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Transcription activity of lactic acid bacterial proteolysis-related genes during cheese maturation

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ABSTRACT

The catabolism of milk protein in cheese is one way how the microorganisms influence the sensorial characteristics of the final product. In this investigation, we paid attention to four genes [*prtP* (cell-envelope proteinase gene), *pepX* (X-prolyl dipeptidyl aminopeptidase gene), *pepN* (aminopeptidase gene) and *bcaT* (branched chain aminotransferase gene)] responsible for the production of volatile aroma-active compounds from milk proteins by lactic acid bacteria (LAB). We studied the dynamics of these genes and their corresponding LAB host, during the maturation of a raw ewes' milk-based cheese, using metagenomics and metatranscriptomics approaches. The transcriptome-oriented experiments included the analysis of total RNA (at three stages of cheese maturation) and also the construction of specific cDNA sub-libraries of the abovementioned genes. The proteolytic transcriptome analysis was supported by following the transcription activity of 16S rRNA gene and by metagenomic investigation. The combination of the described methods permitted to screen the dynamics of targeted genes throughout the cheese production. Lactococci were the major players in the LAB group, but the analysis provided also information on the role and properties of members of the genus *Lactobacillus*, such as *Lb. rhamnosus*, *Lb. helveticus*, *Lb. pentosus*, *Lb. curvatus*, *Lb. parabuchneri*, *Lb. plantarum*, *Lb. brevis*, *Lb. delbrueckii*, *Lb. paracasei*, *Lb. fermentum* and *Lb. heilongjiangensis*, proteolysis-related genes of which were active during cheese ripening.

1. Introduction

Various ewes' cheeses are produced across Europe, being typical for individual regions. Several of them deserved the appellation PDO or IGP. Some of these cheeses have a longer repining such as Pecorino Romano or Manchego (Di Cagno et al., 2003; Nieto-Arribas et al., 2010), while a large group is formed by cheeses with short maturation (Rantsiou et al., 2008; Pangallo et al., 2014). Bryndza is a spreadable cheese made by milling from ewes' lump cheese with short maturation of 10–14 days (Valík, 2004).

In the production of all of these cheeses, the present microbiota have an important role in formation of organoleptic characteristics of the final product, in particular its flavor and taste. The microbiota with its enzymatic ability confer typical features to cheeses. Different kinds of enzymes, mainly proteases and lipases, contribute to these features. In this contest, the lactic acid bacteria (LAB) are among the protagonists having different kinds of extracellular and intracellular proteases and lipases (Ozturkoglu-Budak et al., 2016).

The proteolytic abilities are mediated by different genes present in LAB, such as *prtP* which encodes an important proteinase able to cleave casein into smaller peptides (Steele et al., 2013). Several intracellular peptidases, generally identified with the suffix Pep (PepP; PepN; PepX etc.), are also involved in this proteolytic system and they are deputed to degrade the peptides (Savijoki et al., 2006). Consequently, the produced amino acids are converted to their corresponding α-keto acids by aminotransferases (AraT and BcaT). The conversion of amino acids to

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α-keto acids is the first step in developing aroma compounds (Smid and Kleerebezem, 2014).

During the last decade, it was possible to deeply investigate the microbiota present during cheese manufacturing. Various molecular biology approaches and high-throughput sequencing permitted to know better the occurring microbiota (Gobbetti et al., 2018). For many cheeses we know which kind of microorganisms are dominant and the LAB is the group of prokaryotes always detected, being the main responsible for promoting the aromas and flavors during the cheese maturation (Steele et al., 2013; Stefanovic et al., 2017; Gobbetti et al., 2018). However, until now the connection between the presence of LAB and the proteolytic genes conferring aroma characteristics was not investigated by modern molecular methods.

In this study we have started to investigate the proteolytic transcriptome of various LAB during the maturation of ewes' cheese bryndza. Several genes of the LAB proteolytic system, *prtP* (gene for the cell-envelope proteinase), *pepX* (gene encoding X-prolyl dipeptidyl aminopeptidase), *pepN* (gene for a general aminopeptidases) and *bcaT* (gene encoding branched chain aminotransferase) were taken as specific markers. Two different high-throughput sequencing (HTS) approaches, the analysis of total transcriptome and the analysis of the amplicons of abovementioned genes, were compared in order to investigate the transcriptional dynamics of these genes during the maturation of bryndza cheese.

2. Materials and methods

2.1. Primers design

Approximately 250 sequences (present in the online GenBank database until March 3rd, 2016) were collected and aligned using the software Vector NTI Advance v11.5.2 (Thermo Fisher Scientific, Waltham, USA). The alignment permitted to discover various consensus regions from which it was possible to design specific pairs of primers for each targeted gene (*pepN*, *pepX*, *bcaT* and *prtP*). In the frame of sequence comparison the targeted LAB were divided in two groups (A and B) and each group required a specific pair of primers. Eight pairs of primers were designed and used in our investigation (Table 1). These pairs of primers were experimentally evaluated with a panel of collection and isolated LAB (Čaplová et al., 2018).

2.2. Extraction of DNA and RNA, PCR amplification

DNA and RNA were extracted from ewes' cheese ripened for 4 h, 24 h (D1) and 8 days (D8), samples being taken from different cheeses during a stable regime production process. Each sample of 1 g was extracted by shaking at 45 °C during 30 min in 20 ml of 2% sodium citrate solution with glass beads, with subsequent removal of the fat layer. The DNA extraction was performed by the chaotropic solid phase extraction method using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

RNA was extracted using NucleoSpin RNA Midi kit (Macherey-Nagel, Düren, Germany) according to the protocol of the producer. A portion of extracted RNA was treated by DNase I (Thermo Fisher Scientific) and directly transcribed to cDNA. In vitro transcription of RNA was done by cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer. Obtained DNA and cDNA were used as templates for PCR amplification.

The second portion of extracted RNA was used for the analysis of total transcriptome. In order to remove the vast majority of the most abundant ribosomal RNA molecules (rRNA) and to enrich the whole spectrum of RNA transcripts, the extracted RNA was depleted of rRNA using the kit RiboMinus (Invitrogen) following the protocol provided by the manufacturer.

2.3. PCR amplification of DNA and cDNA

The bacterial 16S rRNA gene was amplified using primers 27f (AGA GTT TGA TCC TGG CTC AG) and 685r (TCT ACG CAT TTC ACC GCT AC). PCR mixture contained 1 × PCR buffer, 200 μmol l^{-1} dNTPs, 50 pmol of each primer, 1.5 U SuperHot-Taq DNA polymerase (Bioron, Ludwigshafen, Germany), and 3 μl of template DNA/cDNA in the total reaction volume of 25 μl. The following thermocycling program was used: 5 min denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 54 °C and 1 min at 72 °C; final extension was run at 72 °C for 10 min.

