Direct Identification of Bifidobacteria from Probiotic Supplements

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Abstract

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The DNA of 14 probiotic supplements was isolated directly from various products without previous cultivation. The bifidobacteria composition declared by the manufacturer was determined by species-specific PCR. Such species found were in accordance with those listed on the products. This approach can be performed in less than 5 h and is applicable to other genera of probiotic bacteria. Bifidobacteria were also detected by culture-dependent analysis and MALDI-TOF MS. These methods drew the same results; however, they are more expensive and time consuming. So, we propose direct identification of bifidobacteria for routine quality control inspections of species composition in probiotic supplements.

Keywords: *Bifidobacterium*; species-specific identification

Probiotics are defined as live microorganisms which when consumed in adequate amounts confer a health benefit on the host (FAO/WHO 2002). They represent a fast evolving field which has expanded in the last years from the traditional health benefit areas of digestive comfort and immune protection to diverse applications in various, sometimes unexpected, health benefit areas (Makinen *et al.* 2012). The administration of probiotic bacteria as nutraceuticals is a business with a global market, which is predicted to be worth $32.6 billion (Cook *et al.* 2012). Probiotic products should contain viable, defined microorganisms in sufficient numbers. Moreover, correct identification of the bacterial species used and agreement between the species declared on the label and those actually present in the product are both necessary for a probiotic product to be considered reliable (Fasoli *et al.* 2003; Temmerman *et al.* 2003). As stated above, there are still no official or generally accepted ISO standards for analysing the composition of bacterial species in probiotic products in Europe. In order to obtain functional probiotic products for human consumption, rapid and reliable quality control of such products is crucial (Matijasic *et al.* 2010). The health benefits provided by bifidobacteria to the host, as supported through clinical trials, have resulted in their being widely applied as probiotic components in health promoting products (Cronin *et al.* 2011). Nevertheless, clearer regulations are expected in the field of probiotics, and it is important to develop accurate methods for quantifying the probiotics in different products. The analysis of most probiotics is still based on culture-dependent methods involving the use of specific selective media and identifying a limited number of isolates, which makes this approach relatively insensitive, laborious and financially demanding (Temmerman *et al.* 2003; Masco *et al.* 2005). In addition, cultivation methods allow the detection of bifidobacteria only to the genus level. The use of molecular techniques in food microbiology has offered various possibilities in the field of microbial detection and identification. For example, DGGE analysis has been successfully used to identify probiotic microorganisms in food products

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and supplements (Temmerman *et al.* 2003; Masco *et al.* 2005; Theunissen *et al.* 2005). Furthermore, the method known as Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) has been introduced into routine microbiological diagnosis with marked success. This has successfully been used to identify different species of bacteria (Veloo *et al.* 2011; Duskova *et al.* 2012). In some cases, MALDI-TOF MS can identify bacteria to a subspecies level (Carbonnelle *et al.* 2011).

The aim of this study was to create and evaluate a rapid molecular methodology for direct identification of bifidobacteria in probiotic supplements without previous cultivation.

MATERIAL AND METHODS

Probiotic products and type strains. A total of 12 different probiotic supplements (lyophilised capsules and drops) commercially available on the European market (Table 1) were collected and analysed. All the probiotic products were tested prior to the expiration date indicated on the labels of the product. The strains used as type strains were as follows: *B. bifidum* DSMZ 20456 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), *B. breve* DSMZ 20213, *B. longum* ssp. *infantis* DSMZ 20088, *B. longum* ssp. *longum* DSMZ 20219, and *B. animalis* ssp. *lactis* DSMZ 10140.

