Safety features of lactobacilli from bryndza cheese and whey inferred from whole genome sequencing

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Summary

Lactobacillaceae are candidates for starter or adjunct cultures for the production of bryndza cheese with traditional organoleptic properties, in particular well adapted strains isolated from bryndza cheese or from ewes' whey. Whole genome sequencing of 34 such strains was carried out, their taxonomic classification was done and the genomes were analysed for the presence of genes responsible for antibiotic resistance or production of biogenic amines. Taxonomic classification of the strains was achieved using several bioinformatic tools, with most weight assigned to Type Genome Server (TYGS). Based on this, qualified presumption of safety (QPS) status as defined by European Food Safety Authority could be assigned to 25 strains, which belonged to species *Lacticaseibacillus paracasei/casei, Limosilactobacillus fermentum, Lactobacillus helveticus, Lactiplantibacillus plantarum* and *Leuconostoc lactis*. None of the strains was found to harbour in the genome any acquired antimicrobial resistance genes to clinically relevant antimicrobials. None of the strains contained genes conferring production of the two most hazardous biogenic amines, histamine and tyramine. All strains were predicted to be non-pathogenic for humans by PathogenFinder (Technical University of Denmark, Copenhagen, Denmark). Based on the results, 25 out of 34 tested strains fulfilled safety requirements for the development of starter or adjunct cultures for the production of bryndza cheese with traditional organoleptic properties.

Keywords

ewes' cheese; bryndza; lactic acid bacteria; antibiotic resistance; biogenic amine

Lactobacilli (genus *Lactobacillus sensu lato*) and leuconostocs (genus *Leuconostoc*) are lactic acid bacteria that play a major role in the production of various types of cheese. They proliferate in milk and curd, metabolically converting saccharides, proteins, lipids and other components of the substrate to acids and organoleptically active compounds. Although their role is mainly positive from the point of view of safety and quality of the food product, they may be connected also with negative effects. Specifically, certain species or strains may be responsible for the production of harmful biogenic amines or may be a source of genes mediating resistance to antibiotics [1–3].

European Food Safety Authority (EFSA) has evaluated the safety of microorganisms intentionally added to food or feed, including food fermentation starter cultures. The list of microorganisms generally considered safe is available since 2007 and has been regularly updated [4]. It provides a principal guide to food producers by listing microbial species with qualified presumption of safety (QPS), such as *Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Lactobacillus helveticus, Limosilactobacillus fermentum, Levilactobacillus brevis* or *Leuconostoc lactis* in the group of lactic acid bacteria used in dairy industry.

A lower safety status is given to a group of species that should be investigated at the individual strain level to demonstrate that they meet

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safety requirements. Presence of antimicrobial resistance genes located on plasmids or transposons should be avoided in lactic acid bacteria used in food production as they could be transferred to pathogenic bacteria. On the other hand, intrinsic resistance of certain lactic acid bacteria to antibiotics (i.e. not plasmid- or transposon-borne) does not pose a problem from the EFSA regulatory standpoint [4–7].

The potential of microorganisms involved in food production to form biogenic amines is another important safety topic, as biogenic amines have adverse health effects. Histamine and tyramine are the most common compounds of this group that cause intoxication when consuming food products (most often fish, cheese, wine or beer) that contain excessive amounts of them. Histamine intoxication is characterized by vascular type headache, pruritus, rash and urticaria. Tyramine intoxication is characterized by headache, sweating, agitation and chest pain. Although the limit for histamine is not legally regulated in cheese, Regulation (EC) No. 2073/2005 [8] regarding microbiological criteria for foodstuffs establishes a limit of 200 $mg \cdot kg^{-1}$ histamine in fish. Other biogenic amines, such as 2-phenylethylamine, putrescine, cadaverine, agmatine, spermine or spermidine, can also be toxic, if their content in food is high. Also, they can potentiate the effects of histamine or tyramine. Biogenic amines are formed in food mainly by decarboxylation of corresponding amino acids. Histidine decarboxylase catalyses the formation of histamine, tyrosine decarboxylase catalyses the formation of tyramine, lysine decarboxylase catalyses the formation of cadaverine and ornithine decarboxylase as well as agmatine deiminase catalyse the formation of putrescine. The enzymes responsible for the formation of biogenic amines are of microbial origin and they should be avoided in the production of fermented foods. Presence of various amino acid decarboxylases has been documented in many species of lactic acid bacteria, including lactobacilli [9–11].

