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A PCR method for detection of bifidobacteria in raw milk and raw milk cheese: comparison with culture-based methods

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Abstract

Bifidobacteria are well known for their beneficial effects on health and are used as probiotics in food and pharmaceutical products. As they form one of the most important groups in both human and animal feces, their use as fecal indicator organisms in raw milk products has recently been proposed. Bifidobacteria species isolated in humans are different from those isolated in animals. It should therefore be possible to determine contamination origin (human or animal).

A method of detecting the *Bifidobacterium* genus was developed by PCR targeting the hsp60 gene. The genus *Bifidobacterium* was identified by PCR amplification of a 217-bp hsp60 gene fragment. The degenerated primer pair specific to the *Bifidobacterium* genus used was tested for it specificity on 127 strains. Sensitivity was measured on artificially contaminated samples. Food can however be a difficult matrix for PCR testing since it contains PCR inhibitors. So an internal PCR control was used. An artificially created DNA fragment of 315 bp was constructed. The PCR detection method was tested on raw milk and cheese samples and compared with three culture-based methods, which comprised enrichment and isolation steps. The enrichment step used Brain Heart Infusion medium with propionic acid, iron citrate, yeast extract, supplemented with mupirocin (BHMup) or not (BH) and the isolation step used Columbia blood agar medium, supplemented with mupirocin (CMup) or not (C). The method using mupirocin at both enrichment and isolation steps and the PCR method performed from the culture in BHMup enrichment medium were shown to be the most efficient. No significant difference was observed in raw milk samples between PCR from BHMup and the culture-based method BHMup/CMup, while a significant difference was noticed between the same methods in raw milk cheese samples, which would favor using PCR.

The results suggested that PCR on the hsp60 gene was convenient for a rapid detection of bifidobacteria in raw milk and raw milk cheese samples and that bifidobacteria always present throughout raw milk cheese production could be efficiently used as fecal indicators.

Keywords*:* PCR; Hsp60 gene; *Bifidobacterium;* Detection; Fecal indicators; Raw milk; Raw milk cheese; Mupirocin

1. Introduction

Bifidobacteria are Gram-positive, non-motile and non-spore-forming bacteria. They had been considered as anaerobic, until one species was defined as aero-anaerobic (Simpson et al., 2004a). They are part of normal intestinal flora in humans and animals and are generally non-pathogenic bacteria.

Fecal contamination of raw milk on farm has been shown by Beerens et al. (2000), who detected the same and most frequent *Bifidobacterium* species in milk as in cow dung. Raw milk can be assumed to be the first critical point in an HACCP analysis of the raw milk cheese industry, but a follow-up of contamination during the cheese-making process is also of interest. The standard in Europe for fecal contamination control of raw milk cheese is *Escherichia coli.*

Bifidobacteria have been proposed as a fecal indicator since they represent one of the most important bacterial groups in human and animal feces (Matsuki et al., 1998; 1999). Moreover, as the dominant *Bifidobacterium* species are different in human and animal flora (Gavini et al., 1991), one should be able to determine contamination origin (human or animal). This bacterium has been recently proposed as a fecal indicator in water

(Lynch et al., 2002; Nebra et al., 2003; Gilpin et al., 2003) and in meat and raw milk samples (Beerens, 1998; Gavini and Beerens, 1999; Beerens et al., 2000).

Numerous culture-based methods for bifidobacteria detection have been described for these above-mentioned applications and for others, such as knowledge of the genus *Bifidobacterium* and its evolution within gastrointestinal flora (human or animal) (Martineau, 1999; Rada and Petr, 2000; Petr and Rada, 2001) and the use of bifidobacteria as probiotics in food or pharmaceutical products (Nebra and Blanch, 1999; Pacher and Kneifel, 1996; Payne et al., 1999).

The culture-based method using propionic acid (Beerens, 1990) and paromomycin as selective agents (Beerens, 1998) to detect bifidobacteria in meat products and in raw milk samples is not sufficiently efficient to eliminate contaminating flora such as lactobacilli in raw milk or Clostridia in meat samples. Using the culture-based detection method requires knowledge of the contaminating flora and the researched *Bifidobacterium* species in the samples.

Several molecular methods that alleviate this inconvenience have recently been described: PCR-Elisa method based on the 16S rRNA to detect the most common *Bifidobacterium* species in humans (Malinen et al., 2002); pulsed-field gel electrophoresis (PFGE) and PCR targeting the 16S rRNA (Roy et al., 1996; Bonjoch et al., 2004); PCR in denaturing gradient gel electrophoresis (DGGE) targeting the transaldolase gene for identification, detection and enumeration of human *Bifidobacterium* species (Requena et al., 2002); PCR-RFLP method based on the 16SrRNA to detect the most common species from animal and human origins (Delcenserie et al., 2004; Roy and Sirois, 2000), and real-time quantitative PCR from the 16S or the transaldolase gene (Requena et al., 2002). They have also been used in the detection of human *Bifidobacterium* species from feces (Matsuki et al., 2002; Requena et al., 2002, Mullié et al., 2003; Venema and Maathuis, 2003), of bifidobacteria as probiotics (Brigidi et al., 2003; Fasoli et al., 2003) or as fecal indicators in waters (Bernhard and Field, 2000).

Most of these molecular methods have been applied to detect *Bifidobacterium* species in human feces, rather than in the detection of bifidobacteria of animal origin. Moreover, the 16S rRNA sequences are well conserved among the bifidobacteria and there are multiple copies of the 16S rRNA gene per chromosome. These features might influence quantitative PCR methods (Requena et al., 2002). Another gene, the hsp60 gene, has been sequenced in most *Bifidobacterium* species (Jian et al., 2001, Jian and Dong, 2002). This gene presents species-specific sequences.

This study compares three different protocols of a culture-based method using mupirocin, as recommended by Rada et al. (1999) and Rada and Petr (2000), instead of paromomycin as selective agent in parallel with a PCR method on raw milk samples. Then, utilizing both culture-based and PCR methods, bifidobacteria contamination levels in raw milk cheese samples are determined and compared with those of *E. coli.* Application of bifidobacteria as fecal indicators in raw milk cheese industries is also discussed.

2. Material and methods

2.1. Samples

2.1.1. Raw milk samples

Detection of bifidobacteria was performed from raw milk stored in tanks collected on French farms (Vercors and Courtenay regions).

Samples were diluted until 10^{-4} and presence or absence of bifidobacteria at each dilution was compared statistically by the different detection methods as follows:

(i) 39 samples (195 dilutions) have been analyzed and results compared using three combinations of culture-based methods using mupirocin or not

(ii) 12 samples (60 dilutions) have been analyzed and results compared by PCR from two enrichment broth using or not mupirocin

(iii) 148 samples (740 dilutions) have been analyzed and results compared by the different PCR and culture-based methods.

2.1.2. Raw milk cheese samples

In the industry under study from the Vercors region (France), milk was collected on farms and stored in tanks at the factory at 4°C. Milk is prepared for maturation by addition of cream, ferment and surface flora. Animal rennet is added. On day 1, the following steps are successively performed: molding, a first manual turnover, a manual salting and a second turnover. On day 2, cheeses are removed from the molds and a new manual or mechanical salting is performed. Ripening is carried out for 28 days.

Twenty-five raw milk cheeses at four different steps of the production chain from raw milk to the end product (100 samples) were analyzed by the best culture-based method chosen among the three tested and by the best PCR method. The following production steps were analyzed: raw milk (Step A), after addition of rennet (Step C), after removal from the mold (Step E), during ripening (Step G). Samples were diluted until 10^{-6} to perform semiquantitative detection of bifidobacteria.

2.2. Methods

2.2.1. Culture-based method for E. coli detection

E. coli was numerated on the Coli ID medium (BioMérieux, France; Sueiro et al., 2001).

2.2.2. Culture-based methods for bifidobacteria detection

The methods were performed in two steps, an enrichment and an isolation step. Components of enrichment and isolation media before adding mupirocin have been described by Beerens (1998).

2.2.2.1. Enrichment step medium. The following components were added to the medium Brain Heart Infusion (BHI, 37 g/l, Bio-Rad, Marnes-la-Coquette, France): propionic acid, 5 ml/l; Fe-citrate, 0.5 g/l; cystein chlorhydrate, 0.5 g/l; yeast extract, 5 g/l; agar, 2 g/l. Mupirocin was provided by GSK Laboratories (Lithium mupirocin, GlaxoSmithKline, England) and added (BHMup) or not (BH) at the concentration equal to 80 mg/l (to be added when the medium must be used). The final pH, 5.0, was obtained with the addition of a NaOH solution. The medium (without mupirocin) was dispensed in 9 ml amounts. Sterilization was not needed because of the medium's low pH.

2.2.2.2. Isolation step medium. Columbia blood agar medium (Columbia blood agar, Difco, Elancourt, France) was used with addition of Fe-citrate, 0.5 g/l; glucose, 5 g/l; cystein chlorhydrate, 0.5 g/l. Mupirocin was added (CMup) or not (C) at the concentration equal to 50 mg/l (to be added when the medium must be used). The medium (without mupirocin) was dispensed in 100 ml bottles and autoclaved at 120°C.

2.2.2.3. Protocol. The selective enrichment medium, with mupirocin added or not, was held in boiling water for 20 min to expel oxygen and cooled to 30-40°C.

The milk and the raw milk cheese samples were diluted until 10^{-3} and until 10^{-5}, respectively, in quarter-strength Ringer solution containing cystein chlorhydrate (0.3‰). One milliliter of milk or 1 g of raw milk cheese was transferred in a tube of enrichment medium. Then 1 ml of each of the appropriate sample dilutions was inoculated in tubes of enrichment medium in order to detect bifidobacteria in milk and raw milk cheese until 10^{-4} and at 10^{-6}, respectively. Tubes were incubated at 37 °C for 72 h in aerobiosis, since bacteria were able to grow in depth because of agar present in the medium.

For each enrichment culture, 0.03 or 0.1 ml were spread onto five plates of Columbia blood agar. The plates were incubated at 37 °C for 72 h in jars with an "Anaerogen" (Oxoid, Dardilly, France).

We compared the following different protocols: enrichment broth containing mupirocin (BHMup) or not (BH) and isolation medium containing mupirocin (CMup) or not (C). The combinations used were BH/ CMup (Cultural 1), BHMup/C (Cultural 2), BHMup/ CMup (Cultural 3). Presence of bifidobacteria was confirmed by (1) the production of fructose-6-phosphate phosphoketolase (F6PPK test as described by Scardovi, 1986) tested on the whole culture obtained after the isolation step, (2) by transfer of the isolated colonies into Veillon tubes that contained Columbia agar to eliminate aerobic strains and to perform F6PPK test on Gram-positive bacillar strains.

Table 1: *References of the Bifidobacterium strains used for the validation of the PCR essay*

International or INRA internal reference	Name as received	Isolated from
ATCC 27672	*B. animatis*	Rat feces
P16 (Biavati*a*)	*B. animatis*	Chicken feces
F434 (Biavati)	*B. animatis*	Sewage
RA16 (Biavati)	*B. animatis*	Rabbit feces
RA20 (Biavati)	*B. animatis*	Rabbit feces
NCFB 2242^{T}	*B. animatis*	Rat feces
DSM 20210^{T}	*B. thermophilum*	Pig feces
Cheval 1/1	*B. thermophilum*	Horse feces
Pigeon 1/2	*B. thermophilum*	Pigeon feces
LC 403/1	*B. thermophilum*	Raw milk
LC 458/3	*B. thermophilum*	Raw milk
LC 294/2	*B. thermophilum*	Raw milk
LC 103/1	*B. thermophilum*	Raw milk
B 39/3	*B. thermophilum*	Cow dung
B 105/5	*B. thermophilum*	Cow dung
LC 288/1	*B. thermophilum*	Raw milk
Porc 3/1	*B. thermophilum*	Pig feces
B 42/1	*B. thermophilum*	Cow dung
LC 110/1	*B. thermophilum*	Raw milk
B 25/1	*B. thermophilum*	Cow dung
T 585/1/2	*B. thermophilum*	Raw milk
Pigeon 1/1	*B. thermophilum*	Pigeon feces
Cheval 5/1	*B. thermophilum*	Horse feces
T 528/4	*B. thermophilum*	Raw milk
B 79/3	*B. thermophilum*	Cow dung
LC 102/2	*B. thermophilum*	Raw milk
LC 26/3	*B. thermophilum*	Raw milk
LC 75/1	*B. thermophilum*	Raw milk
F 38/3	*B. thermophilum*	Raw milk cheese
B 25/2	*B. thermophilum*	Cow dung
LC 205/1	*B. thermophilum*	Raw milk
Pigeon 4/1	*B. thermophilum*	Pigeon feces
Pigeon 4/3	*B. thermophilum*	Pigeon feces
DSM 20434^{T}	*B. choerinum*	Pig feces
Internal 1	*B. pseudolongum*[b]	Unknown
Internal 2	*B. pseudolongum*[b]	Unknown
RU 224 (Biavati)	*B. pseudolongum* subsp. *Globosum*	Bovine rumen
Internal 3	*B. pseudolongum*[b]	Unknown
Internal 4	*B. pseudolongum*[b]	Unknown
MB7 (Biavati)	*B. pseudolongum* subsp. *pseudolongum*	Pig feces
LC 287/2	*B. pseudolongum*[b]	Raw milk
LC 289/2	*B. pseudolongum*[b]	Raw milk
LC 302/2	*B. pseudolongum*[b]	Raw milk
LC 407/1/1	*B. pseudolongum*[b]	Raw milk
B 81/1	*B. pseudolongum*[b]	Cow dung
LC 312/2	*B. pseudolongum*[b]	Raw milk
LC 317/2	*B. pseudolongum*[b]	Raw milk
LC 405/3	*B. pseudolongum*[b]	Raw milk
LC 290/1	*B. pseudolongum*[b]	Raw milk

Table 1 ***(continued)***

International or INRA internal reference	Name as received	Isolated from
LC 464/3	*B. pseudolongum*[b]	Raw milk
LC 287/1	*B. pseudolongum*[b]	Raw milk
LC 305/2	*B. pseudolongum*[b]	Raw milk
Poule 1/2	*B. pseudolongum*[b]	Chicken feces
B 86/1	*B. pseudolongum*[b]	Cow dung
B 81/1	*B. pseudolongum*[b]	Cow dung
LC 304/1	*B. pseudolongum*[b]	Raw milk
LC 334/1	*B. pseudolongum*[b]	Raw milk
LC 323/1	*B. pseudolongum*[b]	Raw milk
LC 324/2	*B. pseudolongum*[b]	Raw milk
LC 340/3	*B. pseudolongum*[b]	Raw milk
LC 306/1	*B. pseudolongum*[b]	Raw milk
Internal 5	*B. pseudolongum*[b]	Unknown
LC 240/3	*B. pseudolongum*[b]	Raw milk
LC 229/2	*B. pseudolongum*[b]	Raw milk
LC 232/1	*B. pseudolongum*[b]	Raw milk
LC 172/2	*B. pseudolongum*[b]	Raw milk
LC 147/2	*B. pseudolongum*[b]	Raw milk
LC 160/3	*B. pseudolongum*[b]	Raw milk
LC 109/3	*B. pseudolongum*[b]	Raw milk
LC 99/1	*B. pseudolongum*[b]	Raw milk
LC 123/1	*B. pseudolongum*[b]	Raw milk
LC 26/1	*B. pseudolongum*[b]	Raw milk
LC 120/1	*B. pseudolongum*[b]	Raw milk
B 121/1	*B. pseudolongum*[b]	Cow dung
LC 700/2	*B. pseudolongum*[b]	Raw milk
LC 697/3	*B. pseudolongum*[b]	Raw milk
T 690/1/1	*B. pseudolongum*[b]	Raw milk
T 702/2/2	*B. pseudolongum*[b]	Raw milk
B 116/1/1	*B. pseudolongum*[b]	Cow dung
B 117/1/3	*B. pseudolongum*[b]	Cow dung
B 117/1/1	*B. pseudolongum*[b]	Cow dung
LC 686/1	*B. pseudolongum*[b]	Raw milk
LC 684/3	*B. pseudolongum*[b]	Raw milk
LC 680/2	*B. pseudolongum*[b]	Raw milk
LC 617/2	*B. pseudolongum*[b]	Raw milk
RU 915 B^T	*B. merycicum*	Bovine rumen
RU 687^T	*B. ruminantium*	Bovine rumen
DSMZ 20102^T	*B. minimum*	Sewage
LC 396/4	*B. minimum*	Raw milk
LC 300/1	*B. minimum*	Raw milk
Internal 6	*B. cuniculi*	Unknown
Internal 7	*B. adolescentis*	Unknown
BS3	*B. adolescentis*	Adult feces
CCUG 18363^T	*B. adolescentis*	Adult feces
206 la	*B. adolescentis*	Adult feces
503 le	*B. adolescentis*	Elderly feces
BS5	*B. adolescentis*	Adult feces
BS50	*B. adolescentis*	Adult feces
1604 3a	*B. adolescentis*	Elderly feces
DSMZ 20082	*B. bifidum*	Adult feces
BS95	*B. bifidum*	Adult feces

