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Microbial diversity and volatile odour-active compounds of barrelled ewes' cheese as an intermediate product that determines the quality of winter bryndza cheese

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ABSTRACT

Microbial diversity and volatile odour-active compounds were studied in barrelled ewes' cheese, which is a long-ripened intermediate product in the production of winter bryndza cheese, a type of PGI Slovakian bryndza cheese produced from unpasteurized ewes' milk. Microbial consortia were studied by both culture-based and culture-independent approaches. Microbiological analysis demonstrated that lactococci, lactobacilli and *Geotrichum* spp. were dominant, while levels of coliforms and coagulase-positive staphylococci were acceptably low. Culture-independent analysis facilitated identification of 26 prokaryotic taxa, with the majority of clones being *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*, and 23 eukaryotic taxa, with the majority of clones being members of *Galactomyces*/*Geotrichum* group. Profiles of odour-active compounds were studied using gas chromatography – olfactometry supported by gas chromatography – mass spectrometry. Thirty-nine odour-active compounds, or mixtures in case of co-elution, were identified. Butanoic acid, ethyl butanoate, isovaleric acid, hexanoic acid, octanoic acid, decanoic acid, methyl octanoate, ethyl hexanoate, ethyl octanoate, *p*-cresol, and δ-decalactone were detected at high or moderate intensities in all samples. The data obtained demonstrate that barrelled ewes' cheese is a product with specific microflora and a very specific rich profile of odour-active volatile compounds.

1. Introduction

Long-ripened barrelled ewes' cheese is an intermediate product in the production of winter bryndza cheese, which is a type of PGI Slovakian bryndza cheese produced from unpasteurized ewes' milk. Traditional production starts at small mountain farms (salaš), where ewes' milk is processed immediately after milking by renneting at 29–31 °C for 30 min using chymosin or chymosin-identical rennet. No starter cultures are used. The curd is drained at 18–22 °C for 24 h, and then left to ripen for 3 days at 18–20 °C. After that, the curds are transported to a bryndza-producing dairy for processing. Here individual lumps are pressed, milled and loaded in layers mixed with salt in barrels, the NaCl content being 4%–6% (w/w) meaning up to 12% (w/v) in the water phase. The barrels are closed and the cheese is left to ripen at 2–10 °C for at least 2 months, with intermittent removal of foam formed on the top of the cheese. After ripening, the barrelled cheese is milled and mixed with lump cheese made from pasteurized cows' milk, at a ratio of 51:49%, forming winter bryndza, an aromatic cheese with a spreadable texture (Council Regulation, 2007; Görner, 1980; Palo & Kaláb, 1984).

Historically, the product was developed to utilize the excess ewes' lump cheese that could not be immediately processed and marketed in summer and, on the other hand, to provide a kind of ewes' cheese in the winter season. Mixing of barrelled ewes' cheese with cows' lump cheese was developed to partly neutralize a very strong odour of the former component. Recently, the product is gaining new interest as long-term ripening provides an apparently

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safer product, which is rich in specific odours that are absent in shorter-ripened ewes' cheeses. Long-term ripening in barrels in microaerobic conditions, at 4%–6% (w/w) NaCl and a temperature of 2–6 °C, provides conditions for the development of specific microbial consortia, producing specific profiles of volatile aroma compounds (Görner & Valík, 2004; Palo & Kaláb, 1984).

The aim of this study was to obtain, for the first time, parallel information on microbial consortia and principal odour-active compounds of barrelled ewes' cheese as an intermediate product in the production of winter bryndza cheese. For this purpose, we used effective methods developed in our previous research on May bryndza cheese, involving culture-based and culture-independent techniques to analyse the microflora (Pangallo et al., 2014), and gas chromatography – olfactometry supported by gas chromatography – mass spectrometry to analyse the odour-active compounds (Sádecká, Kolek, Pangallo, Valík, & Kuchta, 2014).

2. Materials and methods

2.1. Samples

Eight samples of barrelled ewes' cheese, and one sample of cows' lump cheese (100 g each) were obtained from Brysyrt (Tisovec, Slovakia). Each barrelled cheese was produced from ewes' lump cheeses from 1 to 5 mountain farms and ripened for 2–3 months in wooden barrels with a volume of 150 l at 10 °C. Fresh samples, stored for a maximum of 48 h at 4 °C, were analysed by culture-based microbiological methods and by solid phase microextraction coupled to gas chromatography. Samples frozen at −20 °C for a maximum of 3 weeks were analysed by culture-independent DNA-based methods.

2.2. Culture-based microbiological analysis

Content of presumptive lactobacilli was estimated on de Man – Rogosa – Sharpe agar (Merck, Darmstadt, Germany) incubated at 30 °C for 72 h, taking into account colony morphology. Content of presumptive lactococci was estimated on M17 agar (Merck) incubated at 30 °C for 72 h, taking into account colony morphology. Total mesophilic aerobic counts were determined on glucose-tryptone-yeast extract agar (Merck) incubated at 30 °C for 48 h. Coliforms and *E. coli* were cultured and quantified on Chromocult C medium (Merck) incubated at 37 °C for 24 h. Bacteria from the genus *Staphylococcus* were grown and quantified on Baird–Parker agar (Merck) incubated at 37 °C for 48 h. Coagulase activity of staphylococci was determined by rabbit plasma tube coagulase test (Bio-Rad, Marnes-la-Coquette, France) incubated at 37 °C for 24 h. Yeasts, fungi and *Geotrichum* spp. were grown and quantified on yeast extract-glucose-chloramphenicol agar (Merck) incubated at 25 °C for 5 days with colony morphology evaluated by microscopy.

2.3. DNA extraction and first PCR step

DNA was extracted from 1 g of barrelled ewes' cheese (samples 1, 2, 6, 7 and 8) by shaking at 45 °C for 30 min in 20 ml of 2% sodium citrate solution with glass beads, removal of the fat layer and subsