

Biohydrogenation of C₂₀ polyunsaturated fatty acids by anaerobic bacteria

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Running foot line: Discovery of novel C₂₀ polyunsaturated fatty acids

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1

2 **Abbreviations**

3 Arachidonic acid, AA; Docosahexaenoic acid, DHA; Eicosapentaenoic acid, EPA; Fast
4 atom bombardment, FAB; ^1H clean-total correlation spectroscopy, TOCSY; ^1H - ^1H -chemical shift
5 correlation spectroscopy, COSY; High-performance liquid chromatography, HPLC; Gas-liquid
6 chromatography, GC; Linoleic acid, LA; Mass spectroscopy, MS; Non-methylene-interrupted
7 fatty acids, NMIFA; Polyunsaturated fatty acids, PUFAs; Proton nuclear magnetic resonance,
8 ^1H -NMR; Vaccenic acid, VA; Two-dimensional nuclear Overhauser effect spectroscopy, NOESY

9

Abstract

The polyunsaturated fatty acids (PUFAs) include many bioactive lipids. The microbial metabolism of C₁₈ PUFAs is known to produce their bioactive isomers, such as conjugated fatty acids and hydroxy fatty acids, but there is little information on that of C₂₀ PUFAs. In this study, we aimed to obtain anaerobic bacteria for the ability to produce novel PUFA from C₂₀ PUFAs. Through the screening of about 100 strains of anaerobic bacteria, *Clostridium bifermentans* JCM 1386 was selected as a strain with the ability to saturate PUFAs during anaerobic cultivation. This strain converted arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid) and eicosapentaenoic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid) into *cis*-5,*cis*-8,*trans*-13-eicosatrienoic acid and *cis*-5,*cis*-8,*trans*-13,*cis*-17-eicosatetraenoic acid, giving yields of 57% and 67% against the added PUFAs, respectively. This is the first report of the isolation of a bacterium transforming C₂₀ PUFAs into corresponding non-methylene-interrupted fatty acids. We further investigated the substrate specificity of the biohydrogenation by this strain and revealed that it can convert two *cis* double bonds at the ω6 and ω9 positions in various C₁₈ and C₂₀ PUFAs into a *trans* double bond at the ω7 position. This study should serve to open up the development of novel potentially bioactive PUFAs.

Supplementary key words: arachidonic acid, fatty acid/metabolism, omega-3 fatty acids, lipids/chemistry, diet and dietary lipids, anaerobic bacteria, eicosapentaenoic acid, conjugated fatty acid, non-methylene-interrupted fatty acids (NMIFA)

1 INTRODUCTION

2 The polyunsaturated fatty acids (PUFAs) include many bioactive lipids that play an
3 important role in the maintenance of biological functions in mammals (1, 2). The vast majority of
4 PUFAs has 2 or more *cis* double bonds that are separated from each other by a single methylene
5 group (known as methylene-interrupted fatty acids). They include two major subgroups (the ω 3
6 and ω 6 PUFAs) that have different functions (1-3). Arachidonic acid
7 [*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4, ω 6), AA], which is the C₂₀ PUFA of the ω 6
8 class and is made from linoleic acid [*cis*-9,*cis*-12-octadecadienoic acid (18:2, ω 6), LA], is
9 involved in many cellular signaling mechanisms, and is also the precursor for the formation of
10 2-series of prostaglandins. On the other hand, eicosapentaenoic acid
11 [*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid (20:5, ω 3), EPA], which is a C₂₀ PUFAs of
12 the ω 3 class and is made from α -linolenic acid [*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3,
13 ω 3)], is the precursor for the formation of 3-series of prostaglandins, and can competes with the
14 effects of AA, such as the AA conversion to the prostaglandins. Unlike methylene-interrupted
15 fatty acids, rare isomers of PUFAs, which have at least two double bonds that are separated by a
16 single carbon-carbon bond (known as conjugated fatty acids) (4-7) or 2 or more methylene
17 groups [known as non-methylene-interrupted fatty acids (NMIFA)] (8-10), have been found in
18 several materials including plant oil. These rare PUFAs have been also reported to show
19 interesting physiological effects (9, 11-15). Therefore, they have gained considerable attention,
20 but natural sources rich in them are limited.

21 The partial hydrogenation of PUFAs is the process of converting PUFAs into the more
22 saturated fatty acids and can produce NMIFAs from more readily available PUFAs. They can be
23 mainly performed by chemical hydrogenation in industry and by microbial biohydrogenation in

1 living organisms (16). Chemical partial hydrogenation is widely used to convert vegetable oils
2 into foods such as margarine. The partial hydrogenation of vegetable oils produces various
3 hydrogenated vegetable oils, including several isomers of octadecenoic acid (18:1), depending on
4 the reaction conditions. In contrast, microbial biohydrogenation can selectively produce specific
5 isomers (4-7). Thus, microbial biohydrogenation has several advantages over chemical
6 hydrogenation.

7 Recently, some studies, including ours, have found that many anaerobic bacteria, such as
8 *Lactobacillus* species, can produce conjugated linoleic acids from LA (4, 17-20). Further, we
9 have revealed that lactic acid bacteria produce unique PUFAs from various C₁₈ PUFAs through
10 partial biohydrogenation (21-23). Thus, the biohydrogenation of C₁₈ PUFAs has been widely
11 studied. However, as far as we know, the biohydrogenation of other fatty acids, especially C₂₀
12 PUFAs, has not been extensively studied so far.

13 In this paper, we report about the screening of anaerobic bacteria for the ability to transform
14 C₂₀ PUFAs through biohydrogenation. We found that *Clostridium bifermentans* JCM 1386 can
15 specifically convert AA and EPA into their partially saturated fatty acids with a *trans* double
16 bond at the ω7 position. We further found that other C₁₈ and C₂₀ PUFAs were also converted in a
17 similar manner. Thus, we succeeded in the production of various C₁₈ and C₂₀ NMIFAs with a
18 *trans* double bond at the ω7 position through the biohydrogenation by *C. bifermentans* JCM 1386,
19 leading to the development of novel potentially bioactive PUFAs.

21 MATERIALS AND METHODS

22 Chemicals

23 LA and α-linolenic acid were purchased from Wako Pure Chemical (Osaka, Japan).

1 γ -Linolenic acid (*cis*-6,*cis*-9,*cis*-12-18:3), dihomogamma-linolenic acid
2 [*cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid (20:3)], AA, and EPA were purchased from Sigma (St.
3 Louis, USA). Docosahexaenoic acid [*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid
4 (22:6), DHA] was purchased from Cayman Chemical (MI, USA). All other chemicals used were
5 of analytical grade and are commercially available.

6 **Microorganism and cultivation**

7 The identified anaerobic bacteria used for this study (Supplementary Table S1) were
8 preserved in our laboratory (AKU Culture Collection, Division of Applied Life Science, Faculty
9 of Agriculture, Kyoto University, Kyoto, Japan) and those obtained from other culture collections
10 (JCM, Japan Collection of Microorganisms, Saitama, Japan; and ATCC, American Type Culture
11 Collection, VA, USA). The unidentified anaerobic bacteria used for this study were isolated from
12 pond, wastewater, fish viscera, and so on. The medium was GAM broth (pH 7.0) (Nissui
13 Pharmaceutical co., Ltd., Tokyo, Japan) supplemented with 0.03% (w/v) LA, AA or 0.02% (w/v)
14 EPA. Each strain was inoculated into 15 mL of the medium in screw-capped tubes (16.5 × 215
15 mm) and then incubated in an anaerobic chamber (98% nitrogen and 2% hydrogen) at 37°C for
16 2-3 days. After the cultivation, the culture medium was separated into supernatant and cells by
17 centrifugation (8,000 *g*, 10 min), and the supernatant was used for lipid analysis.

18 **Lipid analysis**

19 Lipids were extracted from the supernatants with chloroform-methanol according to the
20 procedure of Bligh-Dyer (24), and methylated with 4% methanolic-HCl at 50°C for 20 min. The
21 resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid
22 chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped
23 with a flame ionization detector and a split injection system and fitted with a capillary column

(ULBON HR-SS-10, 50 m × 0.25 mm I.D., Shinwa Kako, Kyoto, Japan). The column temperature was initially 180°C and was raised to 220°C at a rate of 2°C/min and maintained at that temperature for 20 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at 0.97 ml/min.

Isolation, derivatization, and identification of products

For the isolation of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with 0.03% (w/v) AA (UK1), its methyl esters were purified by high-performance liquid chromatography (HPLC, monitored at 205 and 233 nm) using a Shimadzu LC-VP system fitted with a Cosmosil column 5C18-ARII (20 × 250 mm, Nacalai tesque, Kyoto, Japan). The mobile phase was acetonitrile-water (80:20, v/v) at a flow rate of 5.0 mL/min and the column temperature of 30°C. The fraction containing UK1 was further purified by HPLC on Inertsil ODS SQ5-1385 (4.6 × 250 mm, GL Science Inc., CA, USA) joined with Capcelpak C18 UG20 (4.6 × 250 mm, Shiseido, Tokyo, Japan). Acetonitrile-water (80:20, v/v) was used as the mobile phase at a flow rate of 1.2 mL/min. For the isolation of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with 0.02% (w/v) EPA (UK2), its methyl esters were purified using a same procedure as described for UK1 except that the mobile phase used for the latter step was acetonitrile-water (80:20, v/v) at a flow rate of 1.0 mL/min.

The chemical structures of purified fatty acids were determined by mass spectroscopy (MS), proton nuclear magnetic resonance (¹H-NMR), ¹H-¹H-chemical shift correlation spectroscopy (COSY), two-dimensional nuclear Overhauser effect spectroscopy (NOESY), and ¹H clean-total correlation spectroscopy (TOCSY).

¹H-NMR, ¹H-¹H COSY, NOESY and TOCSY analyses

All NMR experiments were performed on a BrukerBiospin DX-750 (750 MHz for ¹H)

and chemical shifts were assigned relative to the solvent signal (dichloromethane-d₂).

Preparation of pyrrolidide fatty acids

Pyrrolidide derivatives were prepared by direct treatment of the isolated methyl esters with pyrrolidine-acetic acid (10:1, v/v) in screw-cap tubes for 1 h at 115°C followed by extraction according to the method of Andersson and Holman (25). The organic extract was washed with water and dried over anhydrous Na₂SO₄, and then the solvent was removed by a vacuum in a rotary evaporator.

GC-MS analysis

GC-MS QP5050 (Shimadzu) with a GC-17A gas chromatograph was used for mass spectral analysis. The GC separation of the methyl ester and the pyrrolidide derivatives was performed on an ULBON HR-1 column (25 m × 0.5 mm, Shinwa Kako) at 300°C. MS was used in the electron impact mode at 70 eV with a source temperature of 250°C. Split injection was employed with the injector port at 250°C.

MS-MS analysis

MS-MS analyses were performed on the free acids of the fatty acids with a JEOL-HX110A/HX110A tandem mass spectrometer. The ionization method was fast atom bombardment (FAB), and the acceleration voltage was 3 kV. Glycerol was used for the matrix.

RESULTS

Screening of anaerobic bacteria that have the ability to convert C₂₀ PUFAs

The ability of anaerobic bacteria to convert the C₂₀ PUFAs of EPA and AA during cultivation was investigated together with LA as a reference of C₁₈ PUFA. We tested about 100 strains, including the identified bacteria, which belonged to genera such as *Megasphaera*,

1 *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Clostridium*, *Bacteroides*, *Eubacterium*, and
2 so on (Supplementary Table S1), and the unidentified bacteria. The peaks of the PUFAs were
3 identified by comparison with the retention time of the reference standards on GC analysis.

4 Of these bacteria, 2 strains of *Clostridium bifermentans* (JCM 1386 and JCM 7832)
5 showed the activity to convert AA and EPA, while 5 strains (including the two C₂₀ PUFAs
6 converting strains mentioned above) belonging to the genera of *Clostridium* and
7 *Propionibacterium* were found to have the ability to convert LA to vaccenic acid (*trans*-11-18:1,
8 VA) (Table 1).

9 Figure 1 shows the GC chromatogram of methylated fatty acids produced by *C.*
10 *bifermentans* JCM 1386 from AA, EPA, and LA as examples. When *C. bifermentans* JCM 1386
11 was cultured with AA or EPA, newly generated fatty acids [UK1 from AA (Fig. 1A) and UK2
12 from EPA (Fig. 1B)] were detected on the GC chromatogram of methylated fatty acids. The same
13 reactions were observed when *C. bifermentans* JCM 7832 was cultured with AA or EPA (Table 1).
14 However, *C. sporogenes* JCM 7849, *C. sporogenes* JCM 7850, and *P. acnes* JCM 6473 couldn't
15 convert AA and EPA.

16 As the concentration of the C₂₀ PUFAs added grew, *C. bifermentans* JCM1386 showed
17 higher activity than *C. bifermentans* JCM 7832 (data not shown). *C. bifermentans* JCM1386 was
18 used for further analyses.

