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PCR verification of microplate phenotypic system identification for LAB from traditional Western Balkan raw milk cheeses

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Diana Paveljšek[1], Aljoša Trmčić[1], Biljana Hacin[2], Irena Rogelj[1]*

[1]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia
[2]National Veterinary Institute, Veterinary Faculty, University of Ljubljana, Pri Hrastu 18, SI-5000 Nova Gorica, Slovenia

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Abstract

Fermentation and ripening specificity of traditional cheeses are predominantly directed by the natural microbial community present in milk selected by the cheese-making environment and technology. Therefore the traditional cheeses are unique products with specific microbiota biodiversity. There are several approaches for the identification of microbial population, however all of them have certain advantages and disadvantages. In this study the eligibility and performance of the Biolog phenotypic identification system (Biolog, Inc.) with GEN III microplates was tested. Parallel to this method, polymerase chain reaction with genus- and species-specific primers was performed. One hundred sixty-five isolates from nine types of artisan cheeses were isolated and analysed. Cheeses were produced from raw ewe's milk in Slovenia, Bosnia and Herzegovina, Croatia and Serbia. The Biolog phenotypic identification system identified 90 isolates, but only 55 identifications acquired by the Biolog system were supported by polymerase chain reaction at a genus level and 28 at a species level. The obtained results showed that the reliability of commercial phenotypic identification systems was inadequate when analysing lactic acid bacteria isolates from natural, spontaneous fermentations and needs to be additionally corroborated by genotypic identification methods.

Key words: microbial population, traditional cheese, phenotypic identification, microplate phenotypic system, species specific PCR

Introduction

Screening and identification of microbial community from traditional cheeses is important for maintenance of their biodiversity and specific characteristics. Because the use of starters has diminished the diversity, nowadays there is an interest in searching for potential new starter organisms among bacterial strains, isolated from artisanal cheeses. Together with the research in this field, a wide range of techniques were developed for successful microbial population analysis. These techniques can be divided into three separate groups: cultivation methods followed by phenotypic characterisation, cultivation methods followed by molecular characterisation and cultivation-independent methods based on direct molecular characterisation and classification (Beresford et al., 2001). All of these approaches have certain advantages and disadvantages. The main disadvantage of classical phenotypic methods is the great time-consumption, yet, they provide information about micro-organisms' adaptations to certain environmental factors (Di Cagno et al., 2010). Today there are several rapid phenotypic identification

*Corresponding author/Dopisni autor: Phone/Tel.: +386 1 3203909; Fax: +386 1 7214074; E-mail: diana.paveljsek@bf.uni-lj.si

systems available, which are suitable for routine microbial analysis. However, they lack reliability, when environmental and food samples with great strain variability are to be analysed. Dairy products and their production environment represent a specific ecological niche to which lactic acid bacteria (LAB) have adapted (van de Guchte et al., 2006). Analysing microbial population in traditional dairy products is usually difficult since most rapid commercial tests have been designed for detection and identification of clinical isolates (Truu et al., 1999). Nevertheless some semi-automated systems have been improved and adapted for LAB and application in the dairy environment. The Biolog system enables phenotypic identification at a species and also subspecies level. First it was designed for the identification of Gram-negative bacteria, but its range of use was eventually upgraded to enable identification of LAB and other Gram-positive organisms (Stager and Davis, 1992). The phenotypic fingerprint, obtained on a specific microplate after incubation, is compared with the fingerprints of known micro-organisms deposited as type strains in American Type Culture Collection (ATCC). Most of the published studies that used the Biolog system involve identification of pathogenic and other Gram-negative bacteria (Odumeru et al., 1999; Truu et al., 1999; Becker et al., 2009). The two published studies that dealt with LAB specifically used the Biolog system either to determine the metabolic profile of different *Lactobacillus* (*Lb.*) *plantarum* strains (Di Cagno et al., 2010) or to identify different *Leuconostoc* (*Lc.*) species (Tamang et al., 2008). It is rather difficult to obtain data that would clarify the usefulness and performance of the Biolog phenotypic identification system for identification of LAB.