Table 1 ***(continued)***

International or INRA internal reference	Name as received	Isolated from
BS 119	*B. bifidum*	Adult feces
BS 127	*B. bifidum*	Adult feces
BS 181	*B. bifidum*	Adult feces
NCFB 2257^T	*B. breve*	Infant intestine
Butel 8	*B. breve*	Infant feces
Butel 10	*B. breve*	Infant feces
Butel 5	*B. breve*	Infant feces
Butel 15	*B. breve*	Infant feces
Crohn 16	*B. breve*	Adult feces
CCUG 18367^T	*B. dentium*	Dental caries
BS 16	*B. dentium*	Adult feces
BS 22	*B. dentium*	Adult feces
BS 39	*B. dentium*	Adult feces
BS 72	*B. dentium*	Adult feces
Crohn 24	*B. dentium*	Adult feces
NCTC 11818^T	*B. longum*	Adult feces
BS 175	*B. longum*	Adult feces
BS 52	*B. longum*	Adult feces
A 10c	*B. longum*	Elderly feces
BS 101	*B. longum*	Adult feces
DSMZ 20438^T	*B. pseudocatenulatum*	Infant feces
B2b	*B. pseudocatenulatum*	Adult feces
BS40	*B. pseudocatenulatum*	Adult feces
C19i	*B. pseudocatenulatum*	Child feces
C20b	*B. pseudocatenulatum*	Child feces
C1c	*B. pseudocatenulatum*	Child feces

ATCC: American Type Culture Collection, Rockville, MD, USA; CCUG: Culture Collection, University of Göteborg, Göteborg, Sweden; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Göttingen, Germany; NCTC: National Collection of Type Cultures, Central Public Health Laboratory, London; England); NCFB: National Collection of Food Bacteria, Shinfield, Reading, Berks, England.

[a] Received from B. Biavati, Instituto di Microbiologia Agaria e Tecnica, Università degli Studi di Bologna, Bologna, Italy. [b] Subspecies not determined.

2.2.3. PCR method for bifidobacteria detection

2.2.3.1. Target DNA preparation

Pure strains. One hundred and twenty-seven reference strains belonging to 14 *Bifidobacterium* species (Table 1) and 37 *non-Bifidobacterium* strains belonging to species or genera often food-contaminating (5 *Enterococcus* spp., 5 *Pseudomonas* sp., 5 *Staphylococcus aureus,* 6 *Lactobacillus*, 4 *Clostridium perfringens,* 6 *Bacillus cereus,* 5 *E. coli* and 1 *Salmonella typhimurium)* were tested for primers validation. Before testing, the *Bifidobacterium* strains were withdrawn from frozen storage on Rosenow medium (Sanofi-synthelabo, Marnes-la-Coquette, France) and subcultured on Brain Heart Infusion (BioRad, Marnes-la-Coquette, France) at 37 °C for 48 to 72 h under anaerobic conditions.

One milliliter of bacterial cultures in BHI broth was centrifuged at 12,000 X g for 2 min using a bench-top centrifuge. The pellets were transferred in sterile, demineralized water, and the DNA was extracted using Wizard Genomic DNA purification kit (Prom-ega, Madison, WI, USA) with addition of lysozyme (10 mg/ml, Eurogentec, Seraing, Belgium), as recommended for Gram-positive bacteria. DNA concentrations were spectrophotometrically estimated (GeneQuant pro, Amersham Pharmacia, Roosendaal, Netherlands). DNA samples were diluted with distilled water to obtain a concentration between 25 and 50 µg/ml.

Artificially contaminated samples. Artificially contaminated samples were prepared as follows: 40 ml of UHT milk was added to 360 ml of BHI broth supplemented with mupirocin (80 mg/ml) in a sterile bottle. After

homogenizing, the resulting mixture was distributed in aliquots of 10 ml. They were inoculated with 100 µl of 10-fold serial dilutions of a 48 h culture of *B. pseudolongum* subsp. *globosum* (RU224) and *B. thermophilum* (DSM 20210^T) in peptone sodium solution, and including a negative control without inoculation.

One milliliter of each aliquot was distributed on MRS medium (Oxoid) plates supplemented with mupirocin (50 mg/ml) for bifidobacteria counting after 72 h anaerobic incubation at 37 °C. The other part of aliquot (about 9 ml) was incubated during 24 h in anaerobic conditions at 37 °C. The same procedure was repeated with a 48 h incubation time. After this, 1 ml of each incubation broth was transferred into a microcentrifuge tube and centrifuged at 12,000 X g for 2 min using a bench-top centrifuge. The pellets were transferred in sterile, demineralized water, and the DNA extracted using Wizard Genomic DNA purification kit (Promega) as previously described for pure strains.

Raw milk and raw milk cheese samples. DNA was extracted from cultures obtained after the enrichment step of the cultural-based method (from pure until 10^{-4} dilution for milk and until 10^{-6} dilution for raw milk cheese samples). One milliliter of each homogenized content was transferred in a microcentrifuge tube and centrifuged at 12,000 X g for 2 min using a bench-top centrifuge. The pellets were transferred in sterile, demineralized water, and the DNA extracted using Wizard Genomic DNA purification kit (Promega) as previously described for pure strains. In case of PCR inhibition, the DNA samples were diluted 10 fold.

2.2.3.2. Selection of primers. The sequences of the *hsp60* gene are available on Genbank for several representative *Bifidobacterium* species in human and animal feces (Accession number, *B. adolescentis:* AF210319, *B. animalis:* AY004287, *B. cuniculi:* AY004283, *B. choerinum:* AY013247, *B. pseudolon-gum* subsp. *globosum:* AF286736, *B. pseudolongum* subsp. *pseudolongum:* AF240573, *B. merycicum:* AY004277, *B. pseudocatenulatum:* AY004274, *B. ruminantium:* AF240571, *B. thermophilum:* AF240567). These sequences were aligned (ClustalW, http://www.ebi.ac.uk/clustalw/). From these sequence alignments, *Bifidobacterium* genus-specific degenerated primers were selected using Oligo software (Medprobe). Specificity of the primers for the *Bifidobacterium*-genus was checked realizing a Meg-ablast. Only *Bifidobacterium* DNA was fully complementary to the primers sequences (data not shown, www.ncbi.nlm.nih.gov/BLAST).

The genus-specific amplification of a 217 bp fragment of the *hsp60* gene is generated using primers: B11 up: 5'-GTS CAY GAR GGY CTS AAG AA-3', B12 down: 5'-CCR TCC TGG CCR ACC TTG T-3' (Sigma Genosys, UK).

2.2.3.3. Controls. The following amplification controls were run with each series: positive, i.e. reaction mix containing DNA extract from a positive strain of *B. pseudolongum* (B 116/1/1, Table 1), two reagent controls, i.e. mix containing all reagents without sample DNA and extraction control, i.e. 1000 µl of distilled water processed in the same manner as the samples.

Food can be a difficult matrix for PCR testing because it contains PCR inhibitors. So to be sure that a negative result is indeed due to absence of the target rather than to an inhibition of PCR reaction, we had to construct an internal PCR control.

An artificially created DNA fragment was used as an internal positive control in every reaction mixture, except for the other controls. The control DNA consisted of a fragment of 315 bp of the pGEMT vector, flanked by the target for the *Bifidobacterium*-genus PCR primers. This product was created by a two-step PCR as follows. Chimerical PCR primers flanked with the *Bifidobacterium* genus-specific primers were chosen: CI up: 5'-GTS CAY GAR GGY CTS AAG AAG CAG GAA AGA ACA TGT GAG CA-3' and CI down: 5'-CCR TCC TGG CCR ACC TTG TAC GAC CTA CAC CGA ACT GAG A-3'. The first step comprised amplification of DNA from the pGEMT vector using the chimerical primers by 45 cycles at the following PCR conditions. A 5 µl pGEMT (Eurogentec, Seraing, Belgium) DNA was introduced in a mix containing 0.2 mol 1^{-1} dNTPs, 400 pmol 1^{-1} of each chimerical PCR primers, 0.8 U of Dap Goldstar polymerase (Eurogentec), 1 x buffer: 20 mM Tris-HCl, pH 8.0, 100 mM KC1, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Nonidet P-40 and 0.5% Tween-20 (Eurogentec).

The samples were subjected to an initial step of denaturation at 95 °C for 5 min, followed by 15 denaturation cycles at 95 °C for 30 s, annealing at 50°C for 30 s and extension at 72 °C for 30 s and 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. In the second step, the amplicon of the first amplification was purified (QIAquick PCR Purification Kit, Qiagen, Westburg, The Netherlands), diluted 1/1000 in distilled water and used as a template to perform a second amplification using the *Bifidobacterium* genus-specific primers (in PCR conditions). The final amplicon was purified, diluted and used

as internal control. As measured by optical density using a GeneQuant pro spectrophotometer UV (Amersham Pharmacia), the DNA concentration was 185 µg/ml. The final dilution in distilled water of the internal control target was established empirically to reduce competition with target DNA and corresponded to 1.1 µg/µl of DNA. The control DNA was used as a positive amplification control in all samples.

2.2.3.4. PCR conditions. PCR mix was composed of 0.2 mol l^{-1} dNTPs, 400 pmol l^{-1} of each primer, 1 U of FastStart TaqPolymerase (Roche), 1 x buffer: 500 mM Tris-HCl, 100 mM KC1, 50 mM $(NH_4)_2SO_4$, pH 8.3/25 °C (Roche), 4 µl DNA (50-100 ng), 1 µl internal control and H_2O in a total volume of 20 µl.

PCR was run using the following cycling conditions: 1 x 5 min at 95 °C, 40 x 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final extension (5 min at 72 °C). Samples were kept at 4 °C or stored at -20 °C before analyzing. A sample was considered as positive when the 217 bp amplicon was visible on 2% agarose gels after electrophoresis and ethidium bromide staining.

Two different protocols were tested: PCR from BH (PCR 1) and PCR from BHMup enrichment broth (PCR 2).

2.2.4. Statistical analysis

We chose the Mc Nemar test (Leroy and Farnir, 2000) to statistically evaluate the different methods (culture-based and PCR). Dilutions were tested as separate values. To compare results obtained at different steps of the raw milk cheese production, an ANOVA test (Dagnelie, 1975) was performed.

3. Results and discussion

3.1. Culture-based methods

Thirty-nine raw milk samples (195 dilutions) were analyzed by three culture-based methods using three combinations of enrichment and isolation media: Cultural 1 (BH/CMup), 2 (BHMup80/C) and 3 (BHMup/CMup). Table 2 presents the number of positive dilutions for each cultural method. The highest percentage of positives (95%) was detected for pure and for 10^{-1} dilutions with Cultural 3. Table 3 presents the comparison of the three methods by the Mc Nemar test (based on Chi-square table), which was calculated on the basis of the number of different results obtained on the 195 dilutions, with methods compared 2 by 2. In the Mc Nemar test, Cultural 3 was confirmed to be the best method. A statistical difference was observed between Cultural 2 and Cultural 3 in favor of Cultural 3 (χ^2=5.56; P<0.025). A trend was noticed between Cultural 1 and Cultural 3 in favor of using mupirocin at both steps, enrichment and isolation (χ^2 =2.91; P<0.l). No difference was observed between methods using mupirocin only, either in the isolation medium or in the enrichment medium.