Various PCR assays were utilized for the amplification of the genes *pepN*, *pepX*, *bcaT* and *prtP* using DNA and cDNA samples. The PCR mixture was the same as that described above and the PCR conditions were analogical as that above program, except for the annealing temperature for the specific target genes (Table 1).

Table 1
Specific PCR primers and annealing temperatures used for the amplification of the genes *pepN*, *pepX*, *bcaT* and *prtP*.

Target gene	Pair of primer	LAB target group	Annealing temperature	Amplicon size (approx.)
prtP	AF: TTA YCC CAG CAA YTG GCG GC AR: GCC TGA K CTT TCT TCG TCA ACG	*Lactobacillus acidophilus*, *Lb. delbrueckii*	55 °C	643 bp
	BF: GTC TTG CCR CCA TCA GYA CT BR: CTA TGA CCC TAA GAC CGG GAT TGC	*Lactobacillus casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lactococcus lactis*	58 °C	638 bp
pepN	AF: GTA TTC CAT CAT GTT RGC AR: GCY TTT GCC TTT GGT GA	*Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. brevis*, *Lb. fermentum*	48 °C	413 bp
	BF: GCT GGY GCG ATG GAA AAC TGG GG BR: TTG CTA TTS ARC GGR ATC TGC CA	*Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*	60 °C	683 bp
pepX	AF: CAY GGB TTA AAY GAC TGG AA AR: GTG RSC YTT RGT AAT CA	*Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. brevis*	55 °C	729 bp
	BF: AAY GAT TAY TTT CTS GCS CG BR: AGC CAM ARR TTC ATC ATY TC	*Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*	55 °C	736 bp
bcaT	AF: CCA ACW GGT GAM AAA ACS GCA GC AR: AGA ATC TWT GCM ACT CCW GTT GG	*Lactobacillus acidophilus*, *Lb. helveticus*, *Lb. delbrueckii*	55 °C	436 bp
	BF: TGG AAA GAY GGS GCW TGG BR: GCT GAM CCM ACT TCT TCA AT	*Lactobacillus casei*, *Lb. paracasei*, *Lb. plantarum*, *Lb. rhamnosus*	50 °C	600 bp

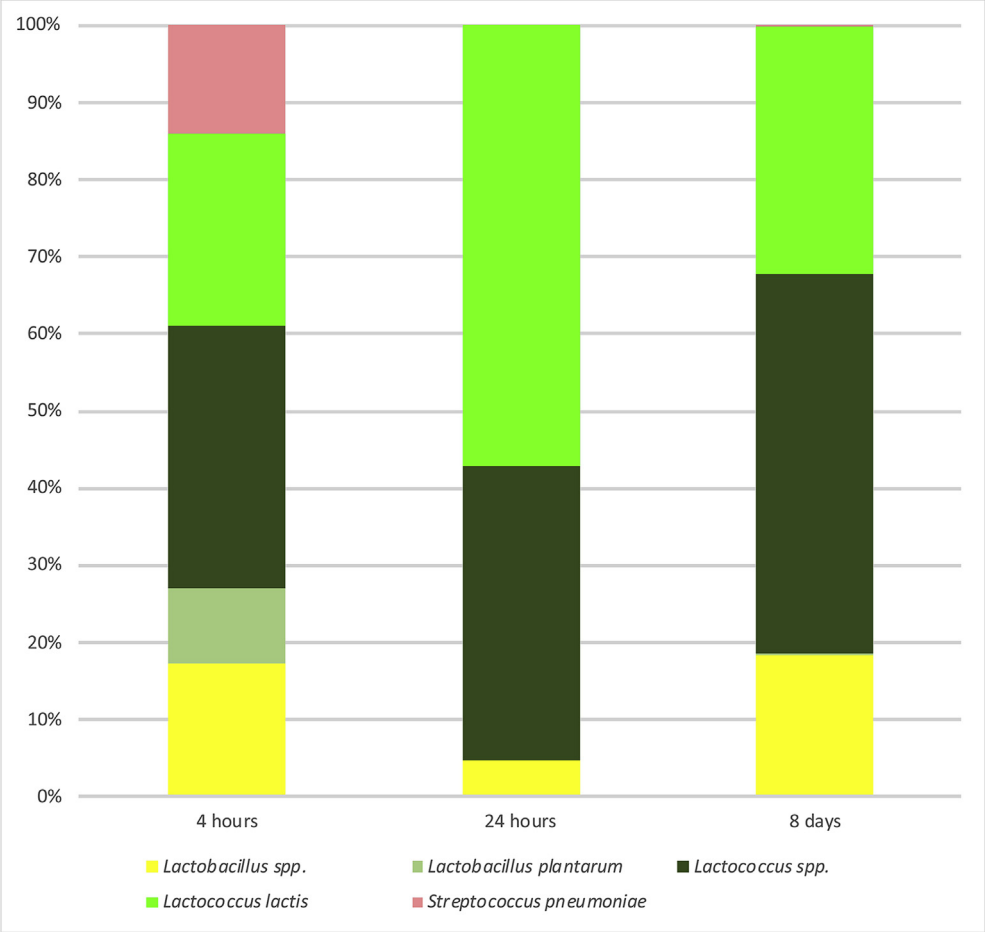

Fig. 1. Metatranscriptome analysis of *prtP* gene on different cheese maturation stages.

2.4. High-throughput sequencing approaches

2.4.1. Specific cDNA sub-libraries and metagenomics libraries construction

Amplicons of different origin were treated with column-based purification system (Monarch PCR & DNA Cleanup Kit; New England Biolabs, Ipswich, USA) and quantified with Qubit dsDNA HS Assay Kit (Invitrogen) using the Qubit 2.0 Fluorometer (Invitrogen). Samples diluted to 0.2 ng/μl were in total amount of 0.5 ng fragmented by transposon-based chemistry Nextera XT DNA library Kit (Illumina, San Diego, USA) and for pooling purposes barcoded by dual-indexing low-cycle PCR according to the manufacturer's protocol. The fragment size of the final libraries purified with 1.8 × Agencourt AMPure XP magnetic beads (Beckman Coulter, MA, USA) was assessed by Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany).

2.4.2. Metatranscriptome analysis

The enriched RNA samples obtained after bacterial rRNA depletion were reverse transcribed into ds cDNA with SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen) using random hexamer primers, subsequently purified with DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA) and eluted into 6 μl of millipore water. After fluorometric quantification (Qubit dsDNA HS Assay Kit) 0.5 ng of ds cDNA was used for transposon fragmentation and low-cycle indexing PCR (Nextera XT; Illumina). The samples were processed identically to PCR products.

