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Quality Control of Fermented Dairy Beverages

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Abstract

The broadening of fermented milk beverage assortment with probiotic products containing bacteria of Bifidobacterium and Lactobacillus gen- era prompts to develop reliable and fast methods for the quantitative and qualitative control. The aim of the present study was, therefore, to apply Polymerase Chain Reaction (PCR) carried out on DNA template extracted directly from beverages (a step of strain isolation excepted) for the detection and identification of Lactobacillus and Bifidobacterium cultures to evaluate commercial kefirs and yoghurts. Bacterial DNA was extract- ed from 3 kefirs and 5 yoghurts of 5 producers. Bacterial species were identified with reference to the type strains using primers specific to the Lactobacillus and Bifidobacterium genera; L. casei group; L. acidophilus, L. delbrueckii subsp. bulgaricus/lactis, L. fermentum, L. johnsonii, L. plan- tarum, L. rhamnosus, B. animalis/lactis, B. bifidum, B. breve, B. longum species. On the basis of positive PCR results, the presence of Lactobacillus cultures was stated in all yoghurts and kefirs, and that of Bifidobacterium cultures – in those with appropriate declaration. The applied primer sets enabled detection of the species of L. acidophilus, L. casei, L. johnsonii, and B. animalis/lactis in kefirs, and those of L. delbrueckii subsp. bulgari- cus, L. casei, L. johnsonii, L. acidophilus, L. fermentum, and B. animalis/lactis in yoghurts. Identification of Lactobacillus species was satisfactory, whereas that of Bifidobacterium species was sporadically unsuccessful, which indicates that the determination of low-number Bifidobacterium cultures demands more efficient DNA extraction and/or more sensitive detection methods to be applied in the fermented milk control.

Key Words: bifidobacterium; lactobacillus; microflora

Introduction:

Probiotic strains of Lactobacillus and Bifidobacterium gen- era recognised as non-pathogenic are increasingly used in dairy production [Reuter et al., 2002]. Yet, they require a strict control due to the uncritical strain selection and false declara- tions of producers, misleading the consumer [McKevith, 2002]. FAO/WHO [2002] recommendations, which define probiotics as precisely identified, characterised and described strains deposited in international culture collections, with effi- cacy proved in double blind, randomised, placebo-controlled (DBPC) phase 2 human trial, provide detailed guidelines for the labelling of probiotic food, with stress put on genus, species and strain designation as well as the minimum num- bers of viable bacteria at the end of the shelf-life claimed. Legal regulations concerning the control of probiotic foods are currently discussed on the European forum, indicating the need for development of reliable analytical methods. Com- monly used culturing methods of determination enable the quantitative evaluation of bacteria present in the product on a genus level, which makes the distinction of technological cultures from the probiotic ones impossible, e.g. numerous Lactobacillus species widely present in fermented dairy prod- ucts and their probiotic strains supplied additionally. To iden- tify species of dairy cultures with traditional methods, the isolation of pure strains is commonly required, followed by unreliable and time-consuming phenotypic assays, including biochemical capabilities, fermentation profile, or profile of proteins extracted from bacterial cells using an SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electro- phoresis) technique [Temmerman et al., 2003; Witthuhn et al., 2005]. Only few molecular tools has recently been available for the rapid and precise identification of species [Ventura et al., 2000]. Of these methods, a partial sequence analysis of DNA fragments containing variable regions V1 and V2 of the 16S rRNA coding gene [Gueimonde et al., 2004] and genus- specific PCR [Coeuret et al., 2004] have proven to be useful tools of identification, however both were preceded by pure strain isolation considerably prolonging the time of analyses. An alternative approach to direct analyses of DNA extracted from even more complex and multi-strain material as human faeces appeared to be successful in the studies of Matsuki et al. [1999] and Schwiertz et al. [2000]. Therefore, the aim of the present study was to apply polymerase chain reaction on DNA template extracted directly from the product for the detection and identification of Lactobacillus and Bifidobacteri- um cultures to evaluate commercial kefirs and yoghurts.