19 **Identification of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386** 20 **with AA**

21 When the lipids extracted from the medium after cultivation of *C. bifermentans* JCM
22 1386 with AA were analyzed by thin-layer chromatography, almost all lipids were present in the
23 free form (data not shown). After complete esterification of the free form fatty acids products, the

1 resulting methyl esters were isolated and used for structural analysis. The mass spectrum of the
2 isolated methyl ester of UK1 exhibited a molecular weight of m/z 320, indicating that UK1 is C₂₀
3 PUFA containing three double bonds. The molecular ion peak ($[M+Na]^+$, 343) obtained by
4 FAB-MS analysis (FAB⁺) of the methyl ester of UK1 was fragmented again by MS-MS [m/z
5 (FAB⁺, 8.00kV), 328(1), 314(2), 300(2), 299(3), 286(3), 285(4), 272(12), 258(1), 257(2), 232(3),
6 218(3), 217(28), 204(33), 190(1), 189(1), 164(35), 163(12), 150(3), 149(4), 124(5), 110(13),
7 109(68), 96(100), 82(6), and 81(40)]. The m/z 124, 150, 164, 190, 232, and 258 were derived
8 from cleavage between single bonds 4-5, 6-7, 7-8, 9-10, 12-13, and 14-15, as numbered from the
9 carboxyl group. The m/z 110, 150, 164, 204, 218, and 272 derived from the cleavage of single
10 bonds between the α and β positions from the double bonds were detected. On the basis of the
11 results of MS analyses, UK1 was identified as the geometrical isomers of 5,8,13-20:3.

12 ¹H-NMR analysis also suggested that UK1 is an isomer of 20:3 (see Fig. 2). The signal
13 intensity of L (5.36 ppm, *m*, 6H) indicates the existence of three double bonds in UK1. The
14 sequence of the protons from the methyl end of the molecule was deduced A, B, E, L, L, J, L, L,
15 G, C, F, L, L, H, D, and I or A, B, E, L, L, F, C, G, L, L, J, L, L, H, D, and I based on the pattern
16 of crosspeaks in ¹H-¹H COSY analysis (see Fig. 2B). The sequence was confirmed as the latter
17 one by the appearance of a crosspeak between J and H, but not J and E in TOCSY analysis (see
18 Fig. 2C). Furthermore, NOESY analysis was carried out to identify the geometric configurations
19 of double bonds. The positive crosspeaks appeared between G and J, and H and J, indicating that
20 the two double bonds of $\Delta 5$ and $\Delta 8$ positions are in the *cis* configuration, whereas no positive
21 crosspeak appeared between E and F, indicating that $\Delta 13$ position is in the *trans* configuration
22 (see Fig. 3A). On the basis of the results of the above spectral analyses, UK1 was identified as
23 *cis*5,*cis*-8,*trans*-13-20:3 (see Fig. 2A).

1 Identification of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 2 with EPA

3 When the lipids extracted from the medium after cultivation of *C. bifermentans* JCM
4 1386 with EPA were analyzed by thin-layer chromatography, almost all lipids were present in the
5 free form (data not shown). After complete esterification of the free form fatty acids products, the
6 resulting methyl esters were isolated and used for structural analysis. The mass spectrum of the
7 isolated methyl ester of UK2 exhibited a molecular weight of m/z 318. This result suggested that
8 UK2 is C₂₀ PUFAs containing four double bonds. The molecular ion peak ($[M+Na]^+$, 341)
9 obtained by FAB-MS analysis (FAB⁺) of the methyl ester of UK2 was fragmented again by
10 MS-MS [m/z (FAB⁺, 8.00kV), 326(4), 312(1), 311(1), 286(2), 272(6), 271(11), 258(1), 257(1),
11 232(2), 218(2), 217(22), 204(18), 190(1), 164(22), 150(2), 149(7), 124(5), 110(10), 109(57),
12 96(100), 82(4), 81(28)]. The m/z 124, 150, 164, 190, 232, 258, 286, and 312 were derived from
13 the cleavage of single bonds 4-5, 6-7, 7-8, 9-10, 12-13, 14-15, 16-17, and 18-19 as numbered
14 from carboxyl group. The m/z 110, 150, 164, 204, 218, 272, and 326 derived from the cleavage of
15 single bonds between the α and β positions from the double bonds were detected. On the basis of
16 the results of MS analyses, UK2 was identified as the geometrical isomers of 5,8,13,17-20:4.
17 ¹H-NMR analysis also suggested that UK2 is an isomer of 20:4 (Fig. 4). The signal intensities of
18 J (5.35 ppm, *m*, 6H) and K (5.42 ppm, *m*, 2H) indicate the existence of four double bonds in UK2.
19 The sequence of the protons from the methyl end of the molecule was deduced A, E, J, J, F, E, K,
20 K, D, B, E, J, J, H, J, J, F, C, and G based on the integration of COSY and TOCSY analyses (see
21 Fig. 4B and C). NOESY spectrum revealed that the positive crosspeaks appeared between F and
22 H, H and E, and F and E, and no positive crosspeak appeared between D and E, indicating that
23 the three double bonds of $\Delta 5$, $\Delta 8$, and $\Delta 17$ position are all in *cis* configuration, and that the

double bond of Δ^{13} position is in the *trans* configuration (see Fig. 3B). On the basis of the results of the above spectral analyses, UK2 was identified as *cis*5,*cis*-8,*trans*-13,*cis*-17-20:4 (see Fig. 4A).

Effects of AA and EPA concentration in the medium on their transformation by *C. bifermentans* JCM 1386

Effects of AA and EPA concentration on their transformation by *C. bifermentans* JCM1386 were investigated (see Fig. 5). When various concentrations of AA were added to the medium, the amount of UK1 production increased with increasing concentration of AA up to 0.42 mg/mL, giving a yield of 57% (0.24 mg/mL) against the added AA (0.42 mg/mL) (see Fig. 5A). When various concentrations of EPA were added to the medium, the amount of UK2 production increased with increasing concentration of EPA up to 0.18 mg/mL, giving a yield of 67% (0.12 mg/mL) against the added EPA (0.18 mg/mL) (see Fig. 5B). However, *C. bifermentans* JCM 1386 no longer produced UK2 when more than 0.24 mg/mL EPA was added.

Substrate specificity of polyunsaturated fatty acid transformation by *C. bifermentans* JCM 1386

To examine the substrate specificity of PUFAs transformation during the cultivation of *C. bifermentans* JCM 1386, free fatty acids of LA, α -linolenic acid, γ -linolenic acid, dihomogamma-linolenic acid, AA, EPA, and DHA were added to the medium (see Fig. 6). *C. bifermentans* JCM 1386 could convert LA, AA, EPA, α -linolenic acid, γ -linolenic acid, and dihomogamma-linolenic acid, but not DHA.

The GC-MS analysis of the products obtained from α -linolenic acid, γ -linolenic acid, and dihomogamma-linolenic acid

The products from α -linolenic acid, γ -linolenic acid, and dihomogamma-linolenic acid were

analyzed by GC-MS (see Fig. 7). The spectrum of pyrrolidide derivative of the product from α -linolenic acid showed a molecular weight of m/z 333 and gaps of 26 amu between m/z 224 and 250, and between m/z 278 and 304, indicating that this is a C₁₈ PUFA with double bonds at the ω 3 and ω 7 positions (11,15-18:2) (see Fig. 7A). The pyrrolidide derivative of the product from γ -linolenic acid showed a molecular weight of m/z 333 and gaps of 26 amu between m/z 154 and 180, and between m/z 222 and 248, indicating that the product is a C₁₈ PUFA with double bonds at the ω 7 and ω 12 positions (6,11-18:2) (Fig. 7B). The pyrrolidide derivative of the product from dihomogamma-linolenic acid showed a molecular weight of m/z 361 and gaps of 26 amu between m/z 182 and 208, and between m/z 250 and 276, indicating that the product is a C₂₀ PUFA with double bonds at the ω 7 and ω 12 positions (8,13-20:2) (Fig. 7C). Thus, *C. bifermentans* JCM 1386 could convert C₁₈ and C₂₀ PUFAs with double bonds at the ω 6 and ω 9 positions into their corresponding NMIFAs by *C. bifermentans* JCM 1386 (see Fig. 8).

