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Research Article

New gene markers for classification and quantification of *Faecalibacterium* spp. in the human gut

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Abstract

Faecalibacterium prausnitzii is a promising biomarker of a healthy human microbiota. However, previous studies reported the heterogeneity of this species and found the presence of several distinct groups at the species level among *F. prausnitzii* strains. Our recent study revealed that methods previously developed for quantification of *F. prausnitzii* were not specific to the species level because of the heterogeneity within the *F. prausnitzii* species and the application of 16S rRNA gene, which is an invalid genetic marker for the species. Therefore, previously available data failed to provide information on different groups, which limits our understanding of the importance of this organism for host health. Here, we propose an alternative gene marker for quantification of *F. prausnitzii*-related taxa. A total of nine group-specific primer pairs were designed by targeting *rpoA* gene sequences. The newly developed *rpoA*-based qPCR successfully quantified targeted groups. Application of the developed qPCR assay in six healthy adults revealed marked differences in abundance and prevalence among the different targeted groups in stool samples. The developed assay will facilitate detailed understanding of the impact of *Faecalibacterium* populations at the group level on human health and to understand the links between depletion of specific groups in *Faecalibacterium* and different human disorders.

Keywords: *Faecalibacterium*, group-specific quantification, qPCR, *recA*, *rpoA*, taxonomy

Abbreviations

MAM: microbial anti-inflammatory molecule
IBD: inflammatory bowel diseases
ANI: average nucleotide identity
qPCR: quantitative PCR
SD: standard deviation

Introduction

Faecalibacterium prausnitzii produces butyrate from the metabolism of carbohydrates in human gut (Louis and Flint 2009). Butyrate is the major energy source of intestinal epithelial cells and has multiple beneficial properties for host health. It exert beneficial effects by different pathways, including regulation of histone acetylation and mitogen-activated protein kinases (Davie 2003, Kida et al. 2006, Macfarlane and Macfarlane 2012). The colonization of *F. prausnitzii* in mice is also related to several metabolites, such as salicylic acid and shikimic acid, which potentially exert beneficial effects (Miquel et al. 2015). Moreover, the microbe produces peptides derived from the microbial anti-inflammatory molecule (MAM) that are associated with the decreased activation of the NF-κB pathways in the host (Quevrain et al. 2016, Breyner et al. 2017, Auger et al. 2022). The beneficial properties of these active molecules are associated with the anti-inflammatory properties, maintenance of gut barrier function, gut immune homeostasis, and induction of apoptosis in colorectal cancer cells in host animals (Kinoshita et al. 2002, Furusawa et al. 2013, Donohoe et al. 2014). The depletion of this microbe in the gut was associated with the development and severity of several diseases, including inflammatory bowel diseases (IBD), metabolic disorders, and psychiatric disorders (Sokol et al. 2009, Lopez-Siles et al. 2017, Borkent et al. 2022, Michels et al. 2022). All these facts underline the importance of this microbe for health maintenance.

Recent studies reported a large genomic heterogeneity among *F. prausnitzii* strains and found eight groups, distinct at the species level being present among the tested strains (Fitzgerald et al. 2018, Tanno et al. 2022). In total, three of the eight groups were very recently reclassified as novel species in the genus *Faecalibacterium* mainly based on genomic level identities determined by average nucleotide identity (ANI) analysis. They were named *Faecalibacterium duncaniae*, *F. hattorii*, and *F. longum* originated from the human gut (Zou et al. 2021, Sakamoto et al. 2022). Other groups have not been studied for reclassification yet. In addition, *Faecalibacterium butyricigenerans*, which was out of the previously mentioned

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eight groups, was also described from the human gut (Zou et al. 2021). Additionally, *Faecalibacterium gallinarum* from the chicken gut was described (Sakamoto et al. 2022). Previous studies reported distinct populations between different groups of *Faecalibacterium* in patients with atopic dermatitis and in paediatric IBD (Song et al. 2016, Zhang et al. 2018). Moreover, a recent study reported that MAM derived from different groups of *Faecalibacterium* displayed distinct anti-inflammatory properties (Auger et al. 2022). These studies suggest that different groups have varying impacts on host health.

The gene encoding 16S rRNA (16S rRNA gene) is the best studied and characterized gene marker for bacterial taxonomy. The species threshold of 16S rRNA gene sequence similarity is around 98.7%–99.0% (Stackebrandt and Ebers 2006), while distinct species sometimes share a similarity of more than 99%. On the other hand, Tanno et al. reported that *Faecalibacterium* species generally possessed six copies of 16S rRNA gene per genome, and sequence similarities among copies in a single genome/strain were sometimes lower than the species threshold of 98.7% (Tanno et al. 2022). These low similarities are due to considerable nucleotide substitutions, particularly in the V6 region of the 16S rRNA gene, among copies. Moreover, 16S rRNA gene copies sharing low sequence similarities sometimes showed higher sequence similarities with strains in other groups, i.e. other species. Due to the heterogeneity of 16S rRNA gene sequences, certain groups have been divided into two or three clusters by a 16S rRNA gene-based phylogenetic tree (Tanno et al. 2022). These findings clearly indicated that 16S rRNA gene is not a well-suited gene marker for the identification and classification of *Faecalibacterium* spp. Very often, 16S rRNA gene is used as a target gene for the quantification of *Faecalibacterium*. A number of primer pairs combined with real-time quantitative PCR (qPCR) have been developed for the quantification of *F. prausnitzii*, and quantitative data were included in studies on the differing abundance of the microbe between healthy subjects and patients with specific disorders (Bartosch et al. 2004, Rinttilä et al. 2004, Balamurugan et al. 2008, Sokol et al. 2009). However, due to the heterogeneity of 16S rRNA gene sequence, several of the primer pairs used targeted only some of the groups in *Faecalibacterium* (Tanno et al. 2022). Even if primer pairs cover all groups, the quantified population is a sum of all groups in *Faecalibacterium* (the former *F. prausnitzii* sensu lato group), not specific groups. Therefore, individual populations of *F. prausnitzii* and related groups in the human gut have not yet been characterized, although the species is regarded as a promising biomarker of a healthy microbiota (Lopez-Siles et al. 2017).

In the present study, housekeeping genes were evaluated as a potential new gene marker for the classification of *Faecalibacterium* spp. The housekeeping genes were also used to design primer pairs specific to each group in *Faecalibacterium* for specific quantification.

Materials and methods

Acquisition of genomic data and grouping of strains based on ANI

The genomic data of 86 strains of *F. prausnitzii* strains, used in our previous study (Tanno et al. 2022), were included in the present study. These were all complete or draft genome sequences of *F. prausnitzii* strains at the time of the analysis (January 2020) in the NCBI database after the exclusion of potential incompleteness and contamination strains (excluded, genome sizes of <2.6 Mbp or >3.5 Mbp). Moreover, the genomic data of *F. butyricigenerans* AF52-21^T and *F. longum* CM04-06^T were obtained from the CNGBdb database and included herein, resulting in a list of 88 strains. Genome level identities of the strains were determined by calculating ANI values, and the ANI values were used to prepare a distance matrix to represent the ANI divergence (100% ANI) and for group separation (threshold of ∼94%), as described previously (Tanno et al. 2022).