Many different selective agents were used for detection of bifidobacteria: lithium chloride, sodium propionate, nalidixic acid, neomycin sulphate, paromomycin sulphate, polymixin B sulphate (Payne et al., 1999). In the case of raw milk samples, an enrichment step was necessary because of the possible relatively low levels of bifidobacteria (10 to 10^6 ml^{-1}) compared to those in human or animal feces (10^7 to 10 g^{-1}). Beerens (1998) recommended using at the enrichment step the BHI medium with addition of propionic acid, yeast extract, iron citrate, and at the isolation step, paromomycin as selective agent. However, the high number of lactobacilli not inhibited by paromomycin hid bifidobacteria at low dilutions.

Table 2: *Number and percentage of positive raw milk samples analyzed by the three culture-based methods*

Dilution/method	Cultural 1	Cultural 2	Cultural 3
Pure	33/39 (85%)	33/39 (85%)	37/39 (95%)
-1	34/39 (87%)	31/39 (79%)	37/39 (95%)
-2	25/39 (64%)	23/39 (59%)	26/39 (67%)
-3	7/39 (18%)	8/39 (21%)	6/39 (15%)
-4	0/39 (0%)	1/39 (3%)	1/39 (3%)

Cultural 1: BH/CMup; Cultural 2: BHMup/C; Cultural 3: BHMup/CMup.

Pure: dilutions analyzed from pure enrichment broth; -1,-2, -3 and -4: dilutions, respectively, analyzed from 10, 10^2, 10^3 and 10^4 fold dilutions of the enrichment broth.

Rada et al. (1997) and Rada and Petr (2000) showed that bifidobacteria were resistant to mupirocin when lactobacilli were susceptible. Mupirocin (pseudo-monic acid A) was originally isolated from *Pseudo-monas fluorescens* and used as a topical antibiotic (Sutherland et al., 1985).

In raw milk samples, addition of mupirocin at the enrichment step can eliminate most of the lactobacilli strains present that could hide bifidobacteria in raw milk. If some lactobacilli strains were still present after the enrichment step, one might suppose that they would be eliminated during the isolation step by mupirocin, when present. Grand et al. (2003) also used mupirocin as selective agent for detection of bifidobacteria in probiotic milk products, as did Mikkelsen et al. (2003) in gastrointestinal samples from piglets and Simpson et al. (2004b) in probiotic animal feed.

The culture-based method presented in this study provides semiquantitative results. As none of culture-based methods are sufficiently selective to detect only bifidobacteria, the F6PPK test must be performed to confirm that isolated strains indeed belong to the genus *Bifidobacterium.* An alternative was to carry out the F6PPK test on the whole culture at the isolation step in order to more rapidly ascertain the contamination level of studied samples.

Table 3 : *Comparison of the three culture-based methods by the Mc Nemar test based on numbers of different results (+/- and - /+) obtained with methods compared 2 by 2 (195 dilutions/39 samples)*

Number of dilutions (samples) analyzed by culture-based method	Compared methods	Percentage of positive dilutions	+/-	-/+	Statistical results
195 (39)	Cultural 1/ Cultural 2	51/49	16	14	NS-$\chi^2 = 0.06$ $P<0.8$
195 (39)	Cultural 1/ Cultural 3	51/55	7	15	NS-;$\chi^2 = 2.91$ $P<0.1$
195 (39)	Cultural 2/ Cultural 3	49/55	4	14	S-;$\chi^2 = 5.56$ $P< 0.025$

Cultural 1: BH/CMup; Cultural 2: BHMup/C; Cultural 3: BHMup/CMup. +/-: Positive dilutions with the first method and negative with the second one. -/+: Negative dilutions with the first method and positive with the second one.
In favor of

Fig. 1. *PCR amplification of Bifidobacterium DNA from some raw milk samples and using an internal control. Legend: T784/2, T785/1, T785/ 2, T786/1, T786/2, T787/1, T787/2: internal numbers of samples. M: 5 µl molecular weight marker (100-200-300-400-500-600-700-800-1000 bp). SM: PCR realized with DNA extracted from pure enrichment broth; -1, -2 and -3, dilutions, respectively, analyzed from 10-, 10^2-and 10 -fold dilutions of the enrichment broth.*

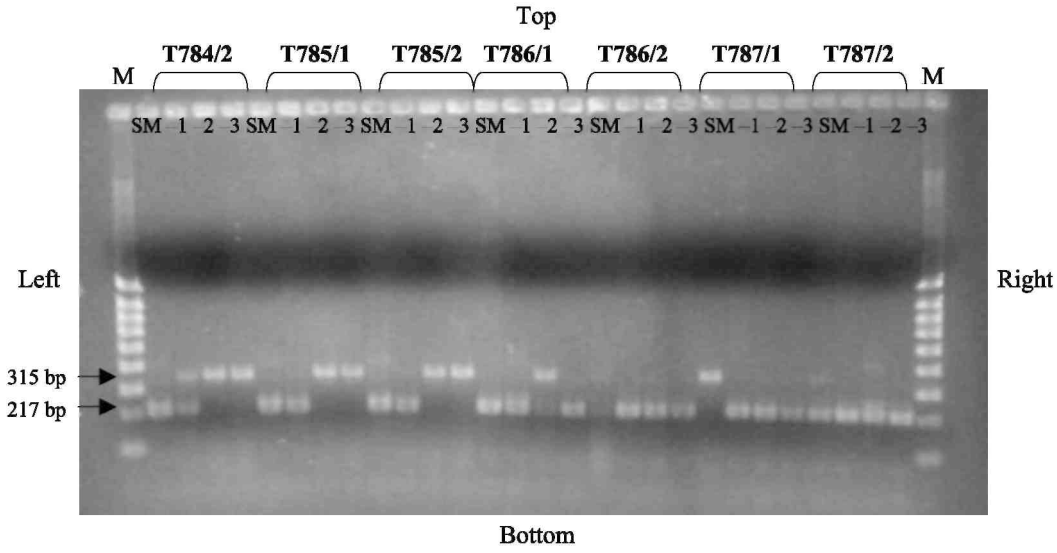

Table 4: *Number and percentage of positive raw milk samples analyzed by the two PCR methods*

Dilution/method	PCR 1	PCR 2
Pure	11/12 (92%)	12/12 (100%)
-1	10/12 (83%)	11/12 (92%)
-2	4/12 (33%)	7/12 (58%)
-3	0/12 (0%)	3/12 (25%)
-4	0/12 (0%)	1/12 (8%)

PCR 1: PCR realized from BH broth; PCR 2: PCR realized from BHMup broth.
Pure: dilutions analyzed from pure enrichment broth; -1, -2, -3 and -4: dilutions, respectively, analyzed from 10, 10 , 10 and 10 fold dilutions of the enrichment broth.

