Quantification of the Flavonoid-Degrading Bacterium *Eubacterium ramulus* in Human Fecal Samples with a Species-Specific Oligonucleotide Hybridization Probe

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To investigate the occurrence of the flavonoid-degrading bacterium *Eubacterium ramulus* in the human intestinal tract, an oligonucleotide probe designated S-S-E.ram-0997-a-A-18 was designed and validated, with over 90 bacterial strains representing the dominant described human fecal flora. Application of S-S-E.ram-0997-a-A-18 to fecal samples from 20 subjects indicated the presence of *E. ramulus* in each individual tested in numbers from 4.4×10^7 to 2.0×10^9 cells/g of fecal dry mass. Six fecal *E. ramulus* isolates were recognized by S-S-E.ram-0997-a-A-18 but exhibited different band patterns when analyzed by randomly amplified polymorphic DNA.

Quercetin is a widespread flavonoid which is of pharmacological interest (4, 7, 21, 27). It is found, usually occurring as a glycoside, in vegetables, fruits, nuts, and tea, and is therefore an integral part of the human diet. The dietary quercetin intake is 16 mg/day (15). It is known that flavonoids are subject to bacterial degradation in the intestinal tract (8, 14). Using quercetin-3-glucoside (isoquercitrin) as a carbon and energy source, Schneider et al. (29) isolated *Eubacterium ramulus* from human fecal dilutions. The organism is able to cleave the flavonoid ring system to 3,4-dihydroxyphenylacetic acid, acetate, and butyrate. However, there is a lack of information on the occurrence and the significance of any of the flavonoid-degrading bacteria in the human intestinal system.

In recent years there has been an increasing effort to de-

TABLE 1. Aligned sequences of the oligonucleotide probe S-S-E.ram-0997-a-A-18 and the 16S rRNA sequences of *E. ramulus* and phylogenetically related organisms[a]

Probe or organism		Sequence	
S-S-E.ram-0997-a-A-18	3′	GGGCCACTGACTTGTACA	5′
Eubacterium ramulus	5′	CCCGGUGACAGAACAUGU	3′
Eubacterium rectale	5′	..UUC....C.GUAC.UA	3′
Eubacterium contortum	5′	..CC....C.GCGU...	3′
Roseburia cecicola	5′	..UUC.....AURU....	3′
Eubacterium uniforme	5′	....A..CA...CUU...	3′
Butyrivibrio fibrisolvens (ATCC 19171)[d]	5′	..A.A...AU..UGGGUA	3′
Butyrivibrio fibrisolvens (strain 49)[b,d]	5′	..UCU.....A.GU....	3′
Butyrivibrio fibrisolvens (OB strains)[d]	5′	..UCU.....A.CU....	3′
Butyrivibrio fibrisolvens (strain BU 43)[e]	5′	..A.A...AUAUCGGGUA	3′
Butyrivibrio fibrisolvens (NCDO 2221)[e]	5′	..A.A...AU..UGGGUA	3′
Butyrivibrio fibrisolvens (NCDO 2222)[e]	5′	..A.A...AUAC.UGGUA	3′
Butyrivibrio fibrisolvens (NCDO 2398)[c,e]	5′	..A.A...AUAC.GGGUA	3′
Butyrivibrio fibrisolvens (NCDO 2399)[e]	5′	...AC.....A.GU....	3′
Butyrivibrio fibrisolvens (NCDO 2434)[e]	5′	..A.A...AUAC.GGGUA	3′
Butyrivibrio fibrisolvens (NCDO 2435)[e]	5′	..A.A...AUA.GUGGUA	3′
Pseudobutyrivibrio ruminis	5′	...AC.....A.GU....	3′
Eubacterium ventriosum	5′	...AC.....GU..GUA	3′
Eubacterium hadrum	5′	..UUC....C.GU.C.UA	3′
Eubacterium fissicatena	5′	...AC....C.GCGUGUA	3′

[a] Nucleotides different from the target sequence are shown. R is an A/G-wobbled nucleotide.
[b] Also strains NCDO 2223, NCDO 2249, NCDO 2397.
[c] Also strain NCDO 2432.
[d] Described by Forster et al. (12).
[e] Described by Willems et al. (35).

