named *Ruminococcus* sp. M-1 (AB125231). This strain was similar to the closest relative of band no. 7 in Fig. 1, and the intensity of band no. 7 increased by DFA III administration. The closest relatives of band no. 3 (*Bacteroides vulgatus* AB050111) and no. 5 (*Bacteroides uniformis* AB050110) were increased by DFA III administration as shown in Fig. 1 but were not isolated as DFA III-assimilating bacteria. The sugar assimilation tests showed that strain M-1 assimilates DFA III, because the color of the medium containing DFA III and glucose (positive control) changed to yellow and that of the negative control remained purple. After cultivation in the GAM modified culture broth (1% DFA III) at 37°C for 4 d in an anaerobic chamber, the concentrations of acetic acid and propionic acid were 105.34 ± 0.85 mM and 0.49 ± 0.10 mM (mean ± SD), respectively. Moreover, TLC analysis of these supernatants showed that DFA III was gradually broken down and disappeared by day 4 (data not shown). Thus, because *Ruminococcus* sp. M-1 assimilated DFA III and mainly produced acetic acid, it is concluded that SCFAs were

increased in DFA III-fed rat cecum.

In conclusion, the effects of DFA III administration (3% DFA III for 4 weeks) on rat intestinal microbiota were determined using DGGE. The DGGE profiles revealed that the number of bacteria related to *B. acidofaciens* and uncultured bacteria within *Clostridium* cluster XI decreased, while that of bacteria related to *B. vulgatus*, *B. uniformis,* and *R. productus* increased in the DFA III-fed rat cecum. In cecal contents of DFA III-fed rats, a lowering of pH and an increase in SCFAs, especially acetic acid, were observed. The DFA III -assimilating bacterium, *Ruminococcus* sp. M-1, was isolated from the cecal contents of DFA III-fed rats. The strain mainly produced acetic acid from DFA IIII consumed. The lower pH owing to acetic acid produced by *Ruminococcus* sp. M-1 probably affected the rat intestinal environment.

A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, which can improve host health (30). DFA III is a non-digestible food ingredient (2, 3), and in this study it was only assimilated by *Ruminococcus* sp. M-1. The improvement of host health means an alteration of the colonic microflora towards a healthier composition, *e. g.* by increasing numbers of saccharolytic species while

reducing putrefactive microorganisms (31). DFA III increased the numbers of saccharolytic bacteria such as *B. vulgatus*, *B. uniformis* and *R. productus* (30, 32, 33), and decreased those of bacteria within the *C. lituseburense* group. Therefore, it is expected that DFA III would be a new candidate as a prebiotic.


## ACKNOWLEDGEMENTS

The authors gratefully acknowledge Nippon Beet Sugar MFG., Co., Ltd. for providing us with DFA III, and Ms. Evelyn B. Elegado for her help during preparation of this manuscript.


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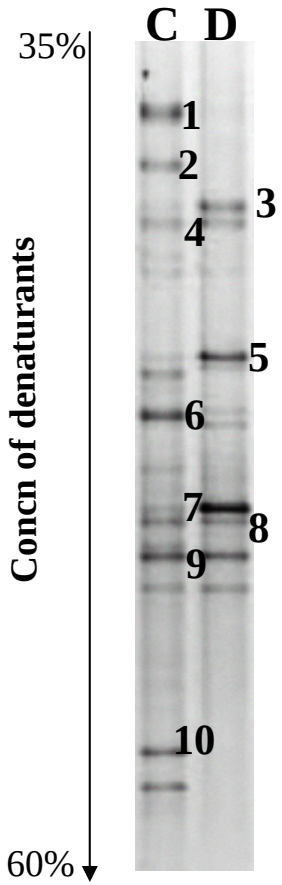
FIG. 1. (a) DGGE profiles of intestinal microbiota obtained from control-fed rats (C) and DFA III-fed rats (D). (b) DGGE profiles of *Bacteroides* spp. from control-fed rats (C) and DFA III-fed rats (D). The identities of bands with closely related bacteria are given in the center table. The picture is the negative image of a DGGE gel stained using SYBR® Green I nucleic acid gel stain.

FIG. 2. Phylogenetic tree showing the relationship between the DGGE bands detected and the type strains of related species. The tree was constructed using the neighbor-joining method based on 16S rDNA gene sequences. The scale bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 100 replications) greater than 60% are shown at branch points.