Table 5*: Comparison of the 2 PCR methods by the Mc Nemar test based on numbers of different results (+/- and -/+) obtained with methods compared 2 by 2 (60 dilutions/12 samples)*

Number of dilutions (samples) analyzed by PCR	Compared methods	Percentage of positive dilutions	+/-	-/+	Statistical results
60 (12)	PCR 1/PCR 2	42/57	3	12	S-χ^2 = 5.4 P< 0.025

PCR 1 : PCR realized from BH broth; PCR 2: PCR realized from BHMup broth. +/-: Positive dilutions with the first method and negative with the second one. -/+: Negative dilutions with the first method and positive with the second one.
In favor of

3.2. PCR methods

3.2.1. Validation of the primers on pure strains

Specificity of the primers was confirmed by PCR using chromosomal DNA extracted from 37 non-*Bifidobacterium* strains and from 127 *Bifidobacterium* strains. The primers were able to detect an expected 217 bp DNA fragment from all *Bifidobacterium* strains using the PCR described conditions. No amplification was obtained for strains of any of the other tested species (5 *Enterococcus* spp., 5 *Pseudomonas* sp., 5 *S. aureus,* 6 *Lactobacillus,* 4 *C. perfringens,* 6 *B. cereus,* 5 *E. coli* and 1 *S. typhimurium).* This validation was realized in triplicate.

3.2.2. Detection limit of the PCR method on artificially contaminated samples

PCR results obtained from enrichment media incubated for 24 and 48 h have been compared in relation with bifidobacteria counts on MRS plates (Oxoid) after 72 h anaerobic incubation at 37 °C. Depending on the incubation time of the enrichment media, the PCR method could detect DNA from 10^2 to 10^3 cfu ml^{-1} present in the sample when the incubation time of the enrichment medium was 24 h, and around 1-10 cfu ml^{-1} when it was 48 h.

3.2.3. Comparison of PCR methods (from BH and from BHMup80 enrichment media)

Bifidobacteria were detected in 12 samples (60 dilutions) by PCR from BH enrichment broth (PCR 1) and from BHMup broth (PCR 2) obtained after the enrichment step of the culture-based methods.

Four possibilities of results were observed, (i) Only the target was positive (217 bp fragment), (ii) The target (217 bp) and the internal control (315 bp fragment) were positive. In these two cases, PCR was considered as positive for bifidobacteria detection, (iii) Only the internal control was positive. In this case, the PCR was indeed negative for bifidobacteria detection, (iv) Finally, if the target and the internal control were negative, it signed PCR inhibition. In this case, it was necessary to do the PCR again on a diluted sample. Some of the results are presented in Fig. 1.

Table 4 presents the percentage of positive dilutions obtained with PCR 1 and PCR 2. The highest percentage of positive dilutions (100%) was detected for pure dilutions with PCR 2. Comparison between the two methods is presented in Table 5. A significant difference was observed between the two PCR methods in favor of PCR 2

(χ^2=5.4; *P*<0.025). This showed that mupirocin in enrichment broth positively affected detection of *Bifidobacterium* by PCR. It suggested that PCR sensitivity was better when a selective agent was used in an enrichment broth. Elimination of most lactobacilli avoided competition with bifidobacteria that could be detected at higher dilutions.

***Table 6**: Comparison of different combinations of culture-based and PCR methods by the Mc Nemar test based on numbers of different results (+/- and -/+) obtained with methods compared 2 by 2 (395 dilutions/79 samples, 255 dilutions/51 samples and 90 dilutions/18 samples)*

Number of dilutions (samples) analyzed by PCR and culture-based methods	Compared methods	Percentage of positive dilutions	+/-	-/+	Statistical results
395 (79)	PCR 1/Cultural 1	51/43	49	23	S-;χ^2 = 9.4 *P*< 0.005
255 (51)	PCR 2/Cultural 2	56/53	21	13	NS-χ^2=1.9 *P*<0.2
90(18)	PCR 2/Cultural 3	61/55	4	2	NS - χ^2 = 0.7 *P*<0.5

Cultural 1: BH/CMup; Cultural 2: BHMup/C, Cultural 3: BHMup/CMup, PCR 1: PCR realized from BH broth; PCR 2: PCR realized from BHMup broth.
+/- : Positive dilutions with the first method and negative with the second one.
-/+: Negative dilutions with the first method and positive with the second one.
In favor of

***Table 7:** Number of Bifidobacterium positive samples (percentage) of raw milk cheese at each step of production (n=25 for A, C, E steps and n=24 for the step G)*

Dilutions/ methods	PCR 2				Cultural 3			
	A	C	E	G	A	C	E	G
Pure	25 (100%)	25 (100%)	24 (96%)	24 (100%)	23 (92%)	24 (96%)	25 (100%)	23 (96%)
-1	22 (88%)	22 (88%)	17 (68%)	24 (100%)	21 (84%)	23 (92%)	17 (68%)	22 (92%)
-2	12 (48%)	15 (60%)	9 (36%)	19 (79%)	3 (12%)	14 (56%)	5 (20%)	18 (75%)
-3	6 (24%)	6 (24%)	1 (4%)	12 (50%)	0 (0%)	1 (4%)	2 (8%)	16 (67%)
-4	3 (12%)	3 (12%)	1 (4%)	6 (25%)	0 (0%)	0 (0%)	1 (4%)	6 (25%)
-5	1 (4%)	2 (8%)	1 (4%)	4 (17%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
-6	0 (0%)	1 (4%)	0 (0%)	2 (8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

PCR 2: PCR realized from BHMup enrichment broth; Cultural 3: BHMup/CMup.
Pure: dilutions analyzed from pure enrichment broth; -1, -2,-3, -4, -5 and -6: dilutions, respectively, analyzed from 10, 10^2, 10^3, 10^4, 10^5 and 10 fold dilutions of the enrichment broth.