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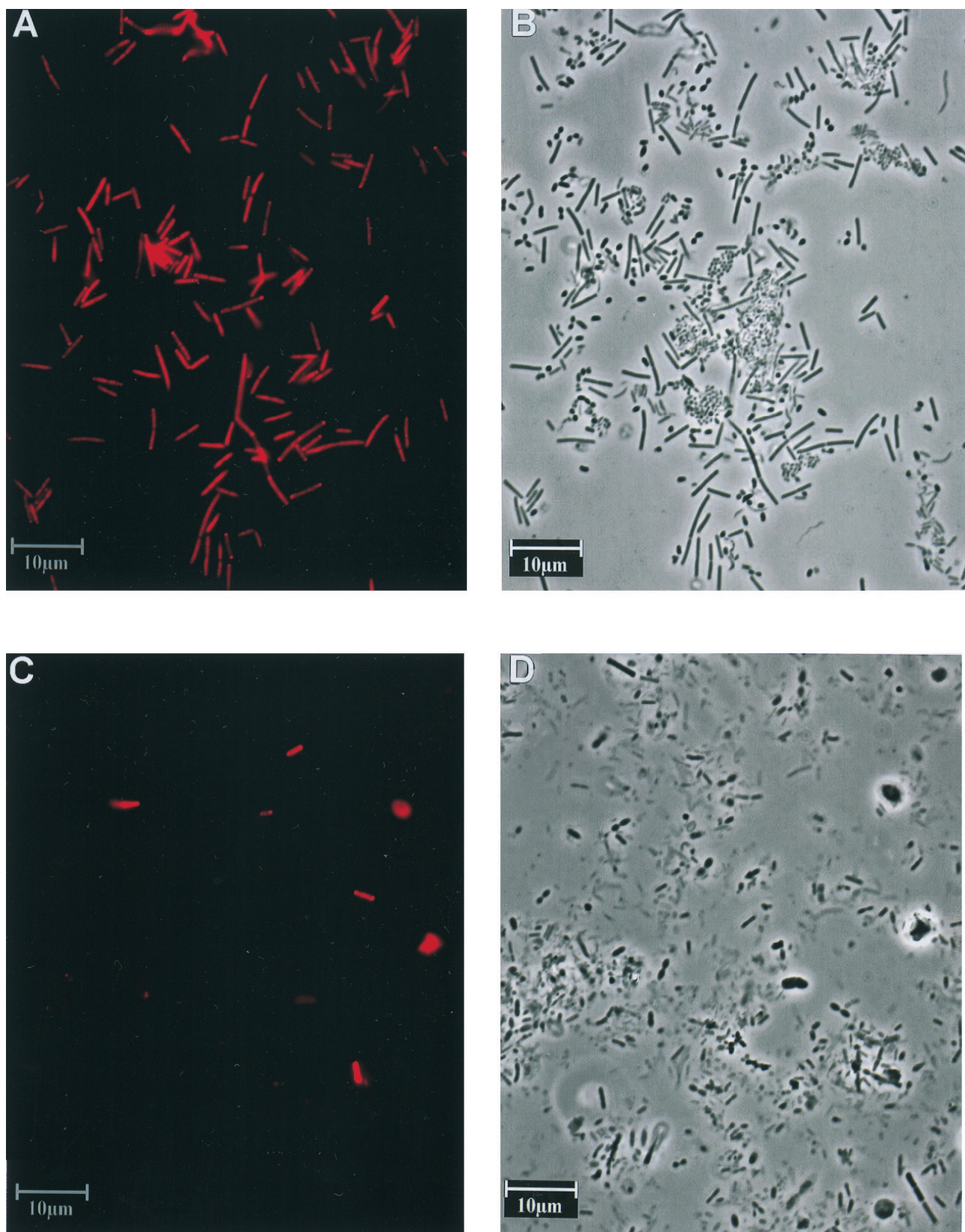
FIG. 1. Epifluorescence and phase-contrast micrographs of a mixed culture and fecal sample. (A and B) Mixed culture of *Butyrivibrio fibrisolvens*, *Eubacterium fissicatena*, *E. ramulus*, *E. rectale*, *Eubacterium uniforme*, and *Streptococcus pleomorphus* hybridized with Cy3-labeled S-S-E.ram-0997-a-A-18. Panels A and B show the same microscopic field as viewed by epifluorescence and phase-contrast microscopy, respectively; (C and D) human fecal sample hybridized with Cy3-labeled S-S-E.ram-0997-a-A-18. Panels C and D show the same microscopic field, viewed by epifluorescence and phase-contrast microscopy, respectively.

