# Detection of genes *prtP*, *pepN*, *pepX* and *bcaT* involved in formation of aroma-active compounds in lactic acid bacteria from ewes' cheese

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**Summary**

Polymerase chain reaction (PCR)-based systems were developed to detect the genes *prtP*, *pepN*, *pepX* and *bcaT*, which are involved in production of aroma-active compounds, and their presence was investigated in 17 strains of lactic acid bacteria isolated from ewes' raw milk-based cheeses. Using the developed PCR systems, along with the previously published PCR systems specific for *Lactococcus*-derived genes, five strains were found to contain all targeted genes (three *Lactobacillus paracasei* strains, one *Lactobacillus casei* and one *Lactococcus lactis*). In 14 *Lactobacillus* strains, *prtP* was detected in 5 strains, *pepN* in 14 strains, *pepX* in 12 strains and *bcaT* in 10 strains. Effectivity of the performance of the PCR systems was checked with whole-genome sequences of the strains, which showed that all strains containing sequences homological (at ≥ 90 %) to primers were correctly amplified. Whole-genome sequences of the strains were used also to determine the representativeness of the PCR systems, which showed that individual genes were correctly detected in all *Lactobacillus* and *Lactococcus* strains by at least one PCR system. Knowledge on the presence of protease genes in bacterial strains is the basis for studying their expression and involvement in formation of volatile aroma-active compounds during cheese ripening.

**Keywords**

protease; lactic acid bacteria; cheese; aroma

Lactic acid bacteria (LAB), mainly *Lactobacillus* spp. and *Lactococcus* spp., play a major role in ripening of cheeses. Besides formation of lactic acid, they produce enzymes that transform various components of the matrix to aroma-active compounds. A range of key volatile aroma-active compounds, mainly $C_3$–$C_6$ alcohols, aldehydes and ketones, are formed by transformation of amino-acids, which are formed from milk proteins by proteolysis. Therefore, genes of LAB whose products are involved in these processes, have a strong impact on aroma of cheeses [1, 2].

Ewes' cheeses represent a matrix with a specific composition. This is reflected also by different microorganisms that are typically involved in ripening of ewes' cheeses, where autochthonous LAB produce the typical aroma profile of ewes' lump cheese, barrelled ewes' cheese and of bryndza cheese [3, 4]. Several aroma-active compounds of cheeses, including raw milk-based ewes' cheeses, are apparently formed by proteolysis and by subsequent transformation of aminoacids [5].

Although LAB are weakly proteolytic, they possess a comprehensive proteinase/peptidase system. Besides the cell envelope-associated proteinase PrtP, they possess several intracellular oligoendopeptidases (PepO, PapF), general aminopeptidases (PepN, PepC, PepG), a glutamyl aminopeptidase PepA, a leucyl aminopeptidase PepL, a prolyl-dipeptidyl aminopeptidase PepX, and several further proteinases/peptidases. PrtP contributes to the formation of small peptides in

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cheese by hydrolysing larger peptides [1, 6]. Other enzymes important for formation of aroma-active compounds by LAB in cheeses (e.g. of methional, 3-methylbutanal, isovaleric acid, benzaldehyde) are transaminases, which transform aminoacids to α-ketoacids. Specific transaminases convert aromatic aminoacids and branched-chain amino acids. An example of the latter is BcaT, which initiates the conversion of major aroma precursors [1, 7].

Presence of genes encoding for proteases and aminoacid-transforming enzymes is a pre-requisite for their expression, subsequent growth of LAB in cheese matrix and for formation of a class of aroma-active compounds. Knowledge on the presence and sequence variability of these genes is important for determination of their expression and for characterization of activities of microflora during ripening of cheese. To facilitate the detection of selected genes, we developed polymerase chain reaction (PCR)-based systems to detect the genes *prtP, pepN, pepX* and *bcaT*. Then, we applied the systems to a panel of representative LAB strains isolated from ewes' lump cheese or similar artisanal cheeses.

**Tab. 1.** Reference strains used for experimental evaluation of primer sets.

| Strain | Source |
|---|---|
| CCM 1815 *Lactobacillus brevis* | CCM |
| CCM 1828 *Lactobacillus rhamnosus* | |
| CCM 3626 *Lactobacillus plantarum* | |
| CCM 3646 *Lactobacillus fermentum* | |
| CCM 3806 *Lactobacillus helveticus* | |
| CCM 4791 *Lactobacillus casei* | |
| CCM 7090 *Lactobacillus paracasei* subsp. *paracasei* | |
| CCM 7191 *Lactobacillus delbrueckii* subsp. *delbrueckii* | |
| CCM 1881 *Lactococcus lactis* subsp. *lactis* | |
| CCM 2106 *Lactococcus lactis* subsp. *cremoris* | |
| CCM 4577 *Pediococcus acidilactici* | |
| *Exiguobacterium undae* | Environmental isolate |
| *Pseudomonas graminis* | |
| *Massillia* sp. | |
| *Bacillus megaterium* | |
| *Bacillus simplex* | |
| *Rhodococcus jostii* | |
| *Streptomyces* sp. | |

CCM – Czech Collection of Microorganisms (Brno, Czech Republic).

## MATERIALS AND METHODS

### Microorganisms and culture conditions

A list of collection strains used for experimental evaluation of primer sets is presented in Tab. 1. These strains comprised those obtained from Czech Collection of Microorganisms (Brno, Czech Republic), and our environmental isolates from previous studies. A panel of isolates from ewes' lump cheese or similar artisanal cheeses is given in Tab. 2. These isolates comprised mostly strains from our previous studies [8, 9], out of which representative strains were selected on the basis of clustering by Fourier transform infrared spectroscopy (data not shown). All strains were cultured in de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany) at 37 °C, with the exception of *Lc. lactis*, which was cultured in M17 (Merck) at 30 °C. Incubation took place for 48 h with shaking of 2 Hz.

### Design of primers

Sequences of *prtP*, *pepN*, *pepX* and *bcaT* genes belonging to various LAB were recovered from GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA) and aligned using the alignment facility offered by the software Vector NTI Advance v11.5.2 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to identify their similarity. Degenerated primers were designed manually on the basis of the found consensus regions. The designed primer sets were supplemented by primer sets previously designed for *Lc. lactis* [10] (Tab. 3).

### DNA extraction and polymerase chain reaction

DNA was extracted from early-stationary phase cultures by chaotropic solid phase extraction using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) by a protocol for G+ bacteria. PCR was carried out in a Veriti thermal cycler (Applied Biosystems, Foster City, California, USA) using a programme consisting of initial denaturation at 94 °C for 2 min, 30 cycles (denaturation at 94 °C for 10 s, annealing at a temperature specified for the given primer pair in Tab. 4, for 20 s, and polymerization at 72 °C for 1 min) and final polymerization at 72 °C for 8 min. The reaction mixture (25 μl) contained 1.25 U thermostable DNA polymerase (Cheetah Hot Start Taq Polymerase; Biotium, Hayward, California, USA), 1× buffer supplied with the polymerase, 1.5 mmol·l$^{-1}$ $MgCl_2$, 340 μmol·l$^{-1}$ dNTP (Applied Biosystems) and 300 nmol·l$^{-1}$ each primer. Amplified products were analysed by agarose gel electrophoresis. Samples with an amplified DNA frag-

**Tab. 2.** Results of polymerase chain reaction with the developed primer pairs and presence of sequences homological to primers in whole-genome sequences of the strains.

| Strain | PCR result | | | | | | | | Presence or homology of primer sequences in genome (forward/reverse) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *prtP* | | *pepN* | | *pepX* | | *bcaT* | | *prtP* | | *pepN* | | *pepX* | | *bcaT* | |
| | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR | AF/AR | BF/BR |
| *Lactobacillus brevis* 2 F1' /2017 | – | – | + | – | + | – | – | – | –/– | –/– | +/+ | –/– | +/+ | –/– | –/– | –/– |
| *Lactobacillus brevis* IV B/8 /2017 | – | – | + | – | + | – | – | – | –/– | –/– | +/+ | –/– | +/+ | –/– | –/– | –/– |
| *Lactobacillus casei* 21L10 | – | + | – | + | – | + | – | + | –/– | +/+ | –/– | +/+ | –/– | +/95% | –/– | +/+ |
| *Lactobacillus fermentum* TIS 2 /2017 | – | – | + | – | – | – | – | – | –/– | –/– | +/+ | –/– | –/– | –/– | 69%/– | –/– |
| *Lactobacillus fermentum* TIS 4/2017 | – | – | + | – | – | – | – | – | –/– | –/– | +/+ | –/– | –/– | –/– | 69%/– | –/– |
| *Lactobacillus paracasei* II A/2 /2017 | – | + | – | + | – | + | – | + | –/– | +/+ | –/– | +/+ | –/– | +/+ | –/– | +/+ |
| *Lactobacillus paracasei* VII B/10 /2017 | – | + | – | + | – | + | – | + | –/– | +/96% | –/– | +/+ | –/– | 95%/+ | –/– | +/+ |
| *Lactobacillus paracasei* X II B2/ 2017 | – | + | – | + | – | + | – | + | –/– | +/96% | –/– | +/+ | –/– | +/+ | –/– | +/+ |
| *Lactobacillus paraplantarum* 251L | – | – | – | + | – | + | – | + | –/– | –/– | –/– | +/+ | –/– | +/90% | –/– | 94%/+ |
| *Lactobacillus plantarum* 17L1 | – | – | – | + | – | + | – | + | –/– | –/– | –/– | +/+ | –/– | +/+ | –/– | 94%/95% |
| *Lactobacillus plantarum* TIS 6/2017 | – | – | – | + | – | + | – | + | –/– | –/– | –/– | +/+ | –/– | +/+ | –/– | +/+ |
| *Lactobacillus plantarum* II B/8 /2017 | – | – | – | + | – | + | – | + | –/– | –/– | –/– | 95%/+ | –/– | +/95% | –/– | +/+ |
| *Lactobacillus plantarum* II B/10 /2017 | – | – | – | + | – | + | – | + | –/– | –/– | –/– | +/+ | –/– | 95%/+ | –/– | +/95% |
| *Lactobacillus plantarum* VI B/7 /2017 | – | – | – | + | – | + | – | + | –/– | –/– | –/– | +/+ | –/– | 95%/95% | –/– | +/+ |
| *Lactococcus lactis* subsp. *tructae* 33S7 | – | + | – | – | – | – | – | – | –/– | 95%/+ | –/– | –/– | –/– | –/– | –/– | –/– |
| *Enterococcus durans* II A/4 /2017 | – | – | – | – | – | – | – | – | –/– | –/– | –/– | –/– | –/– | –/– | –/– | 83%/50% |
| *Leuconostoc mesenteroides* 1 LE 7 | – | – | – | – | – | – | – | – | –/– | –/– | –/– | –/– | 77%/82% | –/– | 78%/78% | –/– |

PCR – polymerase chain reaction; *prtP*, *pepN*, *pep X*, *bcaT* – targeted genes; AF/AR, BF/BR – pairs of primers used in polymerase chain reaction (Tab. 3).
PCR result: (+) – positive, (–) – negative.
Presence or homology in genome: (+) – present (100% homology), (–) – absent or present at homology < 50 %; results are presented as value for forward primer / value for reverse primer.

**Tab. 3.** Sets of primers designed *in silico.*

| Target gene | Primer | Sequence | Lactic acid bacteria target group |
|---|---|---|---|
| *prtP* | AF | TTA YCC CAG CAA YTG GCG GC | *Lactobacillus acidophilus*, *Lb. delbrueckii* |
| | AR | GCC TGA K CTT TCT TCG TCA ACG | |
| | BF | GTC TTG CCR CCA TCA GYA CT | *Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lactococcus lactis* |
| | BR | CTA TGA CCC TAA GAC CGG GAT TGC | |
| *pepN* | AF | GTA TTC CAT CAT GTT RGC | *Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. brevis*, *Lb. fermentum* |
| | AR | GCY TTT GCC TTT GGT GA | |
| | BF | GCT GGY GCG ATG GAA AAC TGG GG | *Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* |
| | BR | TTG CTA TTS ARC GGR ATC TGC CA | |
| | pepN_F | ATK TCT TAY GCW GAY RTY GT | *Lactococcus lactis* [10] |
| | pepN_R | TTK CTT CAA GSM AWG SCC | |
| *pepX* | AF | CAY GGB TTA AAY GAC TGG AA | *Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. brevis* |
| | AR | GTG RSC YTT RGT AAT CA | |
| | BF | AAY GAT TAY TTT CTS GCS CG | *Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* |
| | BR | AGC CAM ARR TTC ATC ATY TC | |
| | pepX_F | TTT GGG TTG AAA GTC CAG T | *Lactococcus lactis* [10] |
| | pepX_R | CCA AGA AGA AAT TCC AGC | |
| *bcaT* | AF | CCA ACW GGT GAM AAA ACS GCA GC | *Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii* |
| | AR | AGA ATC TWT GCM ACT CCW GTT GG | |
| | BF | TGG AAA GAY GGS GCW TGG | *Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* |
| | BR | GCT GAM CCM ACT TCT TCA AT | |
| | bcaT_F | TTT KSH RTG CCD GTW GG | *Lactococcus lactis* [10] |
| | bcaT_R | GGW CCH ACT TCY GTY TC | |

ment of the predicted size were taken as positive results.

#### Analysis of whole genome sequences

Isolated chromosomal DNA from the 17 bacterial strains was used for whole genome sequencing. DNA library was prepared using Nextera kit (Illumina, San Diego, California, USA). Paired-end sequencing was carried out using MiSeq system (Illumina), and *de novo* assembly was carried out on all reads using CLC Genomics Workbench (Qiagen). This software was used also for further analyses.

## RESULTS AND DISCUSSION

Presence of genes *prtP*, *pepN*, *pepX* and *bcaT*, which are known to be involved in formation of aroma-active compounds, was studied in lactic acid bacteria from ewes' lump cheese and similar Slovakian artisanal cheese matrices. At first, we carried out *in silico* analysis of sequences of the abovementioned genes belonging to various LAB. The target LAB were selected according to our previous experience with ewes' lump cheese and bryndza microbiome [3, 4, 7, 8, 11, 12] and included: *Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. brevis*, *Lb. fermentum*, *Lb. casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus* and *Lactococcus lactis*. Approximately 250 sequences (present in the online GenBank database until 3 March 2016) were collected and aligned, which permitted identification of different consensus fragments. From these, specific pairs of primers for each gene (*prtP*, *pepN*, *pepX* and *bcaT*) were designed. The *in silico* analysis facilitated selection of 8 primer pairs, which would be specific for different LAB groups. Three previously published primer sets for *Lc. lactis* genes [10] were added to the list (Tab. 3).

Specificity of the designed primers was experimentally tested by PCR on a panel of reference strains of LAB and other kinds of environmental bacteria (Tab. 1). The most important parameter, with the aim to obtain a reliable PCR assay, was the annealing temperature. This should be optimized to guarantee high stringency at a good yield.

Based on many PCR amplifications at different annealing temperatures for each pair of primers, optimum primer combination for each gene and LAB group was found (Tab. 4).

It was also necessary to establish if the selected pairs of primers really amplified the expected gene. For this reason, the amplicons obtained with different primers and with various LAB were sequenced. The results showed, in all cases, that PCR with selected primers amplified the predicted genes in all *Lactobacillus* and *Lactococcus* strains (data not shown). Thus we obtained several PCR assays able to amplify *prtP*, *pepN*, *pepX* and *bcaT* genes harboured in various lactobacilli. Interestingly, the pair of primers BF/BR specific for *prtP* gene of *Lactobacillus* spp., had a unique feature of permitting amplification of this gene also in *Lc. lactis.*

PCR with the developed primers was used with DNA from a panel of LAB isolates from ewes' lump cheese or similar artisanal cheeses. Results summarized in Tab. 2 demonstrate that PCR with individual primer pairs amplified the target DNA fragment in 0–10 strains. The systems AF/AR targeting *prtP* gene and AF/AR targeting *bcaT* gene failed to produce amplicon from any of the strains. PCR systems pepN_F/pepN_R, pepX_F/pepX_R and bcaT_F/bcaT_R, which were previously designed for lactococcal genes [10], were experimentally positive for *Lc. lactis* and negative with all non-*Lactococcus* strains (data not shown).

In order to check the correctness of the gene detection by the developed PCR systems, whole genome sequences of the strains were searched for the presence and homology of the primer sequences. Full equivalence was found between the experimental PCR results and theoretical *in silico* results, while a minimum of 90% homology (≤ 2 mismatches) of each primer appeared to be a pre-requisite for positive amplification. This corresponds to the well established experience with the number of mismatches allowed for PCR primers.

When the results of PCR for individual genes with various primer sets were combined, a wide presence of genes *prtP*, *pepN*, *pepX* and *bcaT* in the studied strains was demonstrated. Five strains were found to contain all target genes (three *Lactobacillus paracasei* strains, one *Lactobacillus casei* strain and one *Lactococcus lactis* strain). In 14 *Lactobacillus* strains, *prtP* was detected in 5 strains, *pepN* in 14 strains, *pepX* in 12 strains and *bcaT* in 10 strains. These results were found to be representative, as the results of PCR-based detection fully agreed with the results of *in silico* search for the genes in whole-genome sequences of the studied *Lactobacillus* and *Lactococcus* strains (data not shown). In *Enterococcus durans* and *Leuconostoc mesenteroides*, none of our developed primer systems could detect the targeted genes, apparently due to insufficient sequence homology.

The developed primers can be used for detection of the target genes in candidate starter or adjunct cultures, and also for studies of their expression. Based on such studies, it will be possible to select strains capable of production of certain proteases and aminoacid-transforming enzymes during ripening of ewes' lump cheese. LAB strains with these characterized features will facilitate, when used as starter or adjunct cultures, production of cheese with a defined aroma profile.

**Tab. 4.** Sets of primers experimentally optimized with a panel of reference strains.

| Target gene | Primers (forward/reverse) | Species experimentally detected | Amplicon size | Annealing temperature |
|---|---|---|---|---|
| *prtP* | AF/AR | *Lactobacillus fermentum* | 643 bp | 55 °C |
| | BF/BR | *Lactobacillus paracasei, Lb. plantarum, Lactococcus lactis* | 638 bp | 58 °C |
| *pepN* | AF/AR | *Lactobacillus brevis, Lb. fermentum* | 413 bp | 48 °C |
| | BF/BR | *Lactobacillus paracasei, Lb. plantarum* | 683 bp | 60 °C |
| | pepN_F/pepN_R | *Lactococcus lactis* | 482 bp | 50 °C |
| *pepX* | AF/AR | *Lactobacillus brevis* | 729 bp | 55 °C |
| | BF/BR | *Lactobacillus paracasei, Lb. plantarum* | 736 bp | 55 °C |
| | pepX_F/pepX_R | *Lactococcus lactis* | 602 bp | 46 °C |
| *bcaT* | AF/AR | *Lactobacillus brevis, Lb. fermentum* | 436 bp | 55 °C |
| | BF/BR | *Lactobacillus paracasei, Lb. plantarum* | 600 bp | 50 °C |
| | bcaT_F/bcaT_R | *Lactococcus lactis* | 493 bp | 46 °C |

## CONCLUSIONS

PCR primers applicable for detection of *prtP*, *pepN*, *pepX* and *bcaT* genes in genera *Lactobacillus* and *Lactococcus* were developed. Using the developed primers, a wide presence of target genes was detected in ewes' cheese-related LAB isolates. The developed primers can be used in studies of expression of the genes during ripening of ewes' lump cheese.

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