2.4.3. Illumina sequencing

The 4 nM libraries of PCR products (cDNA sub-libraries and metagenomics libraries) and enriched total RNA (metatranscriptome approach) were pooled in equimolar ratio and sequenced using NextSeq 500/550 High Output v2 kit (300 cycles) (Illumina) on Illumina NextSeq 500 platform.

2.5. Bioinformatics investigation

We removed adapters and low-quality ends of sequenced reads using Trimmomatic (Bolger et al., 2014), based on quality control statistics generated by FastQC (Andrews, 2010). After trimming, fragments without sufficient length of both reads (> 35 bp) were removed from read sets.

Filtered reads were assembled into contigs with Matam (Pericard et al., 2017) for each sequenced sample separately. Since Matam did not support paired reads natively, we merged overlapping read pairs with PEAR (Zhang et al., 2013). Then, we aggregated contigs from analysed samples and clustered them together using CD-HIT (Li and Godzik, 2006) with 97% sequence similarity threshold. Each sequence cluster represented a single operational taxonomic unit (OTU). Finally, we compared representative of each cluster with sequences from reference databases using BLAST (Altschul et al., 1990). Taxonomic label of the closest homologue were assigned to sequences of the OTU cluster.

16S rRNA samples were labeled with taxonomy from the Silva database (version 128) (Quast et al., 2012). Since *bcaT*, *pepN*, *pepX* and *prtP* genes lacked high quality reference sequence database, we prepared our own reference set. At first, we downloaded genomes that contained these genes from the NCBI database. Then we extracted taxonomy and gene sequences according to their GenBank annotations.

Transcriptome samples and amplicons of *bcaT* gene were analysed using alternative, mapping based method, since they lacked sufficient

number of reads for assembly. We therefore filtered reads with sequence similarity to reference gene databases using SortMeRNA (Kopylova et al., 2012). Then, we labeled filtered paired reads with taxonomy using Metaxa2 classifier (Bengtsson-Palme et al., 2015, 2018).

We summarized taxonomic labels and read counts in each sample and visualized them in a form of multi-level pie charts generated with KronaTools (Ondov et al., 2011).

3. Results

3.1. Metatranscriptome analysis

The screening of transcriptional activity of *prtP* gene evidenced a certain change in the contribution of LAB members during the maturation of ewes' curd. In fact, at the beginning (stage 4 h) the transcriptional activity was detected for several LAB such as *Lactobacillus* spp., *Lactococcus* spp., *Lactobacillus plantarum*, *Lactococcus lactis* and *Streptococcus pneumoniae*. In the next steps, transcriptional activity was mostly identified for *Lactococcus* species which reached the 95% and 82% at step D1 and D8, respectively (Fig. 1).

The analysis of transcriptional activity of the LAB aminopeptidase N (*pepN*) showed an analogous situation as for the *prtP* gene. The *pepN* of lactococci was the most transcriptionally active, where the species *Lc. lactis* was predominant (Fig. 2). Transcriptional activity was detected also for *Streptococcus* spp. in all samples with a percentage range around 1–2%. Very low level of transcriptional activity was detected for lactobacilli only at step D8.

An interesting point of the *pepN* metatranscriptome investigation was the identification of transcriptional activity for a diverse bacterial community different from LAB. Indeed, the transcriptional activity at the stage 4 h and D1 was identified also for *Actinobacteria* and *Proteobacteria* (especially *Gammaproteobacteria*; Fig. S1).

The dominant contributors of *pepX* transcriptional activity were again *Lactococcus* spp. *Streptococcus* members were transcriptionally active also on stage 4 h and mainly on stage D8, together with a minor contribution of *Lactobacillus* spp. (Fig. 3).

Several *Lactococcus* spp. transcribed the gene encoding for the branched chain aminotransferase (*bcaT*) on all three sampled stages. Other LAB contributors included diverse members of the genus *Streptococcus* together with a minor contribution of *Lactobacillus fermentum* (mainly on step D1) and *Lb. helveticus*. In the first sample (time 4 h), also the *bcaT* gene of *Bacillus halodurans* and *Ramlibacter tataouinensis* was found to be transcriptionally active (Fig. 4).

3.2. Specific cDNA sub-libraries

When the 16S rRNA transcriptome was analysed, lactobacilli (mainly *Lb. helveticus*), lactococci and few *Streptococcus* spp. were found to be active in each analysed step (Fig. 5). The transcriptionally active *Gammaproteobacteria* encompassed *Pseudomonas* spp., *Serratia marcescens*, *Yersinia pseudotuberculosis*, *Escherichia coli* and *Aeromonas* spp. The transcriptionally active members of class of *Flavobacteria* was represented by *Chryseobacterium hominis* and *Myroides gitamensis* (Fig. 5).

The transcriptional activity of *prtP* gene was detected on the stages D1 and D8 only by the pair of primers B, exclusively for *Lb. rhamnosus* (Fig. S2).

The PCR assays A and B for the *pepN* gene revealed the

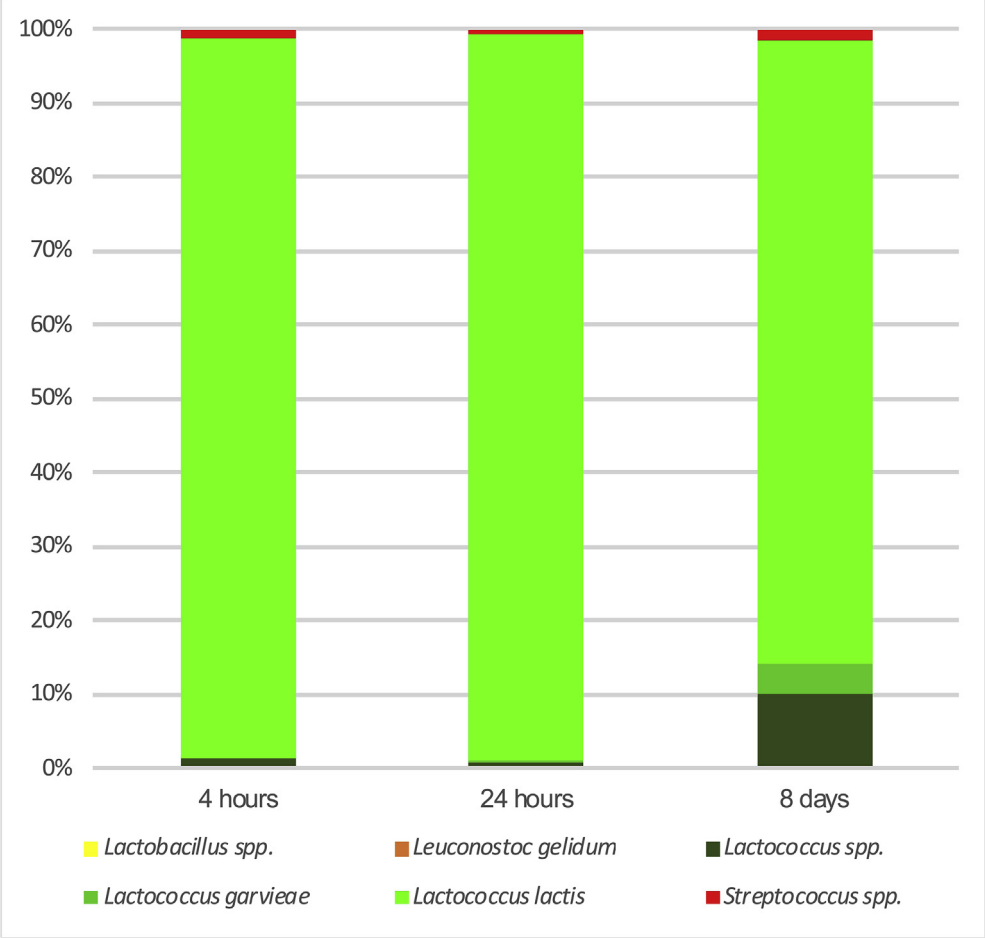

Fig. 2. Metatranscriptome analysis of LAB *pepN* gene on different cheese maturation stages.

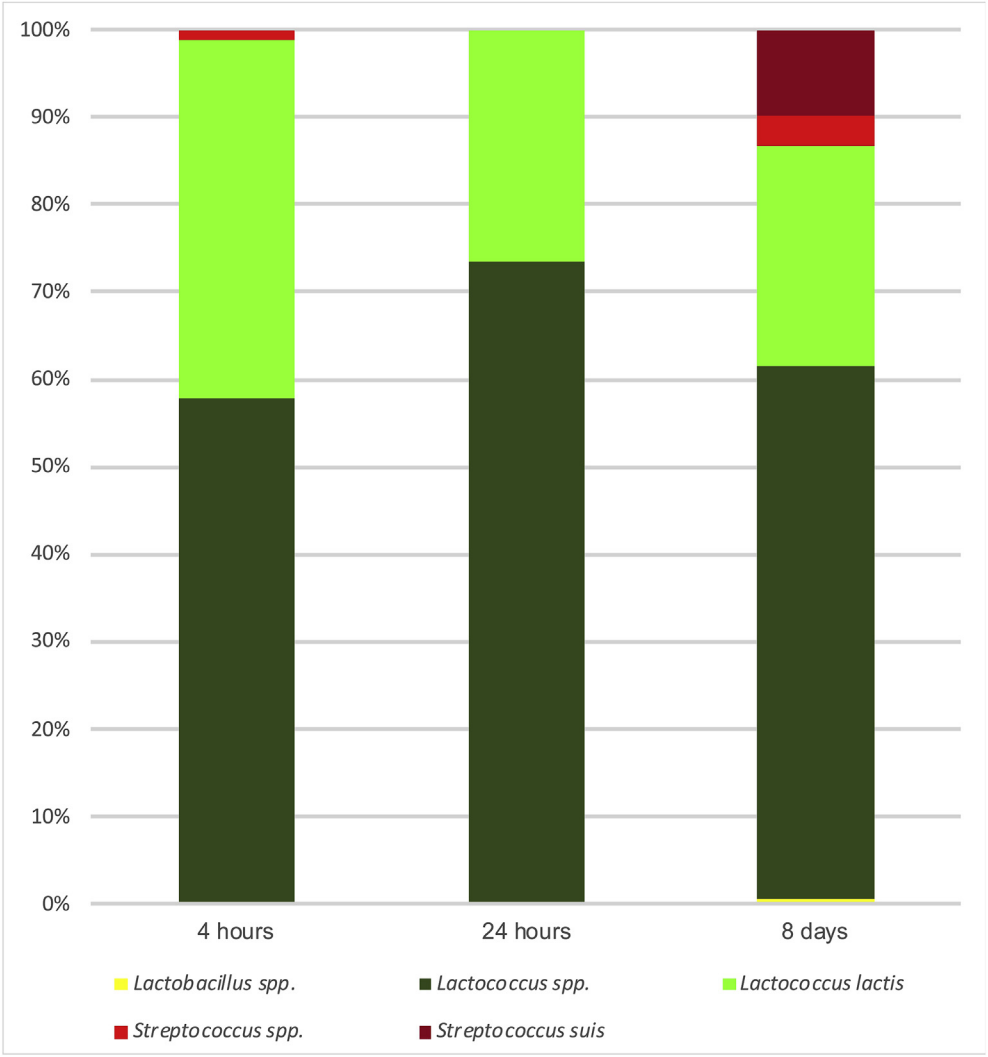

Fig. 3. Metatranscriptome analysis of *pepX* gene on different cheese maturation stages.

transcriptional activity only on stage D8. Several lactobacilli were found to be transcriptionally active, *Lb. pentosus*, *Lb. curvatus* and *Lb. parabuchneri* displaying the biggest number of reads. Moreover, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. brevis* and *Lb. heilongjiangensis* were also found to be transcriptionally active by the two *pepN* PCR assays (Fig. 6). The assay B was able to detect the minor activity for *Enterobacter* spp.

The RNA of *pepX* gene was found to be transcriptionally active exclusively on the last maturation stage with the PCR assay B (Fig. 7), which corresponded to *Lb. helveticus* (80% of the reads), *Lb. delbrueckii* (18%) and *Lb. brevis* (2%).

Unfortunately, the PCR assays for the *bcaT* gene did not amplify any of the investigated samples.

3.3. Metagenomic analysis

The Illumina analysis of DNA 16S rRNA amplicons showed the large presence of LAB in all steps (time 4 h, D1 and D8) of cheese ripening. The LAB were represented mainly by *Lactococcus* spp. and *Lactobacillus* spp., while *Lb. helveticus* was the most detected LAB. A few reads were also revealed for *Streptococcus suis*, *S. agalactiae* and *Enterococcus faecalis* (Fig. 5).

The metagenomic analysis showed also the presence of diverse species of contaminating bacteria, such as *Aeromonas* spp., *Myroides* spp., *Cronobacter universalis*, *Pseudomonas* spp. and *Serratia marcescens*. However, in most cases were these bacteria detected only in the first stages of ripening and were later suppressed, which is important for the safety of the product.

From total DNA, the *prtP* gene was amplified only with the pair of primers B, which were designed and oriented also to *Lc. lactis*. Only *Lb. rhamnosus* and *Lc. lactis* were detected. The biggest number of *Lc. lactis* reads was present at the beginning of the cheese manufacturing (time 4 h) and then, in the stage D1 and D8, the majority of reads were assigned to *Lb. rhamnosus* (Fig. S2).

