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Fusobacterium pseudoperiodonticum sp. nov., Isolated from the Human Oral Cavity

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Abstract

In the present study, three strains (ChDC F213^T, ChDC F251, and ChDC F267) were classified as novel species of genus *Fusobacterium* based on average nucleotide identity (ANI) and genome-to-genome distance (GGD) analysis and chemotaxonomic characterization. 16S rDNA sequences of strains ChDC F213^T, ChDC F251, and ChDC F267 were highly similar to that of *F. periodonticum* ATCC 33693^T (99.6, 99.4, and 99.4%, respectively). ANI and GGD values of the three isolates with *F. periodonticum* ATCC 33693^T ranged from 92.5 to 92.6% and 47.7 to 48.2%, respectively. Considering that threshold of ANI and GGD values for bacterial species discrimination are 95–96% and 70%, respectively, these results indicate that the three isolates represent a novel *Fusobacterium* species. DNA G + C contents of the three isolates were 28.0 mol% each. Cellular fatty acid analysis of these strains revealed that $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ ω6c/$C_{16:1}$ ω7c were major fatty acids. Therefore, these three strains are novel species belonging to genus *Fusobacterium*. Strain ChDC F213^T (= KCOM 1259^T = KCTC 5677^T = JCM 33009^T) is the type strain of a novel species of genus *Fusobacterium*, for which a name of *Fusobacterium pseudoperiodonticum* sp. nov. is proposed.

Introduction

Fusobacterium periodonticum is an obligate, anaerobic, non-spore-forming, nonmotile, and Gram-negative rod bacterium isolated from periodontitis lesion [13]. *F. periodonticum* inhabits the oral cavity and gastrointestinal tract of human [14]. Recently, average nucleotide identity (ANI) and genome-to-genome distance (GGD) analyses instead of DNA–DNA hybridization have become new gold standards for classification of bacteria at species level [1, 11]. Three strains—ChDC F213^T, ChDC F251, and ChDC F267—were isolated from human oral cavity and tentatively identified as *F. periodonticum* by 16S rDNA sequence analysis. Strains ChDC F213^T and ChDC F251 were isolated from the tongues of two male subjects (33- and 41-year old, respectively) who had gingivitis. Strain ChDC F267 was isolated from subgingival plaque of gingivitis lesion of left lower first molar of a male (45-year old). Herein, we proposed these three strains as a novel species of the genus *Fusobacterium* based on the polyphasic taxonomic characterization including whole-genome analysis.

Materials and Methods

Bacterial Strains and Culture Conditions

Three strains—ChDC F213^T (= KCOM 1259^T = KCTC 5677^T = JCM 33009^T), ChDC F251 (= KCOM 1261 = KCTC

Soon-Nang Park and Yun Kyong Lim contributed equally to this work.

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5169), and ChDC F267 (= KCOM 1263 = KCTC 5171)—and *F. periodonticum* ATCC 33693^T were obtained from the Korean Collection for Oral Microbiology (KCOM; Gwangju, Korea) or American Type Culture Collection (ATCC; *Manassas*, *VA,* USA), respectively. All strains were cultured and maintained in tryptic soy agar (TSA; BD Difco Laboratories, Franklin Lakes, NJ, USA) supplemented with 0.5% yeast extract, 0.5 mg/ml of hemin, 0.05% cysteine HCl-H_2O, and 2 µg/ml of vitamin K_1 (TSA-YCHVk) [3] at 37 °C in an anaerobic chamber (Model BACTRONEZ, Sheldon Manufacturing Inc., Cornelius, OR, USA) in an atmosphere of 10% H_2, 5% CO_2, and 85% N_2.