The aim of this study was to test the performance of the Biolog phenotypic identification system on autochthonous LAB isolates from spontaneous fermentations of different traditional raw milk cheeses. To verify any potential misclassification, the results of phenotypic identification were also evaluated by the polymerase chain reaction (PCR) genotypic method with genus- and species-specific primers.

Materials and methods

Cheese samples

Nine artisanal cheeses produced from raw ewes' milk in four countries in the Western Balkans regions were analysed: Karst ewe's cheese, Dolenjski ewe's cheese and Bovški ewe's cheese from Slovenia; Paški ewe's cheese and Krčki ewe's cheese from Croatia; Pirotski ewe's cheese and Sjenički ewe's cheese from Serbia and Travnički ewe's cheese and Livanjski ewe's cheese from Bosnia and Herzegovina. Both cheeses from Serbia were white cheeses ripened in brine. Travnički cheese is a special hard type cheese also ripened in brine while other cheeses belonged to the group of classical hard type ewe's cheeses.

Bacterial strains

Bacterial strains were isolated from cheese samples by homogenising 10 g of cheese in 90 mL 2 % (w/v) tri-sodium citrate di-hydrate solution (Kemika, Zagreb, Croatia) prepared according to IDF standard 122B:1992. Homogenisation in a Stomacher Lab-Blender (Bagmixer R400, Interscience, F-78860 St. Nom, France) was followed by diluting the samples in quarter-strength Ringer's solution (Merck, Darmstadt, Germany) and spreading them on to M17 (Merck, Darmstadt, Germany) and de Man, Rogosa and Sharpe agar plates (MRS, Merck). After incubation (37 °C, 48 h) 10 colonies were randomly picked from each type of agar plate. From Pirotski cheese only isolates that grew on M17 agar plates were included because on MRS agar plates weren't any visible colonies. There were also difficulties with 5 isolates from Krčki cheese which were lost during purification. Because of a poor growth, the final number of isolates was 165 instead of 180. After purification, the isolates were stored at -20 °C in MRS or M17 broth supplemented with 30 % (w/v) glycerol (Merck) until further characterisation.

Phenotypic characterisation by Biolog system

Randomly selected strains from MRS and M17 agar plates were identified using the Biolog system and GEN III microplates (Biolog, Inc., Hayward, USA) designed to identify pure cultures of Gram-negative and Gram-positive bacteria. The entire process of identification was performed according to

manufacturer's instructions. In brief, before inoculation of the Biolog GEN III plates, the isolates were streaked once on MRS or M17 agar plates and twice on BUG agar plates (Biolog, Inc., Hayward, USA) and incubated at 33 °C for 24 h. Based on a growth rate, oxygen and CO_2 preferences of isolates to be identified, the Biolog system offers several different protocols or inoculation fluids to be used with GEN III microplates. In order to determine the most appropriate protocol, twenty isolates were analysed in parallel using two different protocols (A, C1). The protocols A and C1 differ in the inoculation fluid. Of the two inoculation fluids, inoculation fluid A is easier to reduce and develop a colour reaction than inoculation fluid C. According to the manufacturer, protocol A is used for the vast majority of species while protocol C1 is used for slow growing microaerophilic and capnophilic Gram-positive cocci and tiny rods. Protocol C1 was selected as the most appropriate and has been used in majority of the samples. The wells of the Biolog GEN III plates were inoculated with 100 μL of bacterial suspension, adjusted to 90-98 % transmittance (protocol C1). After 22 and 48 h of incubation at 33 °C the plates were read using a MicroStation plate reader and the results analysed with MICROLOG 3 Version 5.2.01 software.

The Biolog system evaluates all comparisons and makes the identification by calculating two different indexes. The SIM index represents how the metabolic profile of an unknown strain is similar to

Table 1. Genus- and species-specific primers used in PCR reactions and corresponding amplicon sizes