Material And Methods:

Fermented milk products. The identification of Lacto- bacillus and Bifidobacterium to the species level was carried out in five commercially available yoghurts and three kefirs (plain products all) produced by leading manufacturers of dairy products on the Polish

market. The presence of live yoghurt bacteria or kefir cultures was declared by all the producers, and that of L. acidophilus and Bifidobacterium sp. – additionally in two yoghurts and two kefirs. All prod- ucts were tested before the expiry date.

Reference strains. The identification was performed with reference to the following type strains: B. animalis subsp. lactis DSM 10140, B. bifidum ATCC 29521, B. breve ATCC 15700, B. longum ATCC 15707, L. delbrueckii subsp. bulgaricus DSM 20081, L. fermentum DSM 20052, L. john- sonii DSM 10533, L. acidophilus DSM 20079, L. rhamnosus DSM 20021, L. plantarum DSM 20174, and L. casei DSM 20011. These strains were also used for the optimisa- tion of PCR conditions and as a positive control in the species-specific PCRs conducted on complex DNA tem- plate of dairy product cultures. Additional strains, used for the determination of L. casei- and L. plantarum-species- specificity of newly-designed primers, were: L. helveticus DSM 20075, L. salivarius subsp. salicinius DSM 20554,L. salivarius subsp. salivarius DSM 20555, L. paracasei subsp. paracasei DSM 5622, L. reuterii DSM 20016,L. crispatus DSM 2058, and L. gasseri DSM 20243.

Isolation of bacterial DNA. Chromosomal DNA of bac- teria was extracted directly from fermented milks without the step of strain isolation. The sample (1 g) was suspended in 9 mL of PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl,

TABLE 1. Primers used in the study. 10 mmol/L Na2HPO4, 2 mmol/L KH2PO4; pH 7.1), mixed and centrifuged at 1700x g for 10 min at 4°C (a 5804R cen- trifuge, Eppendorf, Germany). The supernatant was col- lected and bacterial cells were harvested by centrifugation (10620x g, 10 min), washed twice with PBS buffer, spinned and resuspended in 0.5 mL of TE buffer (Tris-EDTA, 10 mmol/L Tris-Cl, 1 mmol/L EDTA; pH 8.0). Next, about 0.3 g of sterile glass beads (1 mm diameter) was added and the mixture was vortexed for 2 min at the maximal speed (3D Uniprep Gyrator, Germany). A bead/cell mixture was then centrifuged and the upper phase was transferred to a 1.5 mL tube containing proteinase K solution (50 µg/300 µL of 2x T&C Lysis Solution, Epicentre, USA). After incuba- tion (65°C, 15 min), the proteins were removed with MPC Protein Precipitation Reagent (Epicentre, USA). Nucleic acids were precipitated with isopropanol, centrifuged (10 min, 4°C), washed three times with 70% ethanol and after removing the alcohol – suspended in 100 µL of TE buffer. DNA solutions were stored at -20°C until examina- tion. Genomic DNA of the reference strains was extracted according to the method described by Bielecka et al. [2003].

PCR conditions. Amplification was performed with primer pairs described by other authors (Table 1) or designed in the present study. Sequences of L. casei group- and L. plantarum-specific oligonucleotides were retrieved from GenBank database and their complementarity to the target species was confirmed by the Blast algorithm. Ampli- fication reactions were prepared in the total volume of 15 µL, containing: 1.5 µL of 10x PCR buffer (500 mmol/L KCl and 100 mmol/L Tris-HCl, pH 8.8, at 25°C, 0.8% of Nonidet P40; Fermentas, Lithuania), each deoxynucleoside triphosphate at a concentration of 250 µmol/L, a pair of the specific primers at a concentration of 1.0 µmol/L of each primer, 0.4 U of Taq DNA polymerase (Fermentas, Lithua- nia), magnesium chloride at a concentration ranging from 1.5 to 5.0 mmol/L (Table 1), and 0.5 µL of template DNA. PCR amplifications were carried out in Eppendorf Master- cycler Gradient (Germany) applying the following PCR temperature profile: denaturation – 1 cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 15 s, annealing – at temperature dependent on the pair of primers used for 15 s (Table 1), elongation - 72°C for 15 s, and the terminal elon- gation at 72°C for 4 min.

