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Effects of Long-Term Ingestion of Diffructose Anhydride III (DFA III) on Intestinal
Bacteria and Bile Acid Metabolism in Humans

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ABSTRACT

Changes in the intestinal microbiota of 10 human subjects with long-term ingestion of 3 g/d difructose anhydride III (DFA III; 4 persons, 2 months; 3 persons, 6 months; and 3 persons, 12 months) were examined by denaturing gradient gel electrophoresis (DGGE). According to the answers to questionnaires, the subjects were divided into two groups (constipated and normal). The DGGE profile was different for every individual and each subject had unique profiles of intestinal microbiota. In the DGGE profiles of constipated subjects, the intensities of bands related to *Bacteroides* spp. increased. Moreover, the DFA III-assimilating bacteria, *Ruminococcus* sp. were isolated from subjects who ingested DFA III for 12 months. These strains showed 95% similarity of their 16S rDNA sequences with that of *Ruminococcus obeum* ATCC 29174^T (X85101) and produced large amounts of acetic acid.

DFA III ingestion for 2 months tended to increase total organic acids in feces, and tended to decrease fecal pH and the secondary bile acid (SBA) ratio in total bile acids. The SBA ratio in total bile acids corresponded to fecal pH. The production of SBA was decreased by low pH *in vitro*. These results indicated that DFA III ingestion in humans tended to lower intestinal pH, inhibited bile acid 7 α -dehydroxylation activities and also tended to decrease the SBA ratios in total bile acids. Moreover, as another cause for the

decrease in the SBA ratio in total bile acids, it was suggested that the number of bile acid 7 α -dehydroxylating bacteria were decreased by DFA III ingestion.

INTRODUCTION

Difuctose anhydride III (di-D-fructofuranose-1,2': 2,3'-dianhydride; DFA III) is a nondigestible disaccharide that enhances calcium absorption (1-6). It is produced in large quantities from inulin using inulase II from *Arthrobacter* sp. H65-7 (7, 8). DFA III is not assimilated *in vitro* by the type strains of typical intestinal microorganisms: bifidobacteria, lactobacilli and *Bacteroides* spp. (8). However, we isolated a DFA III-assimilating bacterium, *Ruminococcus productus* (AB125231 and AB196512), that produced much of the acetic acid in the cecal contents of 3% DFA III-fed rats (9, 10). *R. productus* was formerly named *Peptostreptococcus productus* (11) and is one of the dominant and indigenous members of the human and rat intestinal floras (11-13). In the cecum of DFA III-fed rats, the pH was lowered below 6.5 (1-3, 9, 10) and an increase in the amount of short-chain fatty acids (SCFAs), particularly acetic acid, was observed.

Secondary bile acids (SBAs) have been found to promote colon carcinogenesis (14-17) and gallstone formation (18). SBAs (deoxycholic acid and lithocholic acid) are converted by bacterial 7 α -dehydroxylation from primary bile acids (PBAs; cholic acid and chenodeoxycholic acid) in the intestine. Bile acid 7 α -dehydroxylase shows an optimum activity at pH 7-8 (19-22) and is inhibited at a pH < 6.5 *in vitro* (19, 20, 23). Therefore, intestinal acidification is expected to reduce bile acid degradation by

7 α -dehydroxylation and prevent colorectal cancer (24). When rats were fed 3% DFA III diets for 2 weeks, PBAs were mostly accounted for in the total bile acids in the feces, whereas most of the total bile acids in the feces of control-fed rats were composed of SBAs (10). In another rat experiment (control diet vs. 3% DFA III diet, 2 weeks ingestion), the SBA ratio in total bile acids was decreased; that of control-fed rats was 0.94 and that of DFA III-fed rats was 0.51 (25). Therefore, DFA III ingestion is expected to also decrease the SBA ratio in total bile acids of human feces.

Another cause of the lowering of the SBA ratio in total bile acids is the decrease in the number of bile acid 7 α -dehydroxylating bacteria. Six species (*Clostridium scindens*, *C. hiranonis*, *C. hylemonae*, *C. bifermentans*, *C. sordellii* and *C. leptum*) are known as bile acid 7 α -dehydroxylating bacteria (26-30). Many of the genes needed for 7 α -dehydroxylation are located on a bile acid-inducible operon (*bai*) in *C. scindens* and *C. hiranonis* and each structure is very similar (31, 32). The *baiCD* gene of three species (*C. scindens*, *C. hiranonis* and *C. hylemonae*), including two species that have high 7 α -dehydroxylase activity (*C. scindens* and *C. hiranonis*), can be amplified using *baiCD* gene-specific primers designed using a part of the similar sequences (33).

When rats were fed 3% DFA III for 4 weeks, the effects of DFA III ingestion on rat intestinal microbiota were examined by denaturing gradient gel electrophoresis (DGGE)

(9). According to DGGE profiles, the intensities of bands related to *Bacteroides acidofaciens* and uncultured bacteria within the *Clostridium lituseburense* group decreased, whereas the intensities of bands related to *B. vulgatus*, *B. uniformis* and *Ruminococcus productus* increased in the DFA III-fed rat cecum. For healthy human subjects who ingested 9 g/d DFA III for 4 weeks, the intestinal microbiota did not change as evidenced by the analysis of DGGE profiles, whereas those in subjects with chronic constipation changed and they obtained relief from symptoms of constipation (34).

The primary purpose of this study was to determine whether intestinal microbiota, fecal pH and organic acids in human subjects undergoing long-term ingestion (2 months) of 3 g/d DFA III would change. An additional aim was to isolate DFA III-assimilating bacteria from human subjects. The secondary purpose of this study was to determine whether the SBA ratio in total bile acids would decrease similarly to rat experiments. To investigate the formation of SBA at a low pH, an *in vitro* 7 α -dehydroxylase assay was done using viable bacteria from glycerol stocks of fecal samples. Because 7 α -dehydroxylase activity is extremely oxygen sensitive (22), it is not possible to directly measure the enzyme activity from fecal samples. Moreover, the increase and decrease in the number of bacteria that had high 7 α -dehydroxylase activity

were examined by PCR amplification of *baiCD*.