DISCUSSION

The studies on PUFAs conversion by anaerobic bacteria have been done with the primary aim to improve the quality of the ruminant products such as milk or meat. In the course of these studies, numerous PUFA-transforming bacteria, such as *Butyrivibrio fibrisolvens* (4), *Lactobacillus plantarum* (20-23), and *Bifidobacterium breve* (19), have been isolated, and their metabolic pathways of C₁₈ PUFAs, such as LA and α -linolenic acid, have been revealed. However, the ability to transform C₂₀ and C₂₂ PUFAs has not been studied in detail, although there have been several reports that EPA and DHA are hydrogenated in the rumen *in vivo* (26) and disappear during incubations *in vitro* with mixed ruminal microorganisms (27, 28).

In this study, we found that *C. bifermentans* JCM 1386 could convert AA and EPA into

1 *cis*-5,*cis*-8,*trans*-13-20:3 and *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4, respectively, which are NMIFAs
2 with a *trans* double bond at the ω 7 position (see Figs. 5 and 8). This is the first report of the
3 isolation of the bacterium transforming C₂₀ PUFAs into corresponding NMIFAs. Considering
4 that similar reactions were observed with LA (see Fig. 1C), this strain can convert two *cis* double
5 bonds at the ω 6 and ω 9 positions in PUFAs into a *trans* double bond at the ω 7 position to
6 generate the *trans* fatty acids regardless of the existence of double bonds at other positions. In
7 addition, similar reactions were also observed of other C₁₈ and C₂₀ free PUFAs (α -linolenic acid,
8 γ -linolenic acid, and dihomogamma-linolenic acid) (see Fig. 6). They might be converted into the
9 corresponding NMIFAs with a *trans* double bond at the ω 7 position. However, *C. bifermentans*
10 JCM 1386 could not convert DHA, indicating that C₂₂ PUFAs might not be a substrate for this
11 strain. Thus, we succeeded in the production of various C₁₈ and C₂₀ NMIFAs with a *trans* double
12 bond at the ω 7 position through the biohydrogenation by *C. bifermentans* JCM 1386.

13 NMIFAs are a class of PUFAs that has received attention because of their unique structure
14 and physiological activity, and they have often been found in plant oils. Pinolenic acid
15 (*cis*-5,*cis*-9,*cis*-12-18:3) and columbinic acid (*trans*-5,*cis*-9,*cis*-12-18:3) are C₁₈ NMIFAs that
16 were found in *Pinus koraiensis* and *Aquilegia hybrida*, respectively (8, 9). They are isomers of
17 γ -linolenic acid and show various effects, such as the reduction of platelet aggregation by
18 prostacyclin production, attenuation of the elevation of blood pressure, LDL-lowering and
19 essential fatty acid activity (9, 11, 12). Podocarpic acid (*cis*-5,*cis*-11,*cis*-14-20:3) is a C₂₀ NMIFA
20 that was found in *Platycladus orientalis* oil (10). It has been reported to show a reduction in the
21 AA concentration in the phosphatidylinositol fraction of rat liver (13), which functions in signal
22 transduction, such as in the phospholipase C-signaling pathway (13, 29). Considering that PUFAs
23 often show an isomer-specific function, novel NMIFAs are expected to show novel interesting

1 physiological effects. Interestingly, several natural plant oils have a high content of PUFAs with
2 a double bond at the ω 7 position (30), and the biohydrogenation of PUFAs often produce PUFAs
3 with a double bond at the ω 7 position, such as VA (6, 7). These observations enable us to
4 consider that a double bond at the ω 7 position may become a key factor for a biological function.
5 In this context, various C₁₈ and C₂₀ NMIFAs obtained in this study could be worthwhile. It is also
6 noted that these NMIFAs were obtained in the high yield (approximately 60%). Therefore, this
7 study could serve to open up the development of novel methods in the preparation of these rare
8 possibly bioactive PUFAs.

9 Lipid metabolism by anaerobic bacteria is an attractive research area from the viewpoint of
10 the role of the gut microbiota in relation to health of the host. Interestingly, obesity induced by a
11 high-fat diet has been suggested as being associated with alterations of gut microbiota
12 composition (31, 32). Dietary fats are metabolized by gut microbiota as well as by the host. It is
13 noted that the biohydrogenation of fatty acids might function as a detoxification mechanism in
14 bacteria, and PUFAs especially are more toxic than saturated fatty acids (18, 33). This suggests
15 that the ability of the biohydrogenation of PUFAs might relate to the survival of gut bacteria
16 when dietary intake of PUFAs is high. In addition, our recent research suggested the possibility
17 that lipid metabolism by gut microbiota affects the health of the host by modifying fatty acid
18 composition (23). Therefore, our evidence-based studies on lipid metabolism by gut bacteria,
19 including *C. bifermentans* (in this study) and *Lactobacillus* (21-23, 34), should serve to maintain
20 and improve the health of the host.

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Figure legends

Fig. 1. GC chromatograms of methyl esters of fatty acids produced by *C. bifermentans* JCM

1386 Cultivations were carried out in GAM broth with 0.03% (w/v) of arachidonic acid (AA) (A), eicosapentaenoic acid (EPA) (B), or linoleic acid (LA) (C) for 3 days. The lipid products were extracted from the supernatant and methylated as described in MATERIALS and METHODS. AA, EPA, and LA are converted to UK1, UK2, and vaccenic acid (VA), respectively.

Fig. 2. ^1H -NMR analysis of UK1 and structure of UK1 identified (A) Structure of methyl ester of UK1. (B) ^1H - ^1H -chemical shift correlation spectroscopic (COSY) spectrum of the methyl ester of UK1. (C) ^1H clean-total correlation spectroscopic (TOCSY) spectrum of the methyl ester of UK1.

Fig. 3. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) spectra of the methyl esters of UK1 (A) and UK2 (B). The negative diagonal peaks are denoted in blue. The positive NOE crosspeaks are denoted in red.

Fig. 4. ^1H -NMR analysis of UK2 and structure of UK2 identified (A) Structure of methyl ester of UK2. (B) ^1H - ^1H -chemical shift correlation spectroscopic (COSY) spectrum of the methyl ester of UK2. (C) ^1H clean-total correlation spectroscopic (TOCSY) spectrum of the methyl ester of UK2.

Fig. 5. Effects of fatty acid concentration for medium on fatty acids transformation by *C. bifermentans* JCM 1386 (A) Arachidonic acid (AA) and (B) eicosapentaenoic acid (EPA).

