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Comparison of culture-dependent and independent approaches to characterize fecal bifidobacteria and lactobacilli

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ABSTRACT

Different culture-dependent and independent methods were applied to investigate the population of bifidobacteria and lactobacilli in the feces of five healthy subjects. Bacteria were isolated on MRS, a complex medium supporting growth of lactobacilli and bifidobacteria, and on three selective media for bifidobacteria and two for lactobacilli. Taxonomic characterization of the isolates was carried out by RAPD-PCR and partial 16S sequencing. The selectivity of genus-specific media was also investigated by challenging colonies from MRS plates to grow onto each medium. In parallel, a quantitative and qualitative description of bifidobacteria and lactic acid bacteria was obtained by FISH, qPCR, TRFLP, and 16S rRNA gene sequencing.

Bifidobacteria did not fail to grow on their specific media and were easily isolated and enumerated, showing comparable quantitative data among culture-dependent and -independent techniques. The *Bifidobacterium* species identified on plates and those extracted from TRFLP and 16S rRNA gene sequencing were mostly overlapping.

Selective media for lactobacilli gave unsuitable results, being too stringent or too permissive. The quantification of lactobacilli through selective plates, qPCR, FISH, and 16S rRNA gene sequencing gave unreliable results. Therefore, unlike bifidobacteria, intestinal lactobacilli are still problematic in terms of quantification and accurate profiling at level of species and possibly of strains by both culture-dependent and culture-independent techniques.

1. Introduction

The mammalian gut is colonized by a complex microbial community composed largely of bacteria, whose numbers exceed 10^{11} cells per gram of intestinal content, belonging to over 1000 species, based on a recent review describing culturable bacteria isolated from feces [1]. Among the natural colonizers of the gut, bifidobacteria and lactobacilli benefit the host through a variety of different mechanisms, and specifically selected strains are claimed as probiotics [2,3].

Much literature has accumulated over the years on the development and utilization of selective cultural media to isolate and enumerate bifidobacteria and lactobacilli from a variety of matrices, such as dairy foods, probiotic-based products, and feces [4–7]. The specificity of some *Bifidobacterium*-selective media, including the commercial Bifidus Selective Medium (BSM), rests on the presence of antibiotics, such as mupirocin, nalidixic acid, neomycin sulphate, norfloxacin, or paromycin [8–11]. Other media, such as Raffinose Bifidobacterium (RB), owe their selectivity to a specific carbon source and to the presence of propionate and lithium chloride as inhibitory agents [12]. The selectivity of LAMVAB for fecal lactobacilli is based on vancomycin, low pH, and the characteristic blue or green color of the colonies resulting from bromocresol color transition [13]. LBS medium (*Lactobacillus* Selective) contains acetate/acetic acid to hinder growth of streptococci and molds, and is buffered at low pH to favor lactobacilli [14].

With the advent of high-throughput sequencing technologies, 16S rRNA gene sequencing has been applied to the investigation of gut microbiota, disclosing the different taxa colonizing the gut and shedding light on the ecology of such a complex environment. Even

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though metataxonomic information on the composition of intestinal microbiota is increasingly available, scarce attention has been focused on mining the abundance of specific microbial groups, such as bifidobacteria and lactobacilli, and to compare the results with those obtained by FISH (Fluorescent *in situ* Hybridization), qPCR (Quantitative Polymerase Chain Reaction), or plating on selective media [15].

FISH and qPCR, providing data mostly at genus level, are the techniques most frequently used to obtain quantitative information on bifidobacteria or lactobacilli [16–19]. The identification and quantification of *Bifidobacterium* and *Lactobacillus* species, and especially the traceability of specific strains or biotypes, still rely on methods that exploit cultivation on selective media, followed by taxonomic characterization [20,21]. TRFLP (Terminal Restriction Fragment Length Polymorphism) analysis could be utilized for quantification of the species, but a comprehensive database is still not available for fecal *Lactobacillus* species, whereas it has been developed for bifidobacteria [22].

The aim of this study was to compare methods providing information on bifidobacteria and lactobacilli of fecal human samples. Isolation and enumeration on selective media, followed by the taxonomic characterization of the isolates by RAPD-PCR and partial 16S sequencing, were carried out. In parallel, a quantitative description was obtained by 16S rRNA gene sequencing, FISH, qPCR, and TRFLP.

2. Materials and methods

2.1. Specimens and chemicals

The fecal specimens that were utilized for bacterial enumeration experiments were collected after obtaining written informed consent from five healthy volunteers (men, aged 20 to 40) who had not been treated with prebiotics and/or probiotics for one month, and antibiotics for at least three months. All the chemicals were supplied by Sigma (Stenheim, Germany), unless otherwise stated.

