Comparison of Four Methods for Identification of Bifidobacteria to the Genus Level

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Abstract

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The identification of bifidobacteria to the genus level is important for the differentiation of these bacteria from other bacteria occurring in the animal and human intestine. The detection of fructose-6-phosphate phosphoketolase (F6PPK-test) is used traditionally for the identification of *Bifidobacterium* sp. The original procedure is time consuming and therefore it was modified several times recently. The aim of the present work was to compare the following methods for the genus identification of bifidobacteria: F6PPK-test, F6PPK-test modified by the addition of triton X-100, F6PPK-test modified by the addition of cetridium bromide (F6PPK-CTAB-test), and PCR using genus specific primers. Bifidobacteria isolated from fermented milk products (3 strains), human faeces (6 strains), and animal intestinal tract (2 strains) were tested. All the methods tested proved to be reliable tests for the genus identification of bifidobacteria. The F6PPK-CTAB-test gave the best results. This procedure is quick and does not require any special laboratory equipment.

Keywords: bifidobacteria; identification; fructose-6-phosphate phosphoketolase test; CTAB; Triton X-100; PCR

Bifidobacteria are Gram-positive nonspore-forming irregular anaerobic rods. The typical habitat of this genus is human and animal intestinal tract (SGORBATI *et al.* 1995). It has been suggested that *Bifidobacterium* species are important in maintaining general health. They suppress harmful bacteria by controlling pH of the large intestine through the production of lactic and acetic acids (GIBSON & WANG 1994). The other health benefits which have been attributed to bifidobacteria include the alleviation of lactose intolerance (FOOKS *et al*. 1999), the reduction of cholesterol levels (MODLER *et al.* 1990; RASIC *et al.* 1992), antitumoral activity (REDDY & RIVENSON 1993), and immune system activation effect (MITSUOKA 1992). Other physiological effects that have been described to this genus include vitamin production (ISHIBASHI & SHIMAMURA 1993). The potential health benefits of bifidobacteria to the host have led to their wide application in dairy products and food additives (GIBSON & ROBERFROID 1995; TANNOCK 1999).

Genus *Bifidobacterium* can be distinguished from other bacterial groups such as lactobacilli, actinomycetes, and anaerobic corynebacteria by a particular metabolic pathway, the bifidus shunt, whose key enzyme is fructose-6-phosphate phosphoketolase (F6PPK). The demonstration of the F6PPK activity serves as a taxonomic tool in the identification of the genus (SCARDOVI 1986). The original procedure is time consuming and therefore it was modified several times, recently by BIBILONI *et al.* (2000) and ORBAN and PATTERSON (2000). The fermentation of glucose by bifidus pathway gives rise to acetic and lactic acids in the theoretical ratio of 3:2. Gas liquid chromatography of the fermentation products thus provides another reliable means for the differentiation of bifidobacteria from other bacterial types (HOLDEMAN *et al.* 1977; BIVATI *et al*. 1992).

Biochemical tests for the identification of the members of the genus *Bifidobacterium* are now largely superseded by the use of the genus-specific PCR primers (KOK *et*

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al. 1996). Genus-specific probes have proved useful in the enumeration of the total population of bifidobacteria in faecal (LANGENDIJK *et al*. 1995) and food samples (KAUFMANN *et al*. 1997).

The aim of the present work was to compare three methods of the F6PPK detection and PCR using genus specific primers (KOK *et al*. 1996).

MATERIALS AND METHODS

Seven strains were isolated from human adult and infant faeces. Four of them were obtained from the American Type Culture Collection (ATCC) or the Czech Collection of Microorganisms (CCM) and were of human origin. Three strains were isolated from the intestine and faeces of animals, five strains from fermented milk products (Table 1). The isolation was performed by the use of Trypticase-phytone-yeast extract (TPY) medium (MTPY; ADSA, Spain) modified by mupirocin at a concentration of 100 mg/l (RADA & PETR 2000).

