METAGENOMIC ANALYSIS OF SLOVAK BRYNDZA CHEESE USING NEXT-GENERATION 16S rDNA AMPLICON SEQUENCING

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Abstract: Knowledge about diversity and taxonomic structure of the microbial population present in traditional fermented foods plays a key role in starter culture selection, safety improvement and quality enhancement of the end product. Aim of this study was to investigate microbial consortia composition in Slovak bryndza cheese. For this purpose, we used culture-independent approach based on 16S rDNA amplicon sequencing using next generation sequencing platform. Results obtained by the analysis of three commercial (produced on industrial scale in winter season) and one traditional (artisanal, most valued, produced in May) Slovak bryndza cheese sample were compared. A diverse prokaryotic microflora composed mostly of the genera *Lactococcus*, *Streptococcus, Lactobacillus*, and *Enterococcus* was identified. *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* were the dominant taxons in all tested samples. Second most abundant species, detected in all bryndza cheeses, were *Lactococcus fujiensis* and *Lactococcus taiwanensis*, independently by two different approaches, using different reference 16S rRNA genes databases (Greengenes and NCBI respectively). They have been detected in bryndza cheese samples in substantial amount for the first time. The narrowest microbial diversity was observed in a sample made with a starter culture from pasteurised milk. Metagenomic analysis by high-throughput sequencing using 16S rRNA genes seems to be a powerful tool for studying the structure of the microbial population in cheeses.

Key words: Lactic acid bacteria, Bryndza cheese, Next-generation sequencing, metagenomics, 16S rRNA genes

1. Introduction

Bryndza is a cheese traditionally produced in the mountainous regions of Slovakia, by ripening, salting and grinding of ewes' cheese, or a mixture of ewes' and cows' cheese. Based on the current legislation, the minimum content of the ewes' component must be more than 50%. Since 2008, Slovak bryndza is on the list of European cheeses with Protected Geographical Indication status.

The quality of bryndza cheese depends on many factors, e.g. the production period, quality of ewes' milk and if the starter culture was used. May bryndza cheese is the

DOI 10.1515/nbec-2016-0003

highest valued variant of bryndza, due to its distinctive flavour (SÁDECKÁ *et al*., 2014). Ewes' milk, in comparison to cows' milk, contains higher amounts of proteins, carbohydrates, traces of copper, panthotenic acid, biotin and vitamin B12 (BURDOVÁ, 1997). Composition and activity of microflora are believed to be important for flavour and aroma of bryndza cheese. Study of lactic acid bacteria present in traditional fermented foods and their diversity is necessary to understand their quality and, eventually, for starter culture selection if pasteurisation of the substrate is necessary for safety reasons. The use of starter or adjunct cultures is also useful to control the fermentation process and to standardize the end product (LEROY and De VUYST, 2004). However, this approach often leads to a loss of the uniqueness of the original product and the loss of the characteristics that have made the product popular (CAPLICE and FITZGERALD, 1999).

Metagenomics deals with the investigation of the whole genetic material isolated from environmental samples to characterise their microbial consortia composition. Metagenome is nowadays mostly sequenced using the technique of next-generation sequencing (NGS), which allows sequencing of huge numbers of different DNA strands at the same time. The use of 16S rRNA genes in targeted metagenomics is suitable for bacteria identification, and their taxonomy and phylogeny analysis because of their presence in almost all prokaryotes and their relatively short length (approximately 1500 bp). Moreover, 16S rDNA sequence contains nine hypervariable regions, which are often conserved within species but different between species.

The microbial composition investigation in dairy products by NGS represents an innovative approach, which provides extensive data while requiring less labour and time. This technology has been used to study the bacterial diversity of various cheeses, such as Irish artisanal cheeses (QUIGLEY *et al*., 2012), Latin-style cheeses (LUSK *et al*., 2012), traditional Polish cheeses (ALEGRIA *et al*., 2012), water buffalo mozzarella cheese (ERCOLINI *et al*., 2012) and Danish semi-hard cheese (MASOUD *et al.,* 2012).

The composition of bryndza cheese microflora was previously studied by various culture-based and culture–independent methods. Enterococci in bryndza cheese were previously identified on the genus and species level by phenotypic methods and confirmed by PCR using species-specific primers for *ddl* genes (JURKOVIČ *et al*., 2006). LAURENČÍK *et al*. (2008) investigated the occurrence and diversity of yeast and filamentous fungi in bryndza cheese collected during four months of the summer production period by conventional taxonomy and sequence comparision of D1/D2 region from 26S rRNA genes. Mostly lactobacilli were characterized using microbiological and biochemical testing, genotyping by RAMP, eventually by 16S rDNA sequencing by BERTA *et al*. (2009). The knowledge about the bryndza cheese microflora was extended by CHEBEŇOVÁ-TURCOVSKÁ *et al*. (2011) using the universal prokaryotic and fungal primers to amplify rDNA internal transcribed spacer regions. Diversity and dynamics of prokaryotes and eukaryotes in selected stages of the May bryndza cheese production were studied by PANGALLO *et al*. (2014) using culture-based and non-culture approach based on amplification of 16S rDNA and internal transcribed spacer region, coupled to denaturing gradient gel electrophoresis and sequencing. Also occurrence of pathogenic bacteria *E. coli* (HOLKO *et al.*, 2006) and *S. aureus* (MAŠLANKOVÁ *et al*., 2009) in bryndza cheese were previously

studied. In this article, the most progressive culture-independent technique based on NGS 16S rDNA amplicon sequencing was used for the first time to analyse microbial consortia in bryndza cheese.

2. Materials and methods

2.1 Sample preparation

Four samples of bryndza cheese from different manufacturers and regions of Slovakia were analysed. Their origin, region of production, ewes´ milk content, use of starter culture, production season and the type of manufacturing process are summarized in Table 1.

Table 1. Samples of bryndza cheese in this experiment used, and their properties.

Sample/Origin	Region of production	Ewes´ milk content	Starter culture use	Production season	Manufacturing process
1./store	Senica	50%	Yes	winter	industrial
2./store	Zvolenská Slatina	50%	Yes	winter	industrial
3./store	Liptovský Mikuláš	50%	Yes	winter	industrial
4./ farm (salaš)	Pružina	100%	No	spring (May)	artisanal

DNA was isolated from each sample by chaotropic solid-phase extraction using DNeasy Food Kit *mericon*, (Qiagen, Hilden, Germany) according to the standard protocol of the manufacturer attached to the kit (sample size, 200 mg). 16S rDNA were amplified in six replicates (to increase the amount of PCR product) using primers 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1062R (5´-ACA GCC ATG CAG CAC CT-3´) to amplify V1-V6 hypervariable regions (GHYSELINCK *et al.,* 2013). Into the mastermix with total volume of 22 μL (0.3 μL of each primer, 2.5 μL Cheetah Taq Dilution Buffer (Biotium, Hayward, California), 2.5 μL 25 mM Magnesium Chloride (Biotium), 4 μL 10 mM dNTP (2.5 mM each); 0.3 μL 500 U Cheetah Hotstart Taq DNA polymerase (Biotium) and 12.1 μL PCR grade water, 3 μL of template DNA solution were added. The PCR program was composed of initial denaturation step (95°C, 2 min), followed by 35 cycles (94 °C for 1 min.; 54 °C for 1 min.; 72 °C for 2 min) and final polymerization step (72 °C for 10 min). Success of PCR was checked by 1.5 % agarose gel electrophoresis using 100 bp DNA Ladder (Biolabs, Ipswich, MA, USA). Products of PCR were pooled and purified by QIAquick PCR Purification Kit (Qiagen).

