Composition of human intestinal flora analysed by fluorescent in situ hybridisation using group-specific 16S rRNA-targeted oligonucleotide probes

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Original article

Composition of human intestinal flora analysed by fluorescent *in situ* hybridisation using group-specific 16S rRNA-targeted oligonucleotide probes

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Abstract – Cultivation methods classically used to describe the faecal flora composition are often too selective for certain groups of bacteria. This study was conducted to determine the human faecal flora composition by fluorescent *in situ* hybridisation, a direct and culture-independent method. Four group-specific probes and a domain probe Eub 338 targeting the 16S rRNA were used to analyse fixed faecal bacterial suspensions from nine healthy adult volunteers. Epifluorescence microscopy and image analysis were performed to evaluate the relative proportion of cells from each group. After optimisation of hybridisation conditions, the reproducibility of the protocol was evaluated to validate the FISH procedure. The domain probe Eub 338 labelled an average of $80 \pm 11\%$ total faecal bacteria. The panel of four probes revealed more than 75% of the flora. The *Clostridium coccoides-Eubacterium rectale* group was the most represented, accounting for $36 \pm 7\%$ of the bacteria. The three other probes used, Bacto 1080, Bif 164 and Fprau 645 labelled $17 \pm 5\%$, $15 \pm 9\%$ and $23 \pm 5\%$ of the total flora, respectively. The two dominant groups belonging to *Clostridium* and relatives constituted nearly 60% of the total flora. FISH coupled with image analysis is a direct and powerful molecular tool to assess the composition of the human faecal flora.

fluorescent hybridisation / 16S rRNA probe / intestinal flora / image analysis / microscopic counts

Résumé – Composition de la microflore intestinale humaine analysée par hybridation *in situ* avec des sondes fluorescentes ciblant l'ARN 16S. Les méthodes de culture classiquement utilisées pour étudier la composition bactérienne au sein de la flore fécale de l'homme sont souvent inadaptées car trop sélectives. Cette étude a pour but d'analyser la composition de la flore intestinale par une méthode

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moléculaire utilisant des sondes oligonucléotidiques fluorescentes ciblant les ARNr 16S. Une sonde de domaine Eub 338 ainsi qu'un ensemble de quatre sondes ciblant des groupes dominants de cet écosystème ont été utilisés. Les sondes spécifiques marquent les groupes phylogénétiques suivants : *Bacteroides-Porphyromonas-Prevotella, Clostridium coccoides-Eubacterium rectale, Fusobacterium prausnitzii* et apparentés ainsi que le genre *Bifidobacterium.* Elles ont hybridé sélectivement avec leurs bactéries cibles préalablement fixées avec une solution de paraformaldéhyde à 4 % puis rendues perméables par incubation en présence de lysozyme à 1 mg/mL. Le marquage des sondes par le Cy3 a permis une visualisation des bactéries en microscopie en épifluorescence. Après optimisation des conditions d'hybridation, la reproductibilité de la méthode a été évaluée sur des échantillons de selles afin de valider l'ensemble de la procédure. Dans toutes les suspensions fécales, 80 ± 11 % des cellules présentes ont été hybridées avec la sonde de domaine Eub 338. L'additivité obtenue avec l'ensemble des quatre sondes de groupe a toujours été supérieure à 75 % pour les neuf individus étudiés. Le groupe *Clostridium coccoides-Eubacterium rectale* était le plus important puisqu'il représentait en moyenne 36 % des cellules bactériennes de la microflore intestinale. Les trois autres sondes, Bacto1080, Bif 164 et Fprau 645 ont marqué respectivement 17 ± 5 % , 15 ± 9 % and 23 ± 5 % de la flore fécale. Les deux groupes dominants mis en évidence par les sondes Erec 482 et Fprau 645 regroupaient à eux seuls près de 60 % de la totalité des bactéries présentes. De plus, la sonde Fprau 645 a permis de mettre en évidence des cellules présentant une morphologie particulière. L'hybridation *in situ* couplée à l'analyse d'images avec un ensemble de quatre sondes ciblant les groupes dominants de la microflore fécale de l'homme est une méthode d'étude directe et puissante. Elle permet le classement des bactéries en grands groupes ainsi que la description de leurs morphologies. L'utilisation de sondes de spécificité plus restreinte permettra une identification plus fine au niveau de l'espèce et rendra possible la mise en évidence des populations moins représentées.