Bifidobacteria species detection by culture-independent methods. The PrepMan®Ultra protocol for pure culture (Applied Biosystems, Foster City, USA) was modified for DNA isolation directly from the probiotic supplements. One gram of each of the products tested was dissolved in 9 ml of sterile saline solution. One ml of the suspension was transferred to a tube and centrifuged (3 min, 14 100 *g*). The supernatant was discarded and 200 µl of PrepMan Ultra sample preparation reagent was added to the sample. The mixture was vortexed for 1 min, incubated at 100°C for 10 min in a heat block and cooled at room temperature for 2 minutes. The samples were centrifuged (3 min, 14 100 *g*) and 50–100 µl of the clear supernatant containing DNA was transferred to a new tube. Then the supernatant was ready for PCR analysis. The DNA from overnight cultures of the type strains was isolated by the standard PrepMan®Ultra protocol (Applied Biosystems).

PCR amplification was carried out using the primer pairs specific to *Bifidobacterium* spp., *B. bifidum*, *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, *B. breve*

Table 1. Tested products and results of bifidobacteria species detection (strains were identified by PCR and MALDI-TOF MS, results obtained by both methods were in accordance)

Product	Manufacturer (Country)	Declared species					Found species				
		B. bifidum	*B. breve*	*B. longum* ssp. *longum*	*B. longum* ssp. *infantis*	*B. animalis* ssp. *lactis*	*B. bifidum*	*B. breve*	*B. longum* ssp. *longum*	*B. longum* ssp. *infantis*	*B. animalis* ssp. *lactis*
NutraBona symba	Nutra-Bona®s.r.o. (Czech Republic)	+	–	–	–	+	+	–	–	–	+
BioLac Baby drops Generica	Probiotical S.p.A. (Italy)	–	+	–	–	–	–	+	–	–	–
Biopron	Valosun a.s. (Czech Republic)	+	+	+	–	–	+	+	+	–	–
Pro+bioticum	Hungaria Dairy Research Institute Ltd. (Hungary)	–	–	–	–	+	–	–	–	–	+
Children Dophilus	Harmonium International (Canada)	+	–	+	–	–	+	–	+	–	–
Adult Dophilus	Harmonium International (Canada)	+	+	+	–	–	+	+	+	–	–
Probio-Fix IMUN	S&D Pharma Ltd. (UK)	–	–	–	–	+	–	–	–	–	+
Laktobacily 6+	Cemio (Switzerland)	+	–	–	–	–	+	–	–	–	–
Bio-Kult	Protexin® (UK)	+	+	+	+	–	+	+	+	+	–
PROBIO7 Advanced Formula	Forever Young International (France)	+	+	+	–	–	+	+	+	–	–
Linex® Baby	Lek Pharmaceuticals d.d. (Slovenia)	–	–	–	–	+	–	–	–	–	+
Infant Acidophillus	Swiss Herbal Remedies (Canada)	+	–	–	+	–	+	–	–	+	–

(+) declared/detected, (–) no declared/no detected

able 2. List of used primers specific to bifidobacteria

Species	Primer	Nucleotide sequence[a]	Annealing temperature (°C)	PCR product (bp)
Bifidobacterium sp.	Bif162 Bif662	GGGTGGTAATGCCGGATG CCACCGTTACACCGGGAA	59	523
B. bifidum	BiBIF-1 BiBIF-2	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAAA	59	278
B. breve	BiBRE-1 BiBRE-2	CCGGATGCTCCATCACAC ACAAAGTGCCTTGCTCCCT	57	288
B. longum ssp. *longum*	BiLON-1 BiLON-2	TTCCAGTTGATCGCATGGTC GGGAAGCCGTATCTCTACGA	59	831
B. longum ssp. *infantis*	BiINF-1 BiINF-2	TTCCAGTTGATCGCATGGTC GGAAACCCCATCTCTGGGAT	59	828
B. animalis ssp. *lactis*	Bflac2 Bflac5	GTGGAGACACGGTTTCCC CACACCACACAATCCAATAC	64	680
B. animalis ssp. *lactis*	LW-1 LW-2	GCACGGTTTCGGCCGTG GGGAAACCGTGTCTCCAC	55	567