Cultivations were carried out with different concentrations of AA or EPA.

Fig. 6. Transformation of polyunsaturated fatty acids by *C. bifermentans* JCM 1386

Fig. 7. GC-MS spectra of pyrrolidide derivatives of the products from α -linolenic acid (A), γ -linolenic acid (B) and dihomogamma-linolenic acid (C) The deduced structures are shown above the spectra.

Fig. 8. Pathway of polyunsaturated fatty acid transformation during cultivation of *C. bifermentans* JCM 1386 LA, linoleic acid. VA, vaccenic acid. AA, arachidonic acid. EPA, eicosapentaenoic acid.

Table 1: Screening results for the ability of transforming polyunsaturated fatty acids

Strain	No.	Produced fatty acid (mg/mL culture broth)		
		VA from LA	UK1 from AA	UK2 from EPA
<i>Clostridium bifermentans</i>	JCM 1386	0.06	0.12	0.11
<i>Clostridium bifermentans</i>	JCM 7832	0.05	0.13	0.10
<i>Clostridium sporogenes</i>	JCM 7849	0.11	-	-
<i>Clostridium sporogenes</i>	JCM 7850	0.16	-	-
<i>Propionibacterium acnes</i>	JCM 6473	0.12	-	-

Cultivations were carried out in GAM broth with 0.03% (w/v) of linoleic acid (LA), arachidonic acid (AA) or 0.02% (w/v) of eicosapentaenoic acid (EPA) for 3 days as described in MATERIALS AND METHODS. -, not detected. VA, vaccenic acid.

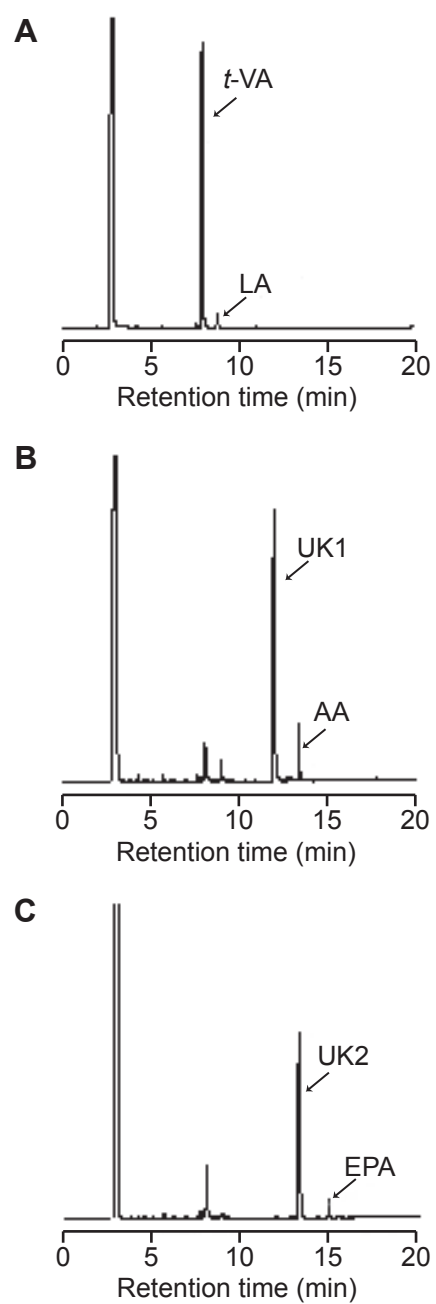


Fig. 1 Sakurama et al.

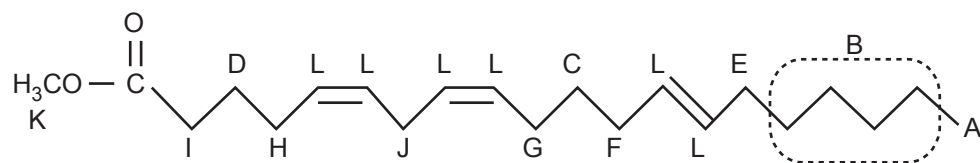
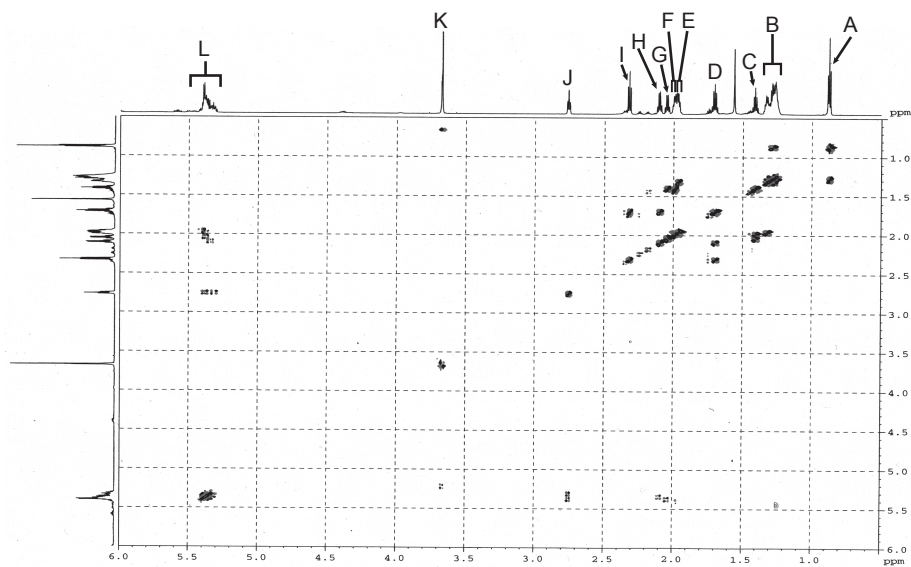
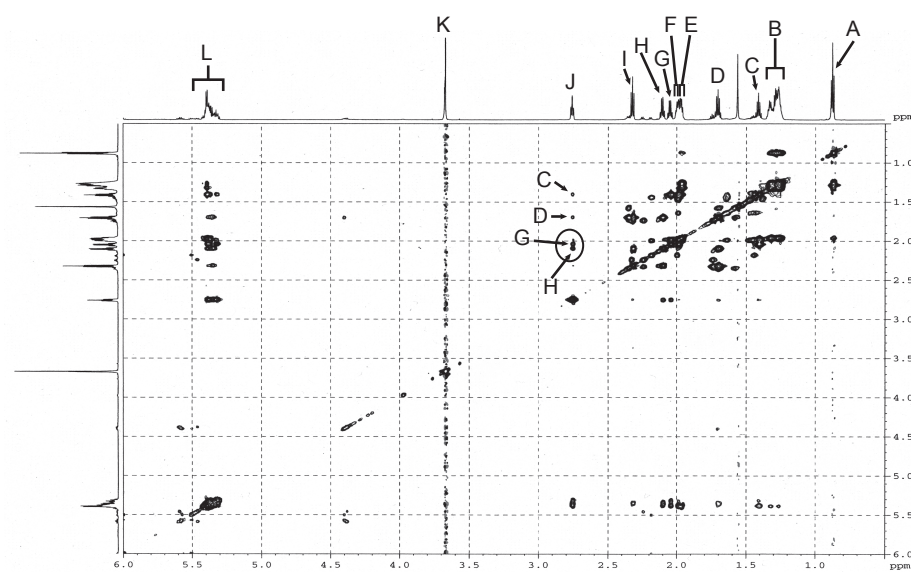
A**B****C**

Fig. 2 Sakurama et al.