All primers were commercially synthesised by TIB Mol- biol Poznañ (Poland). The concentration of magnesium chloride and the temperature of annealing were optimised for all primer pairs specific to Lactobacillus species with ref- erence to DNA of the type strains. The PCR parameters applied to Bifidobacterium species-specific primers were optimised as described previously [Bielecka et al., 2003]. The amplification products were separated in 2.0% (w/v) agarose gel electrophoresis (100 V) in 0.5x TAE buffer, fol- lowed by ethidium bromide staining.

Nomen-clature	Sequence	Target genus/species	Annealing temp. (°C)	$MgCl_2$ concen-tration (mmol/L)	Target sequence	Reference
LR	tggaaacaggtgctaataccg ccattgtggaagattccc	*Lactobacillus spp.*	58	1.5	16S rRNA	McOrist *et al.* [2002]
Aci I Aci II	tctaaggaagcgaaggat ctcttctcggtcgctcta	*L. acidophilus*	62	3.0	16S-23S rRNA	Tilsala-Timisiarvi *et al.* [1997]
LB1 LLB 1	aaaaatgaagttgtttaaagtaggta aagtctgtcctctggctgg	*L. delb. subsp. bulgaricus /lactis*	60	2.5	*pepIP*	Torriani *et al.* [1999]
Joh 16S I 16S II	gagcttgcctagatgatttta actaccagggtatctaatcc	*L. johnsonii*	60	1.5	16S-23S rRNA	Walter *et al.* [2000]
Lfpr Ferm II	gccgcctaaggtgggacagat ctgatcgtagatcagtcaag	*L. fermentum*	60	5.0	16S-23S rRNA	Walter *et al.* [2000]
Rha II Pr I	gcgatgcgaatttctattatt cagactgaaagtctgacgg	*L. rhamnosus*	60	5.0	16S-23S rRNA	Tilsala-Timisiarvi *et al.* [1997]
Lpl-1N Lpl-2N	taggaaccagccgcctaag cggtgttctcggtttcatta	*L. plantarum*	67	5.0	16S-23S rRNA	this study
Lcas-1N Lcas-2N	gcccttaagtggggataac tagagtttgggccgtgtctc	*L. casei* group	64	1.5	16S rRNA	this study
Bif-662 Bif-164	ccaccgttacaccgggaa gggtggtaatgccg	*Bifidobacterium spp.*	66	5.0	16S rRNA	Langendijk *et al.* [1995]

TABLE 1: Primers used in the study.

Results And Discussion

Traditional quantitative evaluation of fermented milk pro- ducts with culturing methods The quantitative determination of cultures is indispen- sable for the evaluation of fermented dairy products, although time-consuming, laborious, and requiring experi- ence. Bacterial colony forming unit (cfu) numbers grown after 48-h incubation of Petri-dishes were verified by the microscopic evaluation of bacterial cell morphology. Colonies of yoghurt cultures obtained in MRS medium had their cells morphologically characteristic for rods or cocci and were classified to the species of Lactobacillus or Strep- tococcus, respectively. Cells of all colonies counted on M17 medium had the form of cocci that is typical of Streptococ- cus and Lactococcus (for yoghurt and kefir, respectively). Two types of colonies considerably differing in size appeared on TOS-agar, however cells of merely larger ones were characterised by the morphology typical of bifidobac- teria [Scardovi, 1986].