2.2 Massively parallel sequencing and sequence data processing

Purified PCR products were diluted to equimolar concentration suitable for library preparation and were used as template for library preparation using transposon based

Nextera Library preparation kit (Illumina, San Diego, CA, USA) according to standard protocol. Four samples were analysed using paired end sequencing on Illumina MiSeq sequencing system (Illumina) in two separate runs targeting at least 250 000 reads and at least 2 x 200 bp read lengths.

Sequence data (sequence lists composed of reads) in fasta format were imported into CLC Genomics Workbench Version 7.5 (Qiagen, Hilden, Germany). The obtained 2 x 250 bp long paired reads from samples 1-3, and 2 x 200 bp paired reads from sample 4 were merged and pre-processed. Before merging, sequence lists of given samples 1-4 contained 2 970 530, 2 202 668, 1 885 378 and 285 398 reads, respectively. Overlapping pairs in paired reads of each sequence list representing a given sample were merged, using default parameters (Alignment scores: Mismatch cost 2; Gap cost 3; Maximum unaligned end mismatches 0; Minimum score 8. After merging, two datasets are produced: merged and not merged). Reads in sequence lists were then trimmed based on their length and quality. Limit of trimming using quality score was set to 0.001 and reads shorter than 150 nucleotides were discarded. Reads with ambiguous nucleotides were discarded with the maximum number of ambiguities set to 2.

2.3 Data analysis and results interpretation

Sequence data were analysed using MiSeq Reporter software (MSR; Illumina) on the genus level using Greengenes 16S rRNA gene database (DeSANTIS *et al.*, 2006) integrated in metagenomics analysis module. Reads were identified based on their homology to reference 16S rRNA genes in NCBI database using Basic Local Alignment Search Tool (BLAST) (ALTSCHUL *et al*., 1990) with following parameters: Number of threads 1; Low complexity filter; Expectation value 10 e^{-50}, word size 50; Match/mismatch: Match 2, Mismatch -3; Gap costs: Existence 5, Extension 2; Maximum number of hit sequences 3. BLAST results were exported to MEtaGenome ANalyzer (MEGAN V5) (HUSON *et al*., 2011), and interpreted using NCBI taxonomy.

3. Results and discussion

3.1 Sequence data analysis and pre-processing

Sequence data quality analysis showed that all raw sequence data, obtained from each sample without any pre-processing step, were of relatively high quality. The percentage of reads with average PHRED score (EWING and GREEN, 1998) above 30 in samples Senica, Zvolenská Slatina, Liptovský Mikuláš and Pružina were more than 89%, 88%, 87 % and 97%, respectively. PHRED score or quality score Q, is defined by the equation: Q= -10 log (p), where p is an error probability. Higher quality values (Q) correspond to lower error probabilities, and conversely. If, for example, quality value is 30, the error probability (p) is 1:1000.

After raw data merging, the number of reads in each sequence list was reduced and the maximum length of reads (in the case of, for example, 250 bp) increased

approximately twice (492 bp). The average PHRED score percentage of each sample increased as well. It is apparent that, for accurate assignment to reference 16S rRNA genes, longer reads including more hypervariable regions of the 16S rRNA gene are of greater information value.

3.2 Metagenomic interpretation using MSR

MSR results in the form of tables with a percentage of reads assigned to each taxonomic rank (from *Kingdom* to *Species*) were transformed to pie charts representing microbial composition of each bryndza cheese sample on the genus level (Fig. 1).

