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ARDRA Analysis on Biodiversity of Lactobacilli Isolated from Bulgarian Raw Buffalo Milk

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Abstract

Lactic acid bacteria are widespread in nature and occur naturally as indigenous microflora in raw milk. Considering that buffalo milk is an excellent medium for the growth of a large variety of lactic acid bacteria, the aim of this study was the isolation of *Lactobacillus* spp. strains from raw buffalo milk originating from different areas and their species identification, using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and a set of five reference strains of the most frequently isolated *Lactobacillus* species. From the analysis of the patterns generated after treatment with *Hae*III it was found that 24 (88.8%) of the isolates had profiles that matched the reference 16S rDNA of *Lactobacillus casei*. The restriction profiles of the remaining three isolates (12.2%) did not match any of the reference strains and they were identified by API 50 CHL as *Lactobacillus fermentum*. This indicates that *L. casei* is highly adaptive and dominates in raw buffalo milk regardless of the climatic conditions and the method of raising animals.
Keywords: *Lactobacillus casei*, *Lactobacillus fermentum*, identification, ARDRA, API.

Резюме

Млечнокиселите бактерии са широко разпространени в природата и се срещат естествено като автохтонна микрофлора в суровото мляко. Като се има предвид, че биволското мляко е отлична среда за растежа на голямо разнообразие от млечнокисели бактерии, целта на това проучване е изолирането на щамове от род *Lactobacillus* от сурово биволско мляко, произхождщо от различни райони и тяхната видова идентификация, с помощта на амплифициращ рибозомна ДНК рестрикционен анализ (ARDRA) и набор от пет референтни щама на най-често изолираните видове *Lactobacillus* spp. От анализа на моделите, генерирани след третиране с Hae III, беше установено, че 24 (88.8%) от изолатите са с профили, които съответстват на референтната 16S рДНК на *Lactobacillus casei*. Рестрикционните профили на останалите три изолата (12.2%) не съответстват на нито един от референтните щамове и те бяха идентифицирани чрез API 50 CHL като *Lactobacillus fermentum*. Получените резултати разкриват, че *L. casei* е силно адаптивен и доминира в суровото биволско мляко, независимо от климатичните условия и метода на отглеждане на животни.

Introduction

Lactic acid bacteria (LAB) are among the most important microorganisms that play a major role in many food and feed fermentations. They are widespread in nature and occur naturally as indigenous microflora in raw milk (Singh and Sharma, 2009; Othman *et al.,* 2017). Their biodiversity is considered a fundamental factor for the features and quality of dairy products. Traditional products fermented under domestic conditions, prepared from cow, sheep, goat and buffalo milk, such as cheese, curd, katyk, and yellow cheese are still a very important part of the daily diet in Bulgaria (Tserovska *et al.,* 2002; Nemska *et al.,* 2016). These products have more intense flavour than those made from pasteurised milk, mainly due to the presence of non-starter lactic acid bacteria (NSLAB) originating from raw milk (Franciosi *et al.,* 2009). The use of industrial starters improves the technological quality of dairy products, but at the same time

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limits their biodiversity as well as the organoleptic variation of the end products (Zamfir *et al.,* 2006; Zhong *et al.,* 2016). For these reasons, a cognitive survey on biodiversity of raw milk LAB, which could include interesting LAB with specific functional properties, deserves deeper attention. In this respect, buffalo milk has become a research subject throughout the world due to its richer nutrient content compared to other milk (Naydenova and Dimitrov, 2003; Gürler *et al.,* 2013).

Buffalo milk is an excellent medium for the growth of a large variety of bacteria, including LAB, which makes it especially interesting in the search for potential starter microorganisms from the pool of wild LAB strains recoverable from raw milk (Boycheva *et al.,* 2002; Wouters *et al.,* 2002; Melia *et al.,* 2018). During the last decade, the use of PCR-based molecular markers revealing biodiversity at the DNA level has been playing an increasing part in genetics analysis (Hristova *et al.,* 2012; Hristova, 2015). However, there is a lack of data on LAB biodiversity in raw buffalo milk produced in Bulgaria, especially lactobacilli. With this in mind, the aim of this study was the isolation of *Lactobacillus* spp. strains from raw buffalo milk originating from different areas and their species identification, using Amplified Ribosomal DNA Restriction Analysis (ARDRA) and a set of five reference strains of the most frequently isolated *Lactobacillus* species.

Materials and Methods

Samples

In this study, eight raw buffalo milk samples from small farms located in different villages in Trakia valley region of Bulgaria (Kovachevo, Topoliane, Pshenichevo, Mladovo, Sadievo, Lozen, Voden and one sample from the town of Kazanlak) were analysed. The samples were collected according to EN ISO 707:2008 from milk batches during the milking period October-November.

Reference strains

The following reference strains were included in this study: *Lactobacillus helveticus* DSM 20075; *L. plantarum* DSM 20174; *L. casei* DSM 20011; *L. delbrueckii* ssp. *bulgaricus* DSM 20081 and *L. delbrueckii* ssp. *lactis* DSM 20072.