Information on the safety of bacterial strains can also be obtained by correlation analysis of whole genome sequences with those of known pathogens. Using PathogenFinder (Technical University of Denmark, Copenhagen, Denmark), the pathogenic potential is predicted on the basis of sequences of a large number of proteins. *Z*-scores for sequence families are summed and when the sum is above a threshold, the strain is considered pathogenic, otherwise it is considered non-pathogenic [12].

Bryndza is a typical Slovakian cheese recognized as a Protected Geographical Indication (PGI) product [13]. It is a natural, white, mature, spreadable cheese in granular form. It has a delicate odour and taste and has a pleasantly sour ewes' cheese taste that is slightly spicy and salty. The basic intermediate for bryndza production is ewes' lump cheese, produced by a precisely defined two-stage ripening process lasting 8–14 days [14]. Most of traditional bryndza cheese is produced from unpasteurized ewes' milk without addition of any starter cultures. To improve the onset of acidification at problematic production conditions, e.g. low outside temperature, whey from previous production is sometimes used as an adjunct culture (backslopping). However, instead of using undefined mixtures of microorganisms, use of a defined adjunct culture could lead to products of a more reproducible quality. Unfortunately, classical commercial starters composed mainly of lactococci caused a shift in aroma of the cheese leading to an atypical product. Lactobacilli originating from traditional bryndza cheese, ewes' lump cheese or whey could be better suitable for the development of adjunct or starter cultures for the production of bryndza cheese with traditional organoleptic properties, in particular regarding the typical aroma [15–17].

In this study, whole genomes of 34 strains of Lactobacillaceae originating from bryndza cheese or ewes' whey from its production were sequenced and analysed for precise taxonomical classification, presence of genes imparting antibiotic resistance and decarboxylase genes responsible for production of biogenic amines. The knowledge gained on the strains was intended as a basic prerequisite for the further development of adjunct or starter cultures for the production of bryndza cheese with traditional organoleptic properties.

MATERIALS AND METHODS

Microorganisms

Microbial strains used in this study originated from bryndza cheese or whey from the production of bryndza cheese in Slovakia. Strains G183–G187 were isolated in 2006–2007 from winter bryndza cheese made from pasteurized milk (Producer G). Strains A188–A192 were isolated in 2006–2007 from summer bryndza cheese made from unpasteurized milk (Producer A). Both these groups were stored in the freeze-dried state. Another group of strains were freshly isolated in 2021–2023 from unfermented or fermented whey (Producers P, U, Z; Tab. 1). The strains were isolated and cultured on de Man, Rogosa and Sharpe (MRS) agar

Tab. 1. Taxonomic classification of strains.

Strain	GenBank accession number	Classification methods					Conclusive classification
		16S rDNA	RAST	PATRIC	KmerFinder	TYGS	
Winter bryndza cheese							
G183	JBDGNW000000000	*Lb. paracasei*	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
G185	JBDGNV000000000	*Lb. paracasei*	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
G186	JBDGNU000000000	*Lb. helveticus*	np	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*
G187	JBDGNT000000000	*Lactobacillus* sp.	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
Summer bryndza cheese							
A188	JBDGNS000000000	*Lb. plantarum*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
A189	JBDGNR000000000	*Lb. collinoides*	np	*Lb. brevis*	*Lb. brevis*	*Lb. brevis*	*Lb. brevis*
A190	JBDGNQ000000000	*Lb. fermentum*	np	np	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*
A191	JBDGNP000000000	*Lb. paracasei* ssp. *paracasei*	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
A192	JBDGNO000000000	*Lb. curvatus*	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
Fermented whey							
P03	JBDGNN000000000	*Lb. parabuchneri*	np	np	np	*Lb. parabuchneri*	*Lb. parabuchneri*
P05	JBDGNM000000000	*Lb. brevis*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
P06	JBDGNL000000000	*Lb. paracasei* or *Lb. casei*	np	np	np	*Lb. paracasei*	*Lb. paracasei* or *Lb. casei*
P20	JBDGNJ000000000	*Lb. parabuchneri*	np	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*
P21	JBDGNI000000000	*Lb. parabuchneri*	np	*Lb. parabuchneri*	np	*Lb. parabuchneri*	*Lb. parabuchneri*
P22	JBDGNH000000000	*Lb. parabuchneri*	np	np	np	*Lb. parabuchneri*	*Lb. parabuchneri*
P23	JBDGNG000000000	*Lb. paracasei* or *Lb. casei*	np	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*
P41	JBAPMB000000000	*Lb. parabuchneri*	*Lb. casei*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*
P43	JBDGNE000000000	*Lb. parabuchneri*	*Lb. fermentum*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*
P45	JBDGND000000000	*Lb. parabuchneri*	*Lb. brevis*	*Lb. brevis*	*Lb. brevis*	*Lb. brevis*	*Lb. brevis*
U13	JBDGNB000000000	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*
U14	JBDGNA000000000	*Lb. fermentum*	np	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*
U18	JBDGMZ000000000	*Lb. helveticus*	*Lb. delbrueckii*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*