[a]according to Kok *et al.* (1996), Ventura *et al.* (2001), and Matsuki *et al.* (2003)

and *B. animalis* ssp. *lactis* (Table 2). DNA amplifications were performed in a thermal cycler (Biometra, Goettingen, Germany) at a total volume of 25 µl containing 50 ng of DNA, 50 pmol of primer and Dream Taq™ Green PCR Master Mix (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA). The cycler programme consisted of an initial denaturation time of 5 min at 94°C; 35 cycles of 20 s at 94°C, 20 s at the appropriate annealing temperature (Table 2), and 30 s at 72°C; a final extension step of 5 min at 72°C, followed by cooling to 4°C. A different PCR programme was used for the subspecies *B. animalis* spp. *lactis* (Mayer *et al.* 2007). The following programme used an initial denaturation time of 5 min at 95°C; 30 cycles of 30 s at 95°C, 1 min at the appropriate annealing temperature (Table 2), 4 min at 72°C and a final extension step of 7 min at 72°C, followed by cooling to 4°C. The electrophoretic separation of amplified PCR products was performed in 1% agarose gels in 0.75× TAE buffer (both Serva Electrophoresis GmbH, Heidelberg, Germany) using a horizontal electrophoresis system (Owl EASYCAST B2; Thermo Fisher Scientific Inc., Waltham, USA) at a constant voltage (80 V/cm) for 50 min at 20°C. The gels were visualised by GelRed (Biotum, Goettingen, Germany) and documented by digital imaging (Quantity One; Bio-Rad, Philadelphia, USA).

Bifidobacteria species detection by culture-dependent methods. Bifidobacteria species composition in probiotic products was also determined by culture-dependent methods. Modified Wilkins-Chalgren agar (WSPmup) supplemented with soya peptone (5 g/l; Oxoid, Hampshire, UK), L-cysteine (0.5 g/l), Tween 80 (1 ml/l) (both Sigma, St. Louis, USA), mupirocin (100 mg/l; Oxoid, Hampshire, UK) and glacial acetic acid 1 ml/l according to (Rada & Koc 2000) was used for bifidobacteria enumeration and isolation. The bifidobacteria were cultivated in anaerobic jars (Anaerobic Plus System; Oxoid, Hampshire, UK) at 37°C for 2 days. Fifteen colonies were isolated from each sample after cultivation. Pure isolates were enriched with Wilkins-Chalgren broth supplemented with soya peptone (5 g/l; Oxoid, Hampshire, UK), and were identified as members of the genus *Bifidobacterium* by the demonstration of fructose-6-phosphate phosphoketolase activity (Orban & Patterson 2000). DNA was isolated from the isolates by the standard PrepMan® Ultra protocol (Applied Biosystems, Foster City, USA). PCR analyses with specific primer pairs were performed for all isolates, as described above. The isolates were identified also by MALDI-TOF MS (Kmet & Drugdova 2012).

RESULTS AND DISCUSSION

The results of direct PCR analysis of commercial probiotic supplements are reported in Table 1. The presence of the genus *Bifidobacterium* was revealed in all the products tested, the most frequently found species was *B. bifidum*. The species found were in accordance with those labelled on the products. The reliability of the PCR method was verified using type

strains as a positive and negative control. The quality of the DNA isolated directly from products according to the authors' modified PrepMan®Ultra protocol was found to be sufficient for the PCR procedure. The results were obtained at intervals of 3–5 h, depending on the PCR programme used. The methodology described could be improved by using the multiplex PCR method (Dong *et al.* 2000). The same method was successfully used in our study for bifidobacteria from probiotic supplements. The species *B. bifidum* and *B. longum* ssp. *longum* or *B. longum* ssp. *infantis* showed different PCR product size, and were clearly distinguished using the same annealing temperature.