The method of the F6PPK detection described by SCARDOVI (1986) was used for the identification of bifidobacteria to the genus level. Microorganisms were cultivated anaerobically in an anaerobic chamber in 20 ml TPY broth at 37°C for 42 hrs. The cells were harvested by centrifugation at 14 000 × g for 3 min. The pellet was washed twice with a phosphate buffer solution 1:(0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/l) and the cells were suspended in 1 ml of buffer. The cells were disrupted by sonication (BANDELIN, SONOPULS HD2070, Germany) in ice for 2 min. The sonicate was mixed with 0.25 ml each of solutions 2 (6 mg NaF and 10 mg Na iodoacetate in 1 ml distilled H_2O) and 7 fructose-6-phosphate 80 mg/l distilled H_2O). After 30 minutes of incubation at 37°C, the reaction was terminated by the addition of 1.5 ml of solution 3 (hydroxylamine HCl, 13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5). The mixture was kept for 10 minutes at room temperature and then 1 ml each of solutions 4 (TCA 15% (w/v) in water) and 5 (4 M HCl) were added. Finally, 1 ml of the colour-developing solution 6[$FeCl_3.6H_2O$ 5% (w/v) in 0.1 M HCl] was added. The development of a reddish-violet colour immediately after shaking the tube indicated the presence of fructoso-6-phosphate phosphoketolase. This enzyme is unique for

Table 1. Comparison of methods for identification of bifidobacteria to the genus level

Name of the strain	Origin of the strain	F6PPK test	F6PPK test with Triton X-100	F6PP-CTAB test
Strains isolated from dairy products				
Bifidobacterium sp. 19	Fermented milk	$0.511^{A} \pm 0.02$	$0.685^{A} \pm 0.14$	$1.188^{B} \pm 0.07$
B. animalis 1	Fermented milk	1.019 ± 0.17	1.035 ± 0.06	0.916 ± 0.03
B. animalis 2	Fermented milk	$0.840^{A} \pm 0.06$	$1.002^{B} \pm 0.05$	$0.958^{B} \pm 0.02$
B. animalis 3	Fermented milk	0.820 ± 0.02	0.814 ± 0.08	0.927 ± 0.10
B. animalis B2	Fermented milk	$0.522^{AB} \pm 0.07$	$0.347^{A} \pm 0.09$	$0.753^{B} \pm 0.12$
Strains isolated from human intestine				
B. breve ATTC 15 700	Type strain	$0.859^{AB} \pm 0.02$	$0.801^{A} \pm 0.05$	$0.951^{B} \pm 0.01$
B. catenulatum CCM 4989	Type strain	0.326 ± 0.05	0.382 ± 0.04	0.410 ± 0.04
B. infantis ATTC 17 930	Type strain	$1.148^{A} \pm 0.07$	$1.490^{B} \pm 0.08$	$1.261^{A} \pm 0.07$
B. longum ATCC 15 707	Type strain	$0.674^{A} \pm 0.11$	$0.828^{A} \pm 0.02$	$1.227^{B} \pm 0.02$
B. longum 1	Infant faeces	$0.483^{A} \pm 0.08$	$0.522^{A} \pm 0.10$	$0.797^{B} \pm 0.09$
B. dentium 21	Human faeces	$0.767^{A} \pm 0.05$	$0.913^{B} \pm 0.05$	$1.212^{C} \pm 0.04$
B. longum 3	Infant faeces	$1.060^{A} \pm 0.08$	$1.154^{AB} \pm 0.05$	$1.222^{B} \pm 0.03$
Strains isolated from animal intestine				
B. globosum 13	Rabbit caecum	$0.437^{A} \pm 0.08$	$0.575^{A} \pm 0.04$	$0.971^{B} \pm 0.03$
Bifidobacterium sp. MA1	Hen caeca	$0.926^{A} \pm 0.06$	$0.989^{AB} \pm 0.10$	$1.219^{B} \pm 0.16$
Bifidobacterium sp. MV4	Hen crop	$0.581^{A} \pm 0.05$	$0.408^{B} \pm 0.05$	$1.320^{C} \pm 0.04$

Results are means (n = 3) of absorbances (435 nm). Values with no common superscripts differ ($P < 0.05$)
ATCC – American Type Culture Collection
CCM – Czech Collection of Microorganisms

bifidobacteria. The result was negative if the colour remained yellow.