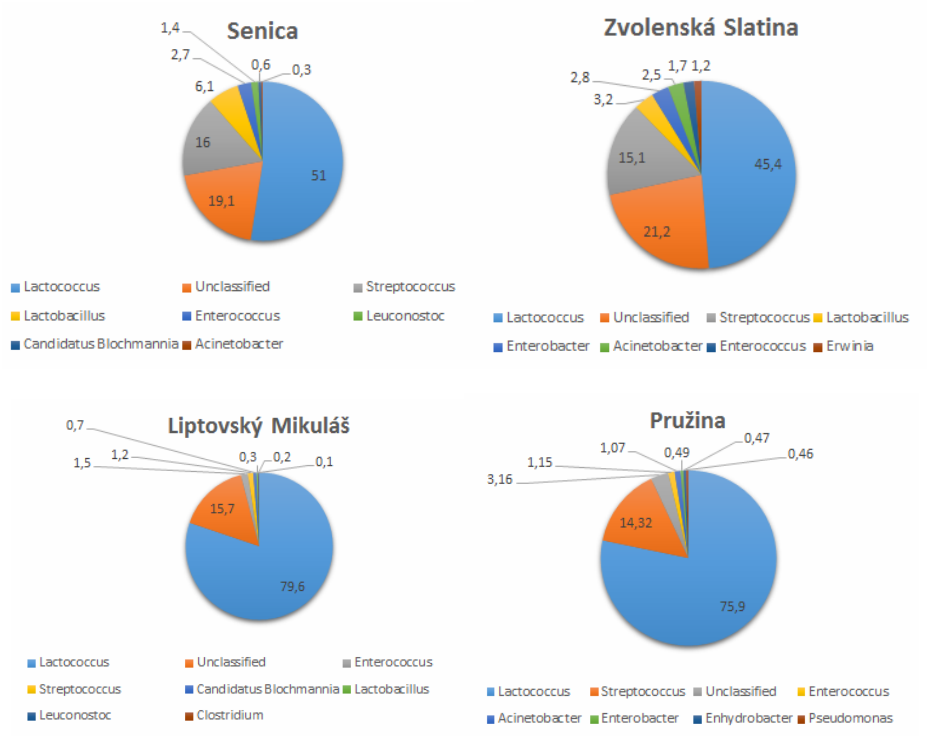

Fig. 1. Percentage of reads assigned to bacteria on genus level (first 8 most abundant) evaluated using MSR and Greengenes database.

The results from MSR showed that the largest percentage of reads, assigned on the genus level, belonged in all bryndza samples to *Lactococci*, followed by the unclassified reads (sample 1-3), which were not assigned to any reference 16S rRNA gene present in Greengenes database. The exception was sample 4, where the second most abundant genus was *Streptococcus*. Streptococci and Lactobacilli, as the third and fourth dominant genera in order, were observed in samples 1 and 2 in similar

amounts (16%; 6.1% and 15.1; 3.2, respectively). Lactobacilli constituted only 0.3% of assigned reads in sample from Liptovský Mikuláš. Lactobacilli were not detected by MSR on the genus level in the sample from Pružina, which confirmed that amount of Lactobacilli depends on the ripening stage of the cheeses and maturation (QUIGLEY *et al*., 2012). The another significant group of lactic acid bacteria observed, were Enterococci in amounts of 2.7%; 1.7%; 1.5%; and 1.15% in the samples 1-4, respectively. The detailed analysis (not illustrated) on the species level showed that the most abundant Lactococci were *Lactococcus lactis* in all samples (22.9%; 20.1%; 37.7%; and 8.6 in samples 1-4, respectively) followed by *Lactococcus fujiensis* in samples 1, 2 and 3 (in the amounts of 5.4%; 5.3%; and 8.6%, respectively) and, in the sample 3 (Pružina), *S. thermophillus* (3.22%) and *L. fujiensi*s (2.54%) on the 2nd and 3rd places. *Lactococcus fujiensis* was detected in bryndza cheese for the first time.

3.3 Metagenomic interpretation using MEGAN 5

Monitoring of microbial diversity on genus level can be used to trace major changes in the structure of the microbial community in foods during fermentation but, in such cases, NGS sequencing at the genus level is not very informative (ERCOLINI, 2013).