Genus/Species	Primers	Amplicon size	References
Enterococcus (*Ent.*)	E1/ E2	737 bp	Deasy et al., 2000
Ent. asini	AS1/ AS2	365 bp	Jackson et al., 2004
Ent. casseliflavus	CA1/ CA2	288 bp	Jackson et al., 2004
Ent. durans	DU1/ DU2	295 bp	Jackson et al., 2004
Ent. faecalis	ddl E. faecalis E1/ ddl E. faecalis E2	941 bp	Dutka-Malen et al., 1995
Ent. faecium	ddl E. faecium F1/ ddl E. faecium F2	550 bp	Dutka-Malen et al., 1995
Ent. gallinarum	GA1/ GA2	173 bp	Jackson et al., 2004
Ent. hirae	HI1/ HI2	187 bp	Jackson et al., 2004
Ent. villorum/porcinus	PO1/ PO2	280 bp	Jackson et al., 2004
Lactobacillus (*Lb.*)	Lbl Ma1-rev/ R16-1	250 bp	Dubernet et al., 2002
Lb. casei	Y2/ CAS1	290 bp	Ward and Timmins, 1999
Lb. bulgaricus	Lb1/ Llb1	1065 bp	Torriani et al., 1999
Lb. helveticus	LheI/ LheII	500-650 bp	Tilsala-Timisjärvi and Alatossava, 1997
Lb. paracasei	Y2/ Para1	290 bp	Ward and Timmins, 1999
Lb. plantarum	Lfpr/ PlanII	200 bp	Walter et al., 2000
Lb. rhamnosus	PrI/ RhaII	186 bp	Walter et al., 2000
Lactococcus lactis	27f/ Lla	100 bp	Barakat et al., 2000
Leuconostoc mesenteroides	Lmes-f/ Lmes-r	1150 bp	Lee et al., 2000
Pediococcus (P.)	Pedio23S_F/ Pedio23S_R	701 bp	Pfannebecker and Fröhlich, 2008
P. acidilactici	PAC23S_F/ P23S_R	213 bp	Collado et al., 2009
Staphylococcus (*Staph.*)	STA I/ STA II	100-200 bp	Forsman et al., 1997
Streptococcus (*Str.*)	STR I/ STR II	150-210 bp	Forsman et al., 1997
Str. thermophilus	ThI/ ThII	205-304 bp	Tilsala-Timisjärvi and Alatossava, 1997

bp - base pair

Table 2. Identification results obtained with Biolog system for isolates from Karst ewe's cheese

Karst ewe's cheese			
isolates from M17		isolates from MRS	
after 22 hours	after 48 hours	after 22 hours	after 48 hours
Lac. lactis subsp. *lactis*	*Lac. lactis* subsp. *lactis*	*Lb. pentosus**	*Lactobacillus/Lb. pentosus**
Lac. lactis subsp. *lactis*	*Lac. lactis* subsp. *lactis*	*Lb. plantarum**	*Lb. plantarum*
Ent. faecalis	*Ent. faecalis**	*Lactobacillus/Lb. plantarum**	*Lb. plantarum**
Ent. faecalis	*Ent. faecalis*	*Lb. plantarum**	*Lb. plantarum**
Ent. faecalis	*Ent. faecalis**	*Lb. plantarum**	*Lactobacillus/Lb. plantarum**
Ent. faecalis	*Ent. faecalis**	*Lb. plantarum**	*Lb. plantarum*
Ent. haemoperoxidus	*Lac. lactis* subsp. *lactis*	*Lb. plantarum**	*Lb. plantarum**
Lac. lactis subsp. *lactis*	*Lac. lactis* subsp. *lactis*	*Lb. plantarum**	*Lb. plantarum*
Enterococcus	*Enterococcus*	*Lb plantarum**	*Lb. plantarum*
Lac. lactis subsp. *lactis*	*Lac. lactis* subsp. *lactis*	*Lb. alimentarius**	*Lb plantarum**

* - most probable identification

the one deposited in the Biolog database under certain identification. The second index (DIST) represents the distance between the unknown strain and proposed identifications. When identification is made, both indexes are also used by the system to evaluate the probability of correct identification. Based on these calculations some isolates analysed in this study were not identified with enough certainty and were only described as most probable.

Genotypic characterisation by genus and species specific PCR

The Biolog phenotypic method was used as a screening method which pointed the way for basic selection of primers and grouped individual isolates together based on their metabolic profile. After initial phenotypic screening by the Biolog system, PCR was used to confirm the identifications of LAB or analyse the strains for some other, related genera or species. Because the focus was on LAB, eleven isolates (out of 165) stayed unidentified with oligonucleotide primers used in this trial.

