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Effects of Difructose Anhydride III (DFA III) Administration on Bile Acids and Growth
of DFA III-Assimilating Bacterium *Ruminococcus productus* on Rat Intestine

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ABSTRACT

The growth of DFA III-assimilating bacteria in the intestines of rats fed 3% DFA III for 2 weeks was examined. Sixty-four percent of the DFA III intake had been assimilated on day 3 of ingestion, and almost all of the DFA III was assimilated at the end of the experiment. The DFA III-assimilating bacterium, *Ruminococcus productus*, in DFA III-fed rats was in the stationary state of 10^8 - 10^9 cells/g dry feces within a week from 10^6 cells/g dry feces on day 1 of DFA III ingestion. The number of *R. productus* cells was associated with the amount of DFA III excreted in the feces. The acetic acid produced from DFA III by *R. productus* lowered the cecal pH to 5.8. In control-fed rats and DFA III-fed rats, 94% of secondary bile acids and 94% of primary bile acids, respectively, were accounted for in the total bile acids analyzed. DFA III ingestion increased the ratio of primary bile acids and changed the composition of fecal bile acids. In conclusion, *R. productus* assimilated DFA III, produced short chain fatty acids, and the cecal pH was lowered. The acidification of rat intestine perhaps inhibited secondary bile acid formation and decreased the ratio of secondary bile acids. Therefore, it is expected that DFA III may prevent colorectal cancer and be a new probiotic candidate.

INTRODUCTION

Difructose anhydride III (di-D-fructofuranose-1,2': 2,3'-dianhydride, DFA III) is a nondigestible disaccharide and is produced in large quantities from inulin using inulase II from *Arthrobacter* sp. H65-7 (1, 2). DFA III has half the sweetness of sucrose, is stable in acid and at high temperature, and absorbs less moisture. DFA III was reported to enhance calcium absorption *in vivo* (3-5) and *in vitro* experiments (6-8). It directly affects the epithelial tissue and activates the passage of tight junctions which are located on the luminal side of adjacent epithelial cells (7).

When rats were fed 3% DFA III for 4 weeks, the effects of DFA III administration on rat intestinal microbiota were examined using denaturing gradient gel electrophoresis (DGGE) (9). According to DGGE profiles, the number of cells of bacteria related to *Bacteroides acidofaciens* and uncultured bacteria within the *Clostridium lituseburense* group decreased, while that of bacteria related to *B. vulgatus*, *B. uniformis* and *Ruminococcus productus* increased in the DFA III-fed rat cecum. These results confirmed that the number of bacterial cells harmful to host health did not increase by DFA III administration. We previously isolated a DFA III-assimilating bacterium, *Ruminococcus* sp. M-1 (AB125231), which showed 98% similarity of its 16S rDNA sequence with that of *R. productus* ATCC 27340^T (L76595) in the cecal contents of

DFA III-fed rats. *R. productus* was formerly named *Peptostreptococcus productus* (10) and is one of the dominant and indigenous members of the human and rat intestinal flora (10-12). The M-1 strain mainly produced acetic acid (9). In the cecum of DFA III-fed rats, a lowering of the pH and an increase in the amount of short chain fatty acids (SCFAs), particularly acetic acid, were observed. This indicated that *Ruminococcus* sp. M-1 assimilated DFA III and produced acetic acid. In this study, the number of *Ruminococcus* sp. M-1 cells in feces was examined over time when rats were fed 3% DFA III. Because *Ruminococcus* spp. are strictly anaerobic bacteria, the number of cells was counted not by a conventional culture technique but by real-time PCR. Moreover, the amount of DFA III excreted in feces was examined over time.

When rats were fed 3% DFA III, the cecal content pH was lowered below pH 6.5 (3-5). The acidification of the intestine changes the intestinal microbiota and affects the activity of microbial enzymes. One enzyme harmful to hosts is 7 α - dehydroxylase. Bacterial 7 α - dehydroxylation converts primary bile acids (cholic acid, chenodeoxycholic acid, α -muricholic acid and β -muricholic acid) to secondary bile acids (deoxycholic acid, lithocholic acid and hyodeoxycholic acid) in the colon. The activity of this reaction is greater in high-risk populations (13) and the incidence of colon cancer appears to be associated with the activity of 7 α - dehydroxylase and the bile

acid concentration (14-17). Bile acids and secondary bile acids have also been found to promote colon carcinogenesis in animal studies (18-20). On the other hand, 7 α -dehydroxylase has optimum activity at pH 7-8, and is inhibited at a pH < 6.5 *in vitro* (21, 22). The consequent colonic acidification would be expected to reduce bile acid degradation by 7 α -dehydroxylation and prevent colorectal cancer (23). Thus, we investigated whether the concentration of bile acids and the ratio of secondary bile acids in feces are decreased by DFA III ingestion.

MATERIALS AND METHODS

Animals and diets Male Sprague-Dawley rats (5-week-old, weighing approximately 130 g; SLC Japan, Tokyo) were housed individually in stainless steel cages in a room with a controlled temperature ($22 \pm 2^\circ\text{C}$), a relative humidity of 40-60%, and lighting (lights on from 8:00-20:00). The rats were freely provided with tap water and a basal diet shown in Table 1 for an acclimatization period of 1 week, and were then divided into two groups ($n = 6$) on the basis of body weight. The rats in one group were fed 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 the test diet without DFA III (control diet). Both test diets were prepared according to an

AIN-93G formulation (24). All the rats were fed the assigned test diets and tap water for 2 weeks. Body weight and food intake were measured daily. At the end of the experiment, the rats were sacrificed after the application of sodium pentobarbital (70 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 the isolation of DFA III-assimilating bacteria was quickly placed into test tubes containing the 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) (25). The remaining cecal contents were immediately frozen in liquid nitrogen and stored at -40°C for subsequent analyses. Fecal samples were collected on days 1, 2, 3, 4, 7 (days 5-7 were pooled) and 14 (days 11-14 were pooled) from DFA III-fed rats and on day 14 (days 11-14 were pooled) from control-fed rats.

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

Isolation and identification of DFA III-assimilating bacteria DFA

III-assimilating bacteria were isolated as described previously (9). Sugar assimilation tests, 16S rDNA sequencing and cell morphology analysis of isolates were also performed as described previously (9). The biochemical features of the isolates were examined using the API 50 CH and API ZYM (bioMérieux sa, Marcy l'Etoile, France) according to the manufacturer's instructions, except for modification of the medium for API 50 CH and incubation conditions. The medium for API 50 CH contained 26.25 g of GAM without dextrose (Nissui Pharmaceutical, Tokyo), 0.5 g of L-cysteine hydrochloride and 40 ml of 0.2% Bromocresol purple (BCP) solution per liter of deionized water (pH 7.0). API 50 CH and API ZYM were incubated in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). The biochemical features of *R. productus* JCM 1471^T were also examined as described and the data were compared with those of the isolates.

DNA extraction The fecal samples were lyophilized and stored at -20°C until use. Metal corns were added and the samples were disrupted at 2500 rpm for 60 s at 4°C using a Multi-Beads Shocker (Yasui Kikai, Osaka). A homogenized sample (0.02 g (dry weight)) was suspended in UltraCleanTM Soil DNA Kit solution (Mo Bio Laboratories, Salana Beach, CA, USA) as described previously (26) with some modifications, such as

lysis using lysozyme, labiase and *N*-acetylmuramidase at 37°C for 20 min.

Real-time PCR DNA extraction from a 10-fold dilution of the culture broth of isolates was also performed as described above. This DNA solution was used to obtain the standard curve for real-time PCR. To evaluate the efficiency of DNA extraction, the culture broth of isolates was added to homogenized fecal samples. The number of isolated cells stained with crystal violet solution was examined by microscopy using a counting chamber. DNA concentration was measured at 260 nm with a UV-VIS spectrophotometer UV mini 1240 (Shimadzu Seisakusyo, Kyoto).

The primers and TaqMan probes were designed according to the guidelines in the ABI Primer Express software program (Applied Biosystems, Foster City, CA, USA).

Among them, specific primers and a probe for isolates were chosen: the forward primer (Rpro-932F: 5' TCG AAG CAA CGC GAA GAA C 3'), the reverse primer (Rpro-999R: 5' GGG AAG GCC CCG TTA CG 3') and the TaqMan probe (Rpro-956T: 5' FAM-CAA GTC TTG ACA TCC CTC TGA CCG GC-TAMRA 3').

Each 50- μ l reaction mixture consisted of 25 μ l of TaqMan Universal Master Mix (Applied Biosystems), 900 nM primers, 200 nM fluorescence-labeled TaqMan probe, and 1 μ l of DNA solution. TaqMan Universal Master Mix contains dNTP, dUTP,

AmpErase UNG, MgCl₂, reaction buffer and Ampli *Taq* Gold. The reactions for the standard curve were performed in triplicate and the reactions for the samples were performed in duplicate in a volume of 50 µl. Real-time PCR was performed using the ABI PRISM[®] 7000 (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed with ABI PRISM 7000 SDS software (Applied Biosystems). PCR products were visualized by electrophoresis on 3% agarose gels stained with ethidium bromide. The PCR product from each sample was sequenced.

DGGE analysis The DGGE analysis targeting the V3 region of the bacterial 16S rDNA was performed as described in a previous paper (26) with some modifications. The second-round PCR was performed using 1 µl of the first-round PCR products as the template and 10% polyacrylamide gels contained a 40% to 55% gradient of urea-formamide. The DGGE fragments (gel pieces) were excised with a razor blade, washed once using autoclaved water, directly subjected to PCR and identified.

The analysis of bile acids Bile acid standards [cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), ursodeoxycholic

acid (UDCA) and hyodeoxycholic acid (HDCA)] of at least 97% purity were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). 7-Dehydrocholic acid (5 β -cholic acid -3 α , 12 α -diol-7-one), 12-dehydrocholic acid (5 β -cholic acid -3 α , 7 α -diol-12-one), α -muricholic acid (α -MCA; 5 β -cholic acid -3 α , 6 β , 7 α -triol) and β -muricholic acid (β -MCA; 5 β -cholic acid -3 α , 6 β , 7 β -triol) were obtained from Steraloids (Newport, RI, USA), while 23-nordeoxycholic acid (NDCA; 23-nor-5 β -cholic acid-3 α , 12 α -diol) was obtained from ICN Biomedicals (Aurora, OH, USA).

Homogenized fecal samples (20 mg) were suspended in ethanol (0.5 ml) containing 1 M NaOH and internal standard (NDCA, 50 nmol), and bile acids were extracted twice at 80°C for 1 h. The samples were centrifuged at 15,000 rpm for 5 min, and then the supernatant was removed. Neutral sterol was removed by hexane, and then the samples were subjected to a solid-phase extraction using a Bond Elut[®] C₁₈ cartridge (Varian, Harbor City, CA, USA). Bile acids were eluted from the cartridge with ethanol, and the organic solution was evaporated to dryness. The acids were redissolved in methanol, and divided into two portions. One portion was used to examine the concentration of total bile acids (kit # 431-15001; Wako Pure Chemical Industries, Osaka) enzymatically by the method of Mashige *et al.* (27) and the other was used for GC-MS. The Samples for GC-MS were methylated with 2.0 M (Trimethylsilyl)

diazomethane solution in hexane (Aldrich Chemical Company, Milwaukee, WI, USA) and silylated with pyridine and the BSA+ TMCS+ TMSI, 3: 2: 3 (SYLON BTZ) kit (Supelco, Bellefonte, PA, USA) at 60°C for 1 h.

The methylated and TMS ester derivatives of bile acids were separated by GC-MS using a 30 m × 0.25 mm DB-1 column (Agilent Technologies, Palo Alto, CA, USA). The bile acids were identified and quantified by GC-MS using a gas chromatograph (GC-2010; Shimadzu Seisakusyo) equipped with a gas chromatograph mass spectrometer (GCMS-QP2010; Shimadzu Seisakusyo) employing the selected ion monitoring mode (SIM). One µl of the final solution of derivatized bile acids was injected in the splitless mode at an injector temperature of 270°C and a column temperature of 220°C. Then, the column temperature was programmed to remain at 220°C for 1 min, to increase to 290°C at a rate of 30°C/min, and then to remain at 290°C for 18 min. The ions selected for the different bile acids were, for CA (m/z 253, 368), DCA (m/z 255, 370), CDCA (m/z 355, 370), LCA (m/z 215, 372), UDCA (m/z 370, 460), HDCA (m/z 405, 460), 7-dehydrocholic acid (m/z 251, 341), 12-dehydrocholic acid (m/z 229, 474), α -MCA (m/z 443, 458), β -MCA (m/z 195, 285) and NDCA (m/z 255, 356). Quantification was carried out by a correction factor obtained using NDCA as an internal standard. Bile acid standards were processed and analyzed in a similar manner.

Ratio of DFA III excreted in feces Homogenized fecal samples (200 mg) were added to 4 ml of deionized water, sonicated for 30 s (Ultrasonic disruptor UD-201; Tomy Seiko, Tokyo) and centrifuged at 13,000 rpm for 10 min. The supernatant was removed and degreased with chloroform. The amount of DFA III was measured by HPLC (Jasco, Tokyo) using a high-performance carbohydrate column (4.6×250 mm; Waters Corporation, Milford, MA, USA) and a RI detector (RI-2031; Jasco).

Analyses of pH and short-chain fatty acids (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 model Argus; Sentron, Roden, Netherlands) to determine the pH of cecal contents. The pools of SCFAs (acetic, propionic, and butyric acids) in the homogenate of cecal contents were measured after sample preparation by the procedure described previously (28) using HPLC (LC-10ADvp; Shimadzu Seisakusyo) with two Shim-pack SCR-102H columns (8 mm i.d. × 30 cm long; Shimadzu Seisakusyo) and an electroconductibility detector (CDD-6A; Shimadzu Seisakusyo).

Statistical analysis Student's *t* test or Welch's test was used to determine whether body weight gain, food intake, pH, SCFA pools in the cecal contents and bile acids in the feces were significantly different between the two dietary groups ($P < 0.05$).

RESULTS

Changes in rat cecal conditions by DFA III ingestion There were no significant differences in body weight gain (7.69 ± 0.13 vs. 7.10 ± 0.26 g) or food intake (18.74 ± 0.36 vs. 17.52 ± 0.50 g) over the experimental period between the groups fed the control and DFA III diets. The pH of the cecal contents in the DFAIII-fed rats (5.82 ± 0.05) was lower than that in the control-fed rats (7.82 ± 0.14 , $P < 0.01$). The pools of total SCFAs (sum of acetic, propionic and butyric acids) in the cecal contents were higher in the DFAIII group (169.3 ± 16.6 $\mu\text{mol}/\text{cecal content}$) than in the control group (135.8 ± 11.0 $\mu\text{mol}/\text{cecal content}$), and, in particular, the acetic acid pool was higher in the DFAIII group (118.5 ± 12.0 $\mu\text{mol}/\text{cecal content}$) than in the control group (88.7 ± 6.9 $\mu\text{mol}/\text{cecal content}$). However, the difference between the two groups did not reach a significant level ($P = 0.057$). These results suggest that the increase in the acetic acid pool mainly contributes to the lowered cecal pH as a result of DFAIII ingestion.

Identification of DFA III-assimilating bacteria DFA III-assimilating bacteria in rat cecal contents in this study were isolated, and the 16S rDNA sequences of the isolates were the same as that of *Ruminococcus* sp. M-1 (AB125231, similarity, 100%) isolated in a previous study (9). The isolates were also strictly anaerobic gram-positive elliptical cocci, and occurred in pairs or in short chains. They showed 98% similarity of their 16S rDNA sequences with that of *R. productus* ATCC 27340^T (L76595). On GAM medium after 48 h, the isolates formed smooth, opaque in the center, cream-colored colonies of approximately 2 mm in diameter. The isolates assimilated DFA III and produced mainly acetic acid. Comparing with the API 50CH and APIZYM data for the isolates and *R. productus* JCM 1471^T, they were similar but some exhibited varying intensities in sugar assimilation and enzyme activities. At different points, the isolates assimilated inulin weakly, and did not exhibit esterlipase C8 activity. On the basis of these results, the isolates were identified as *R. productus* and were named *R. productus* M-2 (AB196512).

Real-time PCR *R. productus* M-2 was present at 1.3×10^9 cells/ml in the

culture broth as determined by microscopy and the concentration of DNA extracted from this broth was 20 ng/ μ l. Five-fold serially diluted DNA solution was used for real-time PCR as a template, and then the standard curve was constructed (Fig. 1). The DNA concentration was linear from 2.56 pg to 40 ng ($R^2 = 0.994$). When the number of *R. productus* M-2 cells in the culture broth was 1.3×10^9 cells/ml as determined by microscopy, it was 1.38×10^9 cells/ml as determined using this standard curve (data not shown). When 1, 10 and 100 μ l of the culture broth were added to the fecal samples, the number of bacterial cells were linear ($R^2 = 0.999$) (data not shown). The PCR products of the fecal samples were of the target length of 70 bp and these sequences were identical to those of the target bacteria (data not shown).

The quantification of *R. productus* in the fecal samples is shown in Fig. 2. *R. productus* cells in control-fed rats varied in individuals from 10^5 - 10^7 cells/g dry feces. *R. productus* cells on day 1 in the DFA III-fed rats also varied in individuals from 10^5 - 10^7 cells/g dry feces. In the DFA III-fed rats (D-3 and D-7), *R. productus* cells were present at 10^7 cells/g dry feces on day 1 and the cells were in the stationary state of 10^8 cells/g dry feces on day 4. In the DFA III-fed rats (D-2 and D-4), *R. productus* was present at 10^5 cells/g dry feces on day 1 and was in the stationary state of 10^9 cells/g dry feces on days 5-7. These results showed that *R. productus* cells in all of the DFA III-fed rats were

in the stationary state of 10^8 - 10^9 cells/g dry feces following a week of DFA III ingestion.

The relationship between the growth of *R. productus* in the DFA III-fed rats and the ratio of DFA III excreted in the fecal samples is shown in Fig. 2. The amount of DFA III excreted in the feces was the highest (36%) on day 3 and then decreased gradually. Finally, DFA III in the feces of three rats was not detected on days 11-14 (data not shown). These results showed that 64% of the DFA III intake had already been assimilated on day 3, and almost all of the DFA III ingested was assimilated by days 11-14. After three days of feeding, the number of *R. productus* cells correlated with the amount of DFA III excreted in feces (correlation coefficient $R = -0.58$, $P < 0.01$) (Fig. 3). When the number of *R. productus* cells increased, the amount of DFA III excreted in feces decreased.

DGGE analysis Representative DGGE profiles of the DFA III-fed rats are shown in Fig. 4. The arrowed bands indicate bacteria related to *R. productus* M-2 (AB196512, 100%). The band representing *R. productus* in the D-3 and D-7 profiles appeared on day 1 and increased in intensity thereafter. The detection and intensity of DGGE bands of *R. productus* corresponded to the results of real-time PCR (Fig. 2). *R. productus* was detected at above 10^6 cells/g dry feces. The growth of *R. productus*

affected changes in the other bands, and, in particular, the bands in the lower part of the DGGE profiles disappeared. These bands were not identified.