The phylogeny relations between taxonomic units of microbial consortia in bryndza cheese samples are illustratively described by taxonomic trees (Fig. 2, 3, 4, 5), which are composed of nodes and roots. Each node represents one taxonomic unit. The size of the node depends on the number of reads assigned.

Firmicutes were the predominant *Phylum* in all four samples tested. Various species of lactic acid bacteria was observed. The species *Lactococcus lactis* predominated in all 4 samples, followed by *Lactococcus taiwanensis*, which was detected in bryndza cheese for the first time. Based on results obtained from MSR, no *Lactococcus taiwanensis* was observed, which could have been caused by the different databases used. After the node uncollapsing to the lowest taxonomic level (showed only in Fig. 4), *Lactococcus lactis* subsp. *lactis* dominated, followed by *Lactococcus lactis* subsp. *cremoris* and *Lactococcus taiwanensis* in all bryndza cheese samples tested. *Lactococcus taiwanensis* seems to be an alternatively assigned operational taxonomic unit instead to *Lactococcus fujiensis* in the case of MSR. The third most abundant species observed was *Streptococcus thermophillus*. *Lactococcus lactis* and *Streptococcus thermophilus* were observed in most bryndza chees samples as major species, regardless of whether the cheese was made from pasteurised or non-pasteurised milk (CHEBEŇOVÁ-TURCOVSKÁ *et al.,* 2011).

In sample 1, the species of *Lactococcus, Streptococcs, Lactobacillus, Enterococcus and Leuconostoc* genera predominated (Fig. 2). This finding was equivocally displayed in results from both approaches (MSR and BLAST + MEGAN 5). Although MSR uses Greengenes database and a proprietary algorithm that provides species-level classification for paired-end reads, the percentages of LAB on the genus level in sample 1 correlated, except for the amount of unknown reads. Only in this sample of winter bryndza cheese, a significant group of Enterococci was observed. These results are different from those of JURKOVIČ *et al.* (2006), which showed that the prevalence of *Enterococcus* species was highest in samples from the winter season.