The gene for the protease PepN was amplified by both designed primer pairs (A and B). This gene was detected in all DNA samples extracted from cheeses at different stages. At time 4 h, the primers A were able to detect mainly *Pediococcus damnosus* and only few reads belonged to *Lb. rhamnosus*. The situation changed during the cheese maturation and, at stage D1, the reads of *P. damnosus* dramatically decreased and those of *Lb. rhamnosus* became the most abundant. *Lb. pentosus* and *Lb. parabuchneri* were also detected. At stage D8, the highest number of reads was attributed to *Lb. pentosus,* followed by *Lb. rhamnosus*, *Lb. plantarum*, *Lb. curvatus* and only a few reads belonged to *P. damnosus* (Fig. 6).

The primers B revealed *pepN* of *Lb. brevis* at each analysed stage being the unique *Lactobacillus* at stage 4 h (Fig. 6). In addition, *Lb. heilongjiangensis* was detected at stage D1 and D8, while at stage D8 further LAB were detected, namely, *Lb. curvatus*, *Lb. parabuchneri* and also *Lc. lactis* and *Lb. rhamnosus*, the latter with a limited number of reads. The primers A and B specific for *pepN* revealed the minor

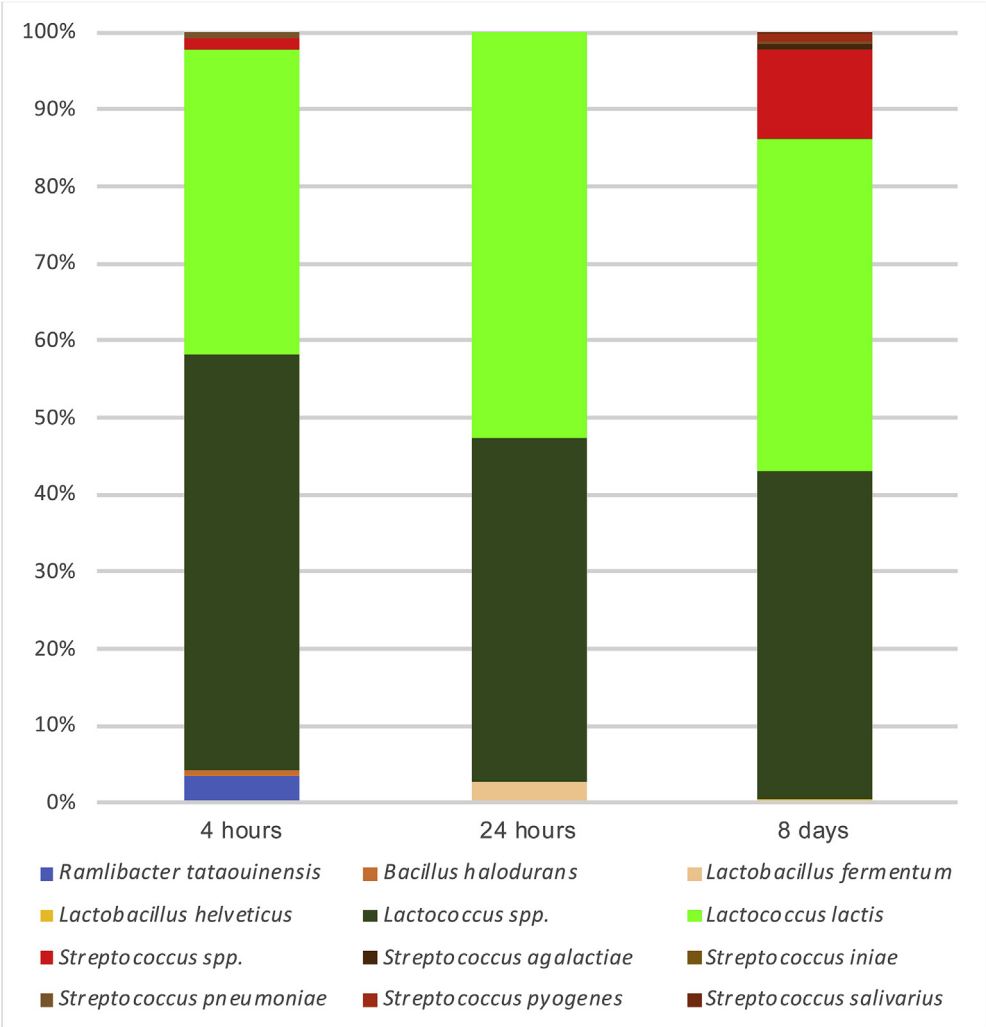

Fig. 4. Metatranscriptome analysis of *bcaT* gene on different cheese maturation stages.

presence of the gene in the members of the order *Enterobacterales* (*Enterobacter* spp., *Raoultella ornithinolytica* and *Pantoea* spp.), based on very few reads.

Regarding the gene *pepX*, it was detected using the primers A for all samples (4 h-D8), while the primers B produced any amplification only in case of the sample D8. The PCR assay A revealed mainly the presence of *Lb. rhamnosus* and *Lb. plantarum*, reads of *Lb. paracasei* being detected only at stage D8. This screening demonstrated a decrease in the presence of *Lb. rhamnosus* along ripening, while the numbers of reads of *Lb. plantarum* increased, accounting for more than 80% in the last stage (D8). At stage D8, the positive PCR assay B revealed the presence mainly of *Lb. delbrueckii* followed by *Lb. helveticus*, with only a few reads of *Lb. brevis* (Fig. 7).

The primers B designed for the gene *bcaT* were able to amplify the DNA from cheese at all three ripening stages and the results showed the large presence of *Lc. lactis* together with different species belonging to the genera *Streptococcus* and *Lactobacillus*. The assay A produced amplicons only with the sample D8 showing a dominant occurrence of *Lb. helveticus*, which reached the percentage of 96% (Fig. S3).

4. Discussion

Studies based on transcriptome analysis were carried out previously on cows' milk cheeses that were produced mainly from pasteurized milk using starter cultures (Dugat-Bony et al., 2015; De Fillipis et al., 2016; Duru et al., 2018). Our study was oriented to the LAB community during the maturation of an unpasteurized ewes' milk cheese and to various genes involved in the proteolytic processes.

The experimental strategy was based on two transcriptomic approaches focused on *prtP*, *pepN*, *pepX* and *bcaT* genes and supported by metagenomics analysis. The approaches involved: i) the analysis of the total extracted RNA depleted of rRNA and ii) the construction of specific cDNA sub-libraries of the abovementioned genes. While the aim of the first approach was to provide general information on proteolytic transcriptome in the context of all mRNA, the aim of the second approach was to characterize it more effectively and specifically. The metagenomic data provided additional information on microbial diversity in the cheese samples at the given maturity stage, without distinguishing live and dead, or active and non-active microorganisms.