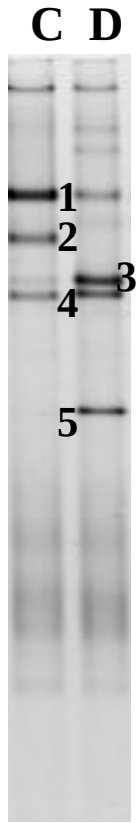
FIG. 3. (a) PCR products obtained with Clus11 primers. Lane M, 100 bp molecular ruler (Bio-Rad); Lane C, control-fed rat; Lane D, DFA III-fed rat. (b) Separation of PCR products using DGGE. Lane C, control-fed rat. The picture is the negative image of DGGE gel stained using SYBR® Green I nucleic acid gel stain. (c) Phylogenetic tree showing the relationship between the detected band no. 11 and the type strains of related species. The tree was constructed using the neighbor-joining method based on 16S

rDNA gene sequences. The scale bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 100 replications) greater than 50% are shown at branch points.



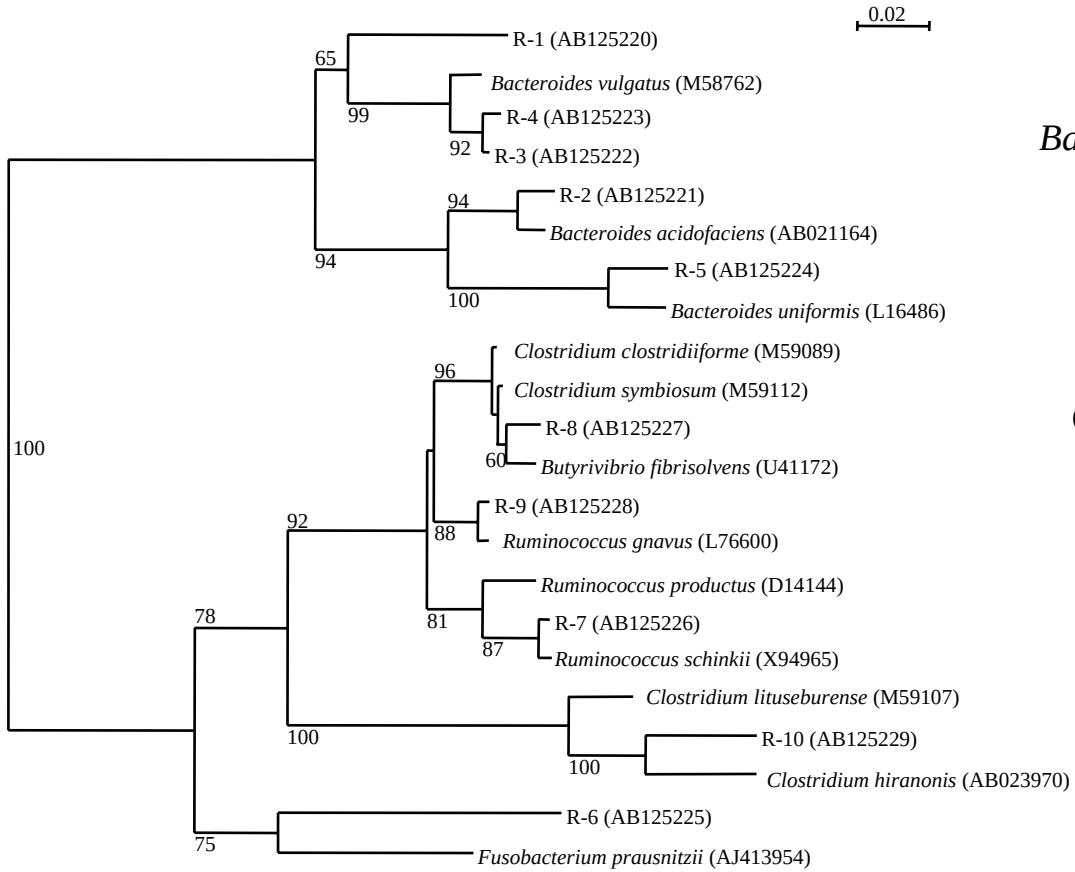

(a) V3 region primer

| Band no. | Closest relative (accession number) | Similarity (%) |
|---|---|---|
| 1 | *Bacteroides acidofaciens* (AB021157) | 100 |
| 2 | *Bacteroides acidofaciens* (AB021158) | 100 |
| 3 | *Bacteroides vulgatus* (AB050111) | 100 |
| 4 | *Bacteroides* sp. CS 21 (AB064917) | 100 |
| 5 | *Bacteroides uniformis* (AB050110) | 100 |
| 6 | Uncultured bacterium (AF371814) | 98 |
| 7 | *Ruminococcus schinkii*（X94964) | 100 |
| | *Ruminococcus productus* (L76595) | 99 |
| 8 | *Clostridium clostridiiforme* (M59089) | 99 |
| | *Clostridium symbiosum* (M59112) | 99 |
| 9 | *Ruminococcus gnavus* (X94967) | 99 |