hybridation *in situ* / sonde ARNr 16S / microflore intestinale / analyse d'images / numération en microscopie

1. INTRODUCTION

The human intestinal microflora represents a complex bacterial ecosystem, mainly composed of obligate anaerobic bacteria [4, 10]. Bacterial cell densities vary along the gastrointestinal tract and reach 10^{11} per gram of intestinal contents in the distal colon. The composition and functioning of the intestinal microflora have a large impact on human health, through their role in nutrition, protection against pathogens and immunology [7,11,12]. Conventional analysis of the intestinal microflora is based on the cultivation of bacteria using anaerobic media. However, direct analysis revealed that the cultivation approach is not representative of the composition of the faecal microflora. Indeed, many bacteria are difficult to culture or non-cultivable [20], and media are not truly specific or too selective for certain micro-organisms [16]. More reliable methods are needed for an objective evaluation of the gut flora composition. In recent years, considerable effort has been devoted to applying molecular techniques to

the identification of specific bacterial groups in complex ecosystems [2,3,19], in a culture-independent way. Whole-cell hybridisation with fluorescently labelled, 16S rRNA-targeted oligonucleotide probes allows the direct detection of single cells within ecosystems. The succession of highly conserved and more variable regions in the 16S rRNA molecule allows the design of universal-, domain-, genus- or species-targeted probes [1,3,13,18,23]. Fluorescent *in situ* hybridisation (FISH) has been used to detect bacteria from various ecosystems [2,8,9] at different phylogenetic levels depending on the probes selected. In the present study, we have used 16S rRNA-targeted oligonucleotide probes to determine the composition of the faecal flora of healthy humans. The domain-probe Eub 338 was used with a panel of four group-specific probes which target the dominant groups of the gut flora.

2. MATERIALS AND METHODS

2.1. Faecal samples

Nine healthy human volunteers, five women and four men, aged 22–65 years, gave their written informed consent to enter the study. All of these donors were on a normal western European diet. None had received antimicrobial treatment for at least 3 months prior to the study and none showed symptoms of gastrointestinal disorders. Fresh faecal samples were collected in sterile vessels and kept at 4 °C for less than 12 h before processing. Stools were homogenised by mechanical kneading for 3 min. Aliquots of 0.5 g (wet weight) of faecal specimen were added to 4.5 mL of sterile Phosphate Buffer Saline (PBS) containing 3 to 5 glass beads (4 mm diameter). The suspension was vortexed for 3 min and centrifuged at 700 g for 1 min to remove large particles.

2.2. Bacterial strains and culture conditions

The reference strains used in this study were obtained from different sources: the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany for *Ruminococcus productus* (DSMZ 2950) or the American Type Culture Collection (ATCC), Rockville, Md., USA for *Bacteroides vulgatus* (ATCC 8482), *Clostridium bifermentans* (ATCC 638), *Bifidobacterium longum* (ATCC 15707T) and *Lactobacillus acidophilus* (ATCC 4356). Cells were grown at 37 °C under strictly anoxic conditions with 100% CO_2 as a gas phase in the complete medium (MC) described by Leedle and Hespell [14], except for *L. acidophilus* which was cultivated in MRS medium. Cells were collected from 5 mL cultures in mid-log phase to maximise the rRNA content. The cell suspensions were centrifuged for 3 min at 3000 g, washed once and resuspended in 1 mL of PBS (pH 7,2).

Table I. Probes used in this study.