DNA was extracted from pure MRS and M17 cultures using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genus- and species-specific primers for DNA amplifications were selected on the basis of preliminary identification with the Biolog system. All PCR mixtures and programmes were carried out according to the instructions of cited authors (Table 1). PCR amplification products were analysed on 1.8 % agarose gels (100 V, 1 h), stained with SYBR Safe (Invitrogen, Oregon, USA) and visualised under a UV transilluminator. The molecular weight of the amplified DNA was estimated by comparison with GeneRuler™ 100 bp DNA Ladder (Fermentas International Inc., Lithuania).

Results and discussion

One hundred and sixty five colonies from nine different cheese samples were isolated and purified. The Biolog identification system with GEN III microplates proved to be easy to use and allowed us to quickly process all 165 isolates. The first problem we encountered with this method involved the flexible time of incubation required to obtain a positive identification. We read the metabolic profile and made the identifications using the MicroStation analysis tool after 22 and 48 h of incubation. Of 165 isolates analysed in this study, only 90 isolates were identified identically after the first and second incubation periods. In cases when the metabolic profiles and identifications made were not identical, each identification was evaluated as a possibly correct one. Fifty-one percent of isolates were successfully identified at a species level, while 4 % of isolates were identified only at genus level. Forty-four percent of isolates analysed in this study were not identified with enough certainty and were only described as most probable. For 1 % of isolates, the

Table 3. Compliance of two types of the Biolog system's identifications with PCR results

number of isolates used in PCR after identification with Biolog system		number of isolates in each group	group	confirmed identification by PCR in % for defined group	not confirmed identification by PCR in % for defined group	not determined [%]
163 isolates at a genus level		90	identifications at a genus level	61	27	12
	163 isolates at a species level	84	identifications at a species level	33	49	18
		79	probable identifications at a species level	17	59	24
		73	probable identifications at a genus level	64	17	19

system was unable to yield any identification at all. Table 2 presents the results obtained with Biolog system for one of the cheeses with best identifications at species level.

Various identification protocols, proposed by the manufacturer, were essayed but all of these protocols had low identification success rate. For most samples, protocol C1 was selected as the most appropriate with the exception of some samples of *Lactobacillus* species where protocol C2 was used. Protocol C2 differs from protocol C1 only in inoculation density.

In parallel with identification by the Biolog system, identifications using the molecular PCR method with genus- and species-specific oligonucleotide primers were preformed (Table 3). The strains analysed by the PCR method were separated into two groups based on the level of identification made by the Biolog system. The first group is represented by 90 isolates for which the Biolog system was able to make the identification, while a group of 73 isolates represents a group for which the metabolic profile was not clear enough to make the identification and was only suggested as the most probable one. Table 3 represents the results of the PCR method analysis of strains within both groups.

Within the first group of isolates, the identifications obtained by the Biolog system were confirmed with the PCR method at a genus level in 61 % of cases and in 33 % of cases at a species level. Different identifications at a genus level were obtained for 27 % of isolates and at a species level for 49 % of the isolates. In table 4 are gathered most common identifications obtained by Biolog system and their confirmation with PCR. It has been shown that strains possessing familiar phenotypes don't always have similar or even remotely related genotype which presents a problem when employing identification methods based on phenotypic features. Temmerman et al. (2004) suggested several phenotypic methods to be used at once so the disadvantages of one method are compensated by another, but this presents a costly and time-consuming approach.

When the second group of isolates was evaluated by the PCR method, 64 % of these most probable identifications were in accordance at a genus level and 17 % at a species level. Different identifications were obtained in 17 % of cases at a genus level and in 59 % of cases at a species level. Both groups of isolates also included a larger share of strains whose identifications and probable identifications could not be analysed by set of oligonucleotide primers used in this study. These include genera *Corynebacterium*, *Micrococcus*, *Bacillus*, *Eikenella*, *Erysipelothrix*, *Dermobacter*, *Cellulomonas*, *Sanguibacter*, *Listeria*, *Microbacterium*, *Gardenerella* and *Actinomyces*. The results represented in Table 3 show that both groups of strains were identified with a similar reliability at a genus level even though the Biolog system designates the strains from the second group as uncertain.