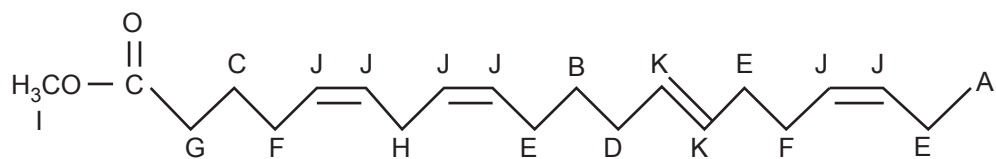
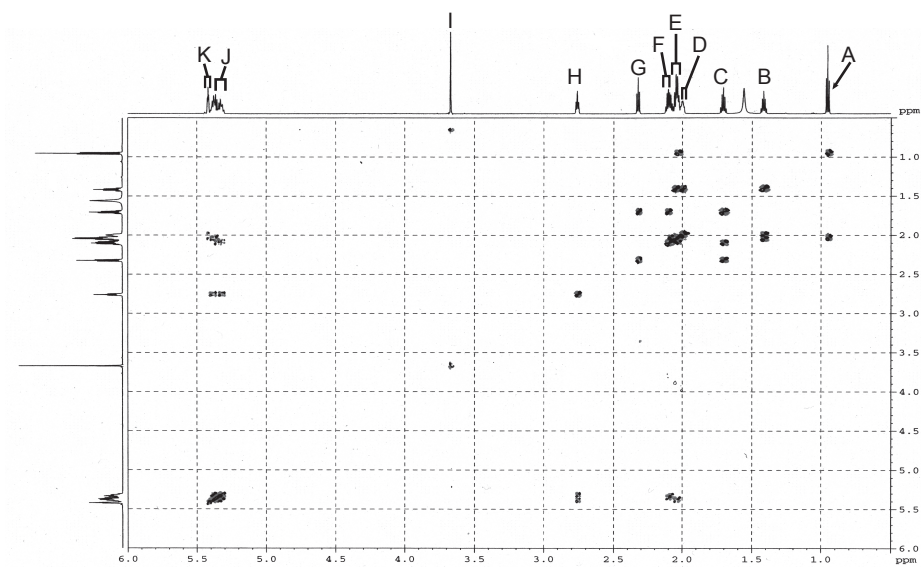
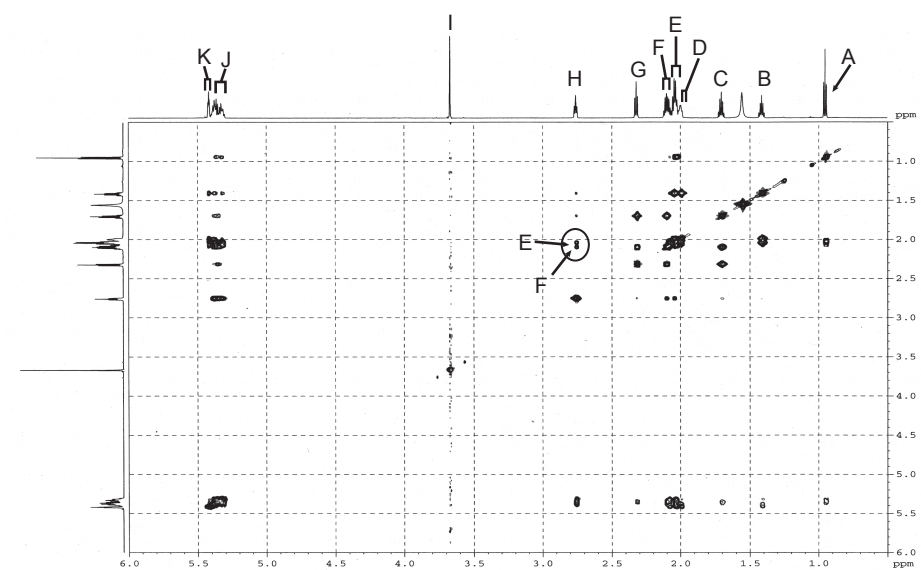
A**B****C**

Fig. 3 Sakurama et al.

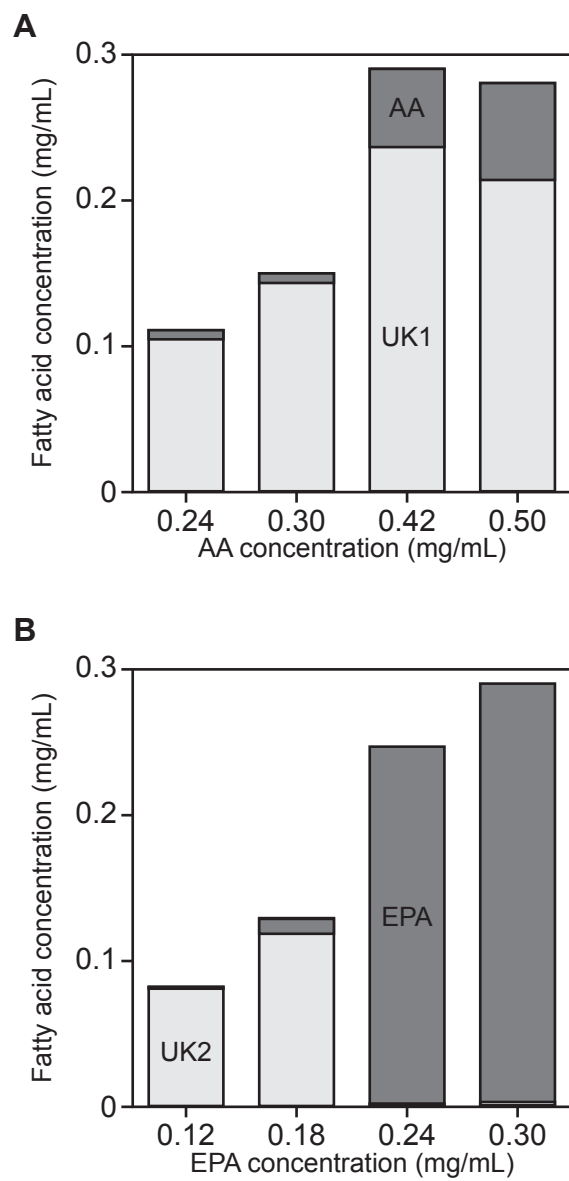


Fig. 4 Sakurama et al.