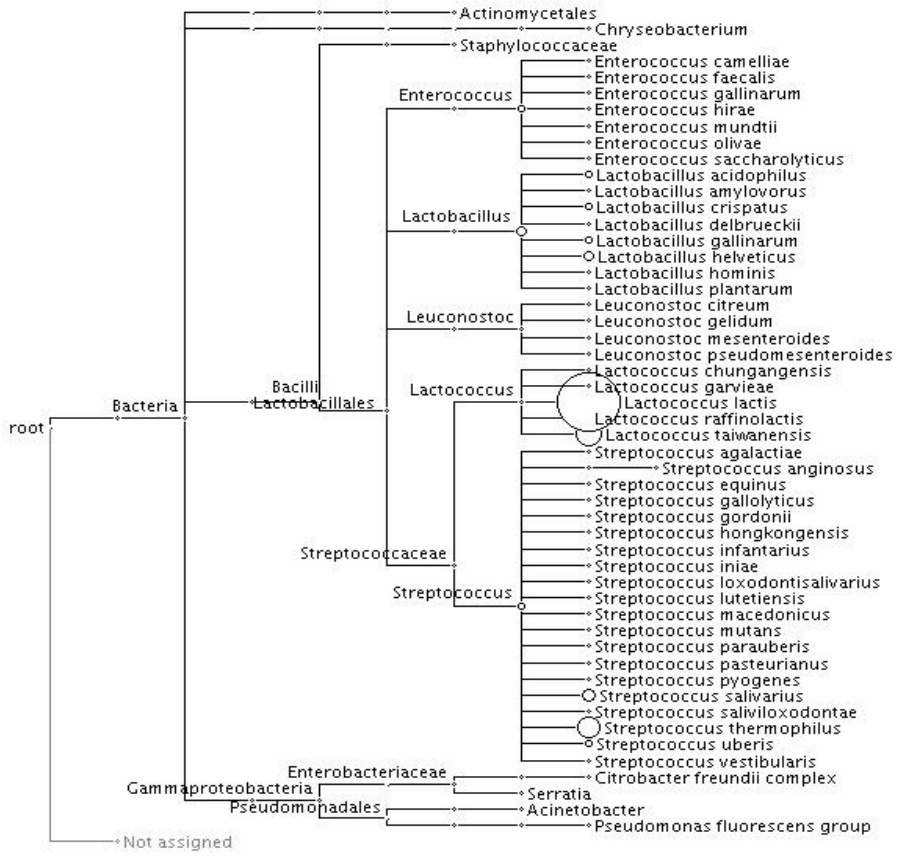

Fig. 2. Taxonomic tree of microflora in bryndza cheese sample from Senica (MEGAN 5).

In sample from Zvolenská Slatina (Fig. 3), lots of *Gammaproteobacteria* were detected including *Enterobacter, Kleibsella, Shigella, Salmonella* and *Yersinia*. These human pathogens could be present in milk before pasteurisation. It must be noted that NGS allows us to detect also bacteria that cannot be cultivated in laboratory conditions, including unviable and dead microorganisms, which cannot affect human health. The group of lactic acid bacteria present in this sample was composed mostly of *Lactococcus, Streptococcus Enterobacter* and *Lactobacillus* species.

The narrowest microbial diversity was observed in the sample from Liptovský Mikuláš (Fig. 4). Only 4 different species including 3 species of lactococci with dominance of *L. lactis followed* by *L. taiwanensis* and *L. raffinolactis*, with contrast to MSR results (Figure 1, Liptovský Mikuláš sample), where genera of *Lactococcus, Enterococcus, Streptococcus, Candidatus Biochmannia, Lactobacillus, Leuconostoc* and *Clostridium* (79,6%; 1,5%; 1,2%; 0,7%; 0,3%; 0,2% 0,1%, respectively) were detected. Loss of some reads could have taken place due to the use of strict assignment identification parameters and reads pre-processing condition. To confirm this, we

additionally analysed raw sequence data, and the taxonomic tree (Fig. 4 B) showed the presence of some small amounts of Enterococci and Streptococci. Short reads, or reads with low quality, could be assigned incorrectly, and therefore could produce false positive results (CHEN *et al.*, 2014). All pre-processed reads were assigned in the sample 3 (Fig. 4) while in MSR results, 15.7% of the total reads were unclassified.