Bile acids in fecal samples The composition of bile acids in the fecal samples on days 11-14 is shown in Table 2. The concentration of HDCA ($1.73 \pm 0.44 \mu\text{mol/g}$ dry feces) was the highest among the components of total bile acids in the control-fed rats and that of β -MCA ($1.34 \pm 0.22 \mu\text{mol/g}$ dry feces) was highest in the DFA III-fed rats. In the control-fed rats, secondary bile acids ($2.61 \pm 0.55 \mu\text{mol/g}$ dry feces) accounted for 94% of the total bile acids ($2.77 \pm 0.56 \mu\text{mol/g}$ dry feces), while primary bile acids ($1.54 \pm 0.26 \mu\text{mol/g}$ dry feces) accounted for 94% of the total ($1.66 \pm 0.30 \mu\text{mol/g}$ dry feces) in the DFA III-fed rats. DFA III ingestion changed the composition of fecal bile acids and increased the ratio of primary bile acids. The concentration of total bile acids in the DFA III-fed rats was lower than that of the control-fed rats using the enzymatic and GC-MS methods (no significant differences).

DISCUSSION

When the rats were fed 3% DFA III for 2 weeks, 64% of the DFA III intake had already been assimilated on day 3 of DFA III ingestion and almost all of the remaining DFA III had been assimilated by the end of the experiment. The DFA III-assimilating

bacterium, *R. productus*, in the DFA III-fed rats was in the stationary state of 10^8 - 10^9 cells/g dry feces from 10^6 cells/g dry feces on day 1 within a week of DFA III ingestion. The number of *R. productus* cells correlated with the amount of DFA III excreted in the feces. The acetic acid produced from DFA III by *R. productus* lowered the pH of cecal contents to 5.8. In the control-fed rats, the secondary bile acids accounted for 94% of the total bile acids, while it was the primary bile acids in the DFA III-fed rats that accounted for 94% of the total. The concentration of total bile acids in the DFA III-fed rats was lower than that in the control-fed rats. DFA III ingestion increased the ratio of primary bile acids and changed the composition of fecal bile acids.

In this study, the cecal contents of the control-fed rats were at pH 7.8 and those of the 3% DFA III-fed rats were at pH 5.8. In previous studies, that of the cecal contents of the control-fed rats was pH 7.1-7.4 and that of the cecal contents of the 3% DFA III-fed rats was pH 5.9-6.2 (3-5). The lowering of the pH of cecal contents was attributed to DFA III ingestion. The acidification of the intestine changes the composition of intestinal microbiota and affects the activity of microbial enzymes. According to DGGE analysis, the fecal microbiota of rat was changed by DFA III ingestion, but the bacterial species whose number of cells decreased following DFA III ingestion were not identified. The optimum pH for 7 α -dehydroxylase activity is 7-8, and it has been shown

to be already inhibited at $\text{pH} < 6.5$ *in vitro* (21, 22). In this study, the cecal pH in the DFA III fed rats was 5.8. Thus, acidification of the cecum also seemed to inhibit bacterial 7 α - dehydroxylase activity in addition to the decrease in the ratio of secondary bile acids and the change in the composition of fecal bile acids.

DFA III ingestion significantly decreased the concentration of secondary bile acids ($P < 0.01$), and slightly decreased total bile acids in rat feces (no significant difference). In previous reports of human trials, the fecal concentration of total and secondary bile acids was significantly decreased by lactulose (0.3 g kg⁻¹ twice daily for 12 weeks) (29), highly resistant amylo maize starch (30), inulin (15 g/d for 3 weeks) (31) and fructo-oligosaccharides (FOS) (15 g/d for 3 weeks) (31). On the other hand, FOS (12.5 g/d for 12 days) (32) and transgalacto-oligosaccharides (TOS) (7.5 and 15 g/d for 3 weeks) (33) slightly decreased the concentration of total bile acids or secondary bile acids, but the difference between the two groups was not significant. Patients with adenomatous polyps had higher concentrations of fecal bile acids compared with control subjects. Patients with colorectal cancer had an increased proportion of secondary fecal bile acids compared with controls. The bile acids may play a role in the development of polyps and colorectal cancer (17). Therefore, it is better to have lower concentrations of secondary and total bile acids. In a preliminary

human trial (administration of 3 g/d DFA III for 11 d to a 40-year-old woman), the pH of the feces and the concentration of total bile acids and secondary bile acids was decreased. A human trial of DFA III administration should be performed and the concentration of bile acids investigated.

In conclusion, when the rats were fed 3% DFA III for 2 weeks, *R. productus* assimilated DFA III, produced SCFAs, and lowered the cecal pH. The acidification of rat intestine may have inhibited secondary bile acid formation, eventually decreasing the ratio of secondary bile acids in the feces, and slightly decreasing the concentration of bile acids in rat feces. Therefore, it is expected that DFA III may prevent the occurrence of colorectal cancer and be a new prebiotic candidate.

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FIG 1. Standard curve for fivefold serial dilutions of *R. productus* M-2 culture broth using TaqMan real-time PCR. The correlation coefficient of the straight line, R^2 , is 0.994.

FIG. 2. The growth of *R. productus* and ratio of DFA III excreted in feces were determined using TaqMan real-time PCR and HPLC as described in Materials and Methods (means \pm SEM, $n=6$). The character D represents the DFA III-fed rats and C represents the control-fed rats.

FIG. 3. Scatter plot of relationship between number of *R. productus* cells and amount of DFA III in feces/intake after 3 d of feeding (Correlation coefficient, $R=-0.58$; DFA III-fed rat, $n=6$).