Tab. 1. *continued*

Strain	GenBank accession number	Classification methods					Conclusive classification
		16S rDNA	RAST	PATRIC	KmerFinder	TYGS	
U19	JBDGMY000000000	*Lb. fermentum*	np	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*	*Lb. fermentum*
Z22	JBDGMU000000000	*Lb. plantarum*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
Z24	JBDGMT000000000	*Lb. plantarum*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
Z33	JBDGMS000000000	*Lb. plantarum*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
Z34	JBDGMR000000000	*Lb. paracasei* or *Lb. casei*	np	*Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
Z55	JBDGMQ000000000	*Lb. plantarum*	np	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*	*Lb. plantarum*
Unfermented whey							
P19	JBDGNK000000000	*Leuconostoc lactis*	np	np	np	*Leuconostoc lactis*	*Leuconostoc lactis*
P40	JBDGNF000000000	*Lb. brevis*	*Lb. brevis*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*	*Lb. parabuchneri*
U04	JBDGNC000000000	*Lb. plantarum* or *Lb. helveticus*	*Lb. delbrueckii*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*
Z07	JBDGMX000000000	*Lb. otakiensis* or *Lb. sunkii* or *Lb. parabuchneri*	np	*Lb. otakiensis*	*Lb. kefiri* or *Lb. buchneri* or *Lb. parabuchneri* or *Lb. fermentum*	*Lb. otakiensis*	*Lb. otakiensis*
Z08	JBDGMW000000000	*Lb. paracasei*	np	*Lb. paracasei* or *Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*
Z11	JBDGMV000000000	*Lb. paracasei* or *Lb. casei*	np	*Lb. paracasei* or *Lb. casei*	*Lb. paracasei*	*Lb. casei*	*Lb. paracasei* or *Lb. casei*

Strain: First letter of strain code indicates the origin: G – producer G, A – producer A, P – producer P, U – producer U, Z – producer Z.
GenBank acession numbers are in the format XXXXXX000000000, which refers to the whole genome sequencing project established in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA) for each strain, including possible future sequence corrections. To see the first version used in this publication, XXXXXX010000000 should be used. Classification methods: 16S rDNA – Sanger sequencing of 16S rDNA gene, RAST – Rapid Annotation using Subsystems Technology Server [20], PATRIC – Pathosystems Resource Integration Center [21], KmerFinder – k-mer-based species identification algorithm within Bacterial Analysis Platform (Technical University of Denmark, Copenhagen, Denmark), TYGS – Type Strain Genome Server [22], species were assigned based on internal ranking of the softwares.
Lb. – *Lactobacillus sensu lato*, np – not performed.

(Merck, Darmstadt, Germany) at 37 °C in anaerobic conditions and liquid cultures were grown in MRS medium (Merck) at 37 °C with shaking of 2.5 Hz.