3.3. Comparison of culture-based and PCR methods

PCR 1 and 2 have been compared to the culture-based methods 1, 2 and 3 (Table 6). Each comparison showed a greater number of positive results by PCR. However, the difference was only significant when PCR 1 was compared to Cultural 1 (χ^2=9.4; P<0.005). No significant difference was observed when PCR 2 was compared to Cultural 2 and 3. For each comparison test, a percentage of false negatives by PCR are present, respectively equal to 6%, 7% and 2%.

Even if the results were not always significant, a trend was observed in favor of PCR, suggesting that the PCR method is more sensitive than culture-based methods. This also indicates that inhibition phenomena apparently do not play a significant role in the given experimental settings. Although they cannot be ruled out entirely, we tried to minimize the effect by a dilution of the DNA extract.

3.4. Contamination of raw milk cheese samples along the production chain

Table 7 presents the number of positive dilutions with the two best methods (PCR 2 and Cultural 3). The highest percentage of positives (100%) was detected for pure (steps A, C and G) and for 10^{-1} dilutions (step G) with PCR 2. Cultural 3 detected

100% of positive dilutions for pure on step E, and respectively, 92%, 96% and 96% of positives for pure on steps A, C and G The two methods were compared on 693 dilutions of 25 samples. The significant difference (χ^2=20.04; P<0.0005) observed in favor of PCR 2 (Table 8) was not in agreement with previous results on raw milk samples (no significant difference). However, it did correspond to the already observed trend in favor of PCR. The highest number of dilutions included in the test calculation on raw milk cheese samples would confirm that PCR 2 was a more sensitive method than the culture-based method.

Additionally, only 4% of false positives were obtained by PCR, which is in agreement with previous results obtained from raw milk samples.

The mean counts of bifidobacteria (Table 9) increased significantly (F=14.4; P<0.0005) from step A (milk) to step G (ripening at D+21), when studying the highest values obtained with the PCR or the culture-based method.

The lowest mean level (2.52 log cfu g^{-1}) of bifidobacteria was found on step E (after removal from the mold), where the pH decreased to 4.35 (on step C, pH was 6.45). This low pH can explain why *E. coli* disappeared from step E and why the level of bifidobacteria was still high, as these organisms can multiply at low pH (Biavati et al., 2000).

***Table 8**: Comparison between the PCR 2 and Cultural 3 methods by the Mc Nemar test based on numbers of different results (+/- and -/+) obtained with methods compared 2 by 2 (693 dilutions/25 samples)*

Number of dilutions (samples) analyzed by PCR	Compared methods	Percentage of positive dilutions	+/-	-/+	Statistical results
693 (25)	PCR 2/Cultural 3	41/35	73	28	S -χ^2 = 20.04 P< 0.0005

PCR 2: PCR realized from BHMup broth; Cultural 3: BHMup/CMup. +/- : Positive dilutions with the first method and negative with the second one. -/+: Negative dilutions with the first method and positive with the second one.
In favor of

***Table 9**: Mean counts (log cfu ml^{-1} or g^{-1} ! standard deviation) of bifidobacteria and E. coli in 25 raw milk cheese samples at four production stens*

Methods	Production steps[a]						
	A	C		E		G	
PCR 2	2.76 ! 1.3	2.96	1.46	2.20	1.12	3.79	1.53
Cultural 3	1.88 ! 0.73	2.48	0.82	2.00	1.00	3.54	1.38
PCR 2 or Cultural 3[b]	2.80 ! 1.26	3.20	1.29	2.52	1.00	4.33	1.27
E. coli	1.58 ! 1.52	1.98	1.34	0.73	1.07	0.18	0.50

PCR 2: PCR realized from BHMup broth; Cultural 3: BHMup/ CMup.
[a] Production steps: A, raw milk; C, after addition of rennet; E, after removal from the mold; G, ripening (Day 21).
[b] Means calculated from the highest values obtained with either PCR 2 or Cultural 3.

4. Conclusion

The culture-based method BHMup/CMup was efficient since it showed that 95% of raw milk and more than 95% of raw milk cheese samples contained bifidobacteria. However, like all culture-based methods, this method is time-consuming and could not be easily applied to food industry controls. The PCR method performed from BHMup enrichment medium does not present this disadvantage and can be used effectively to detect bifidobacteria as fecal indicators in raw milk cheese industries, instead of or with *E. coli.*

Lynch et al.(2002), Nebra et al. (2003) and Bonjoch et al. (2004) proposed the species *B. adolescentis* or *B. dentium* as indicators of fecal pollution. As these species are dominant in human feces, they will indicate a contamination of human origin. Moreover, Rhodes and Kator (1999) enumerated sorbitol-fermenting bifidobacteria to define human fecal pollution in estuarine watersheds. However, in raw milk cheese, the principal contamination was shown to be of animal origin (Beerens et al., 2000), most likely by cow dung on farm, since the same species, *B. pseudolongum,* was isolated from both kinds of samples. Therefore, in food industries, it seems important to define the human or animal origin of the contamination.

Further studies on the identification of bifidobacteria strains isolated from raw milk cheese samples should help to explain the increase of contamination level by bifidobacteria observed along the production chain.

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