

## Effects of Ingestion of Diffructose Anhydride III (DFA III) and the DFA III-Assimilating Bacterium *Ruminococcus productus* on Rat Intestine

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We have isolated a diffructose anhydride III (DFA III)-assimilating bacterium, *Ruminococcus productus* AHU1760, from human. After an acclimation period of 1 week, male Sprague-Dawley rats (5 weeks old) were divided into four groups (control diet, *R. productus* diet, DFA III diet, and *R. productus* + DFA III diet;  $n = 8$ ) and fed the assigned test diets for 2 weeks. The viable count of administered *R. productus* was  $4.9 \times 10^7$  CFU/d in *R. productus*-fed rats and  $4.7 \times 10^7$  CFU/d in *R. productus* + DFA III-fed rats. Survival in cecal content of this strain was confirmed by randomly amplified polymorphic DNA. The ratio of secondary bile acids in feces in *R. productus* + DFA III-fed rats decreased the same as that in rats fed only DFA III. The viable count of lactobacilli and bifidobacteria, known as beneficial bacteria, increased more in *R. productus* + DFA III-fed rats than in control or *R. productus*-fed rats. A combination of *R. productus* and DFA III might improve the balance of intestinal microbiota to a healthier condition.

**Key words:** diffructose anhydride III (DFA III); *Ruminococcus productus*; bile acids

The rate of colon cancer has increased with the consumption of westernized food in Japan. Many causative agents and promoters of colon cancer have been identified. The best known promoters are secondary bile acids.<sup>1–8</sup> Secondary bile acids are converted from primary bile acids in the intestine by  $7\alpha$ -dehydroxylation of intestinal bacteria. Six intestinal  $7\alpha$ -dehydroxylating bacteria have been found.<sup>9–13</sup> While the number of known  $7\alpha$ -dehydroxylating bacteria do not correspond with the amount of secondary bile acids, the presence of many unknown  $7\alpha$ -dehydroxylating bacteria is inferred. To decrease secondary bile acids in the intestine, several ways are suggested: (1) decrease cholesterol as a precursor of bile acids; (2) excrete food fibers which absorb bile acids; (3) decrease  $7\alpha$ -dehydroxylating bacteria; and (4) lower the activity of  $7\alpha$ -dehydroxylation. The activity of  $7\alpha$ -dehydroxylation has

been lowered below pH 6.5<sup>14,15</sup> and it is expected that the amount of secondary bile acids will be decreased by lowering the pH in the intestine.<sup>16</sup>

Diffructose anhydride III (di-D-fructofuranose-1,2':2,3'-dianhydride, DFA III) is a nondigestible disaccharide included in chicory tubers but in small amounts. It is produced in large quantities from inulin using inulase II from *Arthrobacter* sp. H65-7.<sup>17,18</sup> DFA III has half the sweetness of sucrose, and has been reported to enhance calcium absorption in *in vivo* and *in vitro* experiments.<sup>19–24</sup> It directly affects the epithelial tissue and activates passage through tight junctions, which are located on the luminal side of adjacent epithelial cells.<sup>23</sup>

When healthy human subjects were administered 9 g/d DFA III for 4 weeks, the intestinal microbiota did not change,<sup>25</sup> while those in subjects with chronic constipation changed. They obtained relief from symptoms of constipation. When rats were fed 3% DFA III diets for 2 weeks, indigenous *Ruminococcus productus* assimilated DFA III and produced acetic acid, and as a result, the pH of cecum was lowered to 5.8. Secondary bile acids accounted for most of the total bile acids in the feces of control-fed rats, while primary bile acids mostly accounted in the total bile acids in the feces of DFA III-fed rats.<sup>26</sup> Therefore, we thought that the intestinal acidification due to DFA III ingestion had a possibility of decreasing  $7\alpha$ -dehydroxylation activity and the amount of secondary bile acids in feces.

