PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces of human subjects after oral bacteriotherapy and yogurt consumption

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**Abstract**

*Streptococcus thermophilus*, *Bifidobacterium infantis* Y1 and *Bifidobacterium breve* Y8 strains were identified and enumerated by PCR assay in human fecal samples after intake of the pharmaceutical preparation VSL-3 or yogurt. ThI/ThII primer set, specific for *S. thermophilus*, was selected testing its specificity against several strains of enterococci, streptococci and other genera colonizing the human intestine. A culture-independent PCR protocol, developed in this study, allowed to directly detect and enumerate *S. thermophilus* in human feces, excluding culture-based techniques or time consuming DNA isolation and purification procedures. Intestinal persistence of *S. thermophilus* was studied in feces of 10 healthy subjects given VSL-3 or yogurt. Streptococcal population was detected after 3 days of administration and persisted for 6 days after the treatment suspension. In the same trial, the colonization kinetics of *B. infantis* Y1 and *B. breve* Y8 were studied by amplification of colonies with the strain-specific primer sets InfY-BV.L/R and BreY-BV.R/L, showing a host-dependent transient colonization behaviour. PCR analysis of feces from 10 patients affected by inflammatory bowel diseases (IBD) and treated with VSL-3 for 2 months showed a colonization pattern of *S. thermophilus*, *B. infantis* Y1 and *B. breve* Y8 similar to that observed with the healthy subjects.

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1. Introduction

*Streptococcus salivarius* subsp. *thermophilus*, designated as *S. thermophilus* throughout this paper, is used as a starter in the manufacture of fermented dairy products, such as yogurt and cheese, and in probiotic preparations. This microorganism, associated with *Bifidobacterium* strains, is included in the pharmaceutical composition VSL-3, which has been used in the treatment of patients affected by inflammatory bowel diseases (Venturi et al., 1999; Gionchetti et al., 2000).

The recent genetic-based technologies, in particular those related to ribosomal RNA operon polymorphisms, are rapidly replacing conventional bacterial
identification and enumeration methods. PCR is extensively used as detecting and identifying tool for bacteria in different environments including the intestinal microbiota (O’Sullivan, 1999; Vaughan et al., 2000). A PCR-based detection system is highly sensitive and eliminates the need for traditional culture techniques. However, it is well known that fecal components, such as bile salts and complex polysaccharides (Lantz et al., 1997; Abu Al-Soud and Radström, 1998), may interfere with PCR, inhibiting the reaction or reducing the amplification efficiency.

The object of this work was the one of developing a rapid and easy-to-use PCR protocol for directly detecting and enumerating S. thermophilus and Bifidobacterium in human feces of subjects given VSL-3 or yogurt.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1, also including bacteria tested for screening the specificity of the PCR primers used. Strains in bold type were used in the pharmaceutical probiotic VSL-3 which contained 2.0 × 10¹¹ CFU/g of viable, lyophilized bacteria: 2.0 × 10¹¹ CFU/g of S. thermophilus, 9.3 × 10¹⁰ CFU/g of Bifidobacterium (Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve) and 2.8 × 10⁹ CFU/g of Lactobacillus (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus plantarum). The strains originated from the collection of the Institute of Agricultural Microbiology of the University of Bologna and from the collection of our laboratory. All strains were incubated at 37 °C in the anaerobic cabinet (Anaerobic System, Mod. 2028, Forma Scientific, Marietta, OH, USA). Streptococci, enterococci and lactococci were grown in S. thermophilus medium (ST) (Dave and Shah, 1996); Bifidobacterium and Lactobacillus species in MRS broth (Difco, Detroit, MI, USA 0881-17-5) with addition of 0.05% of L-cysteine hydrochloride monohydrate (Merck, Milan, Italy, 102839).

2.2. Optimization of culture media for isolation of S. thermophilus and Bifidobacterium

In order to improve the discrimination of S. thermophilus from other streptococci or enterococci and to prevent overgrowth of Gram-negative rods in analysis of fecal samples, a modified ST medium (ST2) was prepared by adding 30 mg/l of bromocresole purple (Merck, 105225), 100 mg/l of bromocresole green (Merck, 101541) and 30 mg/l of nalidixic acid (Sigma-Aldrich, Milan, Italy, N 4382). Plates were incubated in aerobic conditions for 24 h at 42 °C.

| Bifidobacterium bifidum: ATCC 29521⁴; DSM 20239, B 2662, B 2004, B 2334; B. breve: ATCC 15700⁴, ATCC 15698, B 2456, B 622, B 2429, Y8⁴; B. infantis: ATCC 15697⁴, DSM 20090, B 1824, B 1719, B 1522, Y1⁴; B. longum: ATCC 15707⁴, DSM 20097, B 2352, B 612, B 1429, Y10⁴; B. adolescentis: ATCC 15703⁴, DSM 20239, B 839, B 933, B 618 |
| Bacteroides fragilis: NCTC 10584, ATCC 25285 |
| Clostridium beijerinckii: M 3; C. perfringens: C 112 |
| Enterococcus faecalis: ATCC 29212, ATCC 8043 |
| Escherichia coli: ATCC 11005, ATCC 8739 |
| Lactococcus lactis: MB 406, MB 408; L. lactis subsp. cremoris: DSM 20069, MB 446; L. lactis subsp. lactis biovar diacetylactis: MB 447; L. lactis subsp. lactis: DSM 20481, MB 445 |
| Lactobacillus acidophilus: MB 358, MB 359, MB 442⁴, MB 443⁴, L. Brevis: ATCC 4006; L. casei: ATCC 393, M 264, MB 451⁴; L. crispatus: ATCC 33197; L. delbrueckii subsp. delbrueckii: ATCC 9649, DSM 20074; L. delbrueckii subsp. bulgaricus: MB 453⁴; L. delbrueckii subsp. lactis: DSM 20076; L. plantarum: MB 452⁴ |
| Streptococcus faecium: MB 373, MB 376, MB 454; S. salivarius subsp. thermophilus: DSM 20617, MB 417, MB 418, MB 419, MB 420, MB 421, MB 426, MB 427 MB 428, MB 429, MB 455⁴ |

Table 1
List of the bacterial strains

ATCC, American Type Culture Collection (Rockville, MD, USA); DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

⁴ Type strain.
⁵ Strains in bold are used in VSL-3.
Growth of *S. thermophilus* acidified the ST2 medium, which turned from violet to yellow and the colonies were dark green in the center, specially after successive storage at 4 ºC. To confirm this identification, the single colonies were analyzed by PCR. Bifidobacteria were selected on Raffinose-*Bifidobacterium* agar plates (Hartemink et al., 1996) incubated for 72 h in Anaerobic System. The identification of these colonies was confirmed by genus-specific PCR reaction (Kok et al., 1996) and demonstration of the enzyme fructose-6-phosphate phosphoketolase activity in cell extracts (Biavati et al., 1992).

### 2.3. Subjects and fecal sample collection

Fecal samples were obtained from 10 healthy subjects and 10 patients affected by inflammatory bowel diseases (IBD). The specimens were collected with sterile plastic containers, immediately analyzed or stored at −20 ºC. In the clinical experiment five healthy subjects ingested 250 g of yogurt daily for 10 days, whereas 6 g of VSL-3 daily were administered to the other five subjects for the same period. None of the healthy volunteers used antibiotics in the previous year nor consumed dairy products containing streptococci for a period of 10 days before and after the treatment with yogurt or VSL-3. At 3-day intervals, during yogurt or VSL-3 consumption and after the cessation of consumption, fecal samples were collected for analysis. Ten patients affected by IBD were treated with 6 g of VSL-3 daily and provided fecal samples at the beginning of the trial and after 2 months of treatment.

### 2.4. PCR amplification

Amplification reactions were carried out in a Biometra Thermal Cycler II (Biometra, Göttingen, Germany). Dynazyme II (Celbio, Milan, Italy F-501L) was used as thermostable polymerase at the condition suggested by the supplier. All primers used in this study were supplied by M-Medical-Genenco (Florence, Italy). The total volume of each reaction mixture was 25 µl and cells from plate or from fecal sample were used directly as template, without isolation of chromosomal DNA. Amplification of *S. thermophilus* was obtained using the 16S–23S rDNA primer set ThI/ThII (Timisjärvi and Alatossava, 1997). The PCR reaction mixture was composed of 1.0 µM of each primer, 250 µM of each dNTP (Amersham Pharmacia Biotech, Milan, Italy 272094) and 1 U of Dynazyme II. The thermocycle program used consisted of the following time and temperature profile: (i) 95 ºC for 5 min; (ii) 40 cycles of 1 min at 95 ºC, 30 s at 50 ºC and 1 min at 72 ºC; and (iii) 1 cycle of 1 min at 95 ºC, 30 s at 50 ºC and 5 min at 72 ºC. Bifidobacteria were amplified by using the 16S rDNA genus-specific primer set Bif164/Bif662 (Kok et al., 1996) and the *B. infantis* Y1 and *B. breve* Y8 16S–23S rDNA strain-specific primer sets InfY-BV.L/R and BreY-BV.R/L (Brigidi et al., 2000) as described by the authors. Amplified products were subjected to gel electrophoresis in 2% agarose gels and were visualized by ethidium bromide staining.

### 2.5. Direct quantitative PCR of *S. thermophilus* in feces

Feces, collected at the different times of the study from all healthy subjects and patients consuming yogurt or VSL-3, were analyzed for the quantitative detection of *S. thermophilus*. This enumeration was performed by the culture-independent PCR method used in the present work. One gram of feces was added to 9 ml of sterile phosphate-buffered saline (PBS, 0.05 M, pH 7.4) and mixed by vortexing for 5–10 min. The sample was centrifuged at 2450 × g for 1 min and the supernatant collected. This centrifugation step was repeated three times. The supernatant was then centrifuged at higher speed (8000 × g) for 3 min to collect the bacterial cells. The pellet was washed four times with 2.5 ml of PBS and twice with 1 ml of sterile water, resuspended in sterile water to a final volume of 0.3 ml and serially diluted in Triton X-100 (Sigma-Aldrich, T 9284). All the dilutions were heated at 100 ºC for 5 min and immediately cooled in ice. This step was repeated five times. The dilution tubes were stored at −80 ºC. Two microliters of each dilution were amplified with the primer set ThI/ThII. The *S. thermophilus* titer in fecal samples was determined by considering the sensitivity of this PCR procedure. The sensitivity corresponds to the number of *S. thermophilus* cells present in the highest fecal dilution able to give a positive amplification signal. To evaluate this sensitivity value, the quantification of *S. thermophilus* in five fecal samples was performed...
in a parallel manner by direct PCR analysis and by plate counting in the selective ST2 medium.

2.6. Enumeration of bifidobacteria in feces

Feces of healthy subjects and patients who were given VSL-3 were analyzed for the enumeration of total bifidobacteria and for the specific detection and quantification of B. infantis Y1 and B. breve Y8, present in the preparation VSL-3. One g of each fecal sample was homogenized in 99 ml of Wilkins–Chalgren Anaerobic Broth (Oxoid, Basingstoke, UK, CM 643 B) added with 0.05% of l-cysteine, serially diluted in Anaerobic System and plated onto RB medium. Fifty bifidobacterial colonies, randomly selected from the highest dilution plates, were directly amplified by using the Bifidobacterium genus-specific primer set Bif164/Bif662 and the B. infantis Y1- and B. breve Y8-specific primer sets InfY-BV.L/R and BreY-BV.R/L. The minimal detectable cell concentration of this analytical approach was the ratio between the total number of bifidobacteria present in the sample and the number of colonies examined by PCR.

2.7. Statistical analysis

All results were expressed as mean ± standard deviation. Values of \( P < 0.05 \) were considered statistically significant.

3. Results and discussion

The specificity of the primer set ThI/ThII (Timisjärvi and Alatossava, 1997) was confirmed by testing 71 strains belonging to 24 species which are commonly found in human intestinal microflora and in several dairy products (Table 1). These primers were able to detect the target species, S. thermophilus, providing a PCR product with the expected size of 250 bp. None of the strains belonging to other species were amplified under the reported conditions. Fig. 1 illustrates the specificity of the primer set ThI/ThII in respect to several bacterial strains belonging to the most representative intestinal species.

The strain-specificity of the primer sets InfY-BVL/R and BreY-BVL/L for the VSL-3 bifidobacterial strains B. infantis Y1 and B. breve Y8, was previously demonstrated by Brigidi et al. (2000).

The culture-independent PCR protocol, directly detecting and quantifying S. thermophilus in human feces as described above, included centrifugation and washing steps to remove PCR inhibitors and cell artefacts from feces, excluding time-consuming DNA isolation and purification procedures. Serial dilutions of purified samples were directly amplified for the quantification of S. thermophilus. The fecal streptococcal concentrations were evaluated on the basis of the sensitivity of this PCR procedure. The results of a sensitivity evaluation experiment are reported in Fig. 2, which shows the PCR products obtained by amplification of fecal dilutions with a known streptococcal concentration. The sensitivity value, corresponding to the number of S. thermophilus cells present in the highest fecal dilution able to give a positive amplification signal, was 10 cells. This result was confirmed by analysis of all the five stool specimens tested for the sensitivity determination.

Direct PCR identification of VSL-3 B. infantis Y1 and B. breve Y8 strains, by using the primer sets InfY-BVL/R and BreY-BVL/L, in purified fecal sample of
subjects assuming VSL-3, was unsuccessful. This failed detection can be due to the low sensitivity of the strain-specific primer sets reported by authors (Brigidi et al., 2000), in addition to the high conservation degree of the nucleotide sequence of the L and R primers (Bourget et al., 1996), which recognize universal target regions present in all bacterial ribosomal operons.

Studies of \textit{S. thermophilus}, \textit{B. infantis} Y1 and \textit{B. breve} Y8 intestinal persistence in healthy subjects assuming yogurt or VSL-3 were performed (Figs. 3 and 4). Stool samples were collected from all subjects at different times: after 10 days of \textit{S. thermophilus}-free diet (T0), after 3, 7 and 10 days of yogurt or VSL-3 treatment (T3, T7 and T10), and 3, 6 and 9 days after administration suspension (T13, T16 and T19).

Fig. 3 shows the mean of the \textit{S. thermophilus} concentration values detected by direct PCR in feces of five subjects fed with yogurt and five subjects treated with VSL-3. During yogurt intake, streptococcal population increased, reaching a maximum of $4 \times 10^5$ cells/g feces at time T3. This concentration value remained constant until time T10 and slowly decreased below the detection limit of the direct PCR analysis 9 days after the treatment suspension. VSL-3 administration induced a higher fecal \textit{S. thermophilus} concentration, which increased until time T13 ($5 \times 10^5$ cells/g feces) and drastically decreased below the minimal detectable concentration only 6 days after the treatment suspension. The higher concentration of \textit{S. thermophilus} in the probiotic preparation VSL-3 ($2 \times 10^{11}$ cells/g), compared with the yogurt one ($10^6$–$10^9$ cells/g), could explain the higher streptococcal titer found in feces of VSL-3-treated subjects.

The intestinal persistence of VSL-3 \textit{B. infantis} Y1 and \textit{B. breve} Y8 strains in the five healthy subjects consuming the probiotics were studied by PCR identification of colonies grown on selective \textit{Bifidobacterium} RB plates. Exogenous VSL-3 bифидобактерии gave a different transient colonization behaviour in the subjects treated, evidencing two different colonization trends (Fig. 4), related to the endogenous \textit{Bifidobacterium} concentration (T0). In trend A, T0 \textit{Bifidobacterium} concentration was lower ($6 \times 10^6$ cells/g feces) than that of trend B ($6 \times 10^8$ cells/g feces).
feces), and during the probiotic treatment VSL-3 strains represented a high percentage of the total bifidobacterial population. VSL-3 administration caused a peak of total bifidobacterial titer at time T3 (4 \times 10^8 cells/g feces), evidencing the presence of \textit{B. infantis} Y1 (3 \times 10^7 cells/g feces) and \textit{B. breve} Y8 (8 \times 10^7 cells/g feces). At time T7, the total bifidobacterial concentration decreased to a value of 3 \times 10^7 cells/g feces, which was maintained stable during the VSL-3 treatment. \textit{B. infantis} Y1 was detected at high concentration during the VSL-3 treatment (T7: 9 \times 10^6 cells/g feces; T10: 1 \times 10^6 cells/g feces) and disappeared rapidly after the intake suspension (T13). Differently, \textit{B. breve} Y8 concentration decreased at T7 (2 \times 10^5 cells/g feces) but remained stable for 6 days after the intake cessation (T16). No VSL-3 bifidobacterial strains were evidenced 9 days after the treatment suspension (T19) and the total \textit{Bifidobacterium} titer reassessed to its initial value of concentration after 6 days (T16).

In trend B, no variation in the total bifidobacteria titer was observed during the trial carried out. VSL-3 strains were detected only sporadically at a low concentration close to the minimal detectable cell concentration of the colony-based PCR counting procedure.

These results suggest that the impact of VSL-3 administration on total \textit{Bifidobacterium} concentration is particularly evident in subjects harboring low concentrations of endogenous bifidobacteria, while no visible variation occurs in subjects with a high endogenous bifidobacterial population.

\textbf{Fig. 5} shows the colonization persistence of \textit{S. thermophilus}, \textit{B. infantis} Y1 and \textit{B. breve} Y8 strains in the gut of 10 patients affected by IBD and treated with VSL-3 (6 g/day) for 2 months. Only 30% of patients harbored \textit{S. thermophilus} before the probiotic treatment (9 \times 10^3 cells/g feces), while the T0 bifidobacterial presence (8 \times 10^6 cells/g feces) was observed in 70% of patients, confirming that not all healthy subjects harbor bifidobacteria. After VSL-3
intake, both streptococcal and bifidobacterial population increased significantly ($P < 0.01$ and $P < 0.05$, respectively), reaching a high concentration of 10$^7$ cells/g feces in 100% of the patients examined. VSL-3 B. infantis Y1 and B. breve Y8 strains were found in 40% and 70% of patients at a concentration of $5 \times 10^5$ and $9 \times 10^5$ cells/g feces, respectively. The results related to B. infantis Y1 and B. breve Y8 intestinal persistence indicate a different colonization behaviour of the exogenous industrial strains in the patients treated, emphasizing that only specific strains are able to colonize a particular human host (Kimura et al., 1997; Brigidi et al., 2000).

The results presented in this paper show that direct or colony-based PCR technique, based on the 16S–23S rDNA targeted primers, can be employed to monitor the presence of the species S. thermophilus and specific Bifidobacterium strains in the human gut, offering a picture of the relationship between these probiotic strains in their intestinal niche.

References


