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ENUMERATION, ISOLATION, AND IDENTIFICATION OF BIFIDOBACTERIA FROM DAIRY PRODUCTS

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ABSTRACT

Six dairy products were tested. Bifidobacteria were enumerated and isolated using TPY agar modified by the addition of mupirocin (100 mg l^{-1}). Isolates were identified to the genus level by the detection of fructose-6-phosphate phosphoketolase (F6PPK) and by the FISH method. Bifidobacteria were characterised using API 50 CHL and API ID 32 A Rapid tests. In addition, grow at 46 °C was tested. Subsequently, all strains were identified to the species level using computer program Bacter. The identification was also carried out by PCR method using genus- and species-specific primers.
The bifidobacterial counts in products tested varied from 2.37 to 7.17 log CFU/ml. High selectivity was seen for modified TPY agar from which all isolates were identified as bifidobacteria. While most strains were identified as B. animalis using Bacter program, the same isolates had positive reaction with B. lactis-specific primers. Strain isolated from Rajo yogurt was identified as B. longum by Bacter as well as using PCR, but its bacterial counts were too low. Our results showed, that most of the bifidobacterial strains currently used in food products could be of animal origin.

Key words: milk products / dairy products / microbiology / bifidobacteria / enumeration / identification / isolation

ŠTETJE, IZOLACIJA IN IDENTIFIKACIJA BIFIDOBAKTERIJ V MLEČNIH IZDELKIH

IZVLEČEK

Testirali smo šest mlečnih izdelkov. Štetje bifidobakterij in izolacijo smo izvedli na TPY agarju, ki smo mu dodali muriprocin (100 mg l^{-1}). Izolate smo identificirali na ravni rodu z detekcijo fruktoza-6-fosfat fosfoketolaze (F6PPK) in z metodo FISH. Bifidobakterije smo opisali s hitrima testoma API 50 CHL in API ID 32. Preverili smo tudi rast pri 46 °C. Nato smo vse isolate identificirali na ravni vrst z računalniškim programom Bacter. Dodatno smo izvedli identifikacijo z metodo PCR ob uporabi za posamezen rod, oziroma vrsto specifičnih začetnih oligonukleotidov.
Število bifidobakterij v izdelkih je variralo med 2,37 in 7,17 log ke/ml. Opazili smo visoko selektivnost modificiranega TPY agarja, na katerem smo izolirali izključno bifidobakterije. Kljub temu, da smo večino izolatov z računalniškim programom Bacter identificirali kot *B. animalis*, smo DNA nekaterih izolatov lahko pomnožili z začetnimi oligonukleotidi, specifičnimi za *B. lactis*. Izolat iz jogurta Rajo smo s programom Bacter in vrstno specifično PCR identificirali kot *B. longum*, vendar je bilo število celic zelo nizko. Naši rezultati kažejo, da bi bila lahko večina sevov bifidobakterij, ki so v uporabi prehranski industriji, živalskega izvora.

Ključne besede: mlečni izdelki / mikrobiologija / bifidobakterije / štetje / identifikacija / izolacija

INTRODUCTION

Bifidobacteria are Gram-positive, non-sporeforming, non-motile, anaerobic, irregular rods. The typical habitat of bifidobacteria is human, warm-blooded animal and honeybee intestinal tract (Scardovi, 1986). Members of genus *Bifidobacterium (B.)* are among the most common microorganisms in the human gut, comprising up to 3% of the total faecal microflora of adults (Sghir *et al.*, 2000). They are more numerous in the infant gut, where they form up to 91% of the total microflora in breast-fed babies being supported by bifidogenic factors presented in human milk and up to 75% in formula-fed infants (Harmsen *et al.*, 2000). Using classical culturing methods it has been found that *B. adolescentis* and *B. longum* are major bifidobacterial species in the adult intestine (Gavini *et al.*, 2001; Biavati *et al.*, 1986; Mutai and Tanaka, 1987) and that *B. infantis* and *B. breve* are predominant species in the intestinal tract of human infants (Benno *et al.*, 1984; Biavati *et al.*, 1984; Mutai and Tanaka, 1987). In addition, *B. bifidum*, *B. catenulatum*, *B. pseudocatenulatum*, *B. angulatum, B. gallicum*, and *B. dentium* have also been reported to be human intestinal bifidobacteria (Scardovi, 1986). Matsuki *et al.* (1999) who used for the detection of bifidobacteria in human gut species-specific polymerase chain reaction (PCR) reported, that the most common species in the breast-fed infants are *B. breve*, *B. infantis*, *B. longum*, and *B. bifidum*. In adult intestinal tracts, the *B. catenulatum* group was the most common taxon, followed by *B. longum* and *B. adolescentis*.