Probe	Target	Sequence from 5'to 3'end	OPD code	Reference
Eub 338	338-355	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18	Amann *et al.* [1]
Bacto 1080	1080-1097	GCACTTAAGCCGACACCT	S-*-Bacto-1080-a-A-18	Doré *et al.* [3]
Bif 164	164-181	CATCCGGCATTACCACCC	S-G-Bif-0164-a-A-18	Langendijk *et al.* [13]
Erec 482	482-500	GCTTCTTAGTCARGTACCG	S-*-Erec-0482-a-A-19	Franks *et al.* [5]
Fprau 645	645-667	CCTCTGCACTACTCAAGAAAAAC	S-*-Fprau-0645-a-A-23	This study

R is an (A/G) wobble nucleotide.

2.3. Fixation of samples and storage

One volume of the suspension (from collection strains and faecal samples) was diluted with 3 volumes of 4% paraformaldehyde (PFA) in PBS (pH 7.2), fixed at 4 °C overnight, washed twice in PBS (pH 7.2) and stored in 50% (v/v) ethanol/PBS at –20 °C until use.

2.4. Oligonucleotide probes and specificity testing

The five 16S rRNA-targeted oligonucleotide probes used in this study are listed in Table I. They were covalently linked with indocarbocyanine-3 (Cy3) at the 5′-end and were purchased from Interactiva, Saint Malo, France. They comprised a domain-specific probe for Bacteria, a genus-specific probe for *Bifidobacterium*, and three probes targeting the *Bacteroides-Prevotella-Porphyromonas*, the *Clostridium coccoides* and the *Fusobacterium prausnitzii* groups. The validation of probes Bacto 1080, Bif 164 and Erec 482 was performed by hybridisation experiments with a target strain and a non-target strain (Tab. II). The target strain had 100% sequence homology with the probe target region and the non-target strain presented at least three mismatches inside this region. Hybridisation specificity was optimised by testing several concentrations of formamide (FA) (from 0 to 45%) in the hybridisation buffer to increase the stringency of the annealing reaction.

2.5. Enumeration of bacteria in faecal samples by FISH

Fixed samples were washed twice in sterile PBS (pH 7.2). Pellets of faecal samples were resuspended in 0.5 mL of a 1 mg/mL egg white lysozyme (Serva, Heidelberg, Germany) in 100 mM Tris-HCl (pH 8), 50 mM EDTA and incubated for 10 min at room temperature to permeabilise bacterial cells. Cells were washed twice in 1 mL PBS (pH 7.2) and hybridised for 3 h at 46 °C in 20 μL of hybridisation buffer. The hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS, 15% FA) contained 3 ng·μL^{-1} of fluorescent probe. After centrifugation for 3 min at 5000 g, cells were washed in 200 μL of 0.32 M NaCl, 20 mM Tris-HCl, 0.1% SDS prewarmed to 48°C and then incubated for 20 min at 48°C. After washing, cells were resuspended in 10 to 20 μL of PBS (pH 7.2) according to the size of the pellet. Finally, 5 μL of each sample was spread onto 10-well gelatinised slides (Polylabo, Strasbourg, France), dried at 37°C and dehydrated by successive immersions in a graded ethanol series (50, 80 and 96% (v/v)) for 3 min each. The slides were then mounted with Citifluor (Citifluor Ltd, Canterbury, United Kingdom) supplemented with 220 mM 4′,6-Diamidino-2-Phenylindole Dihydrochloride (Dapi, Sigma, St Quentin Fallavier, France). Image capture was performed with a BX 40 Olympus epifluorescence microscope fitted with a mercury arc lamp (HBO

Table II. 16S rRNA-targeted probes used in this study and the corresponding sequences for target and non-target reference strains.

Probe	Sequence
S-*-Bacto-1080-a-A-18	3′ T C C A C A G C C G A A T T C A C G 5′
Target	5′ A G G U G U C G G C U U A A G U G C 3′
Bacteroides vulgatus (ATCC 8482)	. . . . .
Lactobacillus acidophilus (ATCC 4356)	. . A . . . U . . G . . . . . C .
S-G-Bif-0164-a-A-18	3′ C C C A C C A T T A C G G C C T A C 5′
Target	5′ G G G U G G U A A U G C C G G A U G 3′
Bifidobacterium longum (ATCC 15707)	. . . . .
Lactobacillus acidophilus (ATCC 4356)	A . . . . C . . . . A . . . . . A
S-*-Erec-0482-a-A-19	3′ G C C A T G R A C T G A T T C T T C G 5′
Target	5′ C G G U A C Y U G A C U A A G A A G C 3′
Ruminococcus productus (DSM 2950)	. . . . .
Clostridium bifermentans (ATCC 638	. . . . . G G . G . . . . .

100W/2, Osram, Micromécanique, Évry, France), a 100 (OI UplanApo) immersion objective (Micromécanique, Évry, France), two filter blocks: U-MWU for Dapi excitation and U-MWG for Cy3 excitation. A Peltier cooled LH 750 LL charge-coupled device video camera (Lhesa électronique, Cergy-Pontoise, France) was connected to the microscope. The image analysis software used was Optimas 6.2 (Imasys, Suresnes, France). For probe validation experiments, 10 fields were captured for each condition. This represented 500 to 1000 counted cells. Fluorescence intensities were measured on a 0 to 255 grey level scale. For the other experiments, each selected microscopic field was first excited to observe Cy3 fluorescence, instantaneously captured as an F-image, and then excited for Dapi fluorescence observation and captured as a D-image. Five to ten fields (200 to 2000 counted cells) were treated for each probe. Each sample was analysed in triplicate with each probe. Results are expressed as percentages with standard errors. The reproducibility of the procedure was evaluated by repeating the FISH protocol using 16 aliquots of the same homogenised faecal sample.

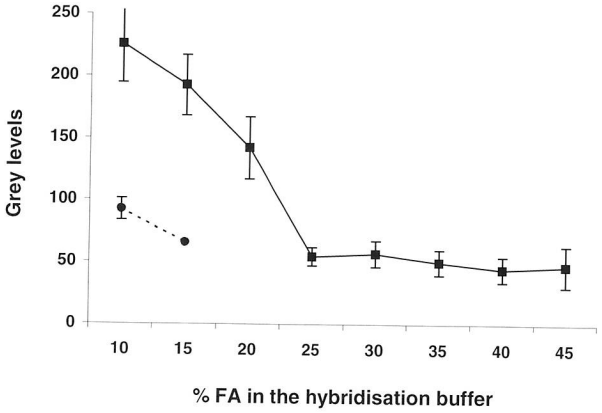

Figure 1. Denaturation curve obtained with the Erec 482 probe. Target strain was *Ruminococcus productus* (■) and non-target strain was *Clostridium bifermentans* (•) presenting three mismatches within the probe target sequence. Percentage of added formamide (FA) was increased from 0 to 45. For each hybridisation condition, means were calculated for 500 to 1000 counted cells.

3. RESULTS

3.1. Optimisation of hybridisation conditions

The specificities of the fluorescently labelled probes used in this study were experimentally determined by whole-cell hybridisation on target and non-target strains. Results obtained with the Erec 482 probe hybridised to *Ruminococcus productus* and *Clostridium bifermentans* are shown in Figure 1. The higher intensities of fluorescence (proportional to the grey levels) were observed with the target strain *R. productus* when the hybridisation buffer contained 10 and 15% formamide (FA). Under the same conditions, the Erec 482 probe weakly hybridised to the non-target strain *C. bifermentans*. The condition with 15% FA was also found to be optimal for the probes Bacto 1080 and Bif 164 (data not shown). The target strain for the Fprau 645 probe, *Fusobacterium prausnitzii*, is not available in culture collections, so we could not determine the optimal hybridisation condition for this probe. As 15% FA was the optimal condition for all of the probes tested, it was selected for subsequent hybridisation experiments.