FIG. 4. DGGE profiles of fecal microbiota obtained from DFA III-fed rats (D-3, D-7). The picture is the negative image of the DGGE gel stained using SYBR[®] Green I nucleic acid gel stain. The arrows indicate the bands of *R. productus* M-2 (AB196512) (relative similarity, 100%).

TABLE 1. Composition of basal and test diets

	Basal diet (g/kg diet)	Test diet (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
<i>tert</i> -Butylhydroquinone	0.014	0.014	0.014
Crystallized cellulose ^d	80	80	50
DFA III	—	—	30

^aCasein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).

^bDextrin (TK-16; Matsutani Chemical Industry, Hyogo).

^cMineral and vitamin mixture were prepared according to the AIN-93G formulation (24).

^dCrystallized cellulose (Avicel PH102; Asahi Chemical Industry, Tokyo).

Minamida *et al.* TABLE 1

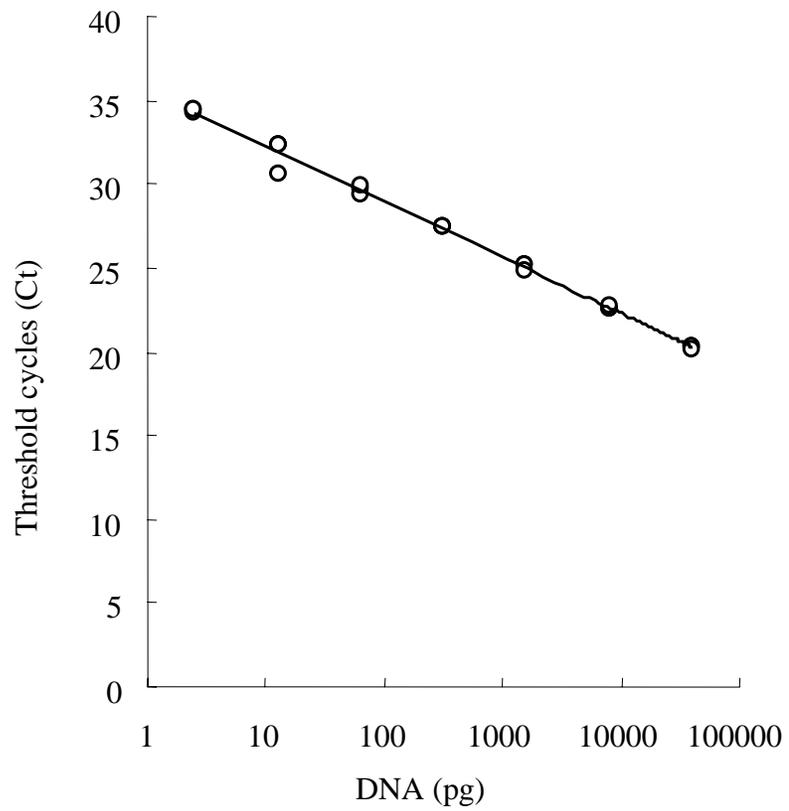
TABLE 2. Composition of fecal bile acid^a ($\mu\text{mol/g}$ dry feces)

	Control	DFA III
Cholic acid	0.00 \pm 0.00	0.07 \pm 0.03
Chnodeoxycholic acid	0.01 \pm 0.01	0.00 \pm 0.00
α -Muricholic acid	0.00 \pm 0.00	0.13 \pm 0.06
β -Muricholic acid	0.14 \pm 0.02	1.34 \pm 0.22 ^{b**}
Primary bile acids	0.16 \pm 0.02	1.54 \pm 0.26 ^{b**}
7-Dehydrocholic acid	0.00 \pm 0.00	0.09 \pm 0.04
Deoxycholic acid	0.42 \pm 0.07	0.03 \pm 0.03 ^{b**}
Lithocholic acid	0.46 \pm 0.05	0.00 \pm 0.00 ^{b**}
Hyodeoxycholic acid	1.73 \pm 0.44	0.00 \pm 0.00 ^{b*}
Secondary bile acids	2.61 \pm 0.55	0.12 \pm 0.06 ^{b**}
Total bile acids	2.77 \pm 0.56	1.66 \pm 0.30
Primary bile acids /Total bile acids	0.06 \pm 0.01	0.94 \pm 0.03 ^{b**}