TABLE 2. Counts of *E. ramulus* and total bacteria per gram of dry feces in 20 human fecal samples determined by FISH with S-S-E.ram-0997-a-A-18 and the Eub probe mix[a]

Volunteer	Mean cell count (standard deviation)[b]		
	Total bacteria (10^{11}) with Eub probe mix	*E. ramulus* (10^8) with S-S-E.ram-0997-a-A-18	% of total bacteria
DlfEH1	3.34 (0.23)	8.74 (1.02)	0.26 (0.01)
DlfEB1	8.05 (0.92)	12.60 (1.47)	0.16 (0.00)
DlfEL1	5.44 (0.87)	7.82 (1.15)	0.14 (0.00)
DlfEN1	4.84 (0.50)	2.55 (0.44)	0.05 (0.01)
DlfEG1	4.13 (0.19)	3.56 (0.03)	0.09 (0.00)
DlfEA1	4.61 (0.04)	0.44 (0.11)	0.01 (0.00)
DlfEZ1	6.54 (0.06)	12.80 (0.90)	0.20 (0.01)
DlfES1	7.74 (1.67)	6.09 (0.39)	0.08 (0.01)
DlfER1	7.86 (1.48)	10.10 (0.74)	0.13 (0.00)
DlfED1	6.96 (0.06)	16.90 (0.52)	0.24 (0.01)
DlfEA2	1.47 (0.25)	1.10 (0.18)	0.08 (0.02)
DlfEB2	1.55 (0.11)	5.31 (0.04)	0.34 (0.09)
DlfEK1	2.18 (0.36)	3.76 (0.05)	0.17 (0.02)
UNIF1	5.46 (0.06)	3.81 (1.62)	0.07 (0.00)
UNIF2	5.18 (0.61)	8.38 (0.95)	0.16 (0.02)
UNIF3	2.94 (0.32)	1.85 (1.30)	0.06 (0.01)
UNIF4	1.98 (0.17)	1.74 (1.36)	0.09 (0.02)
UNIF7	5.63 (0.04)	20.40 (3.69)	0.36 (0.04)
UNIF10	5.43 (0.32)	11.30 (2.49)	0.21 (0.02)
UNIF15	4.43 (1.52)	1.40 (0.85)	0.03 (0.00)

[a] Kleessen et al. (18).
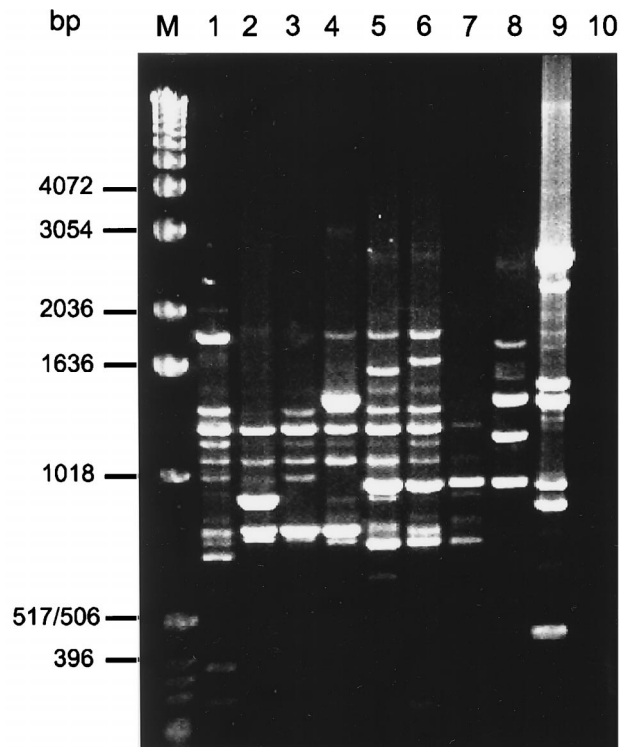[b] The standard deviation was calculated from four enumerations.

FIG. 2. RAPD profiles obtained with genomic DNA of *E. rectale*, *B. fragilis*, and different strains of *E. ramulus* by using the M13-core as the random primer. Lanes: M, molecular mass marker (1-kb ladder; Gibco-BRL); 1, *E. ramulus* ATCC 29099; 2, *E. ramulus* WK1; 3, *E. ramulus* WK5; 4, *E. ramulus* WKBK; 5, *E. ramulus* WKDT; 6, *E. ramulus* WKJN; 7, *E. ramulus* WKRS; 8, *E. rectale*; 9, *B. fragilis*; 10, DNA-free control (H₂O). No amplification products were observed in the absence of template DNA (H₂O control).

scribe complex environments by in situ hybridization with 16S rRNA-targeted oligonucleotide probes (5, 10, 22, 25, 34). The aim of this study was to determine the occurrence of *E. ramulus* in the human intestinal tract by whole-cell hybridization. Therefore, an oligonucleotide probe (S-S-E.ram-0997-a-A-18 [1]) targeting a hypervariable region of the 16S rRNA from *E. ramulus* was designed by using the Arb software package (33), the Check_Probe function of the RDP software package (24), and the EMBL database. Table 1 depicts an alignment of S-S-E.ram-0997-a-A-18 and of the 16S rRNA target sequences of *E. ramulus* and related organisms. The dissociation temperature of S-S-E.ram-0997-a-A-18 as determined according to De Los Reyes et al. (9) was 55.2°C ± 0.3 (mean ± standard deviation).

To ensure the specificity toward the target organism, S-S-E.ram-0997-a-A-18 was checked at 46°C by fluorescent in situ hybridization (FISH) and at 53°C by dot blot hybridization with over 90 reference species (see Appendix). All bacterial species used for validation were grown at 37°C under strictly anoxic conditions with N_2 and CO_2 (80:20 [vol/vol]) as a gas phase (6, 16) in a complex medium (17) or on Columbia blood-agar plates (BioMérieux, Nürtingen, Germany) incubated in anaerobic jars. For whole-cell hybridization, the bacteria were fixed as described elsewhere (3, 28) and hybridized on silanized (23), Teflon-surfaced microscopic slides with 5′-end-Cy3-labeled probes according to the procedure of Roller et al. (28). As a positive control, an equimolar mixture of five bacteria-specific probes (Eub338, Eub785, Eub927, Eub1055, Eub1088 [3, 18]) was used. The fluorescing cells were viewed with either an Optiphot-2 (Nikon, Düsseldorf, Germany) or an Axioplan-2 (Zeiss, Jena, Germany), equipped with filters for epifluorescence microscopy.

The rRNA used for dot blot hybridization was extracted by the procedure of Stahl et al. (32) as modified by Doré et al. (10). Three micrograms of denatured RNA was blotted on positively charged nylon membranes (Boehringer, Mannheim, Germany) by using a Minifold dot blot apparatus (Schleicher & Schuell, Dassel, Germany), cross-linked for 3 min with a UV Stratalinker (Stratagene, La Jolla, Calif.), and hybridized overnight with 5′-end-digoxigenin (DIG)-labeled probes as described by Boehringer, Mannheim, Germany. After the membranes were washed twice at 56°C for 20 min, the DIG-labeled DNA-RNA hybrids were detected with the DIG luminescent detection kit with CSPD (Boehringer). The bacteria-specific probe Eub338 was applied as a positive control (2).

By both techniques S-S-E.ram-0997-a-A-18 hybridized exclusively with the target organism *E. ramulus*. All other organisms tested, including closely related bacteria, such as *Butyrivibrio fibrisolvens* and *Eubacterium rectale*, did not hybridize with S-S-E.ram-0997-a-A-18 (Fig. 1A and B). When the probe was applied to feces, some unspecific binding of the probe to undefined material was observed (Fig. 1C and D). However, this could be easily distinguished from the fluorescent cells. It must be remembered that with oligonucleotide probes in complex ecosystems, unknown bacteria unrelated to the target organism may be detected unspecifically. This, however, applies in general and is not restricted to the probe used here.

To determine the occurrence of *E. ramulus* in the human intestinal tract, S-S-E.ram-0997-a-A-18 was applied to fecal samples. From 20 healthy volunteers aged 23 to 59 years, who consumed a Western diet and had not received any antibiotics at least for 3 months prior to the study, fresh fecal samples were collected and fixed as described above. The cells detected