3.2. Reproducibility of the FISH treatment

In order to evaluate the FISH protocol, a fresh faecal sample was diluted and dispensed in 16 different tubes for PFA fixation, storage and further analysis. Hybridisation experiments and image analysis were performed separately for each subsample. The fixation, permeabilisation and hybridisation steps of the

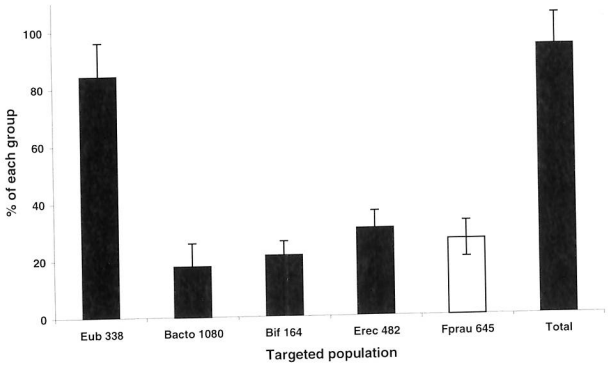

Figure 2. Analysis of the predominant groups and their variations within the same faecal flora. Blocs and bars represent means and standard error for 16 experiments performed using 16 aliquots of a single homogenised faecal sample.

protocol were reproducible, as indicated by results obtained with the domain probe Eub 338 which hybridised with more than 70% of the Dapi-stained bacteria (from 71 to 102%, mean $84 \pm 12\%$) (Fig. 2). This indicated that the protocol was efficient for both Gram-negative and Gram-positive cells. Moreover, the composition of the faecal sample studied was equally reproducible. Firstly, the sum of the four groups labelled with the panel of specific probes was very close to 100% ($94 \pm 11\%$). Secondly, the proportions of the four groups were close to 20% for probes Bacto 1080 ($18 \pm 8\%$) and Bif 164 ($21 \pm 5\%$) and to 30% with probes Erec 482 ($30 \pm 6\%$) and Fprau 645 ($26 \pm 6\%$). The standard deviations were below 12%.

3.3. Analysis of the faecal microfloras of nine healthy donors

When Cy3-labelled probes were used, a low level of background and autofluorescence was observed with the nine faecal samples analysed. Epifluorescence micrographs of an adult (age 25 years) faecal sample hybridised with the domain probe Eub 338 and the panel of specific probes are shown in Figure 3. Most of the bacterial cells visualised by Dapi staining were fluorescently labelled with the domain probe Eub 338 (Fig. 3, panels a and b). A large range of hybridisation signal intensities was observed with this general probe. Hybridisation of faecal samples with the Bacto 1080 probe showed a low level of fluorescence (Fig. 3, panel d). However, the fluorescence signal observed with this probe was sufficient to identify bacterial cells of this group. Conversely, high levels of fluorescence were detected with the group-specific probes Erec 482 and Fprau 645, and with the genus-specific probe Bif 164. With the probes Bacto 1080 and Bif 164, rod-shaped bacteria were hybridised, whereas rod-shaped and coccoid cells were detected with Erec 482. Interestingly, a typical morphology was consistently evidenced with Fprau 645, similar to an asymmetrical double droplet. The nine faecal samples were treated in triplicate with

Table III. Composition of the faecal flora of nine healthy human volunteers.

Donor	Eub 338[1]	Bacto 1080	Bif 164	Erec 482	Fprau 645	Total[2]
1	88	13	7	33	22	75
2	63	18	16	27	27	88
3	83	18	5	52	20	96
4	85	23	8	42	27	99
5	69	13	16	36	19	86
6	90	23	8	37	32	100
7	74	16	26	36	22	100
8	91	10	26	35	16	89
9	67	23	24	29	26	102
Range	63-91	10-23	5-26	27-52	16-32	75-102
Mean	79	17	15	36	23	93
SD	11	5	9	7	5	9

[1] Results obtained with the Eub 338 probe are expressed as percentages of total cells visualised after Dapi staining. The results with the group-specific probes are expressed as percentages of total Eub 338 positive cells.
[2] The total represents the addition of percentages obtained with the four group-specific probes. SD is the standard deviation.

the 5 Cy3-probes. Faecal flora composition assessed by FISH is presented in Table III. In general, 80% of the Dapi-positive cells hybridised to the Eub 338 probe. A proportion of 75 to 102% of the bacterial cells of the different faecal microflora was detected using the four specific probes. For five donors the sum of the percentages obtained with the 4 group-specific probes was close to 90%. Donor 1 had the lowest sum of bacterial cells detected with these probes (75%). Inter-individual variations obtained with the group-specific probes Bacto 1080, Erec 482 and Fprau 645 were quite moderate. In contrast, the percentage of bacteria detected with the Bif 164 probe varied from 5 to 26% of the total bacteria. Donor 3 had the lowest proportion with Bif 164 and the highest proportion with Erec 482.