2.2. Culture-dependent enumeration of bifidobacteria and LAB

Fresh feces were homogenized (10% w/v) and serially diluted in isotonic buffered peptone water (BPW, Fluka–Sigma) supplemented with 0.5 g/L L-cysteine · HCl, then spread onto plates (10^{-4} to 10^{-9} dilutions). Lactobacilli MRS agar (BD Difco, Sparks, USA) supplemented with 0.5 g/L L-cysteine · HCl (hereinafter referred to as MRS) was utilized for both lactobacilli and bifidobacteria. BSM (Bifidus Selective Medium Agar, Fluka–Sigma), MRS supplemented with 50 mg/L mupirocin (hereinafter referred to as MUP) [10], and RB agar [13] were utilized to select bifidobacteria. LBS agar (BD Difco) and LAMVAB [14] were utilized to isolate fecal lactobacilli. The plates were incubated for 48 h at 37 °C in anaerobic cabinet under a 85% N_2, 10% CO_2, 5% H_2 atmosphere.

For each subject, 50 colonies from each of the *Bifidobacterium*-selective media were subjected to *Bifidobacterium*-specific PCR, utilizing the 16S rRNA gene primers Bif164/Bif662 according to literature [23]. In order to validate the selectivity of *Lactobacillus*-selective media, 100 colonies per subject per medium, obtained by direct plating in LBS and LAMVAB, were randomly picked, clustered through RAPD-PCR analysis, and classified by 16S partial sequencing. Furthermore, for each subject, 200 colonies were randomly picked from MRS plates at the lowest dilutions giving single colonies, and were seeded onto RB, BSM, MUP, LBS, and LAMVAB plates. The bacteria grown on the MRS plates at the dilution of 10^{-5} were replica-plated onto LBS and LAMVAB plates. All the colonies isolated by replica plating were taxonomically characterized through RAPD-PCR clustering and 16S partial sequencing.

2.3. Taxonomic attribution of bacterial isolates

The gDNA was extracted from bacterial colonies using Instagene matrix (Bio-Rad) and was subjected to RAPD-PCR amplification with M13-RAPD primer (5′-GAGGGTGGCGGTTCT-3′). The reaction was performed in 15 μL of DreamTaq Buffer, containing 50 ng of template gDNA from the isolates, 7.5 pmol of primer, 1.5 nmol of each dNTP, and 0.75 U of DreamTaq polymerase. The thermocycle was the following: 94 °C for 4 min; 45 cycles at 94 °C for 1 min, 34 °C for 1 min, and 72 °C for 2 min; 72 °C for 7 min. The PCR products were electrophoresed for 4 h at 160 V in a 25 × 25 cm 2% (w/v) agarose gel in TAE buffer. RAPD-PCR fingerprints were digitally captured and were analyzed with Gene Directory 2.0 (Syngene, UK) software, which calculated similarities and derived a dendrogram with an unweighted pair group method with arithmetic means (UPGMA).

To attribute each biotype to a species, a portion of 16S rDNA was amplified with the universal primers (forward primer: 59-TGGAGAGTTTGATCCTGGCTCAG-39; reverse primer: 59-TACCG CGGCTGCTGGCAC-39) spanning positions 5–532 (inclusive) of *Escherichia coli* K-12 (GenBank accession no. NC_000913) [24], sequenced, and compared with Genbank database. Amplification was performed in 50 μL of PCR Master Mix (Thermo Fisher Scientific), containing 10 pmol of each primer, and 50 ng of gDNA. The thermocycle was the following: 94 °C for 4 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 7 min.

2.4. Fluorescence in situ hybridization (FISH)

Bacteria belonging to *Bifidobacterium* genus and *Lactobacillus-Enterococcus* group were quantified by FISH, using the probes Bif164 (5′-CATCCGGCATTACCACCC-3′) and Lab158 (5′-GGTATTAGCAYCTGTTTCCA-3′), respectively [16,25]. The species of human intestinal lactobacilli and enterococci potentially recognized by FISH probe Lab158 according to SILVA database (http://www.arb-silva.de) are presented in Table 1. Feces were suspended (10% w/v) in PBS buffer, homogenized, and gently centrifuged to remove the solids (300 ×g for 1 min). The supernatant was diluted 1:4 with 40 g/L paraformaldehyde and kept at 4 °C for 16 h. The cells, properly diluted with PBS (0–1000 fold), were applied onto gelatin-coated slides and were dehydrated with ethanol. For the enumeration of *Lactobacillus-Enterococcus* group, the cells were treated with 1 mg/ml lysozyme for 15 min at 37 °C. Dehydrated cells were covered with 100 μL of hybridization buffer (20 mM TRIS-HCl, 0.9 M NaCl, 1 g/L SDS) containing 0.75 μM FITC-labeled probe and were kept for 16 h at 53 °C in a dark and moisture-saturated chamber. Hybridized slides were washed with 20 mM TRIS-HCl and 0.9 M NaCl, and were covered with Vectashield (Vector Laboratories, Burlingame, CA). Fluorescent cells were counted with an epifluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged in each slide. Each sample was enumerated in triplicate.

2.5. qPCR

Quantification of *Bifidobacterium* and *Lactobacillus* group was achieved with primer pairs BiTOT-F/BiTOT-R (TCGCGTCYGGTGTGAAAG/CCACATCCAGCRTCCAC) and Lac-F/Lac-R (GCAGCAGTAGGGAATCTTCCA/GCATTYCACCGCTACACATG), respectively [17,26]. The species of human intestinal lactobacilli potentially recognized by qPCR primers Lac-F/Lac-R are presented in Table 1. Reactions were

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