Moreover, some DFA III-assimilating bacteria, *R. productus*, have been isolated from humans and rats.<sup>27</sup> Indigenous *R. productus* can assimilate ingested DFA III. *R. productus* was formerly named *Peptostreptococcus productus*, and is one of the dominant and indigenous members of the human and rat intestinal microbiota.<sup>28–30</sup> However, *R. productus* was not in the intestines of all the humans we have tested. So we administered *R. productus* AHU1760 of human origin to rats. This strain has bile tolerance and a little acid tolerance *in vitro* (unpublished data). Probiotic strain should have the ability to survive (not necessarily grow) in the intestine.<sup>31</sup> A strain with more tolerance to many

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**Table 1.** Composition of Basal and Test Diets (g/kg diet)

	Basal diet	Test diet			
		Control	<i>R. productus</i>	DFAIII	<i>R. productus</i> + DFA III
Casein <sup>1</sup>	200	200	200	200	200
Dextrin <sup>2</sup>	400	400	400	400	400
Sucrose	199.5	199.5	189.5	199.5	189.5
Soybean oil	70	70	70	70	70
Mineral mixture <sup>3</sup>	35	35	35	35	35
Vitamin mixture <sup>3</sup>	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
L-Cystine	3.0	3.0	3.0	3.0	3.0
<i>tert</i> -Butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Crystallized cellulose <sup>4</sup>	80	80	80	50	50
DFA III				30	30
Freeze-dried <i>R. productus</i>			10		10

<sup>1</sup>Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).

<sup>2</sup>Dextrin (TK-16; Matsutani Chemical Industry, Hyogo, Japan).

<sup>3</sup>Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.<sup>34)</sup>

<sup>4</sup>Crystallized cellulose (Avicel PH102; Asahi Chemical Industry, Tokyo, Japan).

harsh conditions, such as *Bifidobacterium lactis* Bb-12, was not enhanced to survive or persist in the gastrointestinal tract by galacto-oligosaccharides containing syrup.<sup>32)</sup> Thus, synbiotics (combinations of probiotics and prebiotics)<sup>33)</sup> might be needed for a strain with poorer survival properties. Hence, we included *R. productus* + DFA III-fed rats as a synbiotic group.

The primary aim of this study was to evaluate whether *R. productus* AHU1760 of human origin has the ability to be a probiotic and synbiotic strain which can survive through the gastrointestinal tract *in vivo*. Because indigenous *R. productus* is also in the rat intestine, to distinguish *R. productus* AHU1760 of human origin from that of rat origin, profiles of randomly amplified polymorphic DNA (RAPD) were analyzed. The secondary aim of this study was to examine whether secondary bile acids in feces are decreased by a combination of DFA III and *R. productus* the same as by ingestion of DFA III only. In addition, we aimed to determine whether the composition of intestinal microbiota is improved by an increase in the viable counts of beneficial intestinal bacteria such as lactobacilli and bifidobacteria.

## Materials and Methods

**Preparation of freeze-dried *R. productus*.** The DFA III-assimilating bacterium *R. productus* AHU1760 of human intestinal origin was precultured in a medium containing 52.5 g of GAM without dextrose (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10 g of DFA III per liter at 37 °C for 16 h in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). Then all the precultured medium was inoculated into batch culture medium containing 52.5 g of GAM without dextrose supplemented with 15 g of DFA III per liter, and cultured to stationary phase in an anaerobic jar fermentor (37 °C, 100 rpm, pH 7.0). The culture broth was centrifuged at 4,500 rpm for 15 min at 4 °C, and

washed once with autoclaved physiological saline with 0.05% L-cysteine hydrochloride (pH 7.0). The pellets were suspended in 20% sucrose solution in an anaerobic chamber and frozen at -80 °C. Then they were lyophilized in a vacuum freeze drier FD-550P (Tokyo Rikakikai, Tokyo, Japan), placed in a plastic bag, and put into a plastic container with dry silica gel at -20 °C until use. The viable count of *R. productus* after freeze-drying was measured in an anaerobic chamber by the conventional culture method.