Isolation and identification of Lactobacillus spp. strains

One milliliter of each sample was homogenized in 9 ml of sterile saline (0.85% NaCl, w/v), supplemented with peptone (0.1%, w/v; Oxoid®, UK) and then serial dilutions from homogenates were prepared. One ml aliquot of the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were pour-plated in MRS agar (Oxoid®, UK) for isolation of *Lactobacillus* strains (each sample was plated in duplicate). After incubation at 37°C for 48 h, the morphology of the cells was observed by light microscopy after Gram staining. The strains were tested for the absence of catalase by direct application of 3% H_2O_2 to the colonies. The Gram-positive and catalase-negative rods were streaked three times on MRS agar (Oxoid®, UK) in order to obtain pure cultures. The bacterial isolates that were judged to be *Lacobacillus* spp. on the basis of the test results were further classified by using ARDRA technique.

DNA extraction

For DNA studies, the *Lactobacillus* isolates were grown in MRS broth for 18 h at 37^0 C, and the genomic DNA was isolated using Genomic DNA Purification Kit (Fermentas), following the manufacturer's instructions.

Genotypic identification

DNA from the reference strains and isolates was used as a template for PCR amplification using universal primers corresponding to the 5'-end fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 3'-end rD1 (5'-TAAGGAGGTGATCCAGGC-3') of the 16S rRNA gene (Weisburg *et al.,* 1991). The PCR product from 16S rDNA amplification was digested with endonucleases *Eco*RI and *Hae*III (NZYTech, Portugal). The restriction fragments were separated electrophoretically in 2% agarose gel (Cleaver Scientific Ltd, Hungary) and visualized by staining with ethidium bromide. Restriction patterns identical to the references led to the identification of the corresponding species

Phenotypic identification

Phenotype identification of three isolates with different restriction profiles was carried out according to the instructions for use of API 50 CHL (API 50CH Strip and API 50 CHL Medium, bioMerieux). API*web*™ identification software was used.

Results and Discussion

Twenty seven pure cultures of rod-shaped bacteria were isolated from the collected samples of raw buffalo milk (Table 1). The PCR amplified products of the 16S rDNA region of all studied bacteria showed the same size as the reference strains (Fig. 1). The PCR products contained approximately 1500 bp and corresponded to the expected size of the 16S rRNA genes for lactic acid bacteria (Weisburg *et al.,* 1991).

Table 1: List of *Lactobacilus* spp. isolates from raw buffalo milk

Isolate	Location	Source	Type of milk
Kc7	Kovachevo	milk batches	Buffalo
Kc7.1	Kovachevo	milk batches	Buffalo
Kv3.2	Kovachevo	milk batches	Buffalo
Kv3.2.3	Kovachevo	milk batches	Buffalo
KK1.1	Kazanlak	milk batches	Buffalo
KK1.5	Kazanlak	milk batches	Buffalo
L1.1	Lozen	milk batches	Buffalo
L1.2.1	Lozen	milk batches	Buffalo
L1.2.2	Lozen	milk batches	Buffalo
L2.1	Lozen	milk batches	Buffalo
L3.1	Lozen	milk batches	Buffalo
L3.2	Lozen	milk batches	Buffalo
M3.3	Mladovo	milk batches	Buffalo
M2.3	Mladovo	milk batches	Buffalo
P3.1	Pshenichevo	milk batches	Buffalo
P3.1.1.2	Pshenichevo	milk batches	Buffalo
P4.3	Pshenichevo	milk batches	Buffalo
S1.3	Sadievo	milk batches	Buffalo
S1.4	Sadievo	milk batches	Buffalo
S3.1	Sadievo	milk batches	Buffalo
S3.1.1	Sadievo	milk batches	Buffalo
T3	Topoliane	milk batches	Buffalo
T3.1	Topoliane	milk batches	Buffalo
T3.1.1.2	Topoliane	milk batches	Buffalo
V2.1	Voden	milk batches	Buffalo
V2.3	Voden	milk batches	Buffalo
V2.3.3.1	Voden	milk batches	Buffalo

According to Giraffa et al. (1998) and Miteva et al. (2001), the amplification product of *L. delbrueckii* ssp. *bulgaricu*s and *L. helveticus* was cut into two fragments by *Eco*RI endonuclease, which was confirmed by the reference strains used (Fig. 2). The amplified 16S rDNA from all milk isolates, however, could not be digested with this enzyme and therefore their affiliation to one of these two species was excluded.

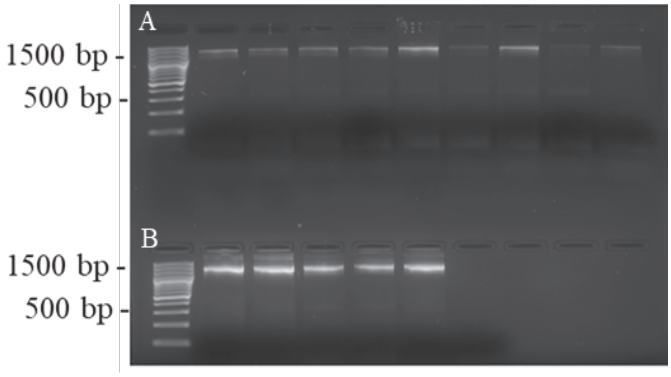

Fig. 1. PCR product from 16S rDNA amplification: A – *Lactobacillus* spp. isolates; B – reference strains

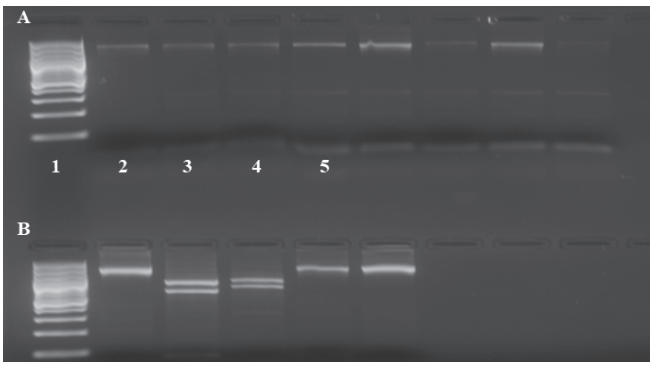

Fig. 2. Restriction patterns derived from digestion of 16S rDNA amplification roducts with *Eco*RI: A – *Lactobacillus* spp. isolates; B – reference strains (1. *L. casei*; 2. *L. delbr.* ssp. *bulgaricus*; 3. *L. helveticus*; 4. *L. delbr.* ssp. *lactis*, 5. *L. plantarum*)

The analysis of the patterns generated after treatment with *Hae*III found that the profiles of 24 (88.8%) of the isolates matched the reference 16S rDNA of *L. casei* (Fig. 3).

Fig. 3: Restriction patterns derived from digestion of 16S rDNA amplification products with *Hae*III: A – *Lactobacillus* spp. isolates; B – reference strains (1. *L. casei*; 2. *L. delbr.* ssp. *bulgaricus*; 3. *L. helveticus*; 4. *L. delbr.* ssp. *lactis*, 5. *L. plantarum*)

The restriction profiles of the remaining three isolates (12.2%) – two from Topolyane region (T3 and T3.1.) and one form Kovachevo village (Kc 7.1), did not match any of the reference strains (Fig. 4) and were identified by API 50 CHL (bioMerieux) as *L. fermentum*.

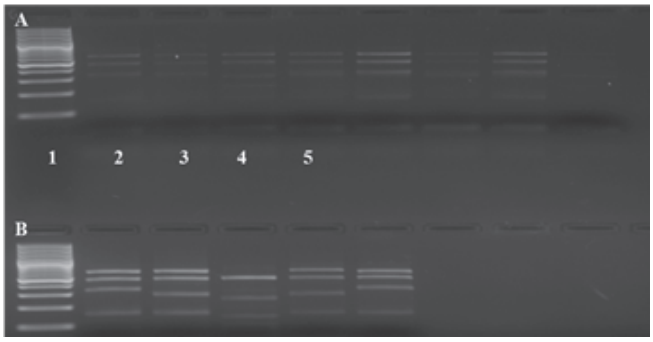

Fig. 4. Restriction patterns of isolates derived from digestion of 16S rDNA amplification products with *Hae*III (the differences in restriction profiles of isolates in lanes 2, 3 and 8 are clearly shown)

A number of authors have reported that *L. casei* is frequently isolated from raw milk in Romania (Zamfir *et al.,* 2005), sheep milk in Pakistan (Aziz *et al.,* 2009), cow milk in Italy (Franciosi *et al.,* 2009), but we could not find in the accessible literature scientific evidence of the dominance of this bacterial species in raw buffalo milk. For example, Rizqiati et al. (2016) did not find any *L. casei* isolates from raw buffalo milk samples from Indonesia, whereas *L. fermentum* is frequently isolated from both raw and pasteurized milk in Latvia (Bluma and Ciprovica, 2015). In another study, *L. fermentum* was found more often in raw milk samples than *L. casei* (Samuel *et al.,* 2016).

L. casei is widely applied in the food industry as a starter culture for cheese, different yogurt types, green olives, etc. as well as in many probiotic products (Wouters *et al.,* 2002; Bernardeau *et al.,* 2006; Ehsani *et al.,* 2018). Many *L. casei* strains have enhanced the probiotic activity, which makes this species an interesting object for study and selection of strains for production of functional foods (Cai *et al.,* 2009; Hill *et al.,* 2018). On the other hand, *L. fermentum* is commonly found in different fermented foods and is considered to be harmless. Some *L. fermentum* strains have beneficial probiotic properties, particularly in relation to gastrointestinal health (Kaewnopparat *et al.,* 2013).

Conclusion

As a result of this study, 27 pure cultures of rod-shaped lactic acid bacteria were isolated. Based on ARDRA analysis, L. casei was found to be the-dominant rod-shaped lactic acid bacteria isolated from raw batch buffalo milk from different regions of Bulgaria. This also indicates that this species is highly adaptive and dominates in raw buffalo milk regardless of the climatic conditions and the method of raising animals.

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