The metatranscrptome approach has the disadvantage that, during the rRNA depletion, a large amount of mRNA may be lost. Therefore, many bacterial representatives may escape detection. The sub-library approach has the advantage to focus our attention to a specific group of bacterial transcripts, but the proportionality between groups of bacteria may become biased by different efficiency of PCR amplification.

Generally, the metatranscriptome investigation evidenced that the proteolytic process is driven by *Lactococcus* species with a minor participation of *Lactobacillus* spp. and *Streptococcus* spp. On the other hand, the 16S rRNA screening displayed a larger presence and also activity of lactobacilli, mainly *Lactobacillus helveticus*, on all three analysed maturation steps. This finding confirmed the previously published data on LAB dynamics in bryndza production (Pangallo et al., 2014).

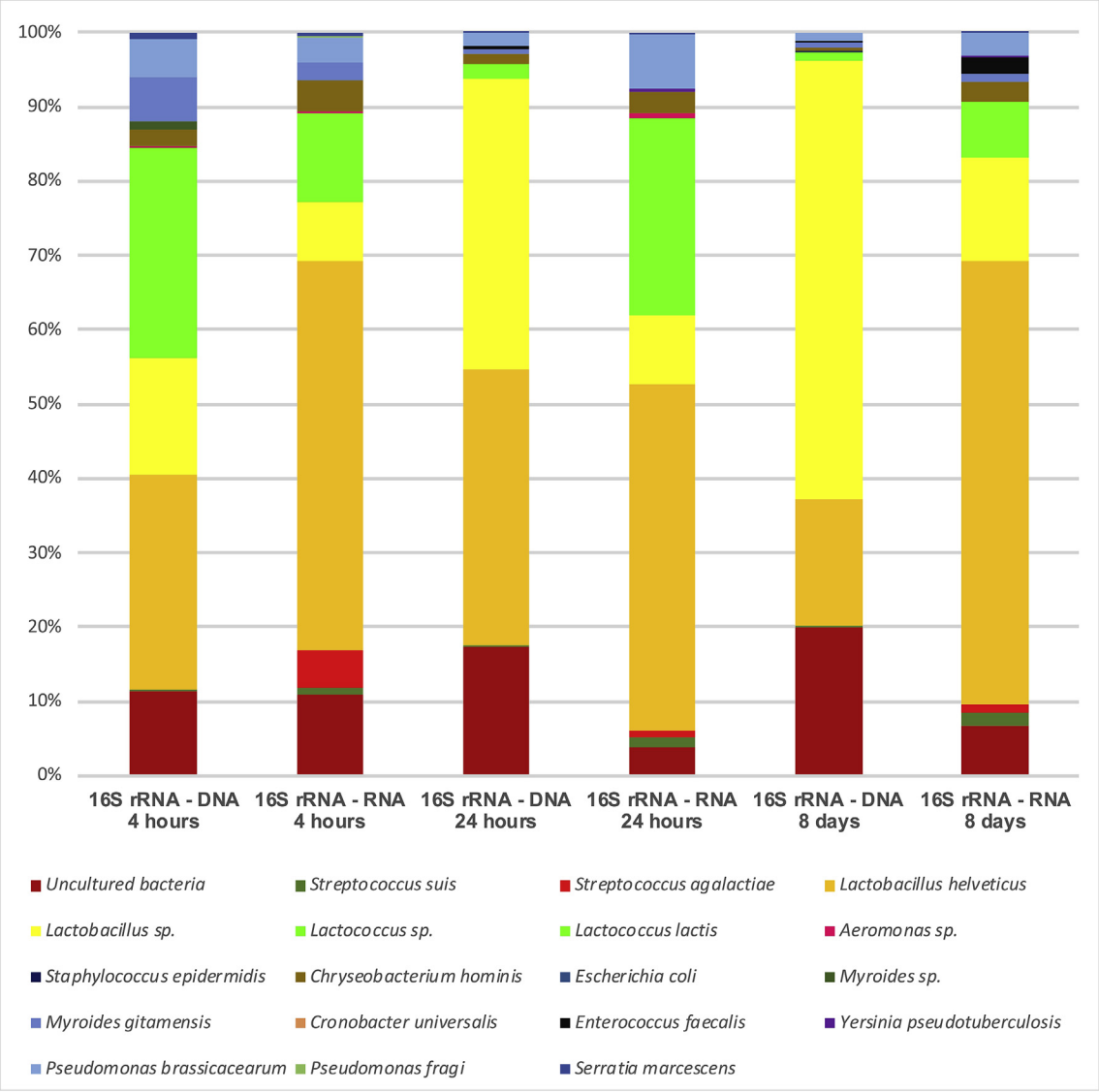

Fig. 5. Bacterial community dynamics described by the detection of 16S rRNA gene using RNA (cDNA) and DNA samples.

Considering the *prtP* gene, there were marked differences between the results of the two approaches. The metatranscriptomic data suggested that lactococci were the main bacteria responsible of cleaving the casein into smaller peptides during the cheese repining already at the first day. The cDNA sub-libraries and metagenomic analyses identified *Lb. rhamnosus* as the principle bacterium with a pronounced PrtP activity in all analysed cheese ripening phases. The role of *Lc. lactis* and its PrtP proteinase in hydrolyzing casein is well known (Steele et al., 2013). The conversion of casein is considered as the most important biochemical pathway for flavour formation in cheese (Smit et al., 2005). *Lb. rhamnosus*, which was also found to participate in this process, was previously found to provide proteolytic activity at Parmigiano Reggiano production, persisting throughout the duration of cheese ripening (1–20 months; Gatti et al., 2008; Bove et al., 2012).

Interesting data, related to diversity of lactobacilli and presence of aminopeptidase genes *pep*N and *pepX*, were produced by the cDNA sub-libraries and metagenomic analyses. In the cheese ripening steps of 4 h and D1, successful amplification was obtained only with total DNA and not with cDNA. However, some species detected at D8 were found to be transcriptionally active in that stage. The *pepN* activity was detected in members of the species *Lb. pentosus*, *Lb.* curvatus, *Lb. parabuchneri*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. brevis* and *Lb. heilongjiangensis*. This suggests the transcriptional activity regarding these genes was negligible at 4 h and D1, becoming detectable at stage D8. The aminopeptidase PepN received a certain attention in previously published transcriptome studies, when it was found to be related to *Lactococcus lactis* in cheese manufacturing (Guédon et al., 2001; Cretenet et al., 2011; Desfossés-Foucault et al., 2014). Lactobacillar aminopeptidase PepN is recognized as important, mainly for biotechnological applications (Stefanovic et al., 2017) but also due to enzymatic activities in ewes' milk cheese production (Tsafrakidou et al., 2016). A novel information provided by our study regards the presence of the taxonomically new *Lb. heilongjiangensis* (Gu et al., 2013) and its PepN aminopeptidase in cheese environment.