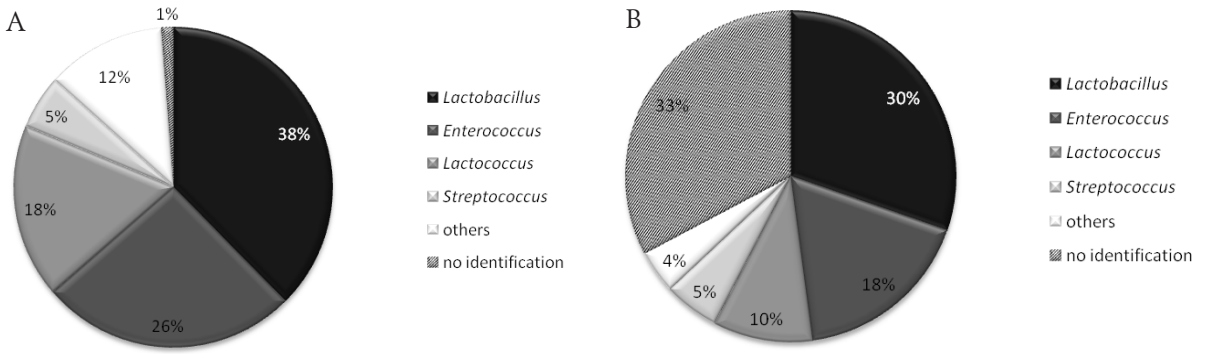
Table 4. Most common identifications obtained by Biolog system and confirmation/different identification with PCR method

Genus/Species	Biolog system	PCR
Lactobacillus	*	✓
Lb. plantarum	*	✓
Lb. rhamnosus	*	×, *Lb. paracasei*
Lb. coryniformis	*	-
Lb. jensenii	*	*Lb. casei*
Lb. brevis	*	-
Lb. pentosus	*	*Lb. plantarum*
Lb. hamsteri	*	*Lb. plantarum*
Lb. sake	*	*Lb. casei*
Weissella viridescens	✓	*Lb. paracasei*
Enterococcus	✓	✓
Ent. faecalis	✓	✓
Ent. casseliflavus	*	×
Ent. faecium	*	✓
Ent. durans	*	✓
Ent. pseudoavium	✓	-
Ent. asini	*	✓
Ent. gallinarum	✓	✓
Ent. hirae	*	×
Ent. haemoperoxidus	*	-
Ent. villorum	*	×
Lactococcus lactis	✓	✓
Streptococcus	✓	✓
Str. bovis	✓	*Str. thermophilus*
Str. vestibularis	✓	*Str. thermophilus*
Str. gallolyticus	✓	-
Staphylococcus	*	✓
Staph. hominis	✓	-
Staph. capitis	✓	-
Staph. haemolyticus	✓	-
Leuconostoc lactis	*	*Leuconostoc mesenteroides*
Pediococcus	*	✓
P. acidilactici	*	×
P. pentosaceus	*	-

✓ successful identification with Biolog system / positive confirmation with PCR, * most probable identification with Biolog system, × different identification with PCR, - not determined with PCR

Of all isolates identified using the Biolog system, most (38 %) were identified as members of the genus *Lactobacillus* (Figure 1), the major representative being species *Lb. plantarum*. Complementary results of prevailing microbial community were obtained with PCR, but there was a larger portion of unidentified samples. Unidentified samples were mostly a consequence of mismatched results with

Figure 1. A: Microbial community (in %) of analysed cheeses obtained with Biolog system;
B: Microbial community (in %) of analysed cheeses obtained with PCR method



Biolog system. PCR was left out for some bacterial strains that were identified with Biolog system in smaller numbers and are not typical for cheese microbiota. A smaller portion of isolates were identified as *Weissella viridescens* by the Biolog system but later on identified as members of the *Lb. paracasei* group by the PCR method. Genus *Weissella* (*W.*) is closely related to *Lactobacillus* (Huys et al., 2012), with a similar metabolic profile which is probably the reason why strains of *Lb. paracasei* group were always identified as *W. viridescens*.