Phylogenetic analysis based on housekeeping genes

Since the 16S rRNA gene was not a suitable gene marker for the classification and identification of *Faecalibacterium* spp. at the group level, six housekeeping genes, including *atpA*, *dnaK*, *groEL*, *pheS*, *recA*, and *rpoA*, which have been characterized for the classification and identification of other Bacillota (formerly known as Firmicutes) members and related bacteria (Torriani et al. 2001, Naser et al. 2007, Neumann and Rehberger 2009, Muñoz et al. 2017, Liu et al. 2018), were included in the initial screening. However, two (*atpA* and *dnaK*) out of the six genes were excluded from this analysis due to the presence of two copies in the genomes of completely sequenced strains (data not shown). The remaining four housekeeping genes were present as a single copy in the genomes and were, thus, included in the phylogenetic analysis. The sequences of housekeeping genes were obtained from the genomes of the 88 strains and were aligned and used to construct phylogenetic trees using ClustalW (Larkin et al. 2007). The number of bootstrapping replicates was 1000. Sequence similarities in the housekeeping genes were assessed using Genetyx software ver. 13 (Genetyx, Tokyo, Japan).

Design and evaluation of *rpoA*-based primer pairs for the specific quantification of each group

The sequences of the *rpoA* gene were used to design specific primer pairs for the different groups previously found in *Faecalibacterium*. In the design of primers, all complete and draft genomes of *F. prausnitzii* and *Faecalibacterium* sp. ($n = 147$) containing the *rpoA* gene sequence, which were deposited in the NCBI database at a time of the analysis (July 2021), were further added to the original 88 strains. This addition was due to the limited number of strains in Groups 2, 5, 7, 8, and 9, originally containing 1, 3, 4, 2, and 1 strain, respectively, when the 88 strains were used. ANI values were determined for the newly added strains, as previously described (Maeno et al. 2016), and the strains were separated into groups based on the values. After this separation, number of strains in Groups 2, 5, 7, 8, and 9 increased to 3, 7, 8, 8, and 4 strains, respectively (data not shown). Moreover, all completely sequenced strains ($n = 21$) available at the time of the analysis (July 2022) were used to count the copy numbers of 16S rRNA gene per genome.

Primers were designed in consideration of no sequence mismatches with the targeted group, but the presence of sequence mismatches with nontargeted groups as much as possible. The specificity of the primers was evaluated using primer-BLAST to the tested strains and BLASTN to all deposited DNA sequences with default settings. A list of the specific primers designed is shown in Table 1.

A qPCR-based primer evaluation was conducted to confirm the specificity of the primer pairs designed using three steps. In the first step, the entire *rpoA* gene sequences (954–969 bp) of 12 strains of *Faecalibacterium* spp. were synthesized by insertion into

Table 1. Primers used in the present study.

Primer	Sequence (5'→3')	Target	References
F-Bact_1369	CGGTGAATACGTTCCCGG	Total bacteria	Lopez-Siles et al. (2016)
R_Prok_1492	TACGGCTACCTTGTTACGACTT	Total bacteria	Lopez-Siles et al. (2016)
Fprau223F	GATGGCCTCGCGTCCGATTAG	*Faecalibacterium genus*	Bartosch et al. (2004)
Fprau420R	CCGAAGACCTTCTTCCTCC	*Faecalibacterium genus*	Bartosch et al. (2004)
Faecali-group1-sp-F	CCTGAGTGGCACATTGCAACT	Group 1 (*F. prausnitzii*)	This study
Faecali-group1-sp-R	TAAATGCTGTCAACGGGAAGG	Group 1 (*F. prausnitzii*)	This study
Faecali-group2-sp-F	CCAAGCTCGTCATGGAGCTC	Group 2	This study
Faecali-group2-sp-R	ATGGTCAGCTTGTCGTAGTCA	Group 2	This study
Faecali-group3-sp-F	AACCTGTCCGATGAGGCAGCC	Group 3	This study
Faecali-group3-sp-R	TCTTCCACCGTGTTGATGCCT	Group 3	This study
Faecali-group4-sp-F	GCCATCATCGAGAAGAATGAC	Group 4 (*F. longum*)	This study
Faecali-group4-sp-R	TGTGCATTGATCGTGCCATCC	Group 4 (*F. longum*)	This study
Faecali-group5-sp-F	GAAATTGTCCTGAACCTGAAA	Group 5	This study
Faecali-group5-sp-R	CCTGTTTGTTGCGCTCAGCC	Group 5	This study
Faecali-group6-sp-F	AAGGGCCGCGGTTATGTGCCT	Group 6 (*F. duncaniae*)	This study
Faecali-group6-sp-R	TAATCGATGGCCTGTCCAACG	Group 6 (*F. duncaniae*)	This study
Faecali-group7-sp-F	CCTGAATGGCACATCGCAACCT	Group 7 (*F. hattorii*)	This study
Faecali-group7-sp-R	ATGCTATCGACGGGAAGCGTA	Group 7 (*F. hattorii*)	This study
Faecali-group8-sp-F	GGTGAATTACAATGTTGAGAA	Group 8	This study
Faecali-group8-sp-R	TCTCGGTGCCAGCGGCCTCA	Group 8	This study
Faecali-group9-sp-F	AATGTCGAGAGCACCCGTGTG	Group 9 (*F. butyricigenerans*)	This study
Faecali-group9-sp-R	GATCTCAGCGCCAGCGGCCTCG	Group 9 (*F. butyricigenerans*)	This study

the pEX-A2J2 vector by Eurofins Genomics (Tokyo, Japan). These 12 strains included at least one strain each of all groups, and three and two strains were included from Groups 1 and 6, respectively (Fig. 2). qPCR was conducted by the combination of the specific primers and 100 pg (= 7.43 $\log_{10}$ *rpoA* gene copies) of synthesized DNA. DNA Calculator (https://www.molbiotools.com/dnacalculator.html) was used to determine the number of molecules in 100 pg of the synthesized DNA. FastStart Essential DNA Green Master Mix combined with the LightCycler 96 system (Roche, Basel, Switzerland) was used for qPCR according to the manufacturer's instructions. The qPCR program consisted of initial denaturation at 95°C for 10 min and 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Standard curves were produced for each primer pair using the synthesized DNA of each targeted group, while ATCC 27768^T and A2165 were used as references for Groups 1 and 6, respectively. A melting curve analysis was conducted to confirm specific amplification. Samples were run in triplicate in the same plate, and the mean and SD were obtained. The detection limit of qPCR was assessed based on the results obtained using serially diluted synthetic DNA.

In the second step of primer specificity confirmation, DNAs isolated from representative strains in each group, which are available in public culture collections or our own collection, were used for qPCR. DNA was isolated from cultures of BCRC 81047^T (= *F. prausnitzii* ATCC 27768^T) in Group 1, CNCM 4541 in Group 2, CNCM 4540 in Group 3, JCM 39211^T (= *F. longum* CM04-06^T) in Group 4, JCM 31915^T (= *F. duncaniae* A2-165^T) in Group 6, JCM 39210^T (= *F. hattorii* APC922/41–1^T) in Group 7, and JCM 39212^T (= *F. butyricigenerans* AF52-21^T) in Group 9, and 10 ng of isolated DNA was used in this study. The BCRC strain and JCM strains were obtained from the Bioresource Collection and Research Center (BCRC) and the Japan Collection of Microorganisms (JCM), respectively. CNCM strains were obtained from our private collection. No strains in Groups 5 or 8 were available in the public culture collection at the time of the analysis (March 2022). qPCR combined with specific primers to each group was conducted using the methods described above. Samples were run in triplicate in the same plate, and the mean and SD were obtained.

In the third step of the primer specificity evaluation, cultures (~10^7 cells) of *F. prausnitzii* BCRC 81047^T (Group 1) or *F. duncaniae* JCM 31915^T (Group 6) were added to 200 mg of infantile stool (9 months old), and DNA was extracted from stool samples with or without the addition of bacterial cultures using the QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan). Sample collection was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Tokyo University of Agriculture. Written informed consent was received from the parent of the infant. Isolated DNAs were used for *rpoA*-based qPCR of the nine groups. The qPCR products were sequenced by the methods as described previously (Endo and Okada 2005). The genus *Faecalibacterium* was also quantified using the 16S-based primer pair of Fprau223F/Fprau420R designed by Bartosh and coworkers (Bartosch et al. 2004) shown in Table 1 and the synthesized 16S rRNA gene of ATCC 27768^T included in a previous study (Tanno et al. 2022) for a standard curve. The primer pair combined with qPCR equally quantified all groups in *Faecalibacterium* in the previous study (Tanno et al. 2022), and the primer pair had substantial sequence mismatches with the 16S rRNA gene of other members in the family *Oscillospiraceae*. qPCR conditions were described elsewhere (Tanno et al. 2022). Moreover, the 16S rRNA gene copy number of total bacteria was quantified to assess the relative abundance of the genus *Faecalibacterium* and each group using the 16S-based bacterial universal primer pair of F-Bact_1369/R_Prok_1492 (Lopez-Siles et al. 2016), as shown in Table 1. The synthesized 16S rRNA gene of ATCC 27768^T was used for the standard curve, and the qPCR program consisted of initial denaturation at 95°C for 10 min, and 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Samples were run in triplicate in the same plate, and the mean and SD were obtained. The detection limit of qPCR was determined based on the results obtained using serially diluted DNA from a *F. prausnitzii* BCRC 81047^T-added stool sample.

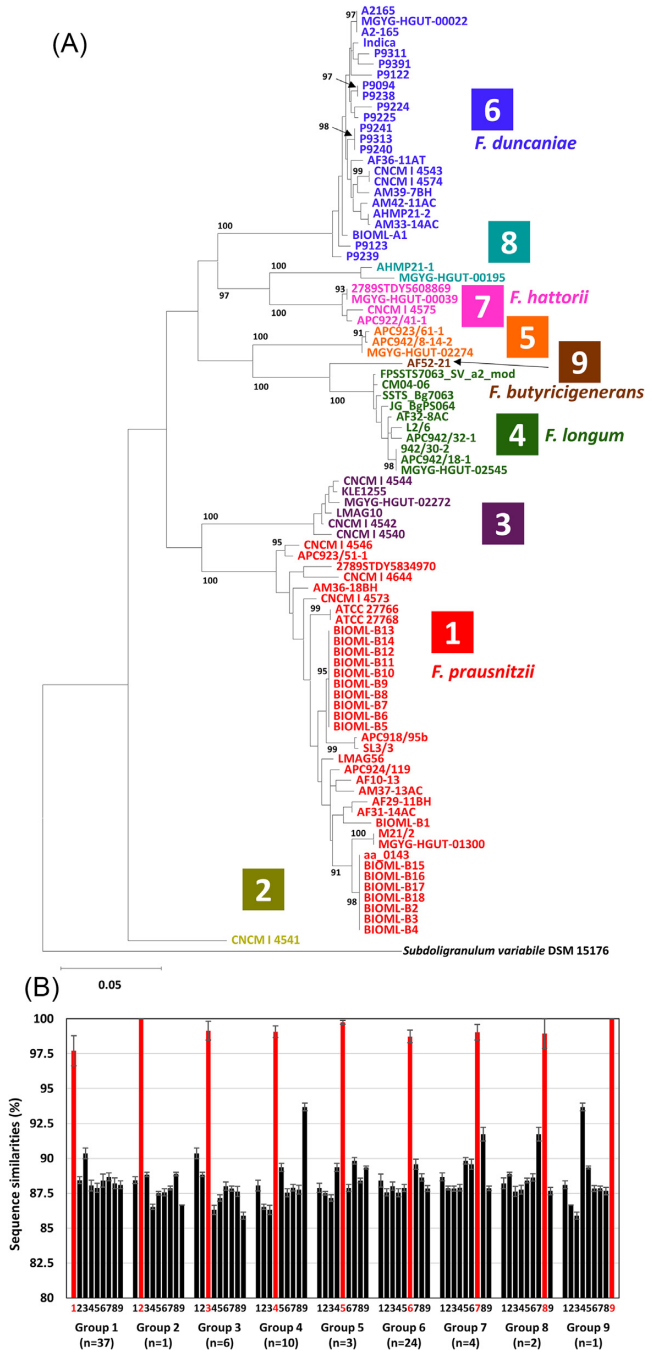


Figure 1. Phylogenetic tree based on *recA* gene nucleotide sequences of 88 strains of *Faecalibacterium* spp. (**A**) and intra- (red bars) and inter- (black bars) group *recA* gene sequence similarities (**B**). The maximum-likelihood tree was constructed using the best-fit evolutionary model. The values on the branches are bootstrap support from 1000 rapid bootstrapping replicates and only values higher than 90% are indicated. *Subdoligranulum variabile* DSM 15176 (GCA_000157955.1) was used as an outgroup. The scale bar means substitution per site. Groups based on ANI-values (Figure S1, Supporting Information) are indicated in different colours, and Groups 1, 4, 6, 7, and 9 are *F. prausnitzii* sensu stricto, *F. longum*, *F. duncaniae*, *F. hattorii*, and *F. butyricigenerans*, respectively. In (B), bars numbered 1–9 indicate the medians of sequence similarities to Groups 1–9, respectively, and error bars indicate SD. The numbers of strains in each group are indicated.

Quantification of different *Faecalibacterium* groups in stool samples of healthy individuals

Volunteers who had not received antibiotics for more than 8 weeks or prebiotics for more than 2 weeks prior to the donation of stool samples were recruited, and fecal samples were collected from six healthy adult men (volunteers A–F, mean ± SD, 26.3 ± 6.7 years old) with a body mass index ranging between 22 and 25 in a previous study (Endo et al. 2020). After defecation, stool samples were immediately placed in anaerobic jars (AnaeroPack-Anaero, Mitsubishi Gas Chemical, Tokyo, Japan), transported to the laboratory, and stored at −80°C within 2 h of collection. Sample collection was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Tokyo University of Agriculture. Written informed consent was received from the volunteers. DNA was extracted from the collected samples within 2 weeks using a previously described method (Takahashi et al. 2014). The quantification of the nine groups in *Faecalibacterium* (*rpoA*-based), the genus *Faecalibacterium* (16S-based), and total bacteria (16S-based) was conducted by the method described above. The relative abundance (%) of each group in *Faecalibacterium* and the genus *Faecalibacterium* was calculated using the total bacterial count. The 16S/*rpoA* ratio was obtained using 16S-based genus *Faecalibacterium* qPCR and the sum of *rpoA*-based nine group-specific qPCR. Samples were run in triplicate in the same plate, and the mean and SD were obtained.

Results

Selection of a potential new gene marker for the classification of *Faecalibacterium* spp

A total of 88 strains were divided into nine groups based on ANI value-based grouping, i.e. eight groups described in a previous study (Tanno et al. 2022) with an additional ninth group of *F. butyricigenerans* (Figure S1, Supporting Information). Group 1 was *F. prausnitzii* sensu stricto, and Groups 4, 6, 7, and 9 were *F. longum*, *F. duncaniae*, *F. hattorii*, and *F. butyricigenerans*, respectively. Other groups were obviously different from *F. prausnitzii* at the species level but have not yet been taxonomically reconsidered and separated from this taxon. Phylogenetic trees based on the nucleotide sequences of the genes revealed that the nine groups produced independent clusters on the trees (Fig. 1a; Figure S2, Supporting Information). Of the four genes, *recA* had the lowest intergroup sequence similarity (Fig. 1b; Figures S1b, S1d, and S1f, Supporting Information). The highest intergroup sequence similarity value of 94.2% on *recA* gene was recorded between strains in Groups 4 and 9 (Table S1a, Supporting Information), whereas Group 4 strains shared more than 98.5% sequence similarities among the strains. The medians of intragroup *recA* gene sequence similarities were at least 97.7% in Group 1, whereas those of the intergroup were less than 93.7% recorded between Groups 4 and 9 (Fig. 1b). This result suggests that the *recA* gene has high discriminatory power and is a suitable gene marker for the classification and identification of *Faecalibacterium* spp.

Design and evaluation of novel specific primer sets for the selective quantification of each group

Among the four housekeeping genes (*groEL*, *pheS*, *recA*, and *rpoA*) tested, *recA* gene has a high power to discriminate groups. However, this gene showed a high level of intragroup sequence

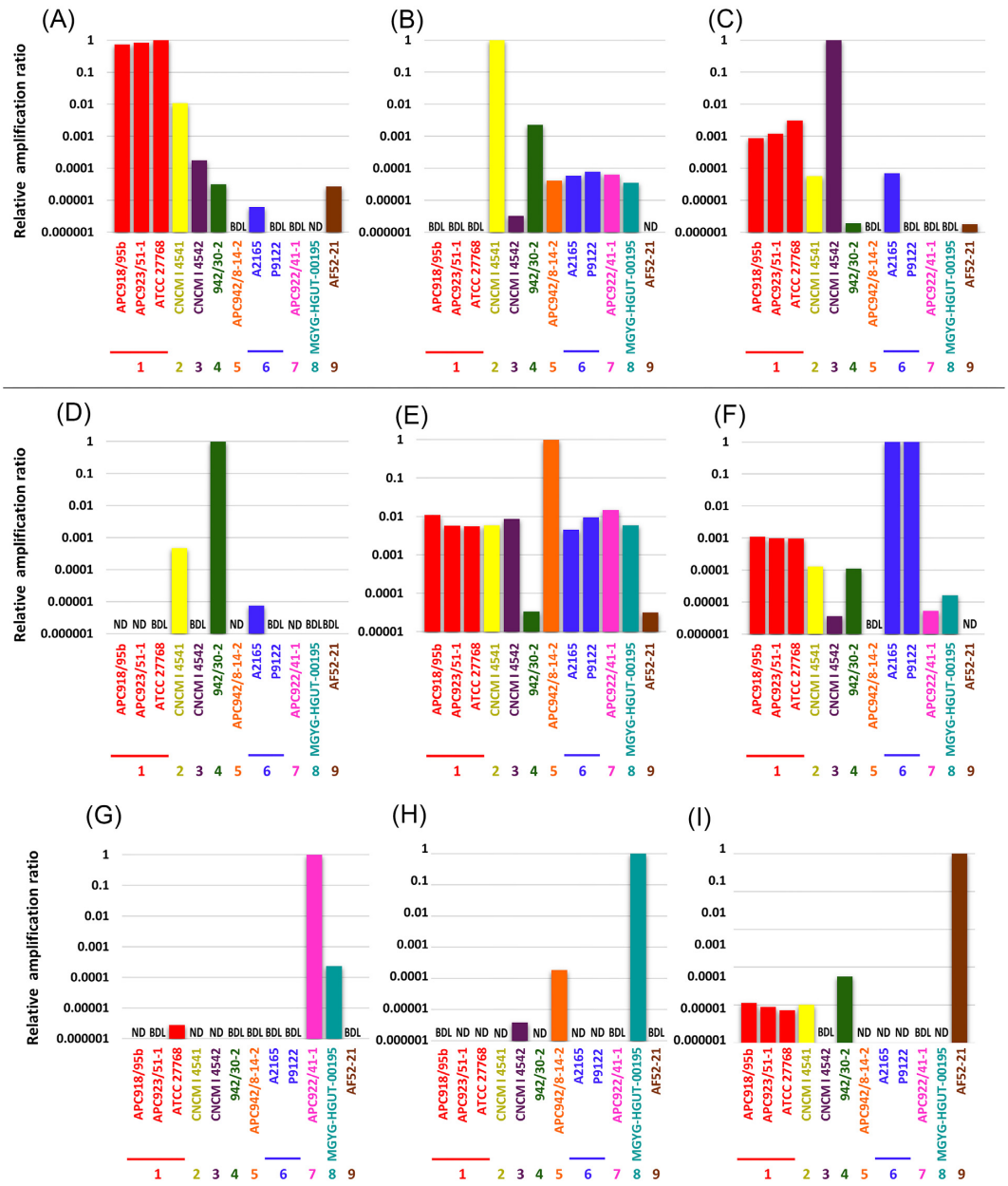


Figure 2. Relative amplification ratio against the targeted group using the synthetic *rpoA* gene and *rpoA*-based group-specific primer pairs. A DNA amount of 100 pg (= 7.43 $\log_{10}$ *rpoA* gene copies) was used in each PCR reaction. (**A**) to (**I**) use specific primer pairs to Groups 1–9, respectively. ATCC 27768 and A2165 were used as references in Groups 1 and 6, respectively. Samples were run in triplicate in the same plate, and the mean and SD were obtained. ND, not detected. BDL, below the detection limit.

divergence, and, thus, designation of group-specific primers targeting *recA* gene sequences was not possible. In contrast, the *rpoA* gene sequences contained low levels of intragroup sequence divergence, maintaining group-specific sequence regions, thus allowing the design of group-specific primers. The designed primer pairs contained no sequence mismatches with the *rpoA* gene sequences of the targeted groups but contained substantial sequence mismatches with the sequences of nontargeted groups (Table 1). BLASTN analysis confirmed that all DNA sequences deposited in the database did not completely match with the primer sequences.

The designed primer pairs were initially evaluated using synthesized DNA. The evaluation revealed that the newly developed *rpoA*-based qPCR assay accurately quantified the targeted group (~7.43 $\log_{10}$ *rpoA* gene copies). The relative amplification ratio of the nontargeted groups against the targeted group was generally less than 1%, and over 50% of the nontargeted groups were not detected or were below the detection limit ($10^{1.7}$ molecules/reaction) in total (Fig. 2).

A primer specificity evaluation was also conducted using isolated DNAs from cultures originating from seven out of the nine groups. The *rpoA*-based qPCR assay accurately quantified the targeted group, but quantified the nontargeted groups less than 1% of the targeted groups (Fig. 3). In total, over 60% of the nontargeted groups were not detected or were below the detection limit.

An evaluation of primer specificity was further conducted by using the addition of cultures to infantile stool (9 months-old infant), which originally contained very low levels (below the

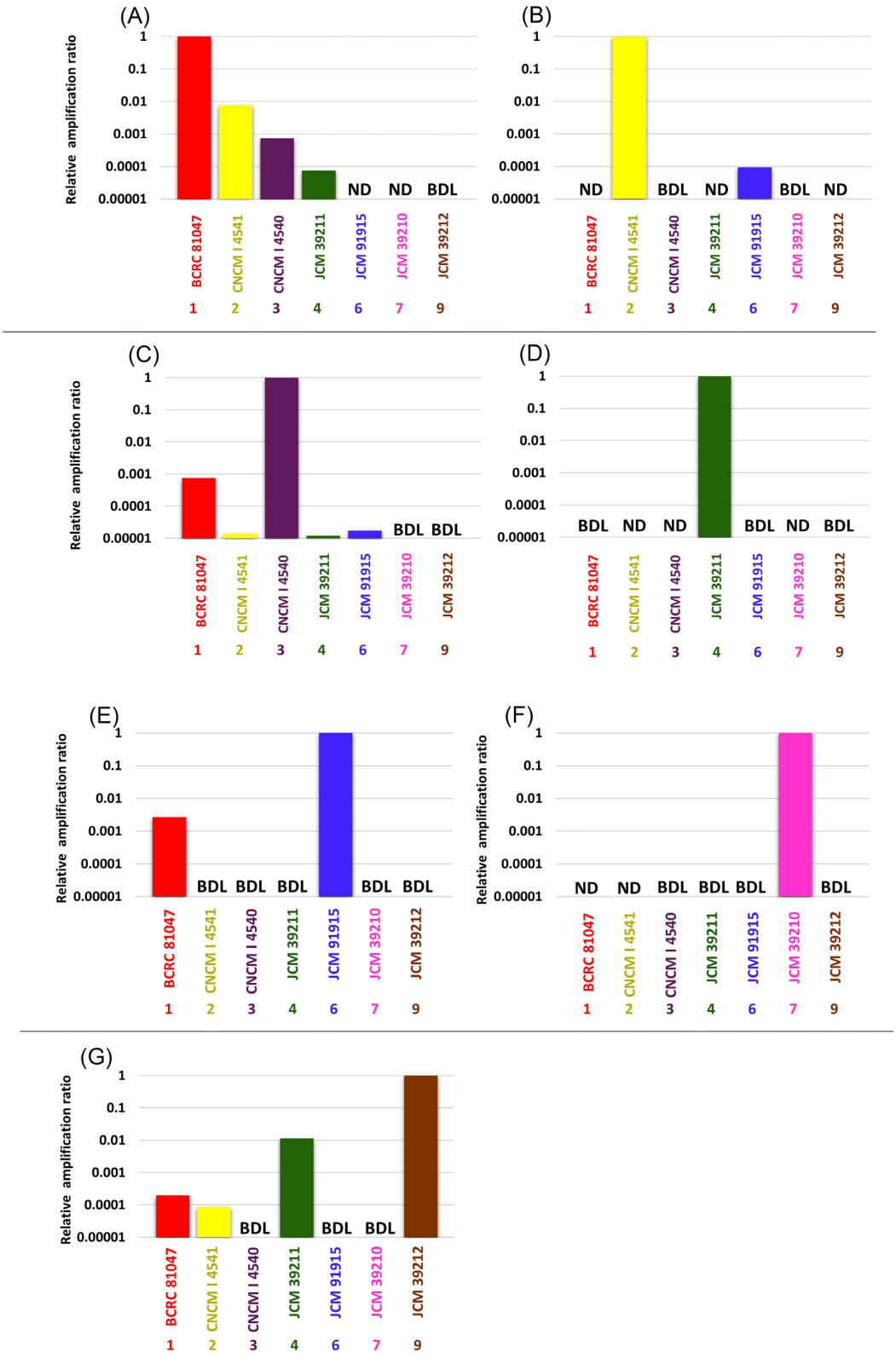


Figure 3. Relative amplification ratio against the targeted group using DNA isolated from cultures and *rpoA*-based group-specific primer pairs. A DNA amount of 10 ng was used in each PCR reaction (**A**) to (**G**) use primer pairs specific to Groups 1–9, respectively. Samples were run in triplicate in the same plate, and the mean and SD were obtained. ND, not detected. BDL, below the detection limit.

Table 2. Relative abundance (%) of each group in infantile stool with or without the addition of the *F. prausnitzii* BCRC 81047^T (Group 1) or *F. duncaniae* JCM 31915^T (Group 6) culture.

	Group								
	1	2	3	4	5	6	7	8	9
Without addition*	BDL	–	BDL	–	–	BDL	–	–	BDL
+ BCRC 81047^T**	100	–	0.2	BDL	–	0.2	–	–	BDL
+ JCM 31915^T***	BDL	BDL	BDL	–	BDL	100	–	–	BDL

–, Not detected; BDL, below detection limit (< $10^{5.6}$ cells/g of feces).
*All groups were not detected or BDL in stool sample without culture addition.
**Relative abundance (%) to Group 1.
***Relative abundance (%) to Group 6.

detection limit) of Groups 1, 3, 6, and 9 (Table 2). After the addition of *F. prausnitzii* BCRC 81047^T (= ATCC 27768^T, Group 1) culture (~10^7 cells) to the stool sample, DNA was isolated and was used for qPCR. The level of Group 1 increased after the culture addition. The relative abundance of Groups 3 and 6 against Group 1 was ~0.2% (Table 2), while other groups were below the detection limit ($10^{5.6}$ cells/g of feces) or were not detected. When a culture of *F. duncaniae* JCM 31915^T (= A2-165^T, Group 6) was added to the stool sample, similar results were obtained. The level of Group 6 increased following the culture addition, whereas other groups were below the detection limit or were not detected (Table 2). The products in the Group 1-speicific qPCR and Group 6-specific qPCR were 100% matched with *rpoA* gene sequences of BCRC 81047^T (accession no. NZ_PXUP00000000.1, LocusTag C7J97_RS08250) in Group 1 and JCM 31915^T in Group 6 (accession number NZ_CP022479.1, LocusTag CG447_RS10630), respectively. Moreover, 16S-based qPCR was applied to quantify the *Faecalibacterium* genus in the infantile stool samples with or without the culture addition, which was compared with the *rpoA*-based qPCR results. The infantile stool was free from the genus *Faecalibacterium* without the culture addition. Cell numbers determined by 16S-based qPCR (as the genus *Faecalibacterium*) increased following the culture addition. The 16S/*rpoA* ratio, calculated using the values obtained from 16S-based genus *Faecalibacterium* qPCR and the sum of *rpoA*-based group-specific qPCR, was 4.5- to 5.3-fold (data not shown). All completely sequenced *Faecalibacterium* strains tested ($n = 21$) possessed six copies of 16S rRNA gene (Table S2, Supporting Information).

Quantification of each *Faecalibacterium* group in healthy adults

The *rpoA*-based group-specific qPCR assays showed marked differences in prevalence and abundance among the groups in the six healthy adults (Fig. 4a). Group 3 was observed in all subjects and was the most abundant group in three (subjects A, D, and E) out of six subjects (Fig. 4b). Group 4 *F. longum* and Group 9 *F. butyricigenerans* were the second most prevalent groups and were detected in five out of six subjects. Group 6 *F. duncaniae* was found in four out of six subjects and was the most abundant group in two (subjects B and F) subjects. Group 1 *F. prausnitzii* and Group 5 were quantified in two out of six subjects. Group 2, Group 7 *F. hattorii*, and Group 8 were not detected or were below the detection limit in all subjects tested. The sum of the relative abundance of the nine groups ranged between 0.152% and 0.759% (median ± standard deviation (SD) = 0.521 ± 0.237%). The relative abundance of the genus *Faecalibacterium* assessed by 16S-based qPCR ranged between 0.86% and 8.48% (median ± SD = 3.37 ± 2.68%, Fig. 4) in the six subjects. The 16S/*rpoA* ratio calculated based on qPCR of the genus *Faecalibacterium* and the sum of the nine specific groups analyzed was between 4.7 and 14.4 (median ± SD = 6.81 ± 3.91).

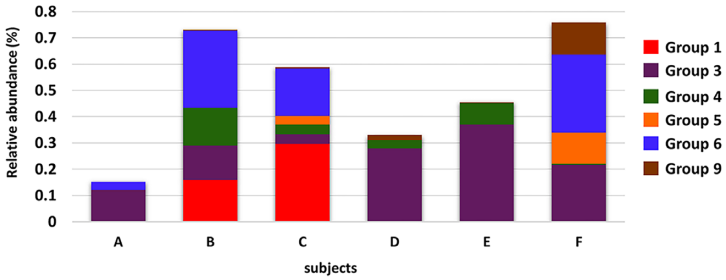
Discussion

Even if *Faecalibacterium* spp. are considered to be of paramount importance for our health status, previous quantification methods of their population contained some issues due to taxonomic concerns and the utilization of a suboptimal genetic marker (i.e. 16S rRNA gene). This fact limited our understanding on the role of these microbes (Tanno et al. 2022). 16S rRNA gene is obviously the best characterized gene for bacterial classification and the study of complex microbiota. It is a reliable marker for more than 97% of bacterial species (Větrovský and Baldrian 2013); however, *Faecalibacterium* spp. belong to the remaining group (Tanno et al. 2022). Therefore, we studied alternative gene markers for the classification and quantification of *Faecalibacterium* spp.

Among the housekeeping genes tested, *recA* gene was the most suitable gene marker for the classification and identification of *Faecalibacterium* spp. because of the lowest intergroup sequence similarities. The *recA* gene is one of 120 bacterial marker genes for suitable phylogenetic inference (Parks et al. 2017) and provides accurate differentiation among phylogenetically related microbes (Pietilä et al. 2000, Torriani et al. 2001, Zbinden et al. 2011). On the other hand, *recA* gene sequences were too divergent within a group to design group-specific primers, whereas *rpoA* was a suitable gene marker to design these primers. qPCR assays targeting housekeeping genes have been applied to quantify bifidobacteria and lactobacilli in the human gut microbiota (Junick and Blaut 2012, Costa et al. 2014). The *rpoA*-based primers designed here, combined with qPCR, accurately quantified the targeted groups with very low cross reactivity (less than 1%), indicating that the qPCR assays developed are useful to quantify *Faecalibacterium* spp. at the group (species) level. Previously reported qPCR assays were unable to quantify *Faecalibacterium* spp. at the species level and relied on the global quantification of the former *F. prausnitzii* sensu lato (Tanno et al. 2022).

In culture-added infantile stool, the 16S/*rpoA* ratio was between 4.5 and 5.3. This was due to different copy numbers between the genes used. A total of six copies of 16S rRNA gene are commonly conserved in a single genome of completely sequenced *Faecalibacterium* spp., whereas *rpoA* is a single copy gene. Therefore, relative abundance assessed by 16S-based qPCR is theoretically 6-fold that evaluated by *rpoA*-based qPCR (the 16S/*rpoA* ratio). Similar findings were reported by Masco et al. (Masco et al. 2007), who quantified bifidobacteria in probiotic products using 16S-based and *recA*-based primer pairs. This bias could be removed if we use DNA from cultures for standard curves based on cellular concentration. However, at the time of analysis (March 2022), strains from

(A)



(B)

	subjects					
	A	B	C	D	E	F
Faecalibacterium genus (16S-based)	0.860	5.782	8.476	3.159	2.591	3.582
Group 1 (*F. prausnitzii*)	BDL	0.160	0.297	BDL	BDL	BDL
Group 2	BDL	BDL	BDL	BDL	BDL	BDL
Group 3	0.121	0.129	0.036	0.279	0.370	0.217
Group 4 (*F. longum*)	ND	0.143	0.038	0.032	0.081	0.004
Group 5	BDL	BDL	0.032	BDL	BDL	0.119
Group 6 (*F. duncaniae*)	0.030	0.295	0.181	BDL	BDL	0.297
Group 7 (*F. hattorii*)	BDL	BDL	BDL	BDL	BDL	BDL
Group 8	ND	BDL	BDL	ND	ND	BDL
Group 9 (*F. butyricigenerans*)	BDL	0.003	0.005	0.020	0.003	0.123
sum of Groups 1 to 9 (*rpoA*-based)	0.152	0.731	0.588	0.331	0.454	0.759
16S/*rpoA* ratio	5.660	7.914	14.40	9.555	5.704	4.718

min max

Figure 4. Relative abundance (%) of each group and the *Faecalibacterium* genus against total bacteria in six healthy adults. Samples were run in triplicate in the same plate, and the mean was obtained. Stacked bars (**A**) indicating the relative abundance of each group (*rpoA*-based) in the six subjects. Colours in the stacked bars indicate Groups. Groups 2, 7, and 8 were not indicated, since they were ND or BDL in all subjects tested. In (**B**), the relative abundance of each group (*rpoA*-based) and the genus *Faecalibacterium* (16S-based) are shown as a heat map, and relative abundance values are indicated. The 16S/*rpoA* ratio was calculated based on the relative abundance of the genus *Faecalibacterium* and the sum of the relative abundance of Groups 1–9. ND, not detected. BDL, below the detection limit.