Cocci predominated in both the examined products – Lactococcus in kefir (2.0´106–2.3´108 cfu/g), and Strepto- coccus in yoghurt (1.0´108–3.1´109 cfu/g) (Table 2). Natu- rally lower counts of Lactobacillus (<105–6.9´107 cfu/g) were assessed in kefir, whereas a considerably lower count of Lactobacillus compared to that of streptococci in yoghurts (1.5´105–2.6´106 cfu/g or even <105) is likely to indicate incorrectly selected yoghurt cultures of L. del- brueckii subsp. bulgaricus and S. thermophilus that did not form synergistic sets [Bielecka et al., 1994], the presence of antibiotic or inhibitory compound residues or problems with survival during shelf-life. Bifidobacterium counts in kefir, 6.1´105 and 7.5´105 cfu/g, were slightly lower than the minimal probiotic level assumed (1´106 cfu/g). Howev- er, taking into consideration that Bifidobacterium cells are able to multiply during kefir production in the presence of carbon dioxide produced by yeasts [Usajewicz et al., 2002], manufacturers might cautiously increase their populations because of unfavourable influence of bifidobacteria on sen- sory attributes (the formation of an unacceptable 'acetic' aftertaste). Bifidobacterium count in yoghurt was deter- mined at a level of 1.2´106 and 3.7´105 cfu/g, hence one product did not meet the assumed minimum probiotic level. Short time of yoghurt incubation determines the amount of Bifidobacterium supplementation, applied directly in the amount required at the end of the expiry date [Bielecka et al., 2000]. The highest counts of Lactobacillus were stated in 'A' and 'D' yoghurts with declared L. acidophilus-supple- mentation (Table 2). The quantitative evaluation of fer- mented milks demonstrated that the kefir and yoghurt of manufacturer A were distinguished by high counts of bacte- rial cultures. The latter product, as the only one of all the yoghurts studied, fulfilled the criteria of the Polish Standard [2002], whereas the others failed to meet recommendations for the count of Lactobacillus.

Qualitative evaluation with molecular technique (PCR)

The presence of Lactobacillus in all the products tested was confirmed using genus-specific primers (Table 2). With the primer sets applied, the species of L. acidophilus,L. johnsonii, L. casei and B. animalis/lactis were detected in kefirs, and those of L. delbrueckii subsp. bulgaricus, L. john- sonii, L. acidophilus, L. casei, L. fermentum and B. animalis/lac- tis – in yoghurts. One, two, or three Lactobacillus species were present in individual kefirs. Four of the five yoghurts contained typical yoghurt cultures of L. delbrueckii subsp. bulgaricus and two other Lactobacillus species – of L. casei, L. johnsonii, L. acidophilus or L. fermentum, whereas one – the species of L. johnsonii, L. acidophilus, L. casei, but not L. delbrueckii subsp. bulgaricus. The presence of Bifidobacterium was confirmed with genus-specific primers in these yoghurts and kefirs (A and D) whose producers declared their presence. In two products, the species of B. animalis/lactis were determined, but not those of B. breve, B. bifidum and B. longum. In single kefirs and yoghurts, the detection of Bifidobacterium species was unsuc- cessful (Table 2).

To recapitulate, with the approach established for the qualitative evaluation of fermented milks, three Lactobacil- lus species were detected in each yoghurt of different pro- ducers, and one to three species – in kefirs. In the investi- gations of commercial fermented milks or probiotic dairy products available on the Western Europe market, the iso- lates (strain isolation step applied) were classified to one or two Lactobacillus species and to one Bifidobacterium species (when declared) [Temmerman et al., 2003; Gueimonde et al., 2004]. In those and our studies, the same species of Lactobacillus and Bifidobacterium were detected, as the experiments are designed in a manner enabling the detec- tion of species of B. animalis/lactis (the only prevalent Bifi- dobacterium species), L. acidophilus, L. delbrueckii subsp. bulgaricus/lactis, L. johnsonii, L. casei, L. fermentum L. plantarum, and L. rhamnosus broadly used in starter cultures and present in fermented milks [Andrighetto et al., 1998; Schillinger et al., 1999; Bielecka et al., 2000; Simova et al., 2002; Gueimonde et al., 2004; Witthuhn et al., 2005]. In tra- ditional kefirs produced using grains, other lactobacilli than those detected in this study were also stated, like the species of L. delbrueckii subsp. bulgaricus that together with L. fer- mentum constituted up to 98.2% of the Lactobacillus popu- lation [Witthuhn et al., 2005], or L. helveticus which togeth- er with L. delbrueckii subsp. bulgaricus and L. casei constituted 24-33% of the microflora [Simova et al., 2002], which confirms the high diversity of kefir microflora.

The results of studies by Temmerman et al. [2003] and Gueimonde et al. [2004] have indicated that the declared bacterial composition of commercial fermented milks or probiotic dairy products did not always reflect the actual content. The Polish Standard [2002] regulates the labelling of additional microflora of yoghurt by demanding species or genus announcement, e.g. L. acidophilus, or Bifidobacterium. Taking into consideration these liberal rules, it should be stated that bacterial species composition of all the yoghurts tested was consistent with the demands of the Polish Stan- dard [2002] and with the producers' declarations as well.