Bifidobacteria species composition was determined also by culture-dependent methods involving the use of selective media in combination with specific PCR identification of isolates. All isolates were identified using the F6PPK test as *Bifidobacterium* sp. WSPMup agar was found to be suitable for the cultivation of all tested bifidobacteria species in probiotic products. The same species were detected in the products as when direct PCR detection was used. However, this methodology is much more time consuming and lasted about 4 days. The procedure can be shortened by 1 day, if DNA is isolated directly from colonies. Recently, spectroscopic techniques have been increasingly used to identify bacteria. The use of MALDI-TOF MS is reported as suitable for the identification of anaerobic bacteria (Veloo *et al.* 2011). MALDI-TOF MS analysis was also used in the study presented here to identify isolates. The results obtained by MALDI-TOF MS analysis enabled reliable identification of selected strains (Biotyper log score > 2.3, highly probable species identification) and corresponded with PCR results (Table 1). This method allows the identification of a culture grown in a broth or directly from a colony. However, a pure culture is needed for MALDI-TOF MS analysis of bacteria, as obtained after selective cultivation. Some authors reported that an extensive database is essential to identify bacteria reliably. For the routine identification of anaerobic bacteria the databases available need to be optimised (Veloo *et al.* 2011).

Probiotic products must contain the species declared. Traditional microbiological methods for studying microbial communities, i.e. counts on differential and selective media followed by identification of isolates, are time consuming and highlight the disadvantages of low selectivity and reciprocal inhibition in plate (Fasoli *et al.* 2003). Only one ISO standard exists for enumerating bifidobacteria in food. ISO 29981:2010 (IDF 220:2010) is the standard for selectively enumerating presumptive bifidobacteria in milk products by using a colony count technique at 37°C under anaerobic conditions. According to this standard, the method is applicable to milk products such as types of fermented and non-fermented milk, milk powders, infant formulas, and starter cultures, where these microorganisms are present and viable, and in combination with other lactic acid bacteria. However, this method does not allow selective determination to the species level. Consequently, the most commonly used species in lyophilised and milk products (*B. animalis* ssp. *lactis, B. adolescentis, B. bifidum, B. breve*, *B. longum* ssp. *longum,* and *B. longum* ssp. *infantis*) cannot be selectively enumerated using this procedure. For verifying the composition of bacterial species in the product it is useful to apply specific PCR methods. PCR with specific oligonucleotide primers represents a powerful, rapid, accurate and sensitive method for detecting target bacteria within complex ecosystems (Matsuki *et al.* 2003). The methodology used in the present study is also applicable when identifying other groups of probiotic bacteria. Culture-independent molecular methods for quantifying probiotic bacteria have been developed extensively; however, they have not been widely applied in routine laboratories yet (Matijasic *et al.* 2010). The culture-independent analysis of probiotic products by DGGE, which can be performed in less than 30 h, is a very laborious procedure (Temmerman *et al.* 2003). Also the quantitative real-time PCR (QRT-PCR) targeting of the 16S rRNA and *recA* genes was evaluated for enumerating bifidobacteria in probiotic products (Masco *et al.* 2007). These assays proved to be rapid and reproducible alternatives for culture-based detection and quantification of bifidobacteria in probiotic products. The QRT-PCR allows the quantification of individual species, but input costs are higher compared to conventional PCR. The approach used in our study is even faster, can be performed in less than 5 h and may be routinely used in food control laboratories. Direct identification of bifidobacteria from probiotic supplements was found to be a time-saving method with low material costs. The method can extend the quantification of bacteria using selective agars described under the ISO 29981:2010 (IDF 220:2010) standard.

CONCLUSION

The result obtained in this study showed that bacterial DNA, isolated directly from probiotic drops or lyophilised products, is applicable to the molecular identification of bifidobacteria by species-specific PCR methods. This culture-independent analysis was found

to be a valuable tool for the quality control of bifidobacterial species composition in probiotic supplements.

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