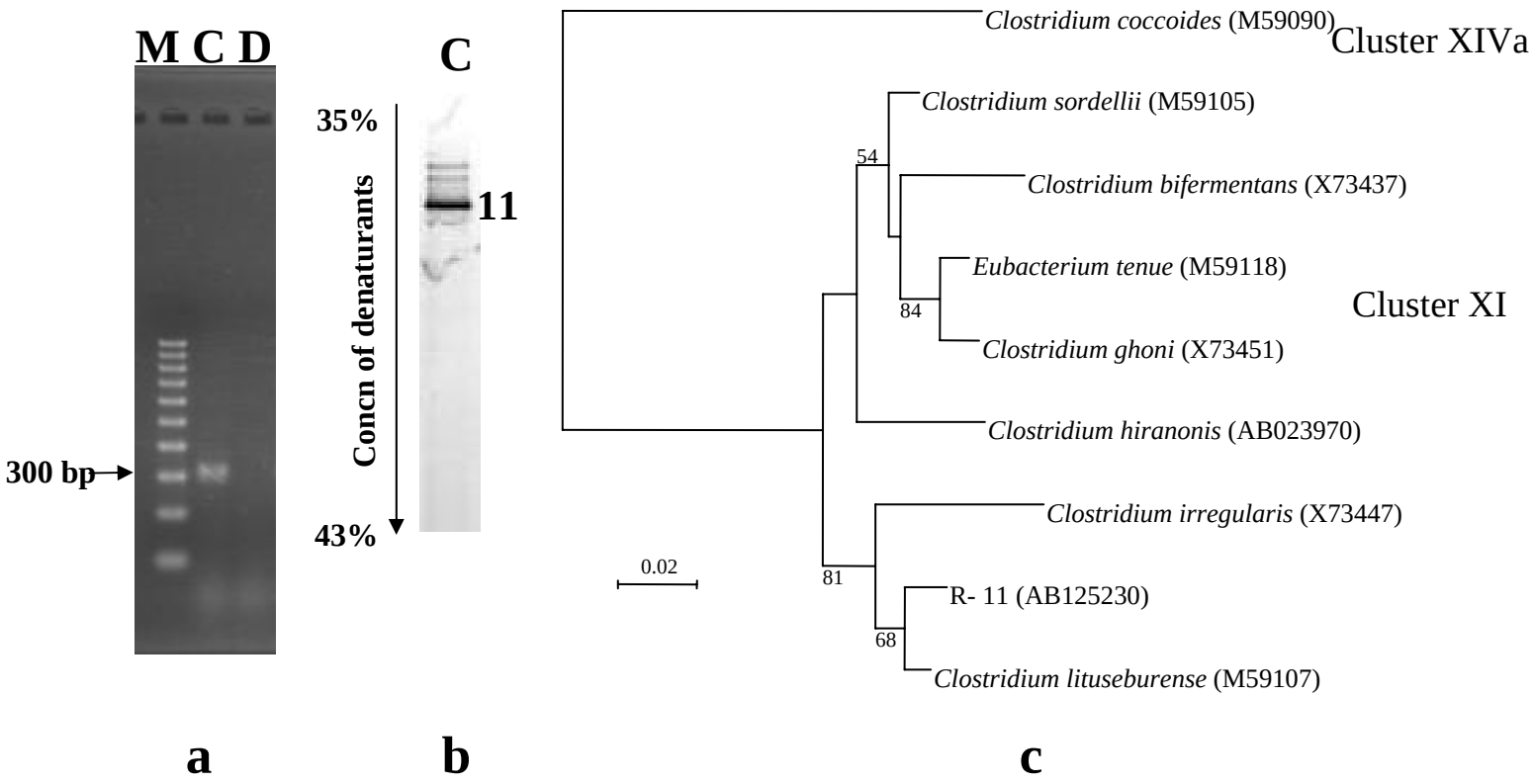



(b) Bacteroides-sp primer

Minamida *et al*. Fig. 1




Minamida *et al*. Fig. 2




Minamida *et al*. Fig. 3

TABLE 1. Composition of basal and test diets

| | Basal diet (g/kg diet) | Test diets (g/kg diet) | |
|---|---|---|---|
| | | Control | DFA III |
| Casein[a] | 200 | 200 | 200 |
| Dextrin[b] | 400 | 400 | 400 |
| Sucrose | 199.5 | 199.5 | 199.5 |
| Soybean oil | 70 | 70 | 70 |
| Mineral mixture[c] | 35 | 35 | 35 |
| Vitamin mixture[c] | 10 | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 |
| L-Cystine | 3 | 3 | 3 |
| *tert* -Butylhydroquinone | 0.014 | 0.014 | 0.014 |
| Crystallized cellulose[d] | 80 | 80 | 50 |
| DFA III | − | − | 30 |

[a]Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).

[b]Dextrin (TK-16; Matsutani Chemical Industry, Hyogo).

[c]Mineral and vitamin mixture were prepared according to the AIN-93G formulation (13).

[d]Crystallized cellulose (Avicel PH102; Asahi Chemical Industry, Tokyo).