PCR approach – advantages, faults, critical points The broadening of probiotic product assortment prompts to work out the adequate standards of quantity and quality control which entail the necessity to develop the reli- able and fast methods for the determination of probiotic bacteria, especially those belonging to the most commonly used Bifidobacterium and Lactobacillus genera. In the pres- ent study, an attempt was made to apply PCR on DNA tem- plate extracted directly from beverages (a step of strain iso- lation excepted) for the detection and identification of Lactobacillus and Bifidobacterium cultures. In PCR analy- ses, the critical factors affecting the number of the species detected are the number and specificity of primer pairs applied, their detection threshold, and adequate amplifica- tion conditions (temperatures of primer annealing and con centration of magnesium chloride). The specificity of the primers reported previously was proved by the respective authors (Table 1), whereas that of the two pairs of hereby designed ones was confirmed in relation to the reference strains (Table 3). The newly-designed Lpl-1N and Lpl-2N primers were solely L. plantarum-specific, whereas Lcas-1N and Lcas-2N primers gave positive results with L. casei DSM 20011, L. paracasei subsp. paracasei DSM 5622 and L. rhamnosus DSM 20021 strains. The positive results obtained for L. rhamnosus strain with L. casei-specific primers can be explained by close affinity of these two species [Reuter et al., 2002]. The identification of the cul- tures to the L. casei group and to the L. rhamnosus species has been performed in two-step PCR, i.e. when positive amplification with Lcas-1N and Lcas-2N primers occurred, the amplification with Pr I and Rha II primers was conduct- ed. A lack of PCR product in the first step makes the sec- ond step pointless and proves the absence of both L. casei and L. rhamnosus species. Regarding all the products test- ed, the positive PCR results with Lcas-1N/2N primers and subsequent negative ones with Pr I/Rha II were obtained, so that the presence of members of L. casei-group but not L. rhamnosus species

was stated. The detection limit of 16S rRNA-specific PCR method applied for the detection of bacteria has been evaluated at a level of 103 cfu/mL [Kok et al., 1996; Matsuki et al., 1999; Furet et al., 2004]. The tech- nique enables, therefore, detecting bacteria present in minority in a mixed population – low-number cultures are hard to determine in the presence of accompanying bacte- ria when cultivated using bacteriological media, whereas directly detectable using the PCR technique. Considering the last critical factor mentioned, the conditions of amplifi- cation were optimised for the thermocycler and polymerase applied, in relation to the reference strains.

The crucial factor affecting the results obtained with the PCR technique is the sufficient quality and quantity of tem- plate DNA which, in turn, depends on the efficiency of DNA isolation method [McOrist et al., 2002]. The applied method of DNA isolation appeared to be excellent for Lactobacillus genus-specific PCR ('strong' product) and sufficient for detec- tion of Lactobacillus species ('weak' or 'very weak' product). The identification of Lactobacillus species was, therefore, sat- isfactory, however that of Bifidobacterium cannot be regarded as entirely successful. The characteristic products of genus- specific PCR were obtained, however two 'weak', and two 'very weak' ones. In consequence, when 'weak' Bifidobacteri- um-specific products appeared, the positive results of PCR species-specific solely to B. animalis/lactis were obtained ('strong' and 'very weak' product), but in the cases of 'very weak' genus-specific products – no further species-specific amplicons were formed. The results indicate that the deter- mination of low-number Bifidobacterium cultures demands more efficient DNA extraction or more sensitive detection methods for the fermented milk control. Modification of the present method towards more efficient Bifidobacterium iden- tification is the subject of an ongoing investigation.

Conclusions

Generally, the usefulness of the PCR method applied to the template DNA extracted directly from fermented milk for the qualitative evaluation of Lactobacillus cultures in kefirs and yoghurts was confirmed. Further optimisation of Bifidobacterium detection method in a combination with plate counting will enable the complex characteristics of fer- mented dairy product microflora.

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