MATERIALS AND METHODS

Subjects Ten healthy female subjects (23-48 years old) participated in this study and ingested 3 g/d DFA III once a day for 2 months. Moreover, following the consent of the subjects, three subjects (subjects C, F and G) ingested DFA III for 6 months and the other three subjects (subjects H, I and K) ingested DFA III for 12 months (Fig. 1). To prevent the subjects from being stressed due to the long-term experiment, we directed the subjects to continue with their usual diets and avoid taking laxatives and antibiotics. Regarding the intake of fermented milk, the subjects routinely taking it continued to do so during the test period. The subjects recorded the frequencies of defecation and the condition of their stools before and after 2 months of this trial. The subjects were divided into two groups: normal defecation group (subjects A, D, E, H, I and J) and chronic constipation group (subjects C, F, G and K) on the basis of the questionnaire results. Because constipation is subjective, constipated subjects were identified on the basis of their responses to the questionnaire. The average frequency of defecation in constipated subjects was 1.75 times/week and in the normal defecation group it was 5.67 times/week. This study was conducted on the basis of the principles of the

Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). Informed consent was obtained from each subject before this study.

Fecal samples Fecal samples were collected immediately after defecation at 0 (before ingestion), 2, 6 and 12 months of DFA III ingestion, and after a washout period of 1 month (Fig. 1). The DNA for DGGE was extracted from fecal samples as described previously (34). For the isolation of DFA III-assimilating bacteria, the fecal samples from subjects (subjects H, I and K) who ingested DFA III for 12 months were immediately added into test tubes containing a diluted solution for anaerobic bacteria (35). DFA III-assimilating bacteria were isolated and identified as described previously (9). The remaining diluted suspensions of feces were added with the same volume of 40% glycerol solution in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) and stored at -80 °C.

DGGE analysis PCR amplification (for targeting the V3 regions of the bacterial 16S rDNA) was performed as described in previous papers (9, 34). The DGGE fragments (gel pieces) were excised using a razor blade, washed once with autoclaved water, directly subjected to PCR and identified. When the sequences were not

determined using pieces of gels as templates, PCR products were cloned using the pGEM[®]-T easy vector system (Promega, Madison, WI, USA). Using the clones, direct colony PCR was carried out, and the sequences of bands were determined. The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession nos. AB222186 to AB222207 and AB239252.

Analyses of pH and organic acids in feces Fecal samples were diluted with 10 volumes of deionized water, vortexed, and then the pH of the suspension was measured using a TOA pH meter (HM-20S; DDK TOA, Tokyo) to determine fecal pH. For the analysis of organic acids, diluted suspensions of fecal samples were centrifuged at 15,000 rpm for 5 min and the supernatants were collected. Fifty mM NaOH was added to the supernatants and they were deoxygenated with chloroform. Total organic acids (succinic acid, lactic acid, acetic acid, propionic acid, butyric acid and valeric acid) were measured by HPLC (organic analysis system; Jasco, Tokyo) equipped with two Shodex RSpak KC-811 columns (8 mm i.d. × 30 cm long; Showa Denko, Tokyo), Shodex RSpak KC-G (Showa Denko) and a multiwavelength detector (MD-1510; Jasco). The mobile phase was 5% acetonitrile in 3 mM HClO₄ at a flow rate of 1 ml/min and the column temperature was at 55°C.

Analysis of bile acids in feces The determination of the composition of bile acids in feces was performed as described in a previous paper (10). PBA consisted of cholic acid and chenodeoxycholic acid, whereas SBA consisted of deoxycholic acid and lithocholic acid. The total bile acids were the sum of PBA and SBA.

Cholic acid 7 α -dehydroxylation activity assay The medium for the cholic acid 7 α -dehydroxylation activity assay modified from ATCC medium 1524 contained 5 g of Bacto™ proteose peptone (Becton Dickinson, Sparks, MD, USA), 5 g of trypticase® peptone (BD Bioscience, Sparks, MD, USA), 10 g of Bacto™ yeast extract (Becton Dickinson), 40 ml of salt solution, 10 ml of 0.05% hemin solution, 0.2 ml of 0.5% vitamin K1 solution and 0.5 g of L-cysteine HCL per 950 ml of 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Dojin, Kumamoto). The salt solution contained 0.2 g of CaCl₂, 0.2 g of MgSO₄, 1.0 g of K₂HPO₄, 1.0 g of KH₂PO₄, 10.0 g of NaHCO₃ and 2.0 g of NaCl per liter of distilled water. The medium was adjusted to pH 7.5 and after autoclaving, the cholic acid solution was added to the medium (final cholic acid concentration: 100 μ M) (33, 36). The same medium with 2-(*N*-morpholino) ethanesulfonic acid (MES; Dojin) instead of HEPES was also utilized

at pH 5.8. When the pH of cecal contents was 5.8, most of the bile acids in rat feces were accounted as PBA (10). Therefore, we used this condition to investigate whether the activities of cholic acid 7 α -dehydroxylase would be inhibited *in vitro*.

The fecal suspensions from subjects I and K who ingested DFA III for 12 months were diluted from 10⁻² to 10⁻⁸ in the media for the cholic acid 7 α -dehydroxylation activity assay in an anaerobic chamber. The samples were incubated anaerobically at 37°C for 24 h. Aliquots of assay media were acidified with 3 M HCl and bile acids were extracted twice with ethyl acetate, dried and dissolved in methanol. TLC was carried out using a silica gel plate (Silica gel 60; Merck, Darmstadt, Germany) with a solvent system of cyclohexane-ethyl acetate-acetic acid (10: 15: 4, v/v) and spots were detected with a reagent containing 10% 12 molybdo (VI) phosphoric acid n-hydrate solution by heating (37). For quantification, aliquots of the assay media were supplemented with 23-nordeoxycholic acid (ICN Biomedicals, Aurora, OH, USA) as an internal standard, extracted as described above, methylated, silylated and analyzed by GC-MS as described previously (10). Protein was measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard protein and the amount of deoxycholic acid production per mg protein was calculated.

Amplification of *baiCD* gene PCR was performed as described previously (33) with some modifications. The reaction mixture (50 μ l) contained 25 pmol of each primer, 5 μ l of dNTP mixture (2.5 mM each), 1 x PCR reaction buffer, 3.0 μ l of MgCl₂ solution (25 mM), 2.5 U of AmpliTaq (Applied Biosystems, Foster City, CA, USA), and 1 μ l of DNA solution. The primers used were *baiCD*-F (5' GGW TTC AGC CCR CAG ATG TTC TTT G 3') and *baiCD*-R (5' GAA TTC CGG GTT CAT GAA CAT TCT KCK AAG 3') (33). To determine the detection limit of the PCR, DNA was also extracted from the culture broth of *C. scindens* (JCM10418) that carried the *baiCD* gene as described previously (10). The number of cells stained with crystal violet solution was counted by microscopy using a counting chamber.

Statistical analysis Values are expressed as mean \pm SEM. Paired-Student's *t* test was used to determine whether total organic acids, pH and the SBA ratio in total bile acids of feces were significantly different between time 0 and after 2 months of DFA III ingestion ($P < 0.05$).