with the S-S-E.ram-0997-a-A-18 probe were enumerated and related to the bacteria detected with the Eub probe mixture (Table 2). The bacterial cells obtained with the Eub probe mix were between 1.98×10^{11} and 8.05×10^{11} per g of dry feces. *E. ramulus* was detected in the feces of all 20 individuals in numbers ranging from 4.40×10^7 to 2.04×10^9 cells per g of dry feces, corresponding to a mean count of 7.03×10^8 cells per g of dry feces. *E. ramulus* constituted, on average, 0.16% of the total fecal flora, which is approximately equivalent to *Escherichia coli*'s contribution to the fecal flora. Finegold et al. (11) detected *E. ramulus* in fecal samples at counts of 3.9×10^8 cells/g dry feces, but only 1 of 141 subjects was positive for *E. ramulus*. Similarly, Moore and Holdeman (26) detected *E. ramulus* at counts of 2.17×10^9 cells/g dry feces but in only 3 of 23 subjects. Hence, the cell counts determined in our study with the probe S-S-E.ram-0997-a-A-18 are in the same range as in these studies, but there is a major difference in the incidence of *E. ramulus* in humans. This discrepancy may be due to the fact that cultural methods were used in the previous studies and that some bacteria are underestimated because of their failure to grow under standard conditions. We noticed that some *Eubacterium* species did not grow on media that are routinely used for culturing anaerobic gut microorganisms. Langendijk et al. (20) and Doré et al. (10) demonstrated that the abundance of bifidobacteria and *Bacteroides*, respectively, in relation to total bacteria was overestimated when plating was used. This was proposed to be due to an underestimation of the total anaerobes by plating. However, it is also conceivable that the observed variance in the occurrence of *E. ramulus* is due to differences in the genetic background, lifestyle, and diet in the human populations studied.

Schneider et al. (29) tested five subjects, who also participated in this study (Table 2), for the occurrence of isoquercitrin-degrading bacteria in the feces. *E. ramulus* was isolated at dilutions of up to 10^{-9} from all of these subjects. The number of organisms detected with S-S-E.ram-0997-a-A-18 is in good agreement with this figure. It is notable that we were able to detect *E. ramulus* in each person tested so far.

The work of Schneider et al. (29) raised the question of whether these isolates (WK1, WK5, WKBK, WKDT, WKJN, and WKRS) obtained from different individuals were identical or different strains. Since the probe technique cannot discriminate between strains of one species, randomly amplified polymorphic DNA (RAPD) analysis was used to investigate the genetic diversity of these strains. Shianna et al. (31) have shown for the protozoan parasite *Cryptosporidium parvum* and Klein et al. (19) have shown for *Bifidobacterium* strains the discriminatory potential of this method. Bacterial DNA was isolated by following protocol number five of the InViTek DNA isolation kit III (InViTek GmbH, Berlin, Germany). Cells were first incubated for 30 min in 25% sucrose in H_2O, centrifuged as described above, and diluted in 2 ml of lysis buffer D (InViTek). The M13-core (5′ GAG GGT GGC GGT TCT 3′) was used as the primer in the reaction mixture (30), and amplification was done in an Omnigene thermocycler (Hybaid, Middlesex, United Kingdom) by using the following conditions: 2 min at 95°C, followed by 30 cycles at 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 6 min. The PCR products were electrophoresed in a 1% ([wt/vol] 0.5× TBE-agarose gel [1× TBE is 90 mM Tris-borate, 2 mM EDTA [pH 8.0]) stained with ethidium bromide and documented with a video printer connected to a video camera (Biometra, Göttingen, Germany). The RAPD profiles of all isolates (Fig. 2, lanes 2 to 7) differed from that of the type strain. None of the banding patterns of the isolates were identical. Whereas some amplification products (e.g., the

1,250-bp band) occurred with all isolates, other products occurred only with three strains (e.g., the 970-bp band), and some occurred only with one strain (the 896-bp band). For comparison, *E. ramulus* ATCC 29099, *Eubacterium rectale*, and *Bacteroides fragilis* were also analyzed. The banding pattern of the closely related species *E. rectale* also showed no similarity with that of *E. ramulus*. This observation indicates that each human individual tested so far has his or her specific *E. ramulus* strain. It can be speculated that individual differences in the intestinal ecosystem promote the colonization of those strains that are optimally adapted to these conditions. This is similar to the situation described for bovine fecal isolates of *Cryptosporidium parvum* which could be assigned to 16 different strains (31). It therefore appears that a broad range of different strains belonging to one species can be found in fecal samples, illustrating that the intestinal flora is even more complex than previously assumed.

Taken together, the data from our study show that FISH with species-specific oligonucleotide probes is a useful technique to investigate the occurrence of bacteria in fecal samples. The usefulness of this experimental approach has also been shown by Franks et al. (13). In addition, this study provides new information on the occurrence of a fecal bacterium that is capable of quercetin degradation.