Members of the *Lb. paracasei* group were expected to be well represented within the genus *Lactobacillus* according to the literature for cheeses made from ewes' milk (Di Cagno et al., 2003; Čanžek Majhenič et al., 2007). Both genera *Lactobacillus* and *Enterococcus* (*Ent.*) are predominant representatives in artisan raw milk cheeses from the Western Balkans area (Čanžek Majhenič et al., 2005; Pogačić et al., 2011; Radulović et al., 2012). This was also shown in this study; 26 % of isolates were identified as genus *Enterococcus* and within this genus species *Ent. faecalis* was the most frequently identified. All the other genera identified in this study by the Biolog system were also in accordance with current literature describing microbial composition of traditional cheeses (Litopoulou-Tzanetaki and Tzanetakis 2011; Golić et al., 2013; Ordiales et al., 2013). Species *Lactococcus lactis* was identified in 18 % of analysed isolates, followed by genera *Streptococcus* (5 %), *Staphylococcus* (3 %), *Pediococcus* (2 %) and *Corynebacterium* (2 %). Other identified genera represented with one or two isolates were *Leuconostoc*, *Micrococcus*, *Bacillus*, *Eikenella*, *Erysipelothrix*, *Dermobacter*, *Cellulomonas*, *Sanguibacter*, *Listeria*, *Microbacterium*, *Gardenerella* and *Actinomyces*.

Conclusions

Appropriate choice of identification method is crucial for the studies of microbial community included in the fermentation process and is dependent on many factors, particularly on the origin and number of samples. The Biolog system was chosen for identification due to its simplicity and its ability to simultaneously analyse a large number of isolates. Although it was possible to obtain a rough picture of the present microbial population, the Biolog system is still not reliable enough to be used as the only method for identification of bacteria that originate from complex natural samples like raw milk cheese. Rapid and high-throughput methods are preferred thus PCR based methods are the most widely used for the identification of unknown strains. Employing PCR with genus- and species-specific oligonucleotide primers is not particularly suitable as a screening method when dealing with a large number of isolates. In this case, combining both phenotypic and genotypic methods for identification proved to be the right way of choice. The single strain isolates that were eventually left unidentified or unconfirmed could be processed by some more sophisticated molecular method like sequencing of 16S rRNA gene or 16S/23S spacer region.

PCR provjera identifikacija fenotipskog sistema s mikropločama za BMK iz tradicionalnih sireva od sirovog mlijeka sa Zapadnog Balkana

Sažetak

Fermentacija i specifično zrenje sira najvećim je dijelom uvjetovano prirodnom mikrobnom zajednicom prisutnom u mlijeku, a koja se selekcionira uz pomoć sirarskog okoliša i tehnologije. Stoga su tradicionalni sirevi unikatni proizvodi sa specifičnom mikrobnom bioraznolikošću. Postoje različite mogućnosti identifikacije mikrobne populacije, međutim svi pristupi imaju svoje prednosti i nedostatke. U ovom istraživanju testirana je prikladnost i učinkovitost fenotipskog identifikacijskog sustava Biolog (Biolog, Inc.) s GEN III mikropločama. Usporedno je izvođena lančana reakcija polimeraze s rodom i vrstom specifičnih početnica. U ovom je istraživanju izolirano i analizirano ukupno 165 izolata porijeklom od devet vrsta tradicionalnih sireva. Sirevi su bili proizvedeni od sirovog ovčjeg mlijeka u Sloveniji, Bosni i Hercegovini, Hrvatskoj i Srbiji. Fenotipski identifikacijski sustav Biolog identificirao je 90 izolata. 55 identifikacija bilo je potvrđeno metodom lančane reakcije polimeraze na razini roda, a 28 na razini vrsta. Rezultati su pokazali da je pouzdanost komercijalnog fenotipskog identifikacijskog sistema neadekvatna kada se analiziraju izolati mliječno kiselinskih bakterija prirodnih i spontanih fermentacija. Taj identifikacijski sistem potrebno je dodatno potvrditi genotipskim identifikacijskim metodama.

Ključne riječi: mikrobna populacija, tradicionalni sir, fenotipska identifikacija, fenotipski sistem sa mikropločama, za vrstu specifične PCR

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