16S rDNA sequencing by Sanger method

Bacterial DNA was isolated from early-stationary phase cultures by chaotropic solid phase extraction using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the instructions for use of the manufacturer, protocol for Gram-positive bacteria. Concentration of DNA was determined fluorimetrically using Qubit instrument with dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). 16S rDNA fragment containing V1–V6 hypervariable regions was amplified using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') [18] and 1062R (5'-ACA GCC ATG CAG CAC CT-3') [19] in a Veriti thermal cycler (Applied Biosystems, Foster City, California, USA). The temperature programme consisted of initial denaturation at 94 °C for 3 min, 35 cycles (denaturation at 94 °C for 15 s, annealing at 54 °C for 30 s, and polymerization at 72 °C for 1 min) and final polymerization at 72 °C for 7 min. The reaction mixture (25 μl) contained 0.5 U thermostable DNA polymerase Kapa Taq HotStart (Kapa Biosystems, Wilmington, Massachusetts, USA), 1× buffer supplied with the polymerase, 1.5 mmol·l^{-1} $MgCl_2$, 200 μmol·l^{-1} dNTP mixture (Applied Biosystems) and 150 nmol·l^{-1} each primer. After amplification, samples were purified using a QIAquick PCR Purification Kit (Qiagen) and analysed by agarose gel electrophoresis to check the size and amount of the amplified product. Then, samples were sequenced at Faculty of Natural Sciences (Comenius University Bratislava, Bratislava, Slovakia). The obtained sequences were compared with those present in the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA) using Basic Local Alignment Search Tool (BLAST).

Whole genome sequencing

Bacterial DNA was isolated from early-stationary phase cultures by Higher Purity Bacterial Genomic DNA Isolation Kit (Canvax Biotech, Córdoba, Spain) according to manufacturer's instructions. Concentration of DNA was measured using Qubit instrument and dsDNA High Sensitivity Assay Kit. DNA sequencing library was prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California, USA) and purified on AMPure XP magnetic beads (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA). The quality of the prepared libraries was checked using electrophoresis in Bioanalyzer 2100 instrument with a high sensitivity chip (Agilent Technologies, Santa Clara, California, USA) and quantified using Qubit instrument and dsDNA High Sensitivity Assay Kit. DNA libraries were normalized to 4 nmol·l^{-1}, denatured and sequenced in MiSeq instrument (Illumina) using 2 × 300 bp paired-end protocol. Obtained reads were assembled de novo by SPAdes algorithm set to standard settings (Center for Algorithmic Biotechnology, St. Petersburg State University, St. Petersburg, Russia). Sequence coverage of 25–360 was achieved (data not shown).

Analysis of genomes

Contigs were annotated and analysed using the online platform Bacterial and Viral Bioinformatics Resource Center (BV-BRC, Chicago, Illinois, USA). For taxonomic classification, Rapid Annotation using Subsystems Technology Server (RAST) [20], Pathosystems Resource Integration Center (PATRIC) [21], k-mer-based species identification algorithm within Bacterial Analysis Platform (KmerFinder, Technical University of Denmark, Copenhagen, Denmark) and Type Strain Genome Server (TYGS) [22] were used. Species were assigned based on internal ranking of the softwares. Antibiotic resistance genes were screened by ResFinder v. 4.4.2 (Technical University of Denmark). Pathogenic potential was screened by PathogenFinder v. 1.1 (Technical University of Denmark).

RESULTS AND DISCUSSION

Species identification

Results of taxonomic classification are presented in Tab. 1. Results of Sanger sequencing of a fragment of 16S rDNA gene were taken as preliminary and sometimes differed from the results of classification using more precise bioinformatic strategies based on whole genome sequences. Finally, we relied most on the assignment obtained by Type Genome Server (TYGS). This classification method performs a comprehensive comparison of whole genomes with type strains and provides genome-based prokaryote taxonomy, thanks to being connected to a large, continuously growing database of genomic, taxonomic and nomenclatural information. It infers genome-scale phylogenies and state-of-the-art estimates for species and subspecies boundaries from user-defined and automatically determined

closest type genome sequences [22]. We classified 2 strains as *Lb. brevis*, 9 strains as *Lb. casei* or *Lb. paracasei*, 4 strains as *Lb. fermentum*, 3 strains as *Lb. helveticus*, 1 strain as *Lentilactobacillus otakiensis*, 8 strains as *Lentilactobacillus parabuchneri*, 6 strains as *Lb. plantarum* and 1 as *Leuconostoc lactis*.

Antibiotic resistance

To evaluate the safety of microorganisms for human use, including food starter cultures, an artificial but practical criterion can be considered – the presence on the list of organisms generally considered safe. Since 2007, European Food Safety Authority (EFSA) has been regularly updating a list of microbial species, which have a "qualified presumption of safety" (QPS) [4]. Among our isolates, the species that do have the QPS status were *Lb. brevis* (our strains C189, P45), *Lb. casei* or *Lb. paracasei* (strains C183, C185, C187, C191, C192, P06, Z08, Z11, Z34), *Lb. fermentum* (A190, U13, U14, U19), *Lb. helveticus* (G186, U04, U18), *Lb. plantarum* (A188, P05, Z22, Z24, Z33, Z55) and *Leuconostoc lactis* (P19). However, for these species, the QPS status comes with a qualification "The strains should not harbour any acquired antimicrobial resistance genes to clinicaly relevant antimicrobials." Such a qualification in the EFSA document was added to avoid contribution to ever increasing antibiotic resistance pool in the

Tab. 2. Presence of genes conferring biogenic amine production in strains.

Strain	Species	QPS	Lysine decarboxylase family gene	Ornithine decarboxylase gene	Agmatine deiminase gene
G183	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
G185	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
G186	*Lb. helveticus*	Yes	No	Yes	Yes
G187	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
A188	*Lb. plantarum*	Yes	Yes	No	No
A189	*Lb. brevis*	Yes	Yes	No	Yes
A190	*Lb. fermentum*	Yes	No	No	No
A191	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
A192	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
P03	*Lb. parabuchneri*	No	Yes	No	Yes
P05	*Lb. plantarum*	Yes	Yes	No	No
P06	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
P19	*Leuconostoc lactis*	Yes	Yes	No	No
P20	*Lb. parabuchneri*	No	Yes	No	Yes
P21	*Lb. parabuchneri*	No	Yes	No	Yes
P22	*Lb. parabuchneri*	No	Yes	No	Yes
P23	*Lb. parabuchneri*	No	Yes	Yes	Yes
P40	*Lb. parabuchneri*	No	Yes	No	Yes
P41	*Lb. parabuchneri*	No	Yes	No	Yes
P43	*Lb. parabuchneri*	No	Yes	No	Yes
P45	*Lb. brevis*	Yes	Yes	No	Yes
U04	*Lb. helveticus*	Yes	No	Yes	No
U13	*Lb. fermentum*	Yes	No	No	No
U14	*Lb. fermentum*	Yes	No	No	Yes
U18	*Lb. helveticus*	Yes	Yes	Yes	Yes
U19	*Lb. fermentum*	Yes	No	No	Yes
Z07	*Lb. otakiensis*	No	Yes	No	Yes
Z08	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
Z11	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
Z22	*Lb. plantarum*	Yes	Yes	No	No
Z24	*Lb. plantarum*	Yes	Yes	No	No
Z33	*Lb. plantarum*	Yes	Yes	No	No
Z34	*Lb. paracasei* or *Lb. casei*	Yes	Yes	Yes	No
Z55	*Lb. plantarum*	Yes	Yes	No	No

None of the strains contained in its genome genes encoding for histidine decarboxylase or tyrosine decarboxylase.
QPS – qualified presumption of safety.

human population. Lactobacillaceae species often contain antibiotic resistance genes. For instance, 81 % of *Lactobacillus sensu lato* strains were intrinsically resistant to vancomycin with resistance genes usually located on chromosome in a study by CAMPEDELLI et al. [23]. In fact, a quick, non-comprehensive search of the genomes of strains in this study revealed the genes encoding methicillin resistance contributor LytH and a glyogalase/dioxygenase involved in bleomycin resistance in *Leuconostoc lactis* P19 (data not shown). However, for the purpose of the EFSA regulation, only the antimicrobial resistance genes acquired by horizontal gene transfer are considered a problem. These are located on plasmids or transposons and therefore have the potential for further transfer to other bacteria, including pathogenic species. For this reason, we used the specialized online software ResFinder to search for acquired antimicrobial resistance genes. None were found in our strains (data not shown). This finding, in combination with the species identification, might help the approval of some strains as components of starter or adjunct cultures for cheese production. These results are consistent with the current knowledge on the occurence of antibiotic resistance in lactic acid bacteria [4–6].