**Animals and diets.** Male Sprague-Dawley rats (5 weeks old, weighing about 130 g; SLC Japan, Tokyo) were housed individually in stainless steel cages in a room under controlled temperature (22 ± 2 °C), relative humidity (40–60%), and lighting (lights on from 8:00–20:00). Rats were freely provided with tap water and the basal diet shown in Table 1 for acclimation of 1 week, and were divided into four groups (control diet, *R. productus* diet, DFA III diet, and *R. productus* + DFA III diet; n = 8) based on body weight. Test diets were as follows: (1) the control diet was the basal diet; (2) the *R. productus* diet had 1% sucrose of the basal diet replaced by 1% freeze-dried *R. productus*; (3) the DFA III diet had 3% cellulose of the basal diet replaced by 3% DFA III; and (4) the *R. productus* + DFA III diet had 1% sucrose and 3% cellulose of the basal diet replaced by 1% freeze-dried *R. productus* and 3% DFA III, respectively. All test diets were prepared according to the AIN-93G formulation.<sup>34)</sup> All rats were fed the assigned test diets and tap water for 2 weeks. Body weight and food intake were measured every day. At the end of the experiment, the rats were killed under sodium pentobarbital anesthesia (70 mg/kg body weight; Nembutal, Abbott Laboratories, North Chicago, IL, USA) and their cecum were obtained. Fecal samples of rats were collected on day 14 (pooled from day 13 to day 14).

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

**Enumeration of intestinal bacteria.** The portion of cecal contents for counting of intestinal bacteria were quickly added into test tubes containing a diluted solution for anaerobic bacteria (4.5 g of  $\text{KH}_2\text{PO}_4$ , 6.0 g of  $\text{Na}_2\text{HPO}_4$ , 0.5 g of Tween 80, 0.5 g of L-cysteine hydrochloride, and 1.0 g of agar per liter).<sup>35</sup> They were diluted with sterilized physiological saline in an anaerobic chamber and inoculated onto plates of modified GAM medium containing 1% DFA III (for DFA III-assimilating bacteria)<sup>27</sup> anaerobically, and inoculated onto the plates of modified TOS medium (for bifidobacteria),<sup>36</sup> modified LBS medium (for lactobacilli),<sup>37</sup> and BL medium (for culturable anaerobic bacteria)<sup>37</sup> aerobically. Colony counts were made after cultivation at 37 °C for 2 d (DFA III-assimilating bacteria) and 3 d (other bacteria) in an anaerobic chamber and colony forming units (CFU) per gram of wet cecal contents were calculated. Modified TOS medium was the modified CPLX medium described by Yuki *et al.*<sup>36</sup> Briefly, TOS propionate agar (Eiken Chemical, Tokyo, Japan) was added with 9 g of yeast extract, 20 g of D(+)-xylose (Wako Pure Chemical, Osaka, Japan), 3 g of lithium chloride, 780 µg of cephalothin sodium salt (Sigma-Aldrich Chemicals, St. Louis, MO, USA), and 25 mg of phosphomycin disodium salt (Sigma-Aldrich Chemicals) per liter. The remaining diluted suspensions of cecal contents were added with the same volume of 40% glycerol solution in an anaerobic chamber and stored in -80 °C.

**Genetic identification of administered *R. productus*.** Colonies with yellow zones on the agar plate selective for DFA III-assimilating bacteria were picked up with a sterilized wooden toothpick, put into 50 µl TE (pH 8.0), and heated at 97 °C for 10 min using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The RAPD-reaction mixture (25 µl) contained 2.5 µl of dNTP mixture (2.5 mM each), 1 × PCR reaction buffer, 1.75 µl of  $\text{MgCl}_2$  solution (25 mM), 0.375 µl of AmpliTaq Gold (Applied Biosystems), 100 pmol amounts of random primer, and 2 µl of the heated colony suspension as template. OPN-6 (5' gag acg cac a 3') or OPN-12 (5' cac aga cac c 3') (Operon Technologies, Huntsville, AL, USA) were used as random primers. RAPD-PCR conditions were as follows: pre-denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 2 min at 34 °C, and extension for 2 min at 72 °C. A final extension of 7 min at 72 °C was added. Amplification was analyzed by electrophoresis on a 1.5% agarose gel. The strain of the same profiles as positive control was examined for the full sequences of 16S rDNA and

was identified as *R. productus* AHU1760 of human origin.

**Analyses of pH and organic acids in cecal contents.** The remaining cecal contents were immediately frozen with liquid nitrogen and stored at -40 °C for subsequent analyses. The cecal contents were diluted with 5 volumes of deionized water and homogenized using a Teflon homogenizer. The pH of these homogenates was measured with a Shidengen ISFET pH meter KS-701 (Shidengen Electric, Tokyo, Japan) to determine the pH of the cecal contents. After sample preparation by the procedure described previously,<sup>38</sup> pools of organic acids (succinic acid, lactic acid, acetic acid, propionic acid, butyric acid, and varellic acid) in the homogenate of the cecal contents were measured using HPLC (organic analysis system; Jasco, Tokyo, Japan) with two Shodex RSpak KC-811 columns (Showa Denko, Tokyo, Japan), Shodex RSpak KC-G (Showa Denko), and a multi-wavelength detector (MD-1510; Jasco). The mobile phase was 5% acetonitrile in 3 mM  $\text{HClO}_4$  at 1 ml/min, and the column temperature was at 55 °C.

**Analysis of bile acids in feces.** Determination of the composition of bile acids in feces was performed as described in the previous paper<sup>26</sup> with some modifications. The selected ions for different bile acids were: cholic acid,  $m/z$  253, 368; deoxycholic acid,  $m/z$  255, 370; chenodeoxycholic acid,  $m/z$  355, 370; lithocholic acid,  $m/z$  215, 372; hyodeoxycholic acid,  $m/z$  255, 370;  $\alpha$ -muricholic acid,  $m/z$  443, 458;  $\beta$ -muricholic acid,  $m/z$  195, 285; and 23-Nordeoxycholic acid,  $m/z$  255, 356. The total primary bile acids consisted of cholic acid, chenodeoxycholic acid,  $\alpha$ -muricholic acid, and  $\beta$ -muricholic acid. The total secondary bile acid included deoxycholic acid, lithocholic acid, and hyodeoxycholic acid.

**Statistical analysis.** Values were indicated as mean  $\pm$  SEM. Two-way ANOVA following Duncan's multiple range test was used to determine whether body weight gain, food intake, the pH values and pools of organic acids in the cecal contents, concentrations of bile acids in feces, and viable counts of culturable anaerobic bacteria, bifidobacteria and lactobacilli (logarithmic value) were significantly different among the four dietary groups ( $P < 0.05$ ). Student's *t* test was used to determine whether viable counts of DFA III-assimilating bacteria (logarithmic value) were significantly different between the DFA III diet only and the *R. productus* + DFA III diet groups ( $P < 0.05$ ).

## Results

### *Body weight, food intake, and counts of administered *R. productus* and DFA III-assimilating bacteria in rats*

Body weight gain did not differ among the four dietary groups, however, food intake was decreased by

**Table 2.** Feed Intake and Body Weight Gain of Rats during the Test Period and the Number of DFA III-Assimilating Bacteria

Group	Feed intake (g/d)	Body weight gain (g/d)	DFA III assimilating bacteria
Control diet	20.49 ± 0.61 <sup>a</sup>	7.55 ± 0.21	≤6
<i>R. productus</i> diet	20.57 ± 0.46 <sup>a</sup>	7.95 ± 0.28	≤6
DFA III diet	18.68 ± 0.72 <sup>b</sup>	7.19 ± 0.31	9.87 ± 0.09
<i>R. productus</i> + DFA III diet	19.56 ± 0.26 <sup>ab</sup>	7.73 ± 0.16	10.15 ± 0.06
ANOVA <i>P</i> -value			
<i>R. productus</i> (P)	0.3844	0.0662	—
DFA III (D)	0.0148	0.2494	—
P X D	0.4597	0.7805	—

Values of feed intake and body weight gain are mean ± SEM (n = 8). Values of DFA III-assimilating bacteria are mean ± SEM of bacterial counts (log CFU/g wet cecal contents, n = 8).

Values not sharing a superscript letter are significantly different according to Duncan's test (*P* < 0.05).

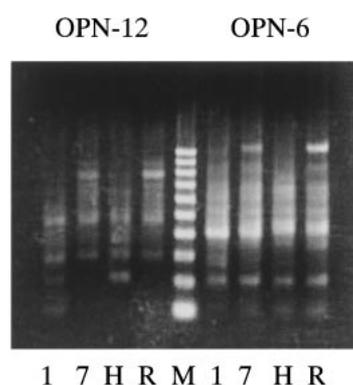
DFA III ingestion (*P* = 0.0148) (Table 2). *R. productus*-fed rats were administered  $4.9 \times 10^7$  CFU/d of *R. productus* AHU1760 of human origin, while *R. productus* + DFA III-fed rats were administered  $4.7 \times 10^7$  CFU/d. The number of DFA III-assimilating bacteria in the cecal contents of *R. productus* + DFA III-fed rats was significantly higher than that of DFA III-fed rats (*P* = 0.020) (Table 2).

*Survival of administered R. productus*

The diluted cecal contents of *R. productus* + DFA III-fed rats were put onto selective medium for DFA III-assimilating bacteria and analyzed by the RAPD method. *R. productus* of rat origin were detected but not much of human origin. The frozen cecal contents of *R. productus*-fed rats and *R. productus* + DFA III-fed rats were then inoculated onto modified 1/4 GAM medium containing 1% DFA III. Because many colonies of *R. productus* were detected, a portion of those was individually picked, isolated and analyzed by RAPD. Most of the colonies examined were *R. productus* of rat origin, while one colony of human origin, designated colony no. 1, was detected in one of the *R. productus*-fed rats. The RAPD profile is shown in Fig. 1. Full sequences of the 16S rDNA of colony no. 1 were the same as that of administered *R. productus* AHU1760 of human origin. Therefore, colony no. 1 was confirmed to be the administered *R. productus* AHU1760 that had reached the gut and survived in the rat cecum.

*Rat cecal characteristics and amounts of organic acids*

With DFA III ingestion, the weights of cecal wall and cecal content increased significantly (*P* < 0.0001) (Table 3) but the pH of cecal contents decreased significantly (*P* < 0.0001) (Fig. 2). The amount of acetic acid and other SCFAs (propionic acid, butyric acid, and varellic acid) in cecal contents increased with DFA III ingestion (*P* < 0.0001) (Fig. 2). The weights of cecal wall and cecal contents, and the amount of propionic acid tended to decrease more on the *R. productus* + DFA III diet than on the DFA III only diet.



**Fig. 1.** RAPD Profiles of DNA of *R. productus*.

RAPD profiles from DNA of representative *Ruminococcus productus* of human origin (H) and rat origin (R) compared to colonies 7 and 1, obtained with primers OPN-12 and 6. Lane M, 100-bp DNA ladder (Bio Rad, Hercules, CA, USA).

**Table 3.** Effects of DFA III and/or *R. productus* on Rat Cecal Wall and Contents

Group	Cecal wall (g)	Cecal contents (g)
Control diet	0.96 ± 0.04 <sup>a</sup>	2.63 ± 0.20 <sup>a</sup>
<i>R. productus</i> diet	0.94 ± 0.07 <sup>a</sup>	2.97 ± 0.23 <sup>ac</sup>
DFA III diet	1.30 ± 0.07 <sup>b</sup>	4.56 ± 0.19 <sup>b</sup>
<i>R. productus</i> + DFA III diet	1.12 ± 0.03 <sup>c</sup>	3.53 ± 0.23 <sup>c</sup>
ANOVA <i>P</i> -value		
<i>R. productus</i> (P)	0.0790	0.1285
DFA III (D)	<0.0001	<0.0001
P X D	0.1892	0.0038

Values are mean ± SEM (n = 8). Values not sharing a superscript letter are significantly different according to Duncan's test (*P* < 0.05).

DFA III ingestion increased the amount of succinic acid in cecal contents (*P* < 0.01), and the combination of *R. productus* and DFA III ingestion tended to decrease to almost half of the amount in DFA III-fed rats ( $3.83 \pm 0.73$  μmol/cecal content in control-fed rats,  $2.10 \pm 0.22$  μmol/cecal content in *R. productus*-fed

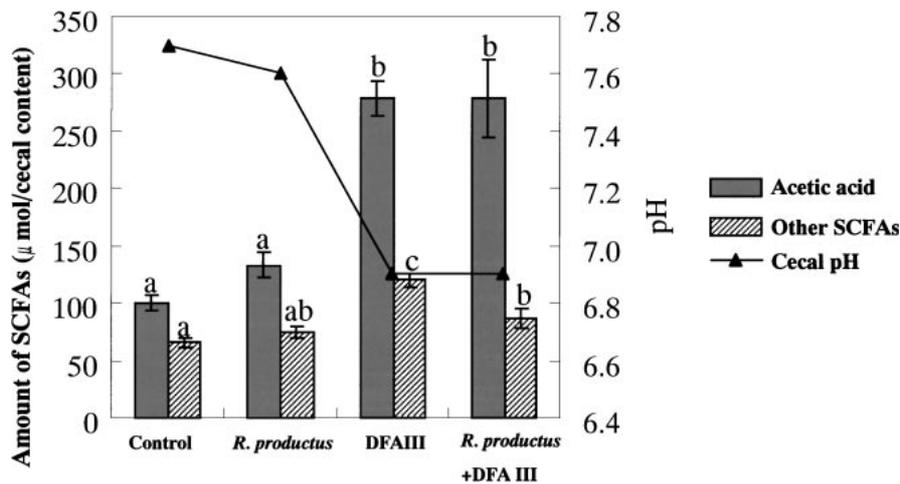


Fig. 2. Cecal pH and the Amount of SCFAs in Cecal Contents.

Each value represents the mean  $\pm$  SEM ( $n = 8$ ).  $P$ -values estimated by two-way ANOVA were 0.7932 (cecal pH), 0.4242 (acetic acid), and 0.0498 (other SCFAs) for the *R. productus* diet; less than 0.0001 (cecal pH, acetic acid and other SCFAs) for the DFA III diet; and 0.6625 (cecal pH), 0.4187 (acetic acid), and 0.0012 (other SCFAs) for the *R. productus* + DFA III diet. The column with dissimilar superscript letter differs significantly ( $P < 0.05$ ) by Duncan's multiple range test. SCFAs, short chain fatty acids; other SCFAs: propionic acid, butyric acid and varellic acid.

Table 4. Effects of DFA III and/or *R. productus* on Cecal Bacteria

Group	Total culturable anaerobes
Control diet	9.78 $\pm$ 0.14 <sup>a</sup>
<i>R. productus</i> diet	9.52 $\pm$ 0.06 <sup>b</sup>
DFA III diet	10.16 $\pm$ 0.10 <sup>c</sup>
<i>R. productus</i> + DFA III diet	10.14 $\pm$ 0.09 <sup>c</sup>
ANOVA $P$ -value	
<i>R. productus</i> (P)	0.1675
DFA III (D)	<0.001
P X D	0.2226

Values are mean  $\pm$  SEM of bacterial counts (log CFU/g wet contents,  $n = 8$ ).

Values not sharing a superscript letter are significantly different according to Duncan's test ( $P < 0.05$ ).

rats, 126.43  $\pm$  41.28  $\mu$ mol/cecal content in DFA III-fed rats, and 52.63  $\pm$  26.66  $\mu$ mol/cecal content in *R. productus* + DFA III-fed rats). The amount of lactic acid was not affected by either of DFA III or *R. productus* ingestion (data not shown).