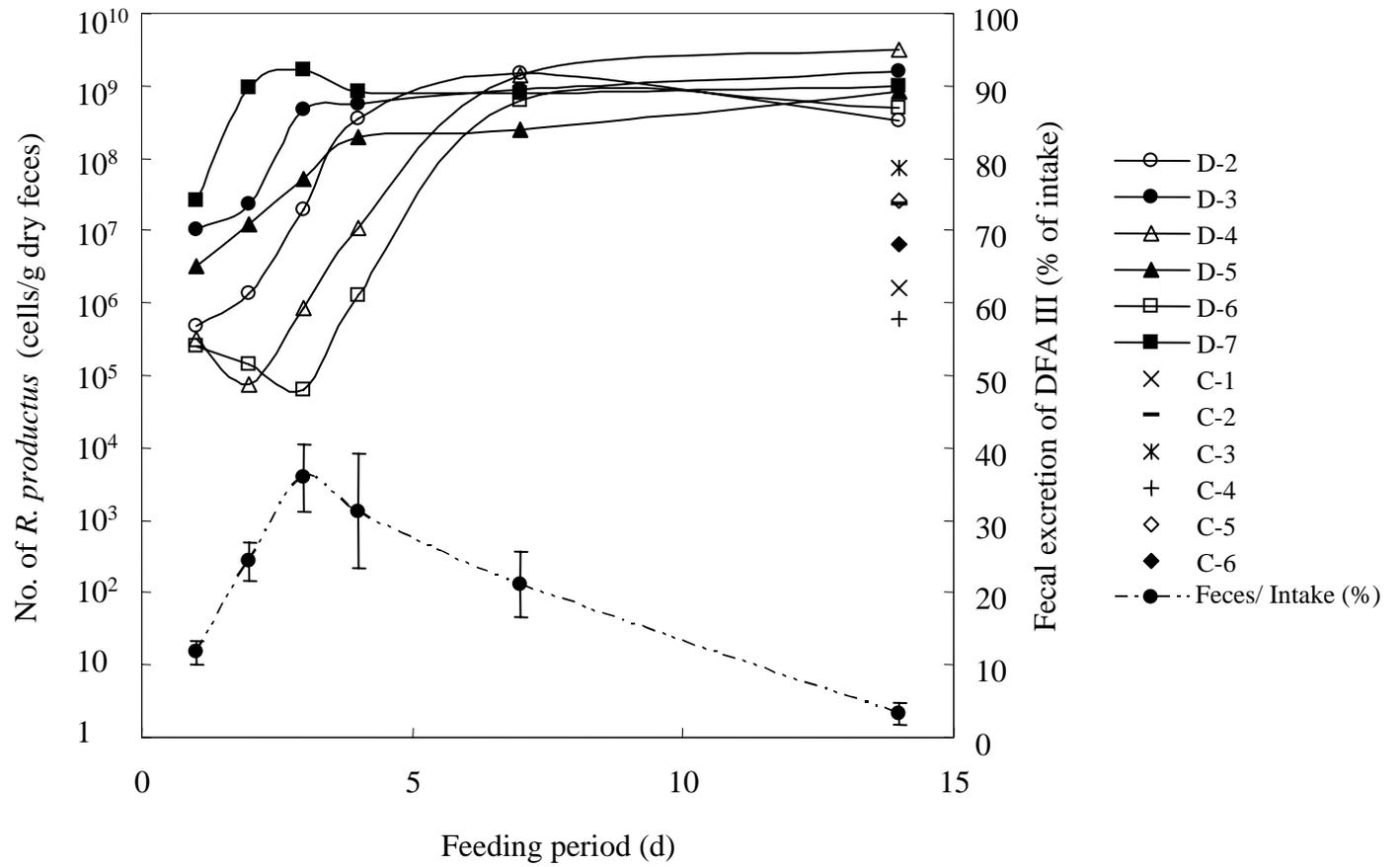
^aValues are means \pm SEM, n=6.

^bSignificantly different from control, * $P < 0.05$, ** $P < 0.01$.