Phylogenetic Analysis

16S rDNA sequences of these three strains were sequenced in the course of genome sequencing. 16S rDNA sequences of type strains of *Fusobacterium* spp. were obtained from GenBank. 16S rRNA accession numbers of all these strains are listed in Fig. 1. Multiple sequences were aligned using CLUSTAL W algorithm. Sequence similarities were calculated using MegAlign program (DNAStar Lasergene^{TM} 8.0, DNAStar Inc., Madison, WI, USA) [4]. Evolutionary distance was calculated in accordance with the Kimura two-parameter model [7]. Phylogenetic trees were constructed with the neighbor-joining method [12] using MEGA 6.06 software [15]. The stability of the phylogenetic tree was assessed by bootstrap analysis [5] with 1000 replicates.

Genome Sequence

Genomic DNAs of these three strains were prepared as described previously [3]. Concentration and quality of these bacterial genomic DNAs were determined using an Epoch™ Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at wavelengths of 260 and 280 nm [3].

Genomic DNAs of ChDC F213^T, ChDC F251, and ChDC F267 were sequenced using a PacBio RSII platform by Macrogen Inc. (Seoul, Korea). DNA libraries (20-kb) were constructed in accordance with the manufacturer's protocol and sequenced using single-molecule real-time sequencing with P6 DNA polymerase and C4 chemistry [6]. From genomic DNAs of ChDC F213^T, ChDC F251, and ChDC F267, 1,175,912,373 bp (487.3 × coverage), 664,376,116 bp (280.0 × coverage), and 1,149,364,741 bp (433.5 × coverage) were generated, respectively. De novo assembly was performed using RS HGAP Assembly 3.0 [2] with default option. Genome annotation was conducted using the NCBI Prokaryotic Genome Annotation Pipeline through the NCBI Genome Submission Portal (https://submit.ncbi.nlm.nih.