The genus *Bifidobacterium* constitutes a significant proportion of the probiotic cultures used in the food industry (Langhendries *et al.*, 1995; Saavedra *et al.*, 1994). The employment of strains belonging to *B. animalis*, *B. longum*, *B. bifidum*, and *B. infantis* as probiotic starter cultures is due to their important role played in the gut (Gibson and Roberfroid, 1995; Modler *et al.*, 1990). They suppress harmful bacteria by controlling pH of the large intestine through the production of lactic and acetic acids (Gibson *et al.*, 1997). Bifidobacteria have antitumoral activity (Reddy and Rivenson, 1993; Rastall and Gibson, 2002), anticholesterolemic (Pereira and Gibson, 2002), and immune system activation effects (Mitsuoka, 1992). Other effects that have been described to this genus are the alleviation of lactose intolerance and vitamin production (Hughes and Hoover, 1995; Fooks *et al.*, 1999).

The presence of high number of bifidobacteria in the large intestine is desirable and can be influenced by dietary supplementation with probiotics and/or prebiotics. Probiotics have been defined as living microorganisms, which beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). Prebiotics are nondigestible dietary supplements that modify the balance of the intestinal microflora, stimulating the growth and/or activity of beneficial organisms and suppressing potentially deleterious bacteria (Gibson and Roberfroid, 1995). In order to exert a beneficial effect, probiotic bacteria should be viable and present at high numbers in the product at time of consumption (McBrearty *et al.*, 2001).

Industrial interest in the use of bifidobacterial strains as food additives in dairy products is rapidly growing. This development leads to the requirement for accurate quantity and quality control of the probiotic products and hence methods for specific identification of probiotic strains. Consequently, the aim of our work was to enumerate and isolate bifidobacteria from dairy products, and to compare biochemical and molecular biology methods for the identification of these isolates.

MATERIAL AND METHODS

Six dairy products were tested, three yogurts and three fermented milk products. Five products were made in the Czech Republic and one (Rajo) in the Slovak republic. The list of products tested is in Table 1.

Table 1. Tested products

Product	Product type	Made in
Activia	yogurt	Czech Republic
Hollandia	yogurt	Czech Republic
Olma – Dr. Bio	fermented milk product	Czech Republic
Yoplait	fermented milk product	Czech Republic
Kefir-like milk	fermented milk product	Czech Republic
Rajo	yogurt	Slovak Republic

Bifidobacteria in dairy products were enumerated and isolated using TPY agar (Sharlou, Barcelona, Spain) modified by the addition of mupirocin at a concentration of 100 mg/L (Rada and Koc, 2000). Pure isolates were enriched in TPY broth, and were identified as members of the genus *Bifidobacterium* by the demonstration of fructose-6-phosphate phosphoketolase (EC 4.1.2.22) activity, as described by Orban and Patterson (2000).

The genus identification was performed also using fluorescence *in situ* hybridisation (FISH) kit for *Bifidobacterium* sp. (RiboTechnologies, Groningen, the Netherlands). The component of the kit is a genus-specific oligonucleotide DNA probe labelled by fluorescein isothiocyanate (FITC), which binds to bifidobacterial rRNA. After the hybridization, the samples were analysed with a Nikon E-800 epifluorescence microscope. Another method used for the genus identification was PCR with genus specific primers which was performed as described previously (Kok *et al.*, 1996).

All isolates were tested for the ability to grow at 46 °C. Testing of grow at this temperature is a method recommended for distinguishing of human and animal strains. Human isolates are not able to grow at 46 °C and most of animal isolates are able to grow at this temperature (Gavini *et al.*, 1991). Subsequently, the isolates were characterised using API 50 CHL and API ID 32 A Rapid kits (BioMérieux, France). On the basis of the results from these tests, all strains were identified to the species level using computer program Bacter (http://kounou.lille.inra.fr, INRA, Lille, France).