two out of the nine groups were not available at public culture collections or in our private collections, and, thus, we used synthetic DNA to prepare the standard curve for all groups. DNA extraction from stool samples always includes different extraction efficiencies (Yang et al. 2020), which has significant impacts on quantification of microbes. Standard curves based on synthetic genes are not appropriate to determine absolute cell numbers in stool samples because synthetic genes are free from extraction biases and different amplification efficiencies. However, relative abundance could be evaluated because the values obtained are normalized by the cell numbers of total bacteria.

The *rpoA*-based qPCR was applied to quantify each group of *Faecalibacterium* in six healthy Japanese adults. Group 3 was the most prevalent and abundant group and was detected in all subjects with a relative abundance of 0.173 ± 0.121% (median ± SD), although only a limited number of subjects was included in the present study. Despite its predominance, this group has not yet been taxonomically characterized. Future studies are essential to establish the taxonomic position of this group and to study its potential impact on human health. Genomes of Group 1 (*F. prausnitzii*) and Group 6 (*F. duncaniae*) were abundant in the public database (Figure S1, Supporting Information), indicating that the two groups have been well-isolated and characterized. Groups 1 and 6 contain the reference strain of ATCC 27768^T (type strain of *F. prausnitzii*) and the most characterized *Faecalibacterium* strain of A2-165 (= JCM 31915^T, type strain of *F. duncaniae*), respectively. These two groups were abundant (relative abundance >0.1%) in two or three out of the six adults but were below the detection limit in the other subjects, suggesting that these two groups are abundant in specific adults, but are not prevalent. Since gut microbiota, including a prevalence and abundance of *Faecalibacterium*, are influenced by several factors, such as age and geography of host (De Filippis et al. 2020, Sang et al. 2022), it would be intriguing to study the population of each group of *Faecalibacterium* considering these factors.

The 16S/*rpoA*-ratio of the six subjects was 6.81 ± 3.91 (median ± SD), which was close to the theoretical value of 6, as described above. On the other hand, the ratios of subjects C and D were markedly higher than the theoretical value (Fig. 4b), suggesting that unidentified group(s) of the genus *Faecalibacterium* were predominant in the subjects. A recent study reported the genomic diversity of *Faecalibacterium* using reference *Faecalibacterium* genomes and *Faecalibacterium*-like metagenome-assembled genomes originating from diverse hosts (De Filippis et al. 2020). This study identified 22 different species-level clades, 11 of which were from humans. The 11 clades included the eight groups detected in the present study, but not Group 9 *F. butyricigenerans*. They also characterized the prevalence of clades in patients with specific diseases or obese subjects by comparisons with healthy subjects. However, the findings obtained were qualitative, not quantitative. Even if prevalence is important, quantitative data will provide better insights into the importance of each group in *Faecalibacterium* for human health.

Numerous studies reported an inverse association of *F. prausnitzii* with several disorders, and, thus, it is considered to be a promising biomarker of a healthy microbiota in human (Lopez-Siles et al. 2017). However, the species is no longer a single species and was recently divided into several species (Zou et al. 2021, Sakamoto et al. 2022), making its role as a biomarker unclear. Here, we established a new assay to quantify the nine groups of *Faecalibacterium* in the complex human gut microbiota. Assays for the quantification of the genus *Faecalibacterium* had already been developed (Bartosch et al. 2004, Ramirez-Farias et al. 2009) for the quantification of '*F. prausnitzii* sensu lato'. The combination

of these assays will provide a more detailed understanding of the impact of the *Faecalibacterium* genus and the specific groups on human health. For instance, application of the assays will support to identify deficiencies on specific group(s) in diseased patients, including Crohn's disease and type 2 diabetes. It is also important to reset a real active biomarker of a healthy gut microbiota.

Author contributions

Hiroki Tanno (Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing), Jean-Marc Chatel (Investigation, Methodology, Resources, Validation, Writing – review & editing), Rebeca Martin (Investigation, Methodology, Resources, Validation, Writing – review & editing), Denis Mariat (Investigation, Methodology, Resources, Validation, Writing – review & editing), Mitsuo Sakamoto (Methodology, Resources, Supervision, Validation, Writing – review & editing), Masao Yamazaki (Project administration, Resources, Supervision, Validation, Writing – review & editing), Seppo Salminen (Conceptualization, Investigation, Supervision, Validation, Writing – review & editing), Miguel Gueimonde (Investigation, Methodology, Supervision, Validation, Writing – review & editing), and Akihito Endo (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data are available at FEMSEC online.

Conflicts of interest. None declared.

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Data availability

All genomic data used in the present study were obtained from the NCBI and CNGBdb databases.

References

Auger S, Kropp C, Borras-Nogues E *et al.* Intraspecific diversity of microbial anti-inflammatory molecule (MAM) from *Faecalibacterium prausnitzii*. *IJMS* 2022;**23**:1705.

Balamurugan R, Rajendiran E, George S *et al.* Real-time polymerase chain reaction quantification of specific butyrate-producing bacteria, desulfovibrio and *Enterococcus faecalis* in the feces of patients with colorectal cancer. *J Gastroenterol Hepatol* 2008;**23**:1298–303.

Bartosch S, Fite A, Macfarlane GT *et al.* Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol* 2004;**70**:3575–81.

Borkent J, Ioannou M, Laman JD *et al.* Role of the gut microbiome in three major psychiatric disorders. *Psychol Med* 2022;**52**:1222–42.

Breyner NM, Michon C, de Sousa CS *et al.* Microbial anti-inflammatory molecule (MAM) from *Faecalibacterium prausnitzii* shows a protective effect on DNBS and DSS-induced colitis model in mice through inhibition of NF-κb pathway. *Front Microbiol* 2017;**8**:114.

Costa GN, Marcelino-Guimarães FC, Vilas-Bôas GT *et al.* Potential fate of ingested *Lactobacillus plantarum* and its occurrence in human feces. *Appl Environ Microbiol* 2014;**80**:1013–9.

Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr* 2003;**133**:2485s–93s.

De Filippis F, Pasolli E, Ercolini D. Newly explored *Faecalibacterium* diversity is connected to age, lifestyle, geography, and disease. *Curr Biol* 2020;**30**:4932–43.e4.

Donohoe DR, Holley D, Collins LB *et al.* A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. *Cancer Discov* 2014;**4**:1387–97.