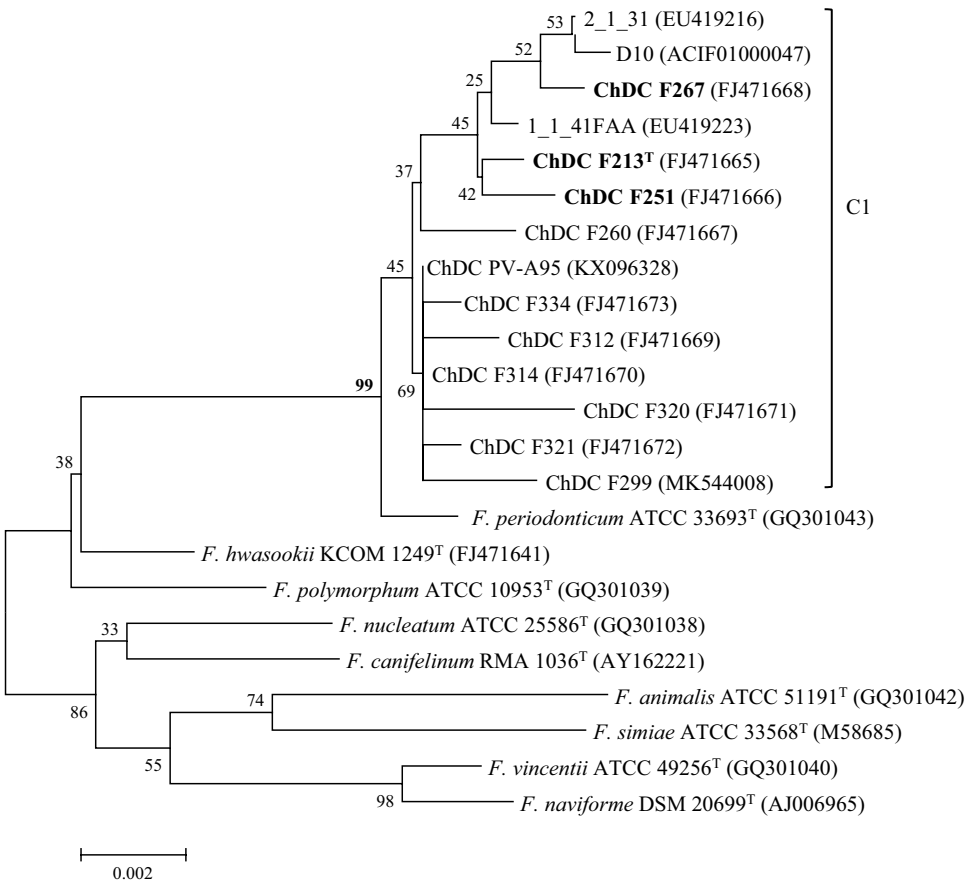

Fig. 1 Neighbor-joining phylogenetic tree based on 16S ribosomal RNA genes. GenBank accession numbers of 16S rDNA of each strain are written in parenthesis. Stability of phylogenetic trees was assessed by bootstrap analysis of 1000 replicates using MEGA version 6.06 [15]. Bar indicates 0.002 changes per nucleotide position

gov/subs/genome) [16]. GenBank accession numbers for genomes of the three strains—ChDC F213^T, ChDC F251, and ChDC F267—are listed in Table 1.

ANI and GGD Analyses

ANI and GGD analyses were conducted using the calculator provided by ChunLab (Seoul, Korea) (http://www.ezbiocloud.net/tools/ani) [9] and the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) (http://ggdc.dsmz.de) [10], respectively, to determine the genome relatedness. Whole-genome sequences of these strains and type strains of *Fusobacterium* spp. were downloaded from GenBank database (https://www.ncbi.nlm.nih.gov/genome/?term=fusobacterium+periodonticum). GenBank accession numbers of these strains are listed in Table 1.

Morphological and Physiological Characterization, Biochemical Analysis, and Chemotaxonomic Characteristics

Cell shape and size of the three isolates were determined by scanning electron microscopy (SEM) as described previously [4]. Growth at different temperatures (25–45 °C at intervals of 5 °C and 37 °C) was determined using TSB-YCHVk medium [4] after incubation for 3 days. Growth at various pH conditions (5–10 at intervals of 0.5) was assessed in TSB-YCHVk medium [4] after culturing at 37°C for 3 days. Growth at various NaCl concentrations was assessed in TP-YCHVk medium [4] containing 0°C, 1°C, 2°C, or 3% (w/v) NaCl (pH 7) after culturing at 37 °C for 3 days.

API ZYM and API 20A test strips (bioMerieux, Marcy-l'Etoile, France) were used to analyze enzyme activities, biochemical traits, and sugar fermentation patterns of these bacterial strains in accordance with the manufacturer's instructions. Cellular fatty acid compositions of these bacterial strains were determined using MIDI/Hewlett Packard Microbial Identification System (MIDI, Microbial ID, Newark, DE, USA) in accordance with the manufacturer's instructions by the Korean Culture Center of Microorganisms (Seoul, Korea). Fatty acids were analyzed using a gas chromatograph (Model 6890N and Autosampler 7683; Agilent, Santa Clara, CA, USA) and identified using Sherlock^{TM} Microbial Identification System (version 6.3).

Table 1 GenBank accession numbers of nucleotide sequences of genomes for isolated strains and closely related type strains of *Fusobacterium* spp