Biogenic amines

The gene encoding for histidine decarboxylase, which is the enzyme responsible for transformation of histidine to histamine, was searched for in the genomes of our strains. We applied both text search of the PATRIC-annotated genomes, as well as BLAST search with known histidine decarboxylase sequences as queries. The queries included examples of both plasmid- and chromosome-borne histidine decarboxylases from Lactobacillaceae (GenBank Accession Numbers WP_057911281.1, AAB59151.1, AAV65956.1, KRM45968.1, EQC57919.1). None of our strains contained a gene for histidine decarboxylase. Similarly, tyrosine decarboxylase gene, which is involved in the production of tyramine and 2-phenylethylamine, was not found in any of our strains. On the other hand, some positive results were obtained when screening for genes encoding three other biogenic amine production enzymes, namely, lysine decarboxylase, ornithine decarboxylase and agmatine deiminase (Tab. 2). Each enzyme type was assigned to a single protein family according to PATRIC. In particular, the lysine decarboxylase family corresponded to a PATRIC global family PGF_02016712. Ornithine decarboxylase was PGF_00027524, agmatine deiminase was PGF_00037006. The detection of genes for lysine decarboxylase family seemed species-dependent in the studied strains. They were found in *Lb. brevis, Lb. paracasei/casei, Lb. otakiensis, Lb. parabuchneri, Lb. plantarum* and *Leuconostoc lactis* strains, while they were not detected in *Lb. fermentum* and *Lb. helveticus* strains. Ornithine decarboxylase gene was found in all *Lb. paracasei/casei* and *Lb. helveticus* strains, and in one out of eight *Lb. parabuchneri* strains. All *Lb. fermentum* strains were negative for ornithine decarboxylase gene. Agmatine deiminase gene was found to be present in all *Lb. brevis, Lb. otakiensis* and *L. parabuchneri*, two out of four *Lb. fermentum* strains and in two out of three *Lb. helveticus* strains. No agmatine deiminase gene was detected in the genome of any *Lb. paracasei/casei* or *Lb. plantarum* strain. These results are consistent with the current knowledge on the occurence of genes involved in the production of biogenic amines in lactic acid bacteria [10, 15, 24]. Based on decarboxylases' gene similarity alone, it could not be deduced if the genes were acquired by horizontal transfer. Absence of the genes conferring the production of histamine and tyramine is significant, as these biogenic amines are considered the most important for food safety.

Pathogenicity potential

The pathogenicity potential of all strains was also tested by analysis of their genomes by PathogenFinder 1.1. All strains were predicted to be non-pathogenic for humans (data not shown).

CONCLUSIONS

All tested strains fulfilled the safety criteria of the absence of acquired antibiotic resistance, genes encoding for the production of histamine and tyramine as well as predicted non-pathogenicity for humans. Twenty-five out of 34 tested strains, specifically, 2 *Lb. brevis*, 9 *Lb. paracasei/casei*, 4 *Lb. fermentum*, 3 *Lb. helveticus* and 6 *Lb. plantarum* strains together with one *Le. lactis* strain were assigned QPS status. These strains can be used for the development of starter or adjunct cultures for the production of bryndza cheese with traditional organoleptic properties. We are not likely to pursue a future approval for the strains belonging to species that do not have the QPS status. Those are *Lb. parabuchneri* and *Lb. otakiensis*, i.e. the group of strains P3, P20, P21, P22, P23, P40, P41, P43 and the strain Z7, respectively. They will be, however, still included in further studies on their metabolic potential because the acquired knowledge may be potentially useful for general understanding of the cheese fermentation biology.