RESULTS

Changes in intestinal microbiota after DFA III ingestion No laxatives or

antibiotics were used during the study period and no subject had diarrhea due to DFA III ingestion. From the DGGE profiles of all the subjects before and after 2 months of DFA III ingestion, the DGGE profiles were different in all subjects and each subject had a unique profile of intestinal microbiota, as in the cases mentioned in a previous paper (34; Figs. 2 and 3).

The DGGE profiles of constipated subjects are shown in Fig. 2. The intensity of the band related to *Collinsella aerofaciens* (band no. 12) decreased in subject C and the intensities of bands related to *Ruminococcus* sp. (band no.9) and *Bifidobacterium catenulatum* (band no. 11) decreased in subject F. The intensities of bands related to *Bacteroides* spp. increased. The intensities of bands related to *Bacteroides uniformis* (band nos. 6, 7 and 17) increased in three out of four subjects, showing the same results as in rats fed 3% DFA III (9). In subject K, the DGGE profiles after the washout period returned to that obtained before DFA III ingestion and the bands related to *Bacteroides* spp. at the upper part of the DGGE gel (band nos. 13-17) disappeared.

The DGGE profiles of normal subjects are shown in Fig. 3. The intensities of bands related to *Bacteroides* spp. were not markedly different from those of constipated subjects. However, the intensities of bands related to *B. uniformis* (band no. 2) increased in three out of six subjects (subjects A, I and J). The intestinal microbiota in subjects

who ingested DFA III for 12 months (subjects H and I) were generally stable for 12 months. In subjects H and I, the intensities of the bands related to uncultured *Clostridium* sp. (band no. 1) decreased after DFA III ingestion; after the washout period, the intensities returned to the level observed before DFA III ingestion.

Isolation and identification of DFA III-assimilating bacterium The DFA III-assimilating bacteria, *Ruminococcus* sp. (AB222208), were isolated from subjects H, I and K. These strains were characterized as mainly producing acetic acid (data not shown) and had 95% similarity of their 16S rDNA full sequences with that of *Ruminococcus obeum* ATCC 29174^T (X85101). The sequences of the V3 region of these DFA III-assimilating bacteria, *Ruminococcus* sp. were the same as those of *R. obeum* (X85101). The intensities of bands related to DFA III-assimilating *Ruminococcus* sp. (band no. 18 in Fig. 2 and band no. 3 in Fig. 3) increased upon DFA III ingestion in subjects H, I and K as compared with those before DFA III ingestion; after the washout period, the intensities returned to the levels observed before DFA III ingestion in subject I.

The pH and total organic acids in feces The fecal pH before DFA III ingestion was 6.96 ± 0.23 and after 2 months was 6.57 ± 0.31 (n=10, $P=0.085$). Comparing the

values before and after DFA III ingestion per individual, fecal pH decreased in nine out of ten subjects (Fig. 4a). The total organic acid concentration in feces before DFA III ingestion was 111.0 ± 10.3 $\mu\text{mol/g}$ wet feces and after 2 months was 145.0 ± 17.3 $\mu\text{mol/g}$ wet feces ($n=6$, $P=0.069$). Comparing the values obtained before and after DFAIII ingestion per individual, the total organic acid concentration increased in four out of six subjects (Fig. 4b). There were no significant differences, however, DFA III ingestion tended to decrease fecal pH and increase total organic acid concentration in feces.

Changes in composition of bile acids in feces The range of concentrations of total bile acids were wide in all subjects (before DFA III ingestion: 10.80 ± 6.58 $\mu\text{mol/g}$ dry feces; after 2 months: 19.25 ± 16.12 $\mu\text{mol/g}$ dry feces, mean \pm SD). Therefore, the overall SBA ratios in total bile acids were compared. Before DFA III ingestion, the SBA ratio was 0.74 ± 0.15 and after 2 months it decreased to 0.48 ± 0.14 . There was no significant difference, however, a downward trend is apparent ($n=6$, $P=0.12$).

Comparing before and after DFA III ingestion per individual, the SBA ratios decreased in five out of six subjects (Fig. 5a). In subject H, who showed an increased the SBA ratio, the ratio was 0.02 before DFA III ingestion and most of the total bile acids consisted of PBA. After 2 months, the SBA ratio increased to 0.18, however, this value

was lower than those for the other subjects.

Cholic acid 7 α -dehydroxylation activity and pH Using the fecal suspensions of two subjects (subjects I and K), we determined whether the formation of deoxycholic acid was inhibited by low pH. The results for subject I are shown in Fig. 6. TLC spots of deoxycholic acid were detected in 10⁻³ and 10⁻⁴ dilutions at pH 7.5 and in a 10⁻² dilution at pH 5.8. The production of deoxycholic acid was 11.2 mg/mg protein in a 10⁻⁴ assay broth of the sample at pH 7.5 and 0.6 mg/mg protein in a 10⁻⁴ assay broth of the sample at pH 5.8. For subject K, the production of deoxycholic acid was 2.6 mg/mg protein in the 10⁻⁴ assay broth of the sample at pH 7.5 and 0.4 mg/mg protein in the 10⁻⁴ assay broth of the sample at pH 5.8. These results imply that the production of SBA was decreased by low pH *in vitro*.

Amplification of *baiCD* gene The result of the amplification of the *baiCD* gene in subjects for whom the concentrations of bile acids were determined is shown in Fig. 7. The *baiCD* gene was detected in subjects E and I before DFA III ingestion, whereas it was not detected after 2 months of DFA III ingestion. In the other subjects D, G, H and K, this gene was not detected before or after DFA III ingestion. It was found that the

number of bacteria which carried the *baiCD* gene in subjects E and I after DFA III ingestion decreased to less than 6.5×10^6 cells/g wet feces because the *baiCD* gene of *C. scindens* was detectable in more than 4.6×10^3 cells.

DISCUSSION

When the intestinal microbiota of 10 human subjects who ingested 3 g/d DFA III were examined by DGGE, the changes in DGGE profiles of all subjects after 2 months were different in individuals as reported previously (34). The intensities of bands related to *B. uniformis* increased in six out of ten subjects. When rats were fed 3% DFA III for 4 weeks and their cecal samples were analyzed by DGGE, the intensities of bands related to *B. vulgatus*, *B. uniformis* and *R. productus* increased. In these bacteria, only *R. productus* was isolated as a DFA III-assimilating bacterium. It seemed that *Bacteroides* spp. could not assimilate DFA III but grew nonetheless (9). In this study, *Bacteroides* spp. could not be isolated as DFA III-assimilating bacteria, but Fig. 2 showed that the growth of *Bacteroides* spp. was stimulated by DFA III ingestion.

The DFA III-assimilating bacteria, *Ruminococcus* sp. were isolated from subjects H, I and K. These strains had 95% similarity of their 16S rDNA full sequences with that of *R. obeum* ATCC 29174^T (X85101) and mainly produced acetic acid. The intensities of

bands related to the DFA III-assimilating *Ruminococcus* sp. (band no. 18 in Fig. 2 and band no. 3 in Fig. 3) increased in subjects H and I after 2 months and in subject K after 12 months compared with before DFA III ingestion. For other subjects, for whom no attempt was made to isolate DFA III-assimilating bacteria, the intensity of the band related to *Ruminococcus* sp. increased in subject C, whereas it did not change or decreased in other subjects. This is perhaps due to the difference in human intestinal microbiota among individuals. When temporal changes in the DFA III-assimilating bacterium *R. productus* in the feces of rats fed 3% DFA III was examined by real-time PCR, the number of *R. productus* cells increased from 10^6 to 10^8 - 10^9 cells/g dry feces in all rats fed DFA III within one week (10). This showed that indigenous *R. productus* in the rat intestine assimilated DFA III and multiplied. In DGGE profiles, the intensities of bands related to *R. productus* increased. Because the intensities of bands related to *Ruminococcus* sp. also increased in DGGE profiles in this study, indigenous *Ruminococcus* sp. seemed to have grown as a result of DFA III assimilation.

In this experiment, DFA III ingestion for 2 months, the tendencies for total organic acid concentration to increase in feces and fecal pH to decrease were highlighted, whereas in rat experiments, the pH and SCFAs of the cecal contents of DFA III-fed rats were significantly different from those of control-fed rats (9, 10, 25). Fermentation by

intestinal bacteria in rat intestine occurs in the cecum and the pHs and amounts of organic acids in rat cecal contents can be directly measured. On the other hand, fermentation products in human intestine from the cecum/right colon can be examined only after passage to the distal colon and excretion. SCFA concentrations are highest in the cecum and right colon of humans and decrease progressively towards the distal colon (38). The pH is lowest in the right colon and increases in the distal colon to neutrality (39). The organic acids produced in the cecum and right colon were absorbed and the concentrations of organic acids decreased upon excretion into the feces. One possible reason why fecal pH and the concentrations of organic acids in this human experiment were not significantly different was that the concentrations of fecal organic acids were less than those of the cecum/right colon. Another reason is the difference in DFA III doses between rat and human experiments. In general, rats eat about 20 g of a 3% DFA III diet per day and the amount of DFA III consumed is about 0.6 g/d. The weight of rats is calculated as 200 g and the dose per weight is 0.003 g. Human subjects ingested 3 g/d and the weight of human subjects is calculated as 50 kg and the dose per weight is 0.00006 g. This was about 50-fold less than the dose for rats. Moreover, the daily ingested amount of DFA III (3 g) for the human subjects was lower than that for rats (3% DFA III diet) and represented only about 0.6% of the total dietary intake per

day.

The concentrations of organic acids, in particular acetic acid, increased; before DFA III ingestion, acetic acid concentration was 79.7 ± 9.9 $\mu\text{mol/g}$ wet feces and after 2 months it was 101.3 ± 17.9 $\mu\text{mol/g}$ wet feces ($P=0.174$, no significant difference).

When rats were fed 3% DFA III diets, the concentration of acetic acid produced by *R. productus* increased (9, 10, 25). In this study, DFA III-assimilating *Ruminococcus* sp. were isolated from three subjects examined. Because these strain also mainly produced acetic acid, the increase in the concentration of acetic acid was higher than those of other organic acids although not significantly different. As a result, fecal pH tended to decrease.

In the rat experiment previously reported, the SBA ratio in total bile acids corresponded to cecal pH (10, 25). In the human experiment, the SBA ratio in total bile acids also corresponded to fecal pH (Fig. 5b; correlation coefficient, $r=0.65$, $P<0.05$). Fecal pH was below 6.5 in four subjects whose SBA ratios in total bile acids were less than 0.6 after DFA III ingestion. When the fecal pH decreased, the SBA ratio in total bile acids decreased. In humans, the production of bile acid 7 α -dehydroxylase is induced by its substrate cholic acid (22, 40, 41) in the cecum and because the cholic acid concentration in the right colon is higher than that in the left colon, consequently

the 7 α -dehydroxylase activity in the cecum/ascending colon is also higher than that in the left colon (41). The 7 α -dehydroxylase activity is affected by pH and is inhibited below pH 6.5 *in vitro* (19, 20, 23). DFA III ingestion tended to decrease the fecal pH and it represented the pH decrease in the cecum/right colon. It seemed that DFA III ingestion lowered the pH of the cecum/right colon and inhibited the 7 α -dehydroxylase activity, and consequently tended to decrease the SBA ratio in total bile acids in the human subjects.

In previous reports of human trials, lactulose ingestion (0.3 g/kg, twice daily for 6 weeks, about 30 g/d for 50 kg weight) decreased fecal pH and the concentration of SBA significantly. The effect is probably mediated through the acidification of colonic contents, thereby reducing 7 α -dehydroxylation of primary bile acids (42). Inulin (15 g/d for 3 weeks) or fructo-oligosaccharide (FOS) (15 g/d for 3 weeks) ingestion decreased the concentration of deoxycholic acid significantly but not fecal pH (43). On the other hand, FOS (12.5 g/d for 12 d) ingestion tended to decrease fecal pH and the concentration of total bile acids (no significant difference). The reason for the latter is that the dose of 12.5 g FOS was low and represented <2% of the total daily intake (44). In our study, because the dose (3 g/d) was very low, this dose resulted in a tendency for the fecal pH and the SBA ratio in total bile acids to decrease.

The number of bacteria that had the *baiCD* gene decreased in two out of six subjects following DFA III ingestion. In these subjects, the SBA ratio in total bile acids decreased after DFA III ingestion and there was the possibility that the number of 7α -dehydroxylating bacteria also decreased due to DFA III ingestion. However, more samples need to be examined. These results suggest that DFA III ingestion in humans lowers the percentage of SBA in total bile acids like other oligosaccharides. However, as another cause for the decrease in the SBA ratio in total bile acids, the possibility of a decrease in the number of bile acid 7α -dehydroxylating bacteria is suggested in addition to the inhibition of bile acid 7α -dehydroxylation activities by intestinal acidification.