Strain	Isolation source	GenBank accession no.
ChDC F213^T (KCOM 1259^T)	Tongue, gingivitis, human	PEQY00000000
ChDC F251 (KCOM 1261)	Tongue, gingivitis, human	CP024699
ChDC F260 (KCOM 1262)	Tongue, gingivitis, human	CP024731
ChDC F267 (KCOM 1263)	Subgingival dental plaque, gingivitis, human	CP024700
ChDC F312 (KCOM 1277)	Subgingival dental plaque, gingivitis, human	CP024701
ChDC F320 (KCOM 1282)	Subgingival dental plaque, gingivitis, human	CP024702
ChDC F321 (KCOM 1283)	Subgingival dental plaque, gingivitis, human	CP024698
ChDC F314 (KCOM 1321)	Subgingival dental plaque, periimplantitis, human	PEQX00000000
ChDC F334 (KCOM 2305)	Subgingival dental plaque, gingivitis, human	CP024703
ChDC F299 (KCOM 2555)	Dental plaque, normal, human	CP024704
ChDC PV-A95 (KCOM 2653)	Tongue, human	CP024705
D10	Oral cavity, human	ACIF01000000
1_1_41FAA	Human[a]	ADGG00000000
2_1_31	Human[a]	CP028108
ATCC 33693^T	Periodontitis, human	ACJY00000000
ATCC 10953^T	Inflamed gingiva, human	NZ_CM000440
ATCC 25586^T	Cervico-facial lesion, human	NC_003454
ATCC 49256^T	Periodontal pocket, human	AABF00000000
ATCC 51191^T	Colon, animal	AFQD00000000
KCOM 1249^T	Subgingival dental plaque, periodontitis, human	ALVD00000000

ATCC 33693^T, *F. periodonticum*; ATCC 10953^T, *F. polymorphum* (previously *F. nucleatum* subsp. *polymorphum*). ATCC 25586^T, *F. nucleatum* (previously *F. nucleatum* subsp. *nucleatum*); ATCC 49256^T, *F. vincentii* (previously *F. nucleatum* subsp. *vincentii*); ATCC 51191^T, *F. animalis* (previously *F. nucleatum* subsp. *animalis*); KCOM 1249^T, *F. hwasookii*

[a]Isolation source was not reported

Results and Discussion

Phylogenetic analysis revealed that 16S rDNAs of all these strains belonged to the same cluster (C1). 16S rDNA sequences of strains ChDC F213^T, ChDC F251, and ChDC F267 were most closely related to that of *F. periodonticum* ATCC 33693^T (identities: 99.6, 99.5, and 99.4%, respectively, Supplementary Table S1). They were clearly separated from *F. periodonticum* ATCC 33693^T with bootstrap value of 99% by neighbor-joining, maximum likelihood, and minimum evolution methods (Fig. 1 and Supplementary Fig. S1). Average percent similarity of 16S rDNAs among strains belonging to cluster C1 was 99.7% (range 99.3% to 100%) (Supplementary Table S1). The genomic DNA sequences of 14 strains belonging to cluster C1 were deposited in GenBank as *F. periodonticum* (https://www.ncbi.nlm.nih.gov/genome/?term=fusobacterium periodonticum). 16S rDNA sequences of these strains closely related to that of *F. periodonticum* ATCC 33693^T (average 99.6%; range 99.4%, and 99.4%, respectively) and *F. hwasookii* KCOM 1249^T (average 99.6%; range 99.4–99.8%) and 99.0% (range 98.8–99.2%) (Supplementary Table S2). These data indicate that these strains in cluster C1 belong to genus *Fusobacterium*.

Genome sizes of strains ChDC F213^T, ChDC F251, and ChDC F267 were 2,413,021 bp, 2,372,833 bp, and 2,651,098 bp, respectively. DNA G + C contents of these three strains were 28.0 mol % each, similar to the G + C content of *F. periodonticum* ATCC 33693^T (27.8 mol%) [3]. ANI value between *F. periodonticum* ATCC 33693^T and strain ChDC F213^T, ChDC F251, or ChDC F267 were 92.7, 92.6, or 92.5%, respectively (Table 2). GGD value between *F. periodonticum* ATCC 33693^T and strain ChDC F213^T, ChDC F251, or ChDC F267 was 48.2, 48.2, or 47.7%, respectively (Table 2). Considering that threshold values of ANI and GGD for bacterial species discrimination are 95–96% and 70%, respectively [9, 10], these results indicate that strains ChDC F213^T, ChDC F251, and ChDC F267 represent a novel *Fusobacterium* species.