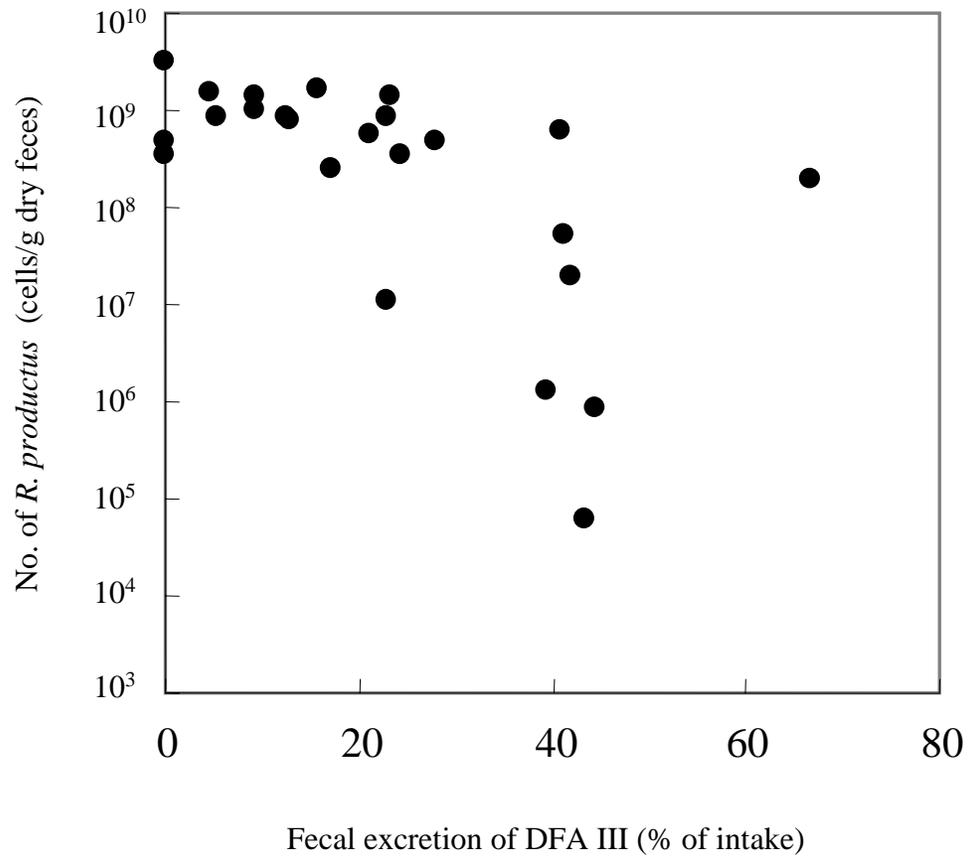
Minamida *et al.* TABLE 2



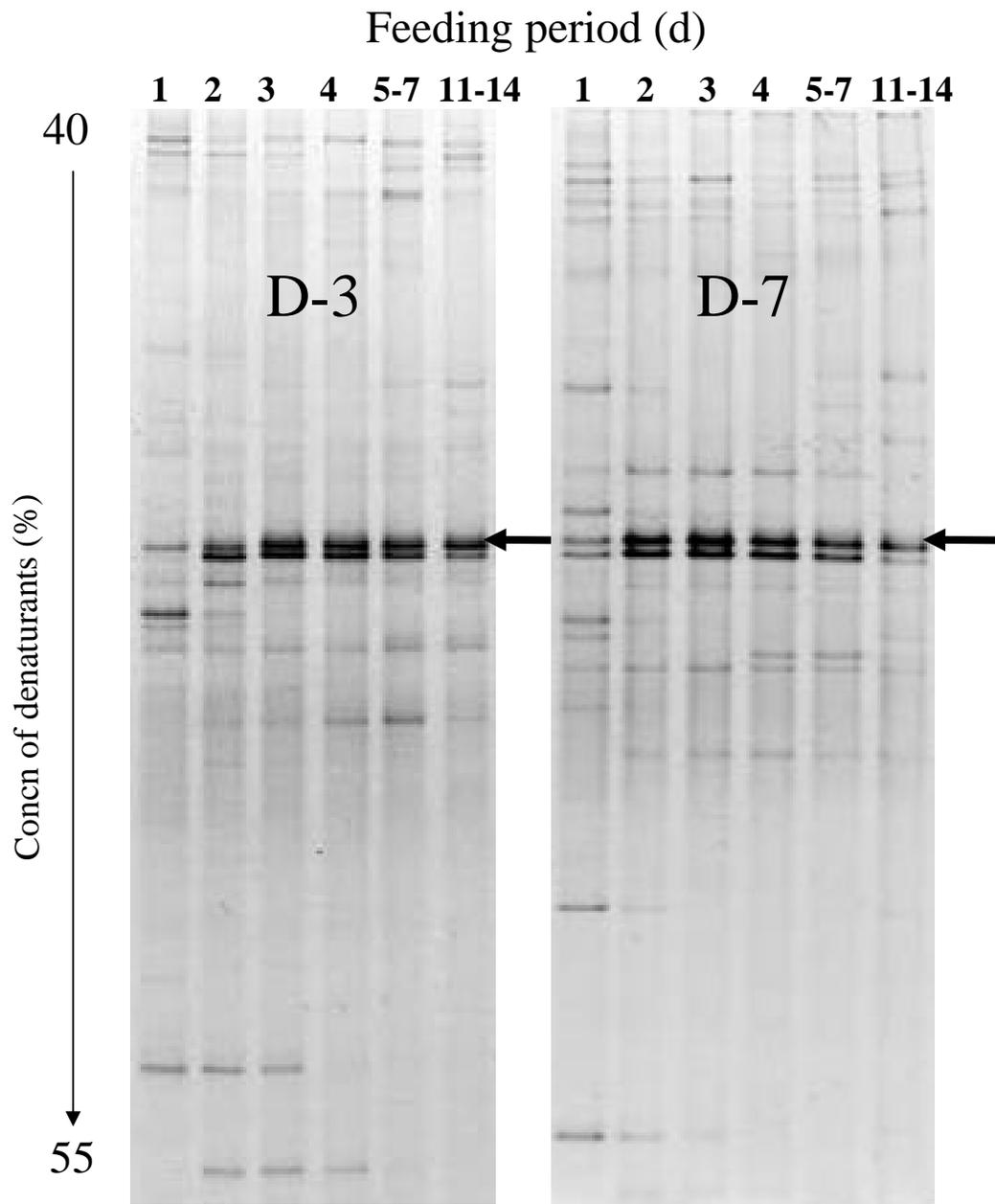
Minamida *et al.* FIG. 1



Minamida *et al.* FIG. 2



Minamida *et al.* FIG. 3



Minamida *et al.* FIG. 4