#### Changes in intestinal microbiota

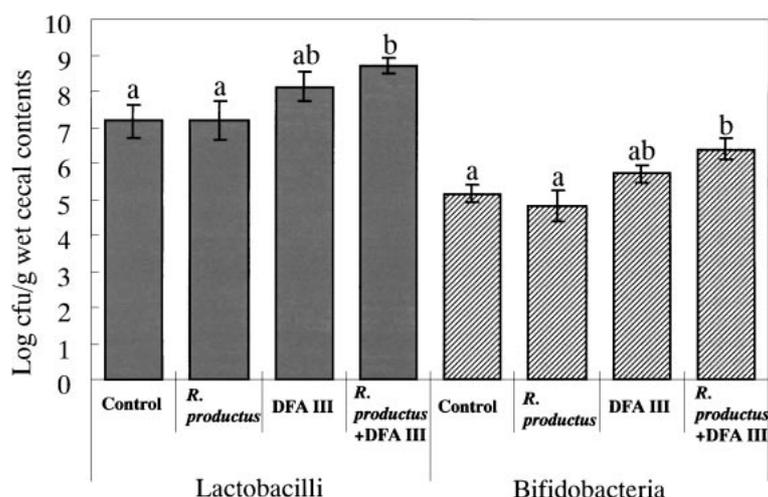
The viable count of culturable anaerobic bacteria increased significantly with DFA III ingestion ( $P < 0.001$ ) (Table 4). The viable count of lactobacilli and bifidobacteria also increased significantly ( $P = 0.0070$ ,  $P = 0.0022$ ) (Fig. 3). Feeding of *R. productus* did not affect the counts. Compared to the control and *R. productus*-fed rats, the combination of *R. productus* and DFA III increased the viable count of lactobacilli and bifidobacteria, which are known to be beneficial bacteria.

#### Changes in composition of bile acids in feces

As shown in Table 5, the concentration of primary bile acids in the fecal contents increased significantly ( $P < 0.0001$ ) and that of secondary bile acids decreased significantly ( $P = 0.0015$ ) with DFA III ingestion. The sum of primary bile acids and secondary bile acids (total bile acids) did not differ significantly. The ratio of secondary bile acids in the total bile acids was 0.94 in the control-fed rats and 0.93 in the *R. productus*-fed rats. In contrast, it was 0.51 in the DFA III-fed rats and 0.60 in the *R. productus* + DFA III-fed rats. These results show that the ratio of secondary bile acids in total bile acids decreased with DFA III ingestion. Moreover, the ratio of secondary bile acids in total bile acids correlated with cecal pH (correlation coefficient  $R = 0.822$ ,  $P < 0.01$ ). When cecal pH decreased, the ratio of secondary bile acids in total bile acids decreased.

## Discussion

*R. productus* AHU1760 of human origin survived slightly through the gastrointestinal tract *in vivo*. The reasons this strain was not well detected by RAPD were: anaerobic bacteria *R. productus* is weak in the presence of oxygen and gastric acid, the viable count of administered freeze-dried *R. productus* AHU1760 was small in amount, and many indigenous *R. productus* already exist in the rat intestine. *R. productus* has the potential to be a probiotic and synbiotic strain, as shown by our results, however, it is necessary to devise a way to protect *R. productus* from oxygen and gastric acid in the next feeding trial. In addition, the number of DFA III-assimilating bacteria in *R. productus* + DFA III-fed rats showed significant difference from DFA III-fed rats. The reason might be an increase in indigenous



**Fig. 3.** Viable Count of Lactobacilli and Bifidobacteria in Cecal Contents.

Values are mean ± SEM of bacterial counts (log CFU/g wet contents, n = 8). *P*-values estimated by two-way ANOVA were 0.4995 (lactobacilli) and 0.5893 (bifidobacteria) for the *R. productus* diet; 0.0070 (lactobacilli) and 0.022 (bifidobacteria) for the DFA III diet; and 0.4897 (lactobacilli) and 0.1115 (bifidobacteria) for the *R. productus* + DFA III diet. The column with dissimilar superscript letter differs significantly (*P* < 0.05) by Duncan's multiple range test.