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FIG. 1. Experimental schedule. Black, intake period; white, washout period; up-arrow, fecal sampling.

FIG. 2. DGGE profiles of intestinal microbiota obtained from constipated subjects (C, F, G and K) before (0) and after 2 months (2), 6 months (6) and 12 months (12) of DFA III ingestion and after washout period (W). The correlation of bands with closely related bacteria is shown in the Table on the right side. Band no.10 is closely related to many bacteria (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. gallinarum* and *L. helveticus*).

FIG. 3. DGGE profiles of intestinal microbiota obtained from normal subjects (A, D, E, J, H and I) before (0) and after 2 months (2), 3 months (3), 6 months (6), 9 months (9) and 12 months (12) of DFA III ingestion and after washout period (W). The correlation of bands with closely related bacteria is shown in the Table

on the right side.

FIG. 4. Effects of DFA III ingestion on fecal pH and total organic acid concentration. (a)

Changes in fecal pH of all subjects (A-K, n=10) before and after (2 months) of

DFA III ingestion. (b) Changes in total organic acids per individual (D-K, n=6)

before and after (2 months) of DFA III ingestion.

FIG. 5. Effects of DFA III ingestion on bile acid composition. (a) Changes in the SBA

ratio in total bile acids of subjects (D-K, n=6) per individual. Before and after (2

months) of DFA III ingestion. (b) Scatter plot of relationship between fecal pH

and the SBA ratio in total bile acids of subjects (D-K, n=6; correlation

coefficient, $r= 0.65$, $P<0.05$).

FIG. 6. *In vitro* formation of deoxycholic acid in two different assay media pHs (5.8 and

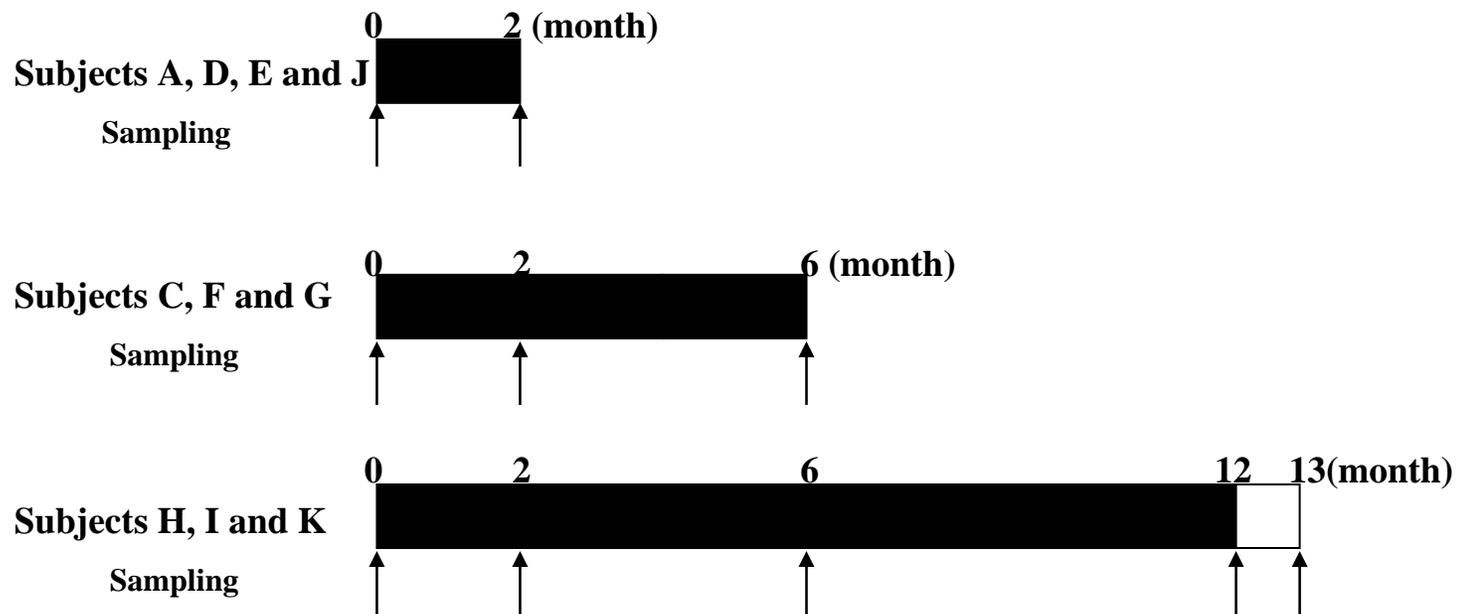
7.5). The assay of deoxycholic acid production and TLC were done as described

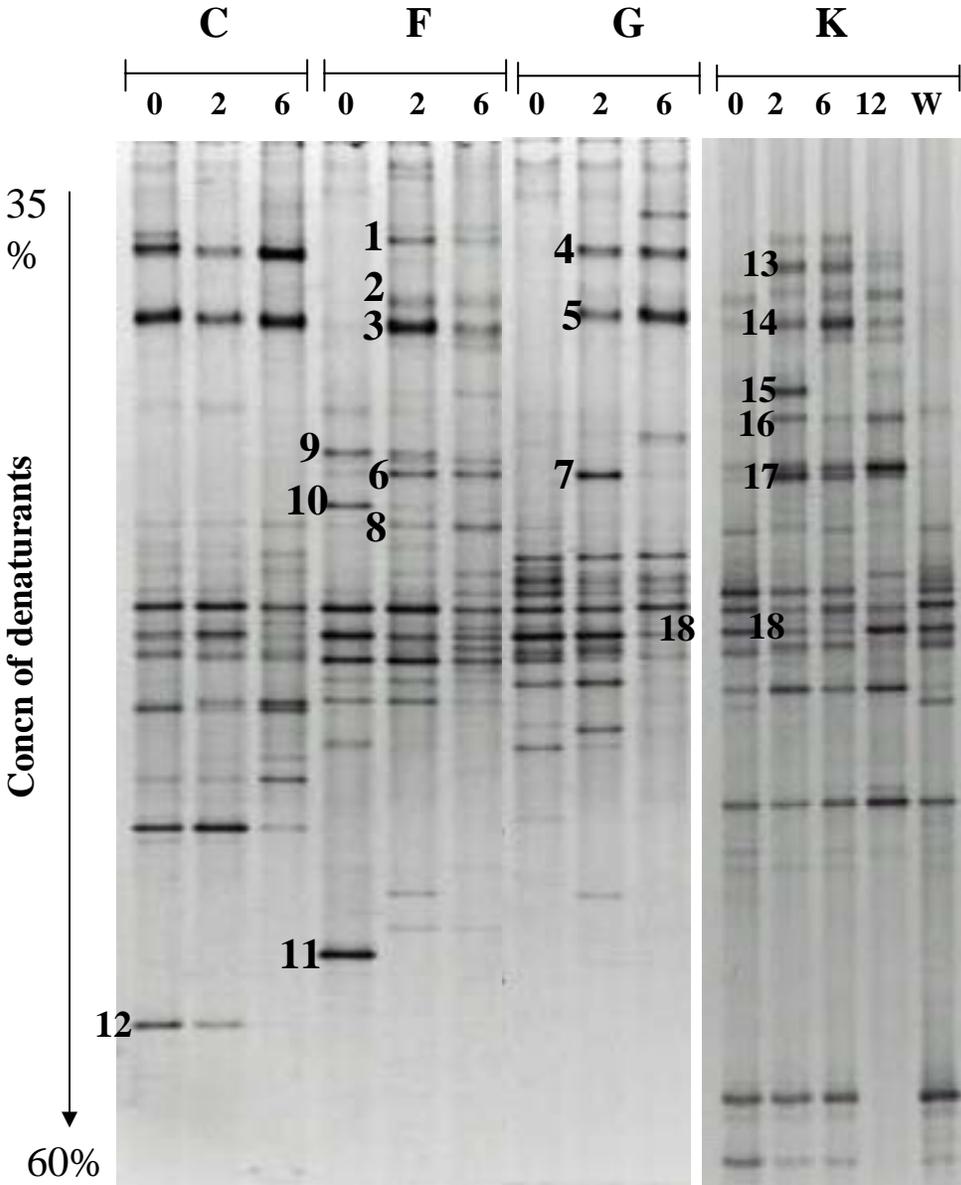
in the Materials and Methods. CA, cholic acid; DCA, deoxycholic acid; and