This F6PPK test was modified by the addition of Triton X-100 (SIGMA) to the phosphate buffer for a better cell disruption. The cells were resuspended in 1 ml 0.25% Triton X-100 in phosphate buffer solution and sonicated in ice for 2 min (BIBILONI *et al*. 2000). Another modification was the addition of cetridium bromide (CTAB). The cell membranes were disrupted using pretreatment with 0.4 ml CTAB detergent in solution containing 0.45 mg CTAB in 1 ml of distilled H_2O, and not by sonication (ORBAN & PATTERSON 2000).

The development of a reddish-violet colour was considered as a positive result. In all three methods, the intensity of this colour was measured spectrophotometrically at 435 nm. The results obtained by the measurement of absorbance were statistically evaluated using one way analysis of variance. The confidence level of 95% was used.

The identification to the genus level was also carried out by PCR method using genus-specific primers Bif164 and Bif662 (KOK *et al*. 1996).

RESULTS AND DISCUSSION

All three methods of the F6PPK detection were used for the testing of all 15 bifidobacteria strains. In all cases, the results were positive and reddish-violet colour was developed proving the presence of fructoso-6-phosphate phosphoketolase. The intensity of colour was measured spectrophotometically at 435 nm. The results of the absorbances determined are in Table 1. The intensity of the developed colour in F6PPK test modified by CTAB cell pretreatment was the highest in ten out of fifteen samples. The results of our work showed that CTAB can be effectively used for the cell disruption in the F6PPK test for the identification of bifidobacteria. The results of the F6PPK tests were confirmed by PCR procedure as all the strains were identified as *Bifidobacterium* sp.

The F6PPK-CTAB test is a less time consuming process of cell disruption compared to the traditional F6PPK test and does not require any special laboratory equipment. In the classic F6PPK test and in the F6PPK test modified by the addition Triton X-100, it is necessary to use a sonicator for the cell disruption. PCR method requires a thermal cycler and electrophoresis. Another possibility of the genus identification is to determine the molar ratio of acetic and lactic acids as the final products of glucose fermentation. This method is reliable but time-consuming and it is necessary to use a chromatograph.

Conclusion

All of the methods tested proved to be reliable tests for the genus identification of bifidobacteria. The F6PPK-CTAB test gave the best results. This procedure is quick and does not require any special laboratory equipment.

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Souhrn

VLKOVÁ E., MEDKOVÁ J., RADA V. (2002): **Porovnání čtyř metod rodové identifikace bifidobakterií**. Czech J. Food Sci., **20**: 171–174.

Identifikace bifidobakterií na rodovou úroveň je důležitá pro jejich odlišení od ostatních bakterií obsažených v trávicím traktu zvířat a lidí. Detekce enzymu fruktoso-6-fosfát fosfoketolasy (F6PPK-test) je tradičně užívanou metodou pro rodovou identifikaci bifidobakterií. Původní procedura je pracovně i časově náročná, proto byla v poslední době několikrát modifikována. Cílem naší práce bylo porovnat následující metody rodové identifikace bifidobakterií: klasický F6PPK test, F6PPK test modifikovaný přídavkem Tritonu X-100, F6PPK test modifikovaný přídavkem detergentu cetridium bromidu (CTAB) a metodu PCR s použitím rodově specifických primerů a nalézt metodu spolehlivou, finančně a časově nenáročnou. Celkem bylo testováno 7 kmenů izolovaných z trávicího traktu lidí, 3 kmeny z animálního trávicího traktu a 5 kmenů izolovaných z mléčných výrobků. Všechny testované metody je možné využít pro rodovou identifikaci bifidobakterií. Jako pracovně nejméně náročná a přitom spolehlivá se ukázala metoda modifikovaného F6PPK testu s přídavkem CTAB. Uvedený postup je i časově nenáročný a na rozdíl od ostatních testů nevyžaduje speciální přístrojové vybavení.

Klíčová slova: bifidobakterie; identifikace; fruktoso-6-fosfát fosfoketolasa test; CTAB; Triton X-100; PCR

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