Endo A, Hirano K, Ose R *et al.* Impact of kestose supplementation on the healthy adult microbiota in in vitro fecal batch cultures. *Anaerobe* 2020;**61**:102076.

Endo A, Okada S. Monitoring the lactic acid bacterial diversity during shochu fermentation by PCR-denaturing gradient gel electrophoresis. *J Biosci Bioeng* 2005;**99**:216–21.

Fitzgerald CB, Shkoporov AN, Sutton TDS *et al.* Comparative analysis of *Faecalibacterium prausnitzii* genomes shows a high level of genome plasticity and warrants separation into new species-level taxa. *BMC Genomics* 2018;**19**:931.

Furusawa Y, Obata Y, Fukuda S *et al.* Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 2013;**504**:446–50.

Junick J, Blaut M. Quantification of human fecal bifidobacterium species by use of quantitative real-time PCR analysis targeting the groEL gene. *Appl Environ Microbiol* 2012;**78**:2613–22.

Kida Y, Shimizu T, Kuwano K. Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. *Mol Immunol* 2006;**43**:1972–81.

Kinoshita M, Suzuki Y, Saito Y. Butyrate reduces colonic paracellular permeability by enhancing ppargamma activation. *Biochem Biophys Res Commun* 2002;**293**:827–31.

Larkin MA, Blackshields G, Brown NP *et al.* Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;**23**:2947–8.

Liu XS, Li WG, Zhang WZ *et al.* Molecular characterization of *Clostridium difficile* isolates in China from 2010 to 2015. *Front Microbiol* 2018;**9**:845.

Lopez-Siles M, Duncan SH, Garcia-Gil LJ *et al. Faecalibacterium prausnitzii*: from microbiology to diagnostics and prognostics. *ISME J* 2017;**11**:841–52.

Lopez-Siles M, Martinez-Medina M, Suris-Valls R *et al.* Changes in the abundance of *Faecalibacterium prausnitzii* phylogroups I and II in the intestinal mucosa of inflammatory bowel disease and patients with colorectal cancer. *Inflamm Bowel Dis* 2016;**22**:28–41.

Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett* 2009;**294**:1–8.

Macfarlane GT, Macfarlane S. Bacteria, colonic fermentation, and gastrointestinal health. *J AOAC Int* 2012;**95**:50–60.

Maeno S, Tanizawa Y, Kanesaki Y *et al.* Genomic characterization of a fructophilic bee symbiont *Lactobacillus kunkeei* reveals its niche-specific adaptation. *Syst Appl Microbiol* 2016;**39**:516–26.

Masco L, Vanhoutte T, Temmerman R *et al.* Evaluation of real-time PCR targeting the 16S rRNA and recA genes for the enumeration of bifidobacteria in probiotic products. *Int J Food Microbiol* 2007;**113**:351–7.

Michels N, Zouiouich S, Vanderbauwhede B *et al.* Human microbiome and metabolic health: an overview of systematic reviews. *Obes Rev* 2022;**23**:e13409.

Miquel S, Leclerc M, Martin R *et al.* Identification of metabolic signatures linked to anti-inflammatory effects of *Faecalibacterium prausnitzii*. *Mbio* 2015;**6**.e00300–15

Muñoz M, Ríos-Chaparro DI, Patarroyo MA *et al.* Determining *Clostridium difficile* intra-taxa diversity by mining multilocus sequence typing databases. *BMC Microbiol* 2017;**17**:62.

Naser SM, Dawyndt P, Hoste B *et al.* Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *Int J Syst Evol Microbiol* 2007;**57**:2777–89.

Neumann AP, Rehberger TG. MLST analysis reveals a highly conserved core genome among poultry isolates of *Clostridium septicum*. *Anaerobe* 2009;**15**:99–106.

Parks DH, Rinke C, Chuvochina M *et al.* Recovery of nearly 8000 metagenome-assembled genomes substantially expands the tree of life. *Nat Microbiol* 2017;**2**:1533–42.

Pietilä J, He Q, Oksi J *et al.* Rapid differentiation of *Borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi sensu* stricto by LightCycler fluorescence melting curve analysis of a PCR product of the recA gene. *J Clin Microbiol* 2000;**38**:2756–9.

Quevrain E, Maubert MA, Michon C *et al.* Identification of an anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient in Crohn's disease. *Gut* 2016;**65**:415–25.

Ramirez-Farias C, Slezak K, Fuller Z *et al.* Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. *Br J Nutr* 2009;**101**:541–50.

Rinttilä T, Kassinen A, Malinen E *et al.* Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004;**97**:1166–77.

Sakamoto M, Sakurai N, Tanno H *et al.* Genome-based, phenotypic and chemotaxonomic classification of *Faecalibacterium* strains: proposal of three novel species *Faecalibacterium duncaniae* sp. nov., *Faecalibacterium hattorii* sp. nov. and *Faecalibacterium gallinarum* sp. nov. *Int J Syst Evol Microbiol* 2022;**72**:35416766.

Sang J, Zhuang D, Zhang T *et al.* Convergent and divergent age patterning of gut microbiota diversity in humans and nonhuman primates. *Msystems* 2022;**7**:e0151221.

Sokol H, Seksik P, Furet JP *et al.* Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. *Inflamm Bowel Dis* 2009;**15**:1183–9.

Song H, Yoo Y, Hwang J *et al.* *Faecalibacterium prausnitzii* subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. *J Allergy Clin Immunol* 2016;**137**:852–60.

Stackebrandt E, Ebers J. Taxonomic parameters revisited: tarnished gold standards. *Microbiol Tod* 2006;**33**:152–5.

Takahashi S, Tomita J, Nishioka K *et al.* Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. *PLoS ONE* 2014;**9**:e105592.

Tanno H, Maeno S, Salminen S *et al.* 16S rRNA gene sequence diversity in *Faecalibacterium prausnitzii*-complex taxa has marked impacts on quantitative analysis. *FEMS Microbiol Ecol* 2022;**98**:fiac004.

Torriani S, Felis GE, Dellaglio F. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Appl Environ Microbiol* 2001;**67**:3450–4.

Větrovský T, Baldrian P. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS ONE* 2013;**8**:e57923.

Yang F, Sun J, Luo H *et al.* Assessment of fecal DNA extraction protocols for metagenomic studies. *Gigascience* 2020;**9**:giaa071.

Zbinden A, Köhler N, Bloemberg GV. *recA*-based PCR assay for accurate differentiation of *Streptococcus pneumoniae* from other viridans streptococci. *J Clin Microbiol* 2011;**49**:523–7.

Zhang X, Deeke SA, Ning Z *et al.* Metaproteomics reveals associations between microbiome and intestinal extracellular vesicle proteins in pediatric inflammatory bowel disease. *Nat Commun* 2018;**9**:2873.

Zou Y, Lin X, Xue W *et al.* Characterization and description of *Faecalibacterium butyricigenerans* sp. nov. and *F. longum* sp. nov., isolated from human faeces. *Sci Rep* 2021;**11**:11340.