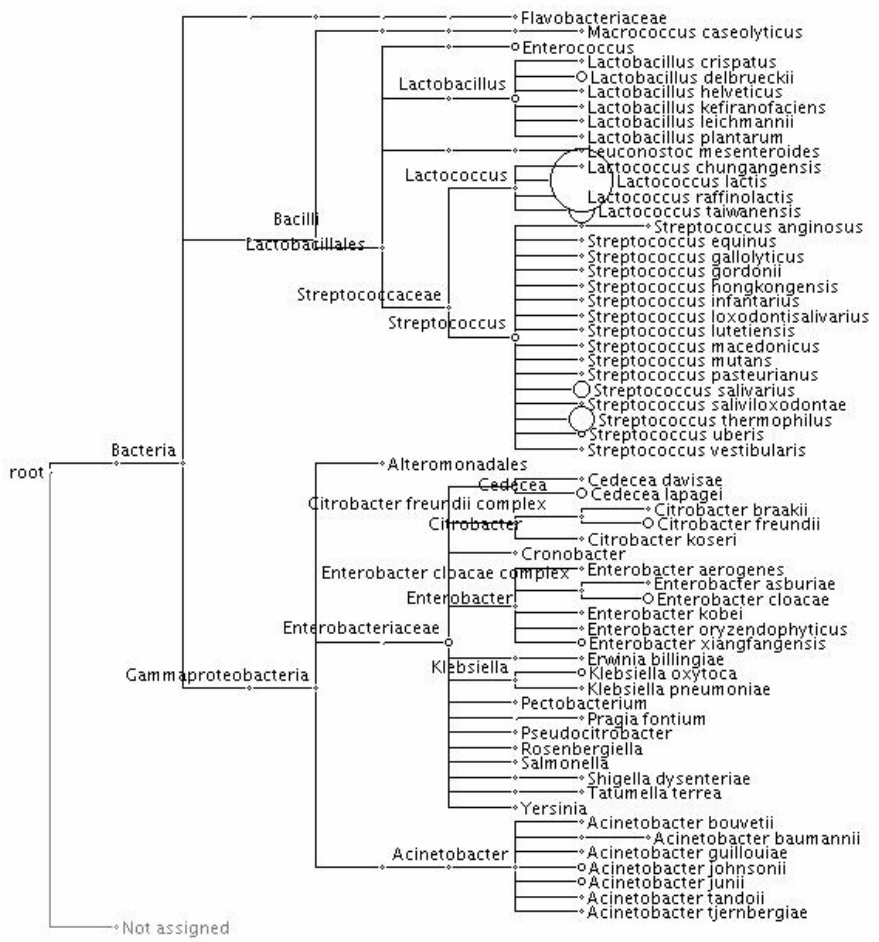

Fig. 3. Taxonomic tree of microflora in bryndza cheese sample from Zvolenská Slatina (MEGAN 5).

The bryndza cheese sample from Pružina differed from the others in the production season and manufacturing process. Apart from the sample number 3, the taxonomic tree of sample from Pružina (Figure 5) seems relatively similar to samples 1 and 2. Therefore we couldn't definitely confirm that the microbial diversity increased in artisanal products in comparison to industrially manufactured cheeses (COPPOLA *et al.*, 2001), also because our samples of bryndza cheese were produced in different seasons.

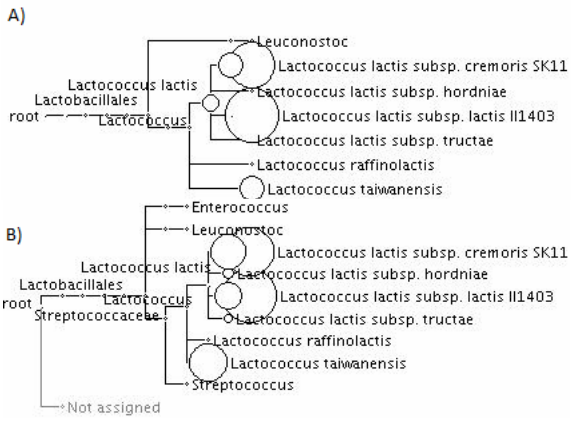

Fig. 4. Taxonomic tree of microflora in bryndza cheese sample from Liptovský Mikuláš (MEGAN 5). A) Pre-processed reads collapsed on strain level; B) Raw reads collapsed on strain level.