7-DehydroCA, 7-dehydrocholic acid.

FIG. 7. PCR products obtained using *baiCD* gene-specific primers. Lane M, 100 bp

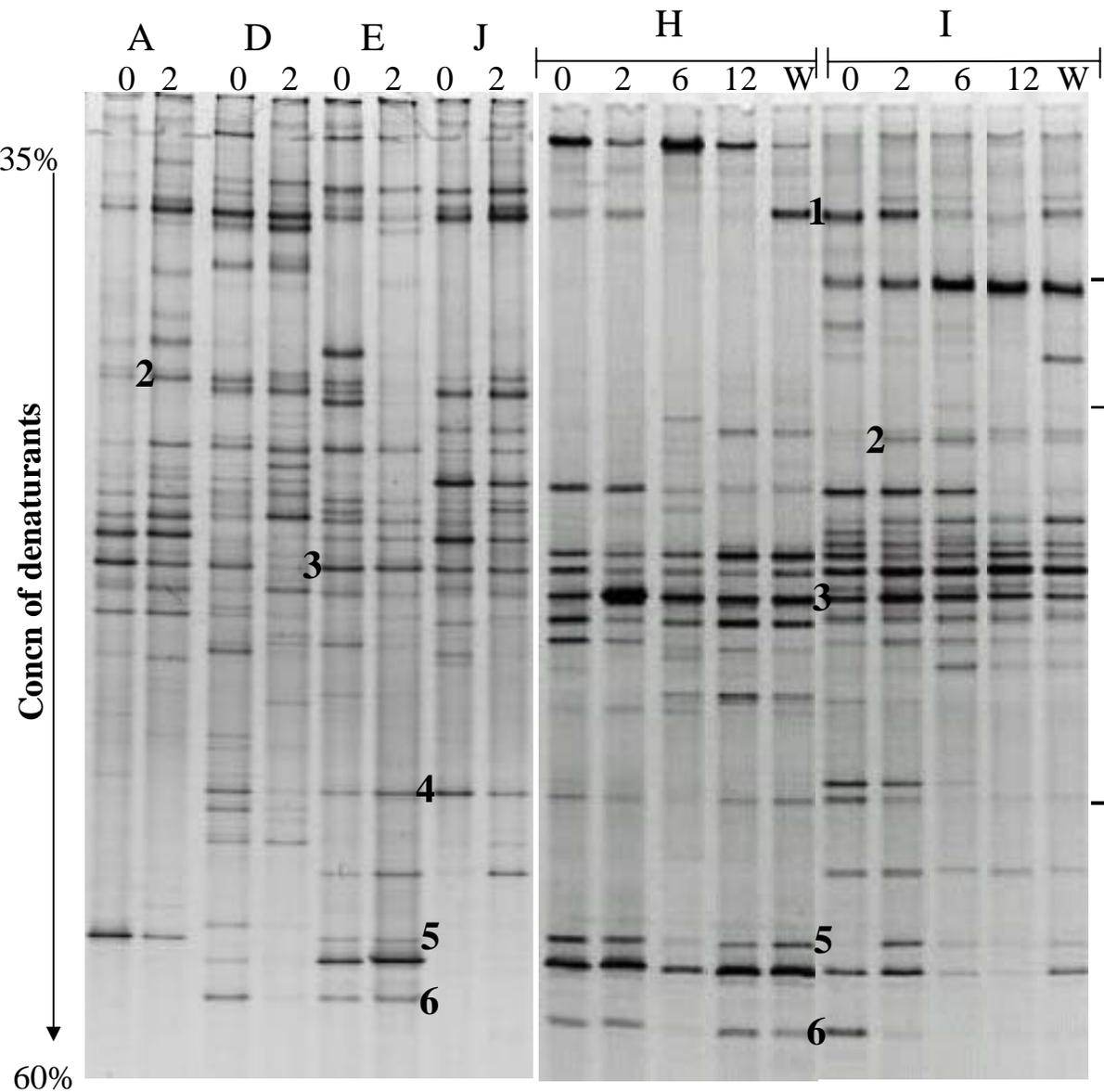
DNA ladder (Sigma Genosys Japan, Ishikari); lane P, *Clostridium scindens* (JCM10418); lanes D, E, G, H, I and K, subjects before (0) and after 2 months (2) of DFA III ingestion.