The identification to the species level was also carried out by PCR method using species-specific primers. The genomic DNA of the strains was extracted by heating at 100 °C for 10 minutes in 1% Triton X-100 (Sigma, USA) by the method described by Wang *et al.* (1996). Amplifications were performed with a thermal cycler (Techne, Techgene, UK) with solutions, species-specific primers and temperature profiles described by Matsuki *et al.* (1999). Amplified PCR products were analyzed by 1% agarose gel electrophoresis at a constant voltage of 7 $V.cm^{-1}$ and visualized with ethidium bromide (0.5 μg/mL) under UV light (wavelength, 260 nm).

RESULTS AND DISCUSSION

Bifidobacterial counts determined in tested products are shown in Table 2. The counts varied from 2.37 to 7.17 log CFU/mL. Recommended lower limit of International Dairy Federation (IDF) for bifidobacterial counts in dairy product is 10^6 CFU per one mL. In Japan this recommendation is even at least 10^7 viable probiotic cells per gram or millilitre (Ishibashi and Shimamura, 1993). Generally, bifidobacteria show poor viability in fermented dairy products and various studies have indicated that not all probiotic products contain the recommended levels of viable microorganisms (Kailasapathy and Rybka, 1997; Dave and Shah, 1997). Only four of our product tested fulfil the recommendation of IDF, while two products did not meet these criteria. In Rajo yogurt the counts of bifidobacteria were only 2.37 log CFU/mL.

High selectivity was observed for modified TPY agar from which all isolates were F6PPK-positive and were identified as bifidobacteria. These results were confirmed by FISH and PCR methods.

Table 2. Bifidobacterial counts and species isolated from dairy products

Product	Bifidobacterial counts	Species isolated
Activia	7.17 ± 0.09^{a}	*B. animalis*/*B. lactis*
Hollandia	6.31 ± 0.38^{a}	*B. animalis*/*B. lactis*
Olma – Dr. Bio	6.22 ± 0.20^{a}	*B. animalis*/*B. lactis*
Yoplait	6.03 ± 0.12^{a}	*B. animalis*/*B. lactis*
Kefir-like milk	5.44 ± 0.41^{b}	*B. animalis*/*B. lactis*
Rajo	2.37 ± 0.47^{c}	*B. longum*

Results are means (n=3) ± SD of log CFU/mL
[a,b,c]Data in column with no common superscripts differ ($P < 0.05$)

While five strains were identified as *B. animalis* using Bacter program, the same isolates had positive reaction with *B. lactis*-specific primers. Hence, it is not clear when *B. lactis* and *B. animalis* are the same species, because Bacter database does not contain species *B. lactis* and on contrary, there are no available *B. animalis*-specific primers. Cai *et al.* (2000) reported that the relative taxonomic position of *B. lactis* is still under discussion and that *B. lactis* could be considered a junior subjective synonym for *B. animalis*. In contrary, Ventura *et al.* (2001) demonstrated clear differences in rDNA sequences between *B. lactis* (DSM 10141) and the type strain of *B. animalis* (ATCC 25227). A decision of this issue by the International Committee on Systematic Bacteriology is still outstanding (Anonym, 2001).

Strain isolated from Rajo yogurt (1?) was identified as *B. longum*, which is the human origin species, by both Bacter as well as using PCR. But its bifidobacterial counts were too low. Five from six dairy-related isolates were identified as species of animal origin, although it is recommended that the bifidobacterial strains used in fermented milk products should be of human origin. Especially *B. animalis* is often found in dairy products (Bonaparte, 1997).

A modified TPY agar was found to be highly selective and suitable for isolation and enumeration of bifidobacteria from dairy products, as all isolates in our study were identified as bifidobacteria. Our results also showed that most of the bifidobacterial strains currently used in food products are probably of animal origin. Only one strain was identified as *B. longum*, which is of human origin, but its survival in fermented milk product was poor. Further investigations should be focused on the selection of human bifidobacterial isolates which are able to survive in milk for longer period of time. Also, the identity and origin of currently used strains should be clearified.

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