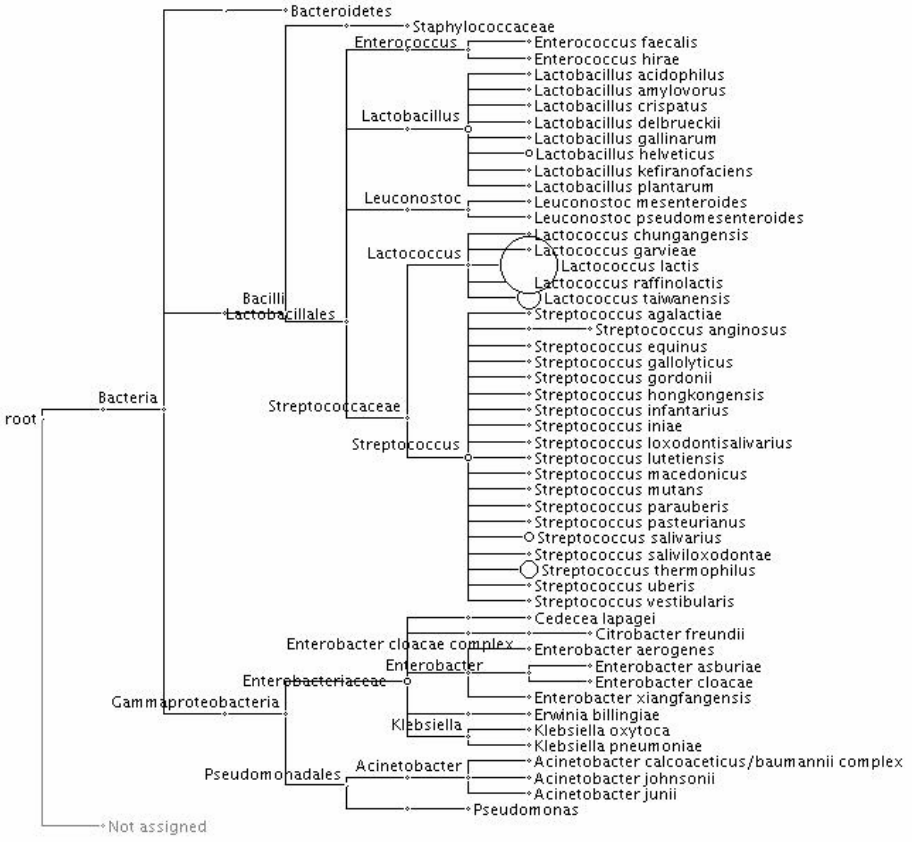

Fig. 5. Taxonomic tree of microflora in bryndza cheese sample from Pružina (MEGAN 5).

A relatively huge part of microflora was composed of *Gammaproteobacteria*, but much less in comparison to sample 2. Some bacteria belonging to *Gammaproteobacteria* were observed in the previous study, mainly during the early stages of May bryndza cheese maturation, namely the strains belonging to the genera *Acinetobacter, Pseudomonas*, *Enterobacter*, *Kluyvera* and *Raoultella* (PANGALLO *et al.*, 2014).

4. Conclusions

Three samples of bryndza cheese purchased in a store (containing 50% ewes´ lump cheese made from pasteurised milk, produced in winter season), and one sample of bryndza cheese purchased in a farm (containing 100% ewes´ lump cheese made from unpasteurised milk, produced in May) were analysed and compared by metagenomics analysis using 16S rDNA amplicons for NGS sequencing. The results showed some differences in the microflora composition in cheeses made from pasteurised and unpasteurised milk but, on the obtained amount of data, it was not possible to clearly determine the specific properties of these two types of bryndza cheese. The most observed genera in all samples of studied bryndza cheese were Lactococci, followed by Streptococci, Lactobacilli and Enterococci. The most abundant species observed were *L. lactis*, *L. taiwanensis*/ *L. fujiensis* (detected by MEGAN 5/ MSR) and *S. thermophillus*. *L. taiwanensis* and *L. fujiensis* were detected in bryndza cheese for the first time at all. The presence of these species in bryndza cheese could be an interesting aim of some future study. The narrowest microbial diversity was observed in pasteurised milk cheese from Liptovský Mikuláš. Some *Gammaproteobacteria* strains were in pasteurised and also in non-pasteurised milk bryndza cheese detected. Contamination may occur after pasteurisation as well. Moreover, we couldn't recognise if the DNA belonged to viable or dead pathogens. Despite this, the metagenomic analysis by high-throughput sequencing using 16S rRNA proved to be a very helpful and suitable tool to make overall picture of the whole microbial composition of various types of Slovak bryndza cheese.

Acknowledgements: This work was supported by the project of the Slovak Research and Development Agency APVV-14-0025.

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Received 27 November 2015
Accepted 5 May 2016