APPENDIX

The following 95 reference species were used for FISH and dot blot hybridization: *Acidaminococcus fermentans* (DSM 20731), *Actinobaculum suis* (DSM 20639), *Bacteroides distasonis* (DSM 20701), *B. fragilis* (DlfE05), *B. fragilis* (DSM 2151), *B. galacturonicus* (DSM 3978), *B. merdae* (ATCC 43184), *B. ovatus* (DSM 1896), *B. thetaiotaomicron* (DlfEBaF1), *B. thetaiotaomicron* (DSM 2079), *B. vulgatus* (DlfEBaF2), *Bifidobacterium adolescentis* (ATCC 15703), *B. angulatum* (ATCC 27535), *B. animalis* (ATCC 25527), *B. bifidum* (ATCC 29521), *B. breve* (ATCC 15700), *B. catenulatum* (ATCC 27539), *B. dentium* (ATCC 27678), *B. infantis* (ATCC 15697), *B. infantis* (ATCC 15702), *B. infantis* (ATCC 25962), *B. longum* (ATCC 15707), *B. longum* (ATCC 15708), *Bifidobacterium* sp. (DlfEBiF4), *Bifidobacterium* sp. (DlfEBiF5), *B. thermophilum* (ATCC 25525), *Butyrivibrio fibrisolvens* (DSM 3071), *Clostridium acetobutylicum* (ATCC 824), *C. acetobutylicum* (DSM 792), *C. barati* (DSM 601), *C. bifermentans* (DSM 46282), *C. butyricum* (DlfEClF1), *C. butyricum* (DSM 10702), *C. cellobioparum* (DSM 1351), *C. clostridiiforme* (DSM 933), *C. coccoides* (DSM 935), *C. innocuum* (DSM 1286), *C. pasteurianum* (DSM 525), *C. perfringens* (DlfEClF2), *C. perfringens* (DSM 756), *C. propionicum* (DSM 1682), *C. sartagoformum* (DSM 1292), *C. sordellii* (DSM 2141), *C. sporosphaeroides* (DSM 1294), *C. tyrobutyricum* (DSM 633), *C. xylanolyticum* (DSM 6555), *Coprococcus catus* (ATCC 27761), *C. eutactus* (ATCC 27759), *Enterococcus casseliflavus* (DlfEEnF1), *E. durans* (DSM 20633), *E. faecalis* (DSM 20478), *E. faecium* (DSM 20477), *E. hirae* (DSM 20160), *Escherichia coli* (DlfEEsF1), *E. hermannii* (ATCC 33650), *Eubacterium aerofaciens* (DSM 3979), *E. barkeri* (ATCC 25849), *E. biforme* (DSM 3989), *E. contortum* (DSM 3982), *E. cylindroides* (ATCC 27528), *E. cylindroides* (DSM 3983), *E. dolichum* (DSM 3991), *E. eligens* (DSM 3376), *E. fissicatena* (DSM 3598), *E. hadrum* (DlfEEuF1), *E. lentum* (DSM 2243), *E. limosum* (DSM 20543), *E. rectale* (ATCC 33656), *E. tenue* (DSM 20695), *E. tortuosum* (DSM 3987), *E. uniforme* (ATCC 35992), *E. ventriosum* (ATCC 27560), *Fusobacterium mortiferum* (ATCC 25557), *F. naviforme* (DSM 20699), *F. necrogenes* (ATCC 25556), *F. nucleatum* (DSM 20482), *F. varium* (ATCC 8501), *Lactobacillus acidophilus* (DlfELaF1), *L. acidophilus* (DSM 20079), *L. fermentum* (DSM 20052), *L. gasseri* (DSM 20243), *L.*

murinus (DSM 20452), *L. plantarum* (DSM 20174), *L. reuteri* (DSM 20016), *Megasphaera* sp. (DlfEMeF3), *Peptostreptococcus anaerobius* (DSM 2949), *P. asaccharolyticus* (DSM 20463), *P. prevotii* (DSM 20548), *Prevotella melanogenica* (ATCC 25845), *Pseudoramibacter alactolyticus* (DSM 3980), *Ruminococcus hansenii* (DSM 20583), *R. productus* (DSM 2950), *Streptococcus pleomorphus* (DSM 20574), *Veillonella parvula* (DSM 2008), and *Veillonella* sp. (DlfEV257).

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