Form	Substrate	Structure	Transformation
Free	Linoleic acid (LA; 18:2 ω6)		+
	α-Linolenic acid (18:3 ω3)		+
	γ-Linolenic acid (18:3 ω6)		+
	Dihomo-γ-linolenic acid (20:3 ω6)		+
	Arachidonic acid (AA; 20:4 ω6)		+
	Eicosapentaenoic acid (EPA; 20:5 ω3)		+
Methyl ester	Docosahexaenoic acid (DHA; 22:6 ω3)		-
	Linoleic acid methyl ester		+
	Arachidonic acid methyl ester		+

Fig. 5 Sakurama et al.

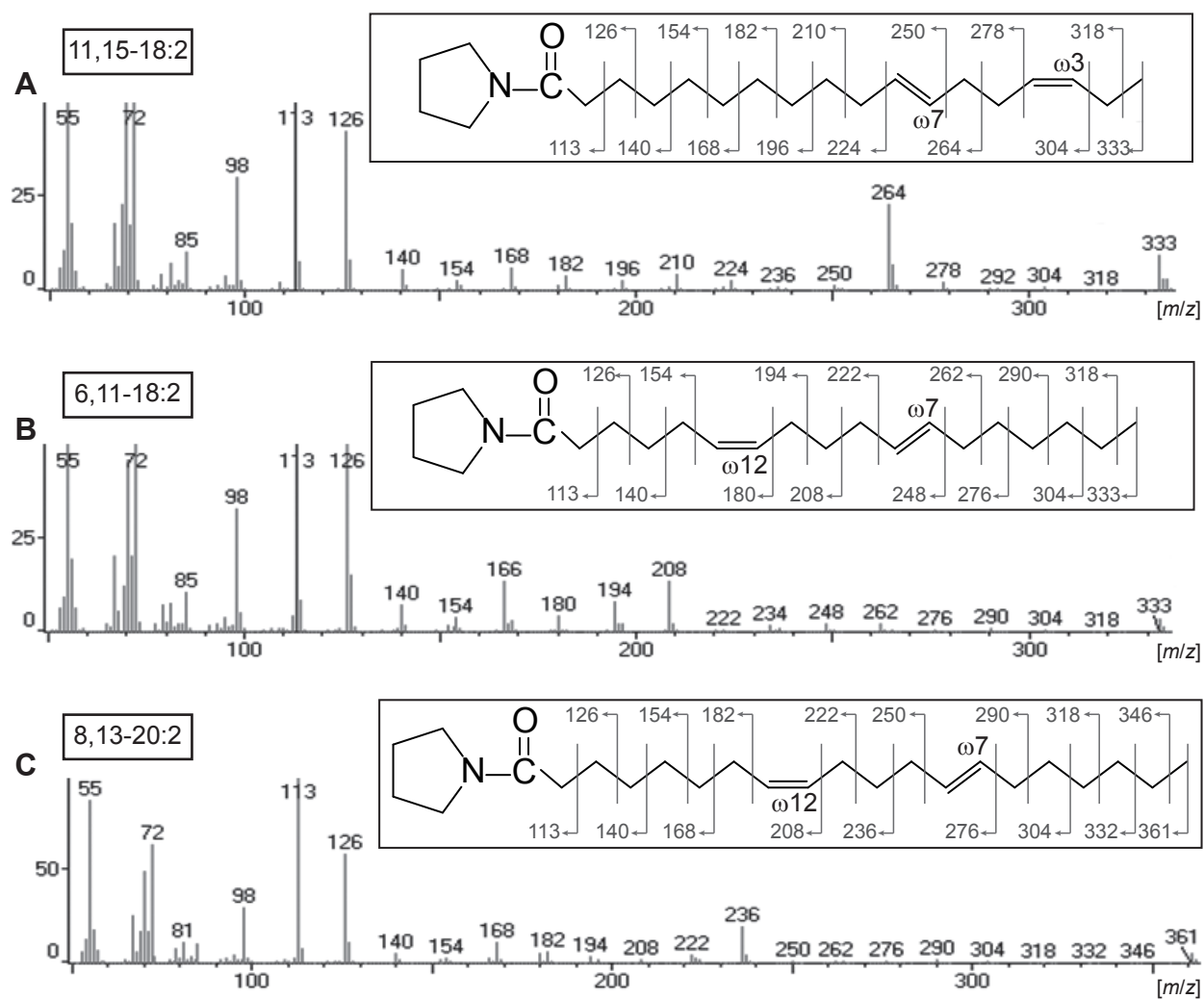
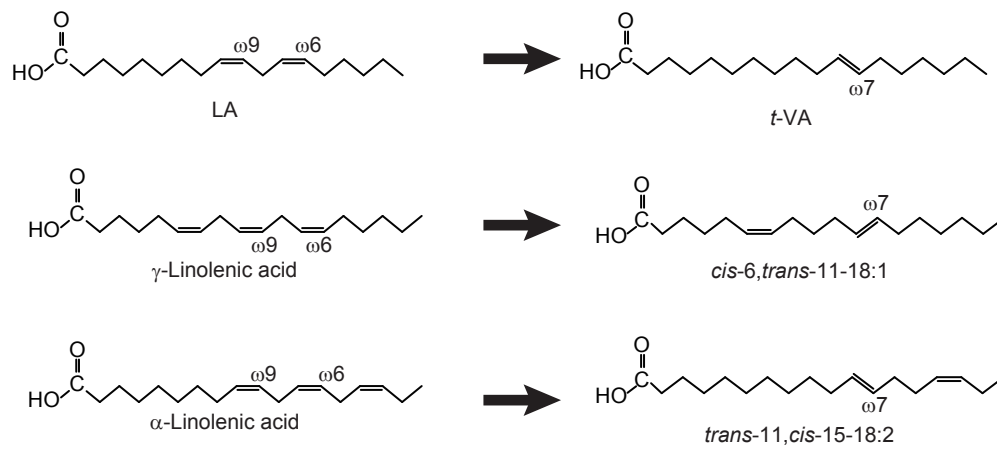


Fig. 6 Sakurama et al.