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REFERENCES

1. Khalid, N. M. – Marth, E. H.: Lactobacilli – Their enzymes and role in ripening and spoilage of cheese: A review. Journal of Dairy Science, *73*, 1990, pp. 2669–2684. DOI: 10.3168/jds.S0022-0302(90)78952-7.
2. Olson, N. F.: The impact of lactic acid bacteria on cheese flavor. FEMS Microbiology Reviews, *7*, 1990, pp. 131–147. DOI: 10.1111/j.1574-6968.1990.tb04884.x.
3. Irlinger, F. – Meunier, J.: Microbial interactions in cheese: implications for cheese quality and safety. Current Opinion in Biotechnology, *20*, 2009, pp. 142–148. DOI: 10.1016/j.copbio.2009.02.016.
4. EFSA Panel on Biological Hazards (BIOHAZ) – Koutsoumanis, K. – Allende, A. – Alvarez-Ordóñez, A. – Bolton, D. – Bover-Cid, S. – Chemaly, M. – De Cesare, A. – Hilbert, F. – Lindqvist, R. – Nauta, M. – Nonno, R. – Peixe, L. – Ru, G. – Simmons, M. – Skandamis, P. – Suffredini, E. – Cocconcelli, P. S. – Fernández Escámez, P. S. – Maradona, M. P. – Querol, A. – Sijtsma, L. – Suarez, J. E. – Sundh, I. – Barizzone, F. – Correia, S. – Herman, L.: Update to the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 19: Suitability of taxonomic units notified to EFSA until September 2023. EFSA Journal, *22*, 2024, article e8517. DOI: 10.2903/j.efsa.2024.8517.
5. Teuber, M. – Meile, L. – Schwarz, F.: Acquired antibiotic resistance in lactic acid bacteria from food. Antonie van Leeuwenhoek – Journal of Microbiology, *76*, 1999, pp. 115–137. DOI: 10.1023/A:1002035622988.
6. Moračanin, S. V. – Djukić, D. – Zdolec, N. – Milijašević, M. – Mašković, P.: Antimicrobial resistance of lactic acid bacteria in fermented food. Journal of Hygienic Engineering and Design, *18*, 2017, pp. 25–35. ISSN: 1857-8489.
7. EFSA Panel on Biological Hazards (BIOHAZ) – Koutsoumanis, K. – Allende, A. – Alvarez-Ordóñez, A. – Bolton, D. – Bover-Cid, S. – Chemaly, M. – De Cesare, A. – Hilbert, F. – Lindqvist, R. – Nauta, M. – Nonno, R. – Peixe, L. – Ru, G. – Simmons, M. – Skandamis, P. – Suffredini, E. – Cocconcelli, P. S. – Suarez, J. E. – Fernández, E. N. – Istace, F. – Aguillera, J. – Brozzi, R. – Liébana, E. – Guerra, B. – Correia, S. – Herman, L.: Statement on how to interpret the QPS qualification on 'acquired antimicrobial resistance genes'. EFSA Journal, *21*, 2023, article e8323. DOI: 10.2903/j.efsa.2023.8323.
8. Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, *48*, 2005, L338, pp. 1–26. ISSN: 1725-2555. <http://data.europa.eu/eli/reg/2005/2073/oj>
9. EFSA Panel on Biological Hazards (BIOHAZ): Scientific opinion on risk based control of biogenic amine formation in fermented foods. EFSA Journal, *9*, 2011, article 2393. DOI: 10.2903/j.efsa.2011.2393.
10. Barbieri, F. – Montanari, C. – Gardini, F. – Tabanelli, G.: Biogenic amine production by lactic acid bacteria: a review. Foods, *8*, 2019, article 17. DOI: 10.3390/foods8010017.
11. Dala-Paula, B. M. – Custódio, F. B. – Gloria, M. B. A.: Health concerns associated with biogenic amines in food and interaction with amine oxidase drugs. Current Opinion in Food Science, *54*, 2023, article 101090. DOI: 10.1016/j.cofs.2023.101090.
12. Cosentino, S. – Voldby Larsen, M. – Møller Aarestrup, F. – Lund, O.: PathogenFinder – distinguishing friend from foe using bacterial whole genome sequence data. PloS One, *8*, 2013, article e77302. DOI: 10.1371/journal.pone.0077302.
13. Commission Regulation (EC) No 676/2008 of 16 July 2008 registering certain names in the Register of protected designations of origin and protected geographical indications (Ail de la Drôme (PGI), Všestarská cibule (PDO), Slovenská bryndza (PGI), Ajo Morado de Las Pedroñeras (PGI), Gamoneu or Gamonedo (PDO), Alheira de Vinhais (PGI), Presunto de Vinhais or Presunto Bísaro de Vinhais (PGI)). Official Journal of the European Union, *51*, 2008, L189, pp. 19–20. ISSN: 1725-2555. <http://data.europa.eu/eli/reg/2008/676/oj>
14. Palo, V. – Kaláb, M.: Slovak sheep cheeses. Milchwissenschaft, *39*, 1984, pp. 518–521. ISSN: 0026-3788.
15. Markusková, B. – Lichvariková, A. – Szemes, T. – Koreňová, J. – Kuchta, T. – Drahovská, H.: Genome analysis of lactic acid bacterial strains selected as potential starters for traditional Slovakian bryndza cheese. FEMS Microbiology Letters, *365*, 2018, article fny257. DOI: 10.1093/femsle/fny257.
16. Sádecká, J. – Čaplová, Z. – Tomáška, M. – Šoltys, K. – Kopuncová, M. – Budiš, J. – Drončovský, M. – Kolek, E. – Koreňová, J. – Kuchta, T.: Microorganisms and volatile aroma-active compounds in bryndza cheese produced and marketed in Slovakia. Journal of Food and Nutrition Research, *58*, 2019, pp. 382–392. ISSN: 1336-8672 (print), 1338-4260 (online). <https://www.vup.sk/download.php?bulID=2043>
17. Pangallo, D. – Kraková, L. – Puškárová, A. – Šoltys, K. – Bučková, M. – Koreňová, J. – Budiš, J. – Kuchta, T.: Transcription activity of lactic acid bacterial proteolysis-related genes during cheese maturation. Food Microbiology, *82*, 2019, pp. 416–425. DOI: 10.1016/j.fm.2019.03.015.
18. Lane, D. J.: 16S/23S rRNA sequencing. In: Stackebrandt, E. – Goodfellow, M. (Eds.): Nucleic acid techniques in bacterial systematics. New York : John Wiley, 1991, pp. 115–176. ISBN: 978-0471929062.