ANI and GGD values of strains in cluster C1, except strains ChDC F213^T, ChDC F251, ChDC F267, and 1_1_41FAA, compared with *F. peridodonticum* ATCC 33693^T were from 90.6% to 93.0% and 47.7% to 48.8%, respectively (Table 2). ANI and GGD values of strains in cluster C1 except for strains ChDC F213^T, ChDC F251, ChDC F267, and 1_1_41FAA, compared with strain ChDC F213^T were from 95.3% to 96.9% and 61.8% to 72.5%, respectively (Table 2). ANI and GGD values between strain 1_1_41FAA and ChDC F213^T were 94.7% and 58.0%, respectively (Table 2). This ANI value is almost borderline ANI value to discriminate bacteria at species level. The phylogenetic tree based on 16S rDNA showed that strain 1_1_41FAA and ChDC F213^T had the same cluster (Fig. 1 and Supplementary Fig. S1). Percent similarity of 16S rDNA between strain 1_1_41FAA and strain ChDC F213^T (99.9%) was higher than that between strain 1_1_41FAA and *F. periodonticum* ATCC 33693^T (99.6%) (Supplementary Tables S1, S2). Based on these data, strain 1_1_41FAA might belong to the same species as strain ChDC F213^T, but not *F. periodonticum* ATCC 33693^T. ANI and GGD values of 14 strains in cluster C1 and type strains of *F. nucleatum*, *F. polymorphum*, *F. vincentii*, *F. animalis*, and *F. hwasookii* that were closely related to strain ChDC F213^T by 16S rDNA

Table 2 Results of average nucleotide identity and genome-to-genome distance analyses of isolated strains and closely related type strains of *Fusobacterium* spp.

Strain	Average nucleotide identity value (%)		Genome–to–genome distance value (%)	
	ATCC 33693^T	ChDC F213^T	ATCC 33693^T	ChDC F213^T
ATCC 33693^T	100	92.7	100.0	48.2 [45.6–50.8]
ChDC F213^T	92.7	100	48.2 [45.6–50.8]	100
ChDC F251	92.6	96.3	48.2 [45.6–50.9]	72.5 [69.5–75.3]
ChDC F260	92.4	95.3	47.7 [45.1–50.3]	61.6 [58.7–64.4]
ChDC F267	92.5	95.3	47.7 [45.1–50.3]	61.6 [58.7–64.4]
ChDC F312	93.0	96.8	48.8 [46.2–51.4]	70.5 [67.5–73.4]
ChDC F320	92.9	96.7	48.3 [45.7–51.0]	69.5 [66.5–72.4]
ChDC F321	92.7	96.9	48.5 [45.9–51.1]	72.5 [69.5–75.4]
ChDC F314	90.6	96.6	48.7 [46.1–51.3]	70.5 [67.5–73.3]
ChDC F334	92.9	96.9	48.8 [46.2–51.4]	71.4 [68.4–74.2]
ChDC F299	92.7	96.0	48.8 [46.2–51.5]	64.3 [61.4–67.2]
ChDC PV-A95	92.9	96.6	48.6 [46.0–51.2]	67.9 [66.7–72.6]
2_1_31	92.2	95.4	47.9 [45.4–50.6]	61.9 [59.1–64.7]
D10	92.4	95.3	48.1 [45.5–50.7]	61.8 [58.9–64.5]
1_1_41FAA	92.7	94.7	48.5[45.9–51.1]	58.1 [55.3–60.9]

ATCC 33693^T, type strain of *F. periodonticum*

sequence analysis were below 93.0% and 31.8%, respectively (Supplementary Tables S3, S4). These results indicate that these 14 strains in cluster C1 are members of a novel *Fusobacterium* spp.