**Table 5.** Effects of DFA III and/or *R. productus* on Fecal Bile Acids (µmol/g dry feces)

Group	PBA	SBA	Total	SBA/Total
Control diet	0.24 ± 0.05 <sup>a</sup>	3.92 ± 0.35 <sup>a</sup>	4.17 ± 0.35	0.94 ± 0.01 <sup>a</sup>
<i>R. productus</i> diet	0.29 ± 0.10 <sup>a</sup>	3.57 ± 0.52 <sup>a</sup>	3.86 ± 0.62	0.93 ± 0.01 <sup>a</sup>
DFA III diet	2.25 ± 0.63 <sup>b</sup>	2.26 ± 0.51 <sup>b</sup>	4.51 ± 0.62	0.51 ± 0.08 <sup>b</sup>
<i>R. productus</i> + DFA III diet	1.47 ± 0.23 <sup>b</sup>	2.23 ± 0.27 <sup>b</sup>	3.70 ± 0.30	0.60 ± 0.05 <sup>b</sup>
ANOVA <i>P</i> -value				
<i>R. productus</i> (P)	0.2912	0.6570	0.8513	0.3863
DFA III (D)	<0.0001	0.0015	0.2614	<0.0001
P X D	0.2346	0.7023	0.6111	0.3129

PBA (primary bile acid): cholic acid, α-muricholic acid, β-muricholic acid, and chenodeoxycholic acid.

SBA (secondary bile acid): deoxycholic acid, lithocholic acid, and hyodeoxycholic acid.

Values are mean ± SEM (n = 8).

Total: the sum of PBA and SBA.

Values not sharing a superscript letter are significantly different according to Duncan's test (*P* < 0.05).

*R. productus* or DFA III-assimilating bacteria.

The weights of cecal wall and cecal contents, and the pool of SCFAs in cecal contents of DFA III-fed rats and *R. productus* + DFA III-fed rats increased but the pH of cecal contents in both groups decreased. This shows that DFA III was fermented by intestinal bacteria in the rat cecum. However, the effects can be attributed only to DFA III, they were not due to *R. productus* ingestion. Also, the decrease in concentration of secondary bile acids in feces was similarly attributed to DFA III but not to *R. productus* ingestion. In the *R. productus* + DFA III-fed rats, the ratio of secondary bile acids decreased with acidification of cecal contents, as observed also in the DFA III-fed rats. However, the fact that the almost half of the total bile acids consisted of primary bile acids was different from the previous study, wherein they accounted for the majority of the total bile acids (94%) in the DFA III-fed rats.<sup>26)</sup> This

was perhaps due to the fact that the pH of cecal contents were higher than pH 6.5 which inhibited the activity of 7α-dehydroxylation in the present study. The cecal contents in the previous study were at pH 5.8, while those of present study were at pH 6.9.

The viable count of culturable anaerobic bacteria, lactobacilli, and bifidobacteria increased significantly with DFA III ingestion. Those of lactobacilli and bifidobacteria increased even more with *R. productus* ingestion. This agrees with reports that although *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in fermented milk are not able to survive through the gastrointestinal tract, ingestion of them increases the number of bifidobacteria.<sup>39-42)</sup> The effects of fermented milk are attributed not only to live bacteria but to compounds of the bacterial cells and fermentation.<sup>43)</sup> When *Lactobacillus* GG yoghurt that survived through the gastrointestinal

tract was administered to healthy volunteers continuously for 90 g/d for 10 d and then 300 g/d for another 10 d, the numbers of lactobacilli and bifidobacteria increased significantly, whereas the number of lecithinase-negative clostridia decreased significantly. The decrease in clostridia might be one of the causes of increases in bifidobacteria.<sup>44)</sup> Recently, it was reported that the mucus-binding ability of *Bifidobacterium lactis* Bb-12 is enhanced in the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus* GG.<sup>45)</sup> The reason there was an increase in the number of bifidobacteria in our study is still being investigated.

In conclusion, when rats were fed *R. productus* ( $4.7 \times 10^7$  CFU/d) + DFA III diets for 2 weeks, *R. productus* produced acetic acid from DFA III and lowered cecal pH. As a result, the ratio of secondary bile acids as a promoter of colon cancer decreased the same as with ingestion of DFA III only. Moreover, when *R. productus* and DFA III were taken together, the balance of intestinal microbiota might be improved by increasing the numbers of lactobacilli and bifidobacteria which are known to be beneficial bacteria.

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