C₁₈ PUFAs



C₂₀ PUFAs

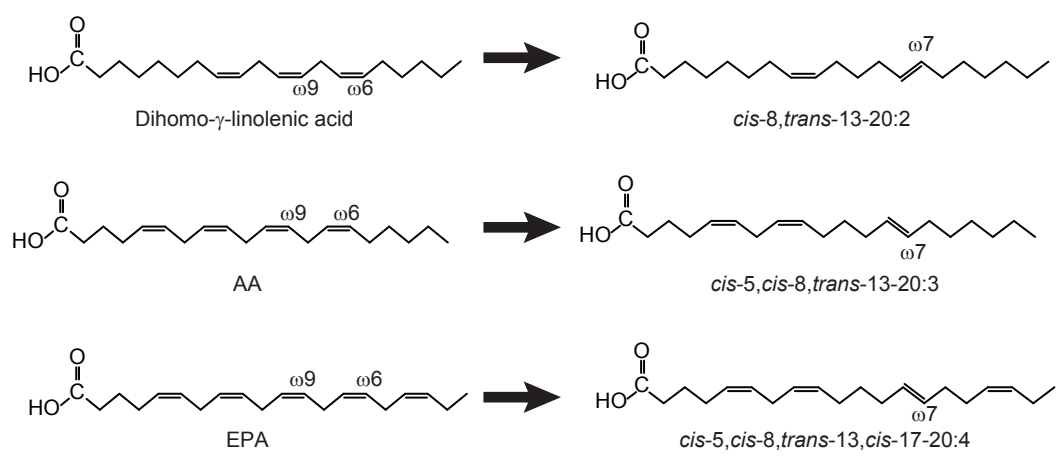


Fig. 7 Sakurama et al.

Supplementary Table S1: List of identified bacterial species used for screening

Acetobacterium	
<i>Acetobacterium wieringae</i>	
Anaerococcus	
<i>Anaerococcus hydrogenalis</i>	<i>Anaerococcus lactolyticus</i>
<i>Anaerococcus prevotii</i>	<i>Anaerococcus tetradius</i>
<i>Anaerococcus vaginalis</i>	
Atopobium	
<i>Atopobium fossor</i>	<i>Atopobium parvulum</i>
Bacteroides	
<i>Bacteroides acidifaciens</i>	<i>Bacteroides caccae</i>
<i>Bacteroides distasonis</i>	<i>Bacteroides fragilis</i>
<i>Bacteroides merdae</i>	<i>Bacteroides ovatus</i>
<i>Bacteroides suis</i>	<i>Bacteroides thetaiotaomicron</i>
Bifidobacterium	
<i>Bifidobacterium adolescentis</i>	<i>Bifidobacterium angulatum</i>
<i>Bifidobacterium animalis</i>	<i>Bifidobacterium asteroides</i>
<i>Bifidobacterium bifidum</i>	<i>Bifidobacterium boum</i>
<i>Bifidobacterium breve</i>	<i>Bifidobacterium catenulatum</i>
<i>Bifidobacterium choerinum</i>	<i>Bifidobacterium coryneforme</i>
<i>Bifidobacterium cuniculi</i>	<i>Bifidobacterium dentium</i>
<i>Bifidobacterium longum</i>	<i>Bifidobacterium merycicum</i>
<i>Bifidobacterium minimum</i>	<i>Bifidobacterium pseudocatenulatum</i>
<i>Bifidobacterium pseudolongum</i>	<i>Bifidobacterium pseudolongum</i> subsp. <i>globosum</i>
<i>Bifidobacterium pseudolongum</i> subsp. <i>pseudolongum</i>	<i>Bifidobacterium pullorum</i>
<i>Bifidobacterium ruminantium</i>	<i>Bifidobacterium subtile</i>
<i>Bifidobacterium thermacidophilum</i>	
Campylobacter	
<i>Campylobacter rectus</i>	
Clostridium	
<i>Clostridium acetobutylicum</i>	<i>Clostridium aminovalericum</i>
<i>Clostridium baratii</i>	<i>Clostridium beijerinckii</i>
<i>Clostridium bifermentans</i>	<i>Clostridium butyricum</i>
<i>Clostridium cadaveris</i>	<i>Clostridium clostridiiforme</i>
<i>Clostridium cochlearium</i>	<i>Clostridium cocleatum</i>
<i>Clostridium difficile</i>	<i>Clostridium ghonii</i>
<i>Clostridium irregularis</i>	<i>Clostridium oceanicum</i>
<i>Clostridium perfringens</i>	<i>Clostridium propionicum</i>
<i>Clostridium ramosum</i>	<i>Clostridium scindens</i>
<i>Clostridium septicum</i>	<i>Clostridium sporogenes</i>
<i>Clostridium symbiosum</i>	<i>Clostridium tyrobutyricum</i>
Collinsella	
<i>Collinsella aerofaciens</i>	<i>Collinsella intestinalis</i>
<i>Collinsella stercoris</i>	
Coprobacillus	
<i>Coprobacillus cateniformis</i>	
Eggerthella	
<i>Eggerthella lenta</i>	

Eubacterium	
<i>Eubacterium barkeri</i>	<i>Eubacterium budayi</i>
<i>Eubacterium callanderi</i>	<i>Eubacterium combesii</i>
<i>Eubacterium cylindroides</i>	<i>Eubacterium fissicatena</i>
<i>Eubacterium hadrum</i>	<i>Eubacterium limosum</i>
<i>Eubacterium moniliforme</i>	<i>Eubacterium multifforme</i>
<i>Eubacterium nitritogenes</i>	<i>Eubacterium nodatum</i>
<i>Eubacterium saburreum</i>	<i>Eubacterium tenue</i>
Fusobacterium	
<i>Fusobacterium necrophorum</i> subsp. <i>funduliforme</i>	<i>Fusobacterium varium</i>
Lactobacillus	
<i>Lactobacillus catenaformis</i>	<i>Lactobacillus crispatus</i>
<i>Lactobacillus hamsteri</i>	<i>Lactobacillus johnsonii</i>
<i>Lactobacillus reuteri</i>	<i>Lactobacillus ruminis</i>
<i>Lactobacillus vitulinus</i>	
Leuconostoc	
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	
Megasphaera	
<i>Megasphaera cerevisiae</i>	<i>Megasphaera elsdenii</i>
Mitsuokella	
<i>Mitsuokella jalaludinii</i>	<i>Mitsuokella multacida</i>
Peptoniphilus	
<i>Peptoniphilus asaccharolyticus</i>	<i>Peptoniphilus lacrimalis</i>
Propionibacterium	
<i>Propionibacterium acidipropionici</i>	<i>Propionibacterium acnes</i>
<i>Propionibacterium arabinosum</i>	<i>Propionibacterium intermedium</i>
<i>Propionibacterium jensenii</i>	<i>Propionibacterium pentosaceum</i>
<i>Propionibacterium peterssonii</i>	<i>Propionibacterium propionicum</i>
<i>Propionibacterium thoenii</i>	<i>Propionimicrobium lymphophilum</i>
Pseudoramibacter	
<i>Pseudoramibacter alactolyticus</i>	
Rarobacter	
<i>Rarobacter faecitabidus</i>	<i>Rarobacter incanus</i>
Rhodospiridium	
<i>Rhodospiridium sphaerocarpum</i>	
Rikenella	
<i>Rikenella microfus</i>	
Ruminococcus	
<i>Ruminococcus productus</i>	
Selenomonas	
<i>Selenomonas artemidis</i>	<i>Selenomonas diae</i>
<i>Selenomonas diana</i>	<i>Selenomonas flueggei</i>
<i>Selenomonas infelix</i>	<i>Selenomonas noxia</i>
<i>Selenomonas ruminantium</i>	<i>Selenomonas sputigena</i>