In the API ZYM panel, tests for acid phosphatase and naphthol-AS-BI-phosphohydrolase were positive for the three strains (ChDC F213^T, ChDC F251, and ChDC F267), but negative for *F. periodonticum* ATCC 33693^T (Table 3). In a previous study, tests for acid phosphatase were negative for four subspecies of *Fusobacterium nucleatum* (now reclassified as four novel species [8]) and *Fusobacterium hwasookii* [3]. Tests for alkaline phosphatase and leucine arylamidase were positive for strains ChDC F213^T and ChDC F251 (Table 3). Esterase lipase (C8) was positive in strains ChDC F213^T and ChDC F267 (Table 3). The remaining 13 tests in the API ZYM panel were negative for the three strains. An API 20A test for indole production was positive (Table 3). Strains ChDC F213^T and ChDC F267 fermented glucose (Table 3). Strains ChDC F213^T and ChDC F267 hydrolyzed gelatin and esculin, respectively (Table 3). The remaining 17 tests, including test for catalase, were negative for the three strains (Supplementary Table S5). Biochemical test results for these three strains were similar to those for *F. periodonticum* ATCC 33693^T.

Morphological characteristics and optimal growth conditions of the three strains are summarized in Supplementary Table S6.

Based on molecular, chemical, and phenotypic evidence presented in the present study, we propose that these three strains—ChDC F213^T, ChDC F251, and ChDC F267—should be assigned to a novel species of *Fusobacterium*, for which a name of *Fusobacterium pseudoperiodonticum* sp. nov. is proposed.

Description of *Fusobacterium pseudoperiodonticum* sp. nov.

Fusobacterium pseudoperiodonticum [Gr. adj. *pseudês*, false; N.L. n. *periodonticum*, a bacterial specific epithet; N.L. n. *pseudoperiodonticum*, a false (*Fusobacterium*) *periodonticum*].

Fusobacterium pseudoperiodonticum is a Gram-negative, anaerobic, and fusiform-shaped bacterium with variable size. Cell size was ranged from 0.3–0.4 × 2.2–106.5 µm. Colonies were pigmented in grayish brown and spread to a diameter of approximately 0.7–1.0 mm after growing on TSA-YCHVk agar at 37°C for 2 days. Growth occurred in the range of 30–37 °C (optimum 35–37 °C). The optimum pH for growth for these strains was 7.0–7.5. Acid phosphatase and naphthol-AS-BI-phosphohydrolase were positive. Indole production test was positive. Cellular fatty acids were mainly composed of $C_{14:0}$, $C_{16:0}$, and $C_{16:1}$ ω6c/$C_{16:1}$ ω7c (Table 4). G + C contents of all strains were 28.0 mol%.

The type strain of *Fusobacterium pseudoperiodonticum* is ChDC F213^T (= KCOM 1259^T = KCTC 5677^T = JCM 33009^T). It was isolated from the tongue of a Korean. It can hydrolyze gelatin. This strain produces esterase (C4). The DNA G + C content is 28.0 mol%.

Table 3 Biochemical characteristics of isolated strains and closely related type strains of *Fusobacterium* spp

Characteristic	1	2	3	4	5	6	7	8	9
Indole production	+	+	+	+	+	+	+	+	+
Acidification									
Glucose	+	−	+	−	−	−	−	−	−
Hydrolysis									
Gelatin	+	−	−	−	−	−	−	−	−
Esculin	−	−	+	−	−	−	−	−	−
Enzyme activity									
Alkaline phosphatase	+	+	−	−	−	−	−	−	−
Esterase (C4)	+	−	−	−	−	−	−	−	w
Esterase lipase (C8)	+	−	+	w	−	−	−	−	w
Leucine arylamidase	+	+	−	+	−	−	−	−	−
Acid phosphatase	+	+	+	−	−	−	−	−	w
Naphthol–AS–BI–phosphohydrolase	+	+	+	w	w	w	w	w	−

Strains: 1, strain ChDC F213^T; 2, strain ChDC F251; 3, strain ChDC F263; 4, *F. periodonticum* ATCC 33693^T; 5, *F. nucleatum* (previously *F. nucleatum* subsp. *nucleatum*) ATCC 25586^T [3]; 6, *F. polymorphum* (previously *F. nucleatum* subsp. *polymorphum*) ATCC 10953^T [3]; 7, *F. vincentii* (previously *F. nucleatum* subsp. *vincentii*) ATCC 49256^T [3]; 8, *F. animalis* (previously *F. nucleatum* subsp. *animalis*) ATCC 51191^T [3]; 9, *F. hwasookii* KCOM 1249^T [3]

Symbols: + positive; *w* weakly positive; − negative

Table 4 Cellular fatty acid compositions of isolated strains and closely related type strains of *Fusobacterium* spp.