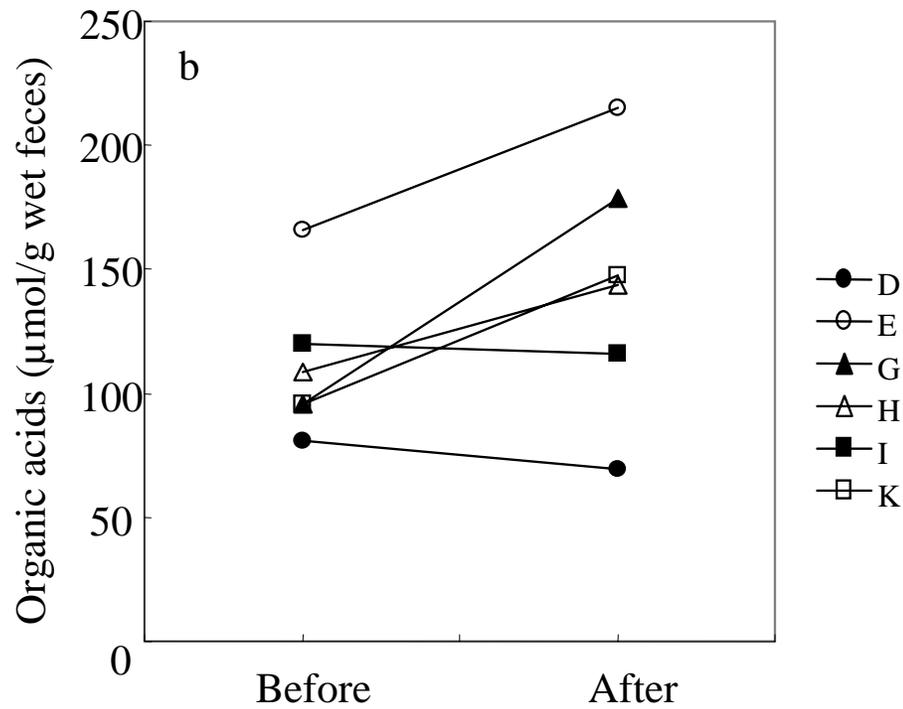
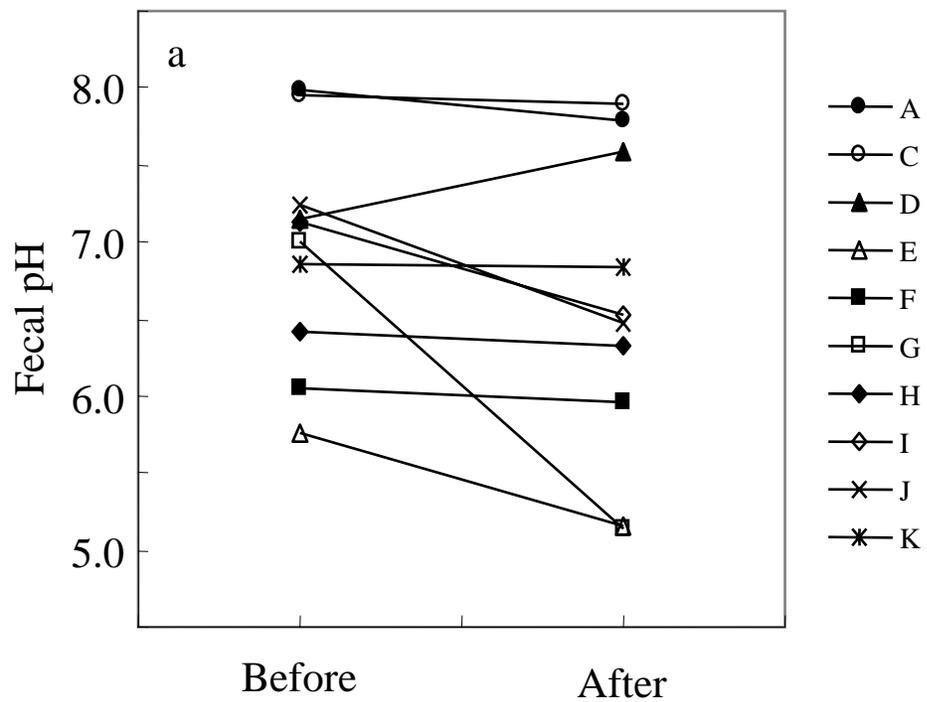
Band no.	Closest relative (accession number)	Similarity (%)
1	<i>Bacteroides thetaiotaomicron</i> (M58763)	100
2	<i>Bacteroides</i> sp. CS19 (AB064915)	100
3	<i>Bacteroides</i> sp.CJ69 (AB080890)	100
4	<i>Bacteroides uniformis</i> (AB215083)	98
5	<i>Bacteroides uniformis</i> (AB215083)	96
6, 7	<i>Bacteroides uniformis</i> (AB117564)	100
8	Uncultured bacterium (AY916228)	100
9	<i>Ruminococcus</i> sp. CO27 (AB064900)	100
10	<i>Lactobacillus</i> sp.	100
11	<i>Bifidobacterium catenulatum</i> (AF432082)	100
12	<i>Collinsella aerofaciens</i> (AJ245920)	100
13	<i>Bacteroides thetaiotaomicron</i> (M58763)	98
14	<i>Bacteroides ovatus</i> (X83952)	98
15	<i>Bacteroides</i> sp.CJ89 (AB080888)	100
16	<i>Bacteroides uniformis</i> (AY349154)	96
17	<i>Bacteroides uniformis</i> (AB117564)	100
18	<i>Ruminococcus obeum</i> (X85101)	100

