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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.

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

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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

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