The gene *pepN* was found to be active also in several other taxa different from LAB, which belonged to the phyla *Actinobacteria* and *Proteobacteria*. The analysis suggested that mainly the members of *Gammaproteobacteria* (*Raoultella* spp., *Klebsiella* spp. and *Escherichia coli*) could contribute, in the first phase of maturation, to the degradation of peptides. At the last step (D8), LAB apparently suppressed the other bacteria, becoming the main responsible for the PepN peptidase activity. The occurrence of *Gammaproteobacteria* at the beginning of ewes' cheese maturation was evidenced also in previous investigations (Schirone et al., 2012). Usually, the members of

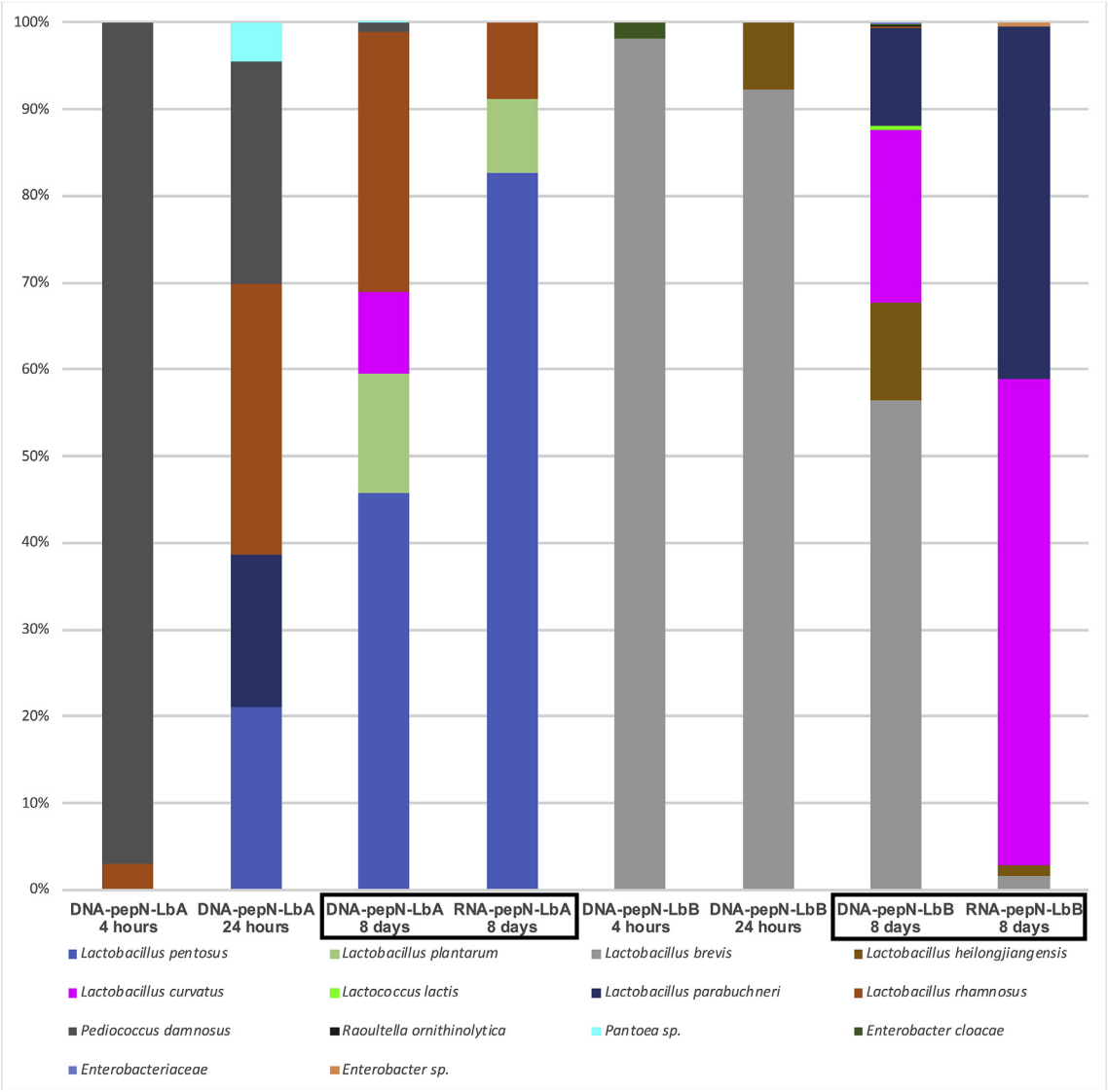

Fig. 6. cDNA libraries and DNA analyses of *pepN* gene during cheese maturation. LbA: PCR amplified by primer pair AF/AR; LbB: PCR amplified by primer pair BF/BR.

Gammaproteobacteria are considered to produce off-flavors, so unpleasant smells (Westling et al., 2016), they can produce biogenic ammines (Schirone et al., 2012; Benkerroum, 2016) and several of them are also pathogens (Coton et al., 2012).

Concerning the PepX aminopeptidase, there is also an unbalance, in literature, between the information on *Lactococcus lactis* and that about lactobacilli in cheese ripening. Many studies were focused on the utilization of the genomic characteristics and enzymatic activities of *Lactococcus lactis* in dairy products and its transcriptome (Cavanagh et al., 2015; Kok et al., 2017). Therefore, the aminopeptidases of other LAB, including PepX, are considered valuable enzymes (Varmanen et al., 2000; Griffiths and Tellez, 2013; Stressler et al., 2016; Wu et al., 2016), but the detection inside a LAB community during cheese production was rarely investigated. Our study demonstrated the transcriptional activity of *pepX* gene from *Lb. brevis*, *Lb. delbrueckii* and *Lb. helveticus* at the last analysed step. It also evidenced the potential involvement, at the beginning of maturation, of *pepX* genes belonging to *Lb. rhamnosus*, *Lb. plantarum* and *Lb. paracasei*. These species are accepted candidates for improving the flavor and organoleptic properties of various cheeses (Azarnia et al., 2010; Desfossés-Foucault et al., 2014; Bozoudi et al., 2015).