Minamida et al. FIG. 2

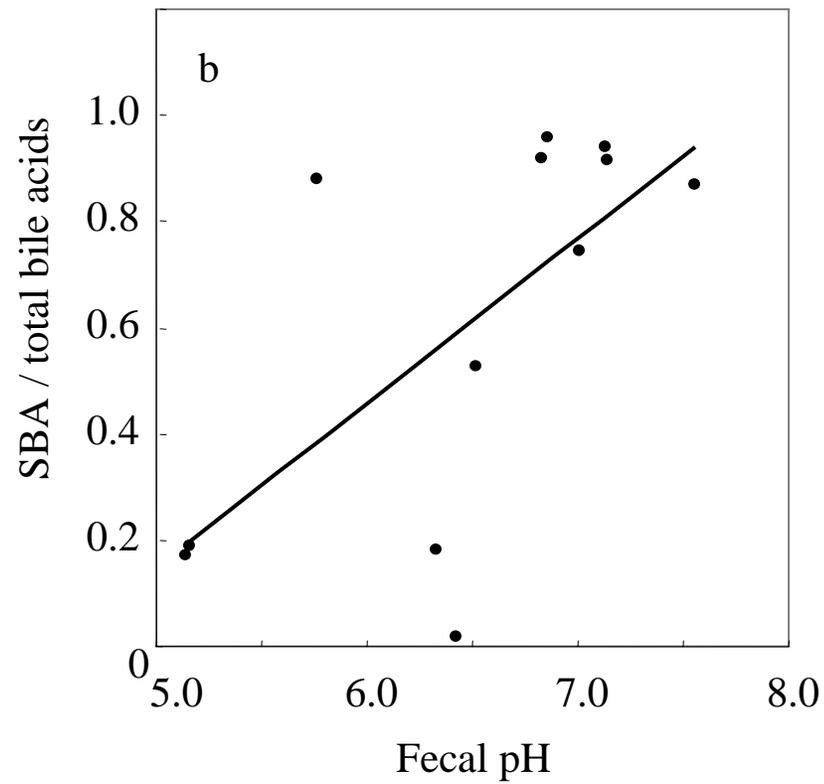
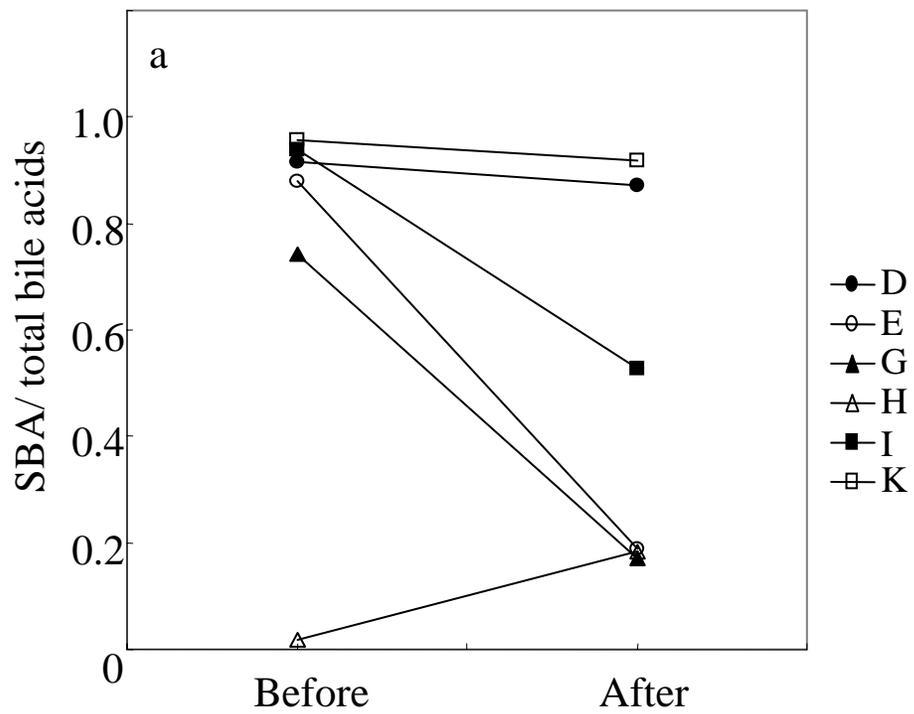


Band no.	Closest relative (accession number)	Similarity (%)
1	Uncultured <i>Clostridium</i> sp. (AB064860)	100
2	<i>Bacteroides uniformis</i> (AB117564)	100
3	<i>Ruminococcus obeum</i> (X85101)	100
4	<i>Bifidobacterium longum</i> (M58739)	100
5	<i>Bifidobacterium catenulatum</i> (AF432082)	100
6	<i>Collinsella aerofaciens</i> (AJ245920)	100

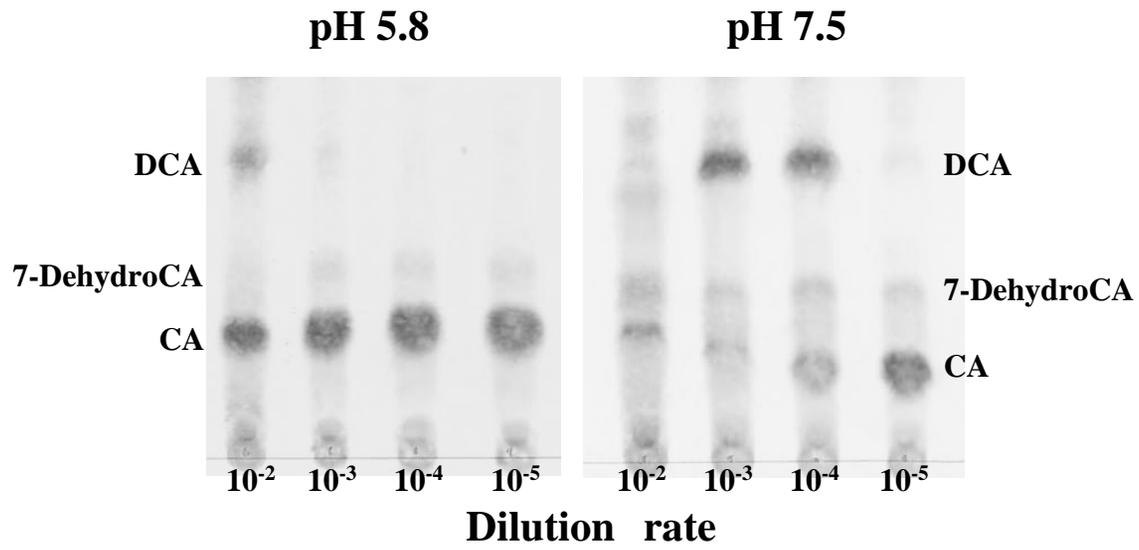
Minamida *et al.* FIG. 3



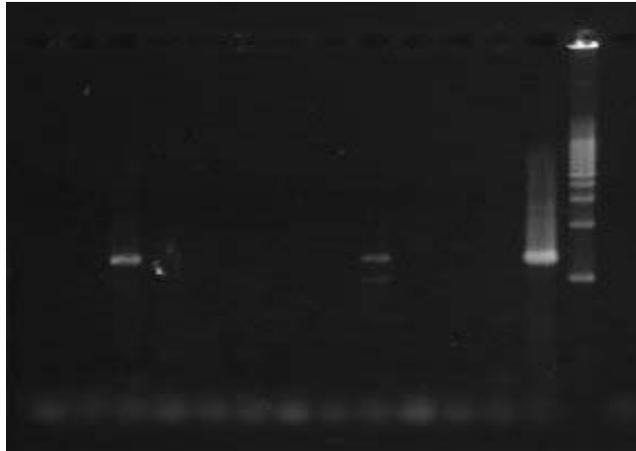
Minamida *et al.* FIG. 4 a and b



Minamida *et al.* FIG. 5 a and b



D E G H I K
0 2 0 2 0 2 0 2 0 2 PM



Minamida *et al.* FIG. 7