4. DISCUSSION

In situ hybridisation was developed to describe directly the structure of the human faecal flora. A set of five fluorescent probes was applied in whole cell hybridisation to detect and enumerate bacterial cells from faecal samples of healthy humans.

The Cy3-Eub 338 probe labelled a mean of 80% of cells from the total flora assessed by Dapi staining. This showed that the permeabilisation protocol was

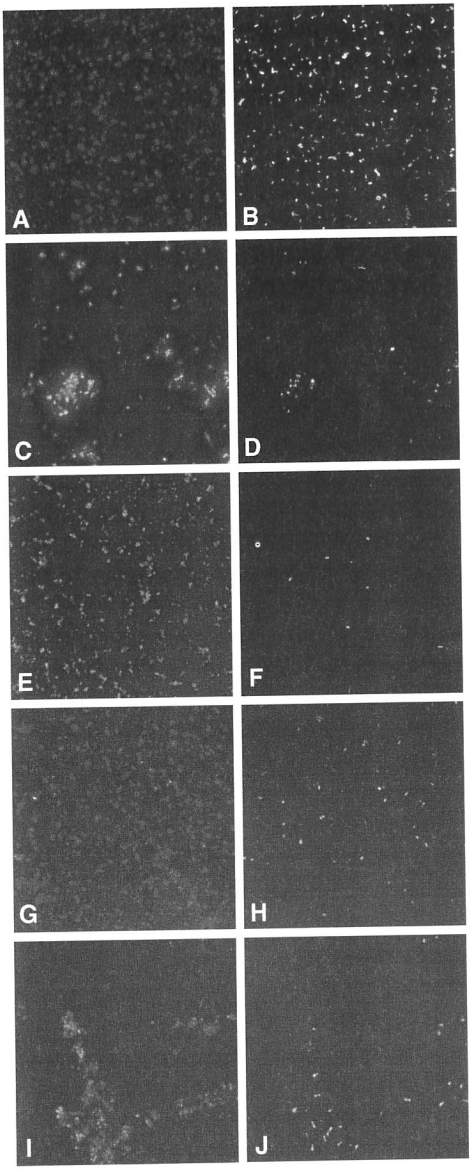

Figure 3. Whole-cell hybridisations of fixed cells from human faecal flora. Left panels (a, c, e, g, i) show Dapi fluorescence of total cells, right panels (b, d, f, h, j) show Cy3-fluorescence of group-specific probe. The two images on the same level show the same microscopic field. (b) Cells hybridised with Eub 338 probe. (d) Cells hybridised with Bacto 1080 probe. (f) Cells hybridised with Bif 164 probe. (h) Cells hybridised with Erec 482 probe. (j) Cells hybridised with Fprau 645 probe. Hybridisations were as described in materials and methods.

well adapted to the study of bacteria from the faecal ecosystem. In particular, it allowed an efficient labelling of the three groups of Gram-positive bacteria, known to be more difficult to detect than Gram-negatives [2]. The ribosome content of faecal bacteria was sufficient to detect cells with Cy3 probes targeting only one region of the 16S rRNA. As observed by Wessendorf and Bretje [21], the probes coupled with Cy3 gave the strongest signals and the lowest autofluorescence background. Indeed, fluorescein and rhodamine were previously tested, but weaker fluorescence signals and higher autofluorescence were observed (data not shown). The combination of horseradish peroxidase (HRP)-labelled probes and the substrate fluorescein-tyramide [17] was also tested as an amplification system. A strong increase in fluorescence intensities was observed but a lower percentage of bacteria labelled with the HRP-Eub 338 probe was detected (data not shown). The difficulty in entering bacterial cells for these large-molecular-weight probes experience may account for the lower counts. Consequently, we selected probes coupled with Cy3 for the subsequent analysis.