19. Allen, A. E. – Booth, M. G. – Verity, P. G. – Frischer, M. E.: Influence of nitrate availability on the distribution and abundance of heterotrophic bacterial nitrate assimilation genes in the Barents Sea during summer. Aquatic Microbial Ecology, *39*, 2005, pp. 247–255. DOI: 10.3354/ame039247.
20. Aziz, R. K. – Bartels, D. – Best, A. A. – DeJongh, M. – Disz, T. – Edwards, R. A. – Formsma, K. – Gerdes, S. – Glass, E. M. – Kubal, M. – Folker, M. – Olsen, G. J. – Olson, R. – Osterman, A. L. – Overbeek, R. A. – McNeil, L. K. – Paarmann, D. – Paczian, T. – Parrello, B. – Pusch, G. D. – Reich, C. – Stevens, R. – Vassieva, O. – Vonstein, V. – Wilke, A. – Zagnitko, O.: The RAST server: Rapid annotations using subsystems technology. BMC Genomics, *9*, 2008, article 75. DOI: 10.1186/1471-2164-9-75.
21. Gillespie, J. J. – Wattam, A. R. – Cammer, S. A. – Gabbard, J. L. – Shukla, M. P. – Dalay, O. – Driscoll, T. – Hix, D. – Mane, S. P. – Mao, C. – Nordberg, E. K. – Scott, M. – Schulman, J. R. – Snyder, E. E. – Sullivan, D. E. – Wang, C. – Warren, A. – Williams, K. P. – Xue, T. – Yoo, H. S. – Zhang, C. – Zhang, Y. – Will, R. – Kenyon, R. W. – Sobral, B. W.: PATRIC: The comprehensive bacterial bioinformatics resource with a focus on human pathogenic species. Infection and Immunity, *79*, 2011, pp. 4286–4298. DOI: 10.1128/IAI.00207-11.
22. Meier-Kolthoff, J. P. – Göker, M.: TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nature Communications, *10*, 2019, article 2182. DOI: 10.1038/s41467-019-10210-3.
23. Campedelli, I. – Mathur, H. – Salvetti, E. – Clarke, S. – Rea, M. C. – Torriani, S. – Ross, R. P. – Hill, C. – O'Toole, P. W.: Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. Applied and Environmental Microbiology, *85*, 2019, article e01738-18. DOI: 10.1128/AEM.01738-18.
24. Wüthrich, D. – Berthoud, H. – Wechsler, D. – Eugster, E. – Irmler, S. – Bruggmann, R.: The histidine decarboxylase gene cluster of *Lactobacillus parabuchneri* was gained by horizontal gene transfer and is mobile within the species. Frontiers in Microbiology, *8*, 2017, article 218. DOI: 10.3389/fmicb.2017.00218.

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