Characteristic	1	2	3	4	5	6	7	8	9
10:0	1.53	0.93	0.63	0.55	–	–	–	–	–
12:0	5.8	3.72	1.58	0.94	2.57	1.25	1.15	0.53	1.27
14:0	28.66	28.67	22.71	16.35	29.97	29.22	25	21.56	29.37
14:0 DMA	–	–	–	–	2.11	1.33	2.5	4.9	–
15:1 ω8c	1.31	–	0.33	–	–	–	–	–	–
16:0	9.61	18.95	21.1	20.3	15.91	21.52	20.05	21.75	28.33
16:0 ALDE	–	–	–	–	1.84	1.71	2.37	2.57	–
16:0 3OH	5.16	4.87	5.48	7.23	8.23	5.78	5.91	6.22	4.9
16:1 cis 9	–	–	–	–	13.02	13.55	5.94	9.55	15.08
16:1 cis 9 DMA	–	–	–	–	1.2	0.73	1.44	1.77	–
16:0 DMA	–	–	–	–	7.37	8.62	12.4	11.42	1.12
16:1 w5c	0.73	1.17	1.99	1.87	–	–	–	–	–
17:0 2OH	1.09	0.61	0.66	–	–	–	–	–	–
17:1 ω8c	–	–	0.16	–	1.32	1.58	1.45	1.96	2.66
18:1 ω7c	2.27	6.04	11.07	10.83	–	–	–	–	–
18:1 ω9c	0.45	0.57	0.58	0.53	1.93	4.04	8.53	4.84	3.81
18:2 cis 9,12	–	–	–	–	–	1.16	2.21	1.2	1.03
19:0 iso	10.03	–	–	10.97	2.93	–	–	–	–
13:1 cis 12 and/or 14:0 ALDE	2.98	0.49	0.14		1.19	0.46	0.69	1.33	–
15:0 DMA and/or 14:0 3OH	–	–	–	–	6.14	5.21	5.4	4.69	4.55
13:0 3OH/15:1 iso H	4.68	1.45	0.52	0.43	–	–	–	–	–
12:0 aldehyde	5.89	5.49	5.21	6.66	–	–	–	–	–
16:1 ω6c/16:1 ω7c	11.29	21.46	22.89	16.91	–	–	–	–	–
18:2 ω6,9c/18:0 ante	0.65	0.59	0.27	0.27	–	–	–	–	–
18:1 ω6c	2.27	6.04	11.07	10.83	–	–	–	–	–
18:1 cis11/trans 9/trans 6.	–	–	–	–	4.27	3.06	3.08	4.46	6.8

Strains: 1, strain ChDC F213^T; 2, strain ChDC F251; 3, strain ChDC F263; 4, *F. periodonticum* ATCC 33693^T; 5, *F. nucleatum* (previous *F. nucleatum* subsp. *nucleatum*) ATCC 25586^T [3]; 6, *F. polymorphum* (previous *F. nucleatum* subsp. *polymorphum*) ATCC 10953^T [3]; 7, *F. vincentii* (previous *F. nucleatum* subsp. *vincentii*) ATCC 49256^T [3]; 8, *F. animalis* (previous *F. nucleatum* subsp. *animalis*) ATCC 51191^T [3]; 9, *F. hwasookii* KCOM 1249^T [3]. Values are expressed as percentages of fatty acids

Symbol: – not detected

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