The fluorescence level obtained with the Cy3-labelled Bacto 1080 probe was relatively low compared to those observed with the other probes. A low accessibility of the region targeted by the Bacto 1080 probe could be responsible for this lower intensity. This was consistent with the study of Fuchs *et al.* [6] where the accessibility of 16S rRNA target sites was evaluated for *Escherichia coli.* In *E. coli* 16S rRNA, the region targeted by the Bacto 1080 probe had a low accessibility [6]. Interestingly, high fluorescence intensities were obtained with the Erec 482 and Fprau 645 probes, while the regions targeted on the rRNA molecule of *E. coli* were equally described as poorly accessible [6]. Differences in the 16S rRNA secondary and tertiary structures between Gram-positive and Gram-negative bacteria could explain these results. A study on the accessibility of 16S rRNA target sites for a Gram-positive organism would help to clarify this point.

Our *in situ* analysis allowed a global subdivision of the bacteria from the faecal ecosystem into four main groups. Species belonging to these four groups were described as dominant when the culture approach was applied [4]. Using these probes, we were able to detect from 75% to 102% of the bacterial cells from the nine samples studied. The major group within the total flora (mean of 36%) was that visualised with the Erec 482 probe, assigning bacteria to the *Clostridium coccoides-Eubacterium rectale* cluster. The minor and more variable group (mean of 15% with a range of 5 to 26%) was detected with the Bif 164 probe. Seventy percent of cells were detected when proportions obtained with the probes Erec 482, Bif 164 and Bacto 1080 were added. Franks *et al.* [5] detected a similar proportion of *Clostridium coccoides-Eubacterium rectale* members (29%) when using the probe Erec 482, but only 3% of *Bifidobacteria* were observed with the Bif 164 probe. Our higher counts with this probe could be due to a higher number of *Bifidobacterium* spp. in the faecal samples analysed. It was also observed that the variation in the percentage of cells from the *Bifidobacterium* genus was quite large in the study of Franks *et al.* The

combination of the Bfra 602 and Bdis 656 probes (respectively specific for the *Bacteroides fragilis* group and for the species *Bacteroides distasonis*) with the Erec 482 and Bif 164 probes detected 53% of bacterial cells. A greater additivity in the present study can be attributed firstly to a higher content of *Bifidobacterium*, and secondly to the use of the Bacto 1080 probe, which allows the estimation of cells belonging to the *Bacteroides, Prevotella* and *Porphyromonas* genera. The second more represented group, targeted by the Fprau 645 probe, accounted for 23% of cells from the faecal flora. The Fprau 645 probe recognises bacteria phylogenetically clustered with the species *Fusobacterium prausnitzii* and belonging to the *Clostridium leptum* subgroup. In recent molecular studies based on the direct analysis of cloned 16S rDNA amplified by PCR, 20% [19] and 50% [22] of the clones belonged to the *Clostridium leptum* subgroup. Furthermore, *Fusobacterium prausnitzii* was found to be the second most frequently cultivated faecal micro-organism by Moore and Holdeman [15]. Our results are consistent with these findings, although we cannot exclude non-specific detection of bacteria, considering the optimisation protocol of this probe. Compared to other molecular methods such as dot blot hybridisation [3] and PCR [19, 22] used to study gut microbial ecology, whole-cell hybridisation has the major advantage of characterising the morphology of bacteria. In particular, the Fprau 645 probe hybridised to a majority of cells forming asymmetrical double droplets. To our knowledge, this morphology has not yet been observed with cultivated cells belonging to this cluster.

In this study, probes targeting four groups of intestinal bacteria were used to obtain a direct and global description of the faecal flora. The relative importance of the dominant fractions of the ecosystem were evidenced and were morphologically discriminated. In the future, the design of probes intended to target groups at a species level would be useful for a better *in situ* description of bacteria from the faecal ecosystem.

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