The aminotransferases, such as BcaT, are crucial for the conversion of amino acids to aroma compounds. BcaT catalyzes the amino acid transamination, which results in the formation of α-keto acids that are subsequently degraded, via one or more additional steps, into various aroma compounds (Smit et al., 2005). The combination of the two transcriptome-based approaches showed that *bcaT* activity, in each maturation phase, is mainly led by three LAB groups: *Lactococcus* spp., *Streptococcus* spp. and *Lactobacillus* spp. A higher diversity of species contributing to transcriptional activity of *bcaT* was detected in the last analysed step (D8). Various *Streptococcus* spp., *Lb. fermentum* and *Lb. helveticus* were found to be transcriptionally active. The results suggested that the LAB species forming aromas by BcaT activity, in our cheese, were mainly *Lc. lactis* and *Lb. helveticus*, in particular in the last step. It was already demonstrated, in a chemically defined medium, that the BcaT enzyme of *Lc. lactis* influenced the aroma formation (García-Cayuela et al., 2012). In addition, the importance of *Lb. helveticus*, as a provider of BcaT activity for converting amino acids into aroma compounds, was displayed by *in vitro* assays (Klein et al., 2001). In bryndza (Pangallo et al., 2014; Šaková et al., 2015; Sádecká et al., 2016) and other ewes' cheeses (Di Cagno et al., 2003; Trmčić et al., 2008; Medina et al., 2011), *Lb. helveticus* was previously frequently detected and the results of this study suggest that it may contribute to aroma formation during the cheese production.

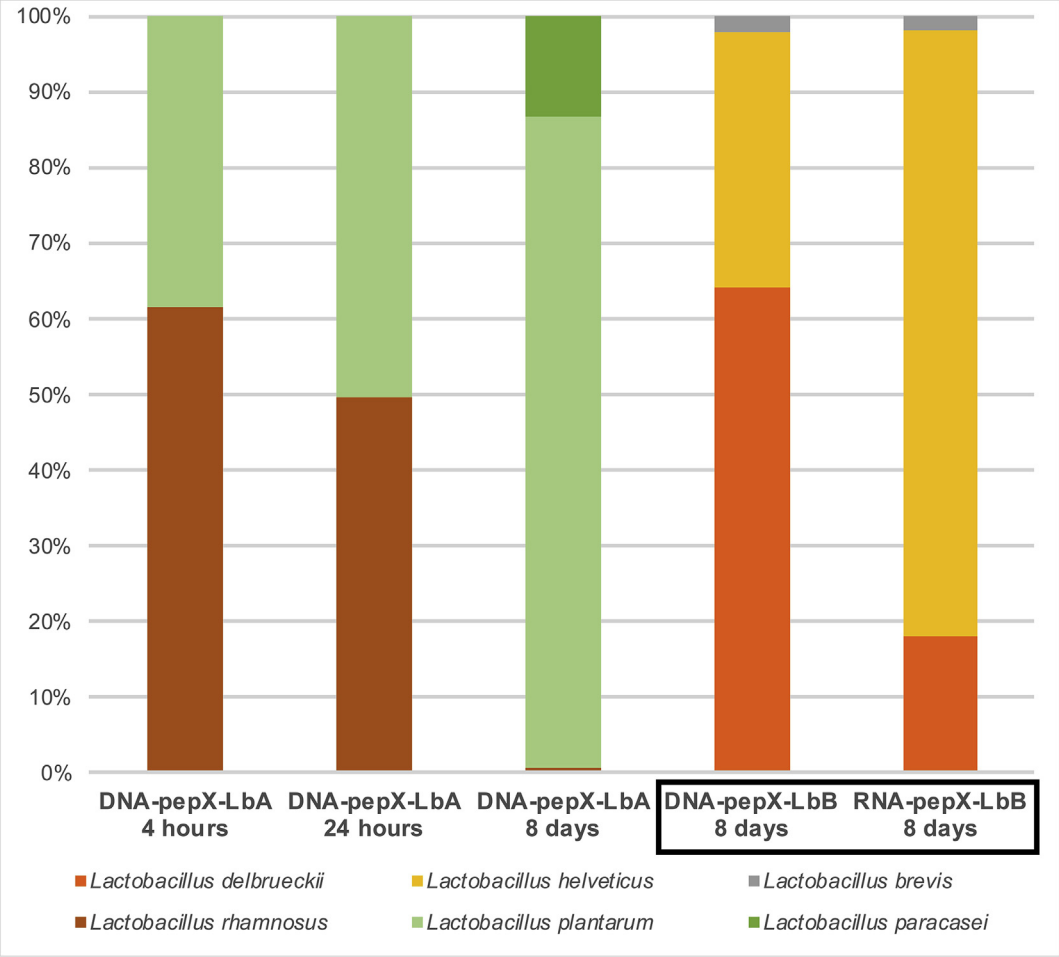

Fig. 7. cDNA libraries and DNA analyses of *pepX* gene during cheese maturation. LbA: PCR amplified by primer pair AF/AR; LbB: PCR amplified by primer pair BF/BR.

Table 2
Presence of the analysed genes in *Lactobacillus* and other LAB species.

Gene	*Lactobacillus* species	Other LAB species
prtP	*Lb. rhamnosus, Lb. plantarum*	*Lactococcus lactis, Streptococcus pneumoniae*
pepN	*Lb. pentosus, Lb.* curvatus, *Lb. parabuchneri, Lb. plantarum, Lb. brevis* and *Lb. heilongjiangensis; Lb. rhamnosus*	*Pediococcus damnosus, Lc. lactis, Lc. garvieae, Leuconostoc gelidum*
pepX	*Lb. brevis, Lb. delbrueckii* and *Lb. helveticus; Lb. rhamnosus, Lb. plantarum* and *Lb. paracasei*	*Lc. lactis, Streptococcus suis*
bcaT	*Lb. fermentum* and *Lb. helveticus*	*Lc. lactis, Streptococcus pyogenes, S. pneumoniae, S. agalactiae, S. iniae, S. salivarius*

Our investigation and developed approaches showed the dynamics of the transcriptional activity of several genes involved in the metabolism of casein, peptides and amino acids throughout the maturation of a raw ewes' cheese. The study demonstrated the limits of 16S rRNA screening, based on cDNA sub-libraries and metagenomic analysis, at tracing a possible connection between the presence of individual species and their function in the cheese environment. The combination of two transcriptomic approaches, the analysis of the total extracted RNA depleted of rRNA and the construction of specific cDNA sub-libraries, gained interesting information on the transcriptional activity of four genes (*prtP*, *pepN*, *pepX* and *bcaT*) harbored by individual LAB species (Table 2).

The results evidenced that both approaches were complementary. The metatranscriptome study permitted to suggest the main protagonists of the proteolytic process (lactococci, streptococci and lactobacilli), while the cDNA sub-libraries construction facilitated a deeper identification of the species involved, mainly for the members of the genus *Lactobacillus*, for which the specific primers were designed.

Such kind of strategy can be also applied to other types of cheeses in order to study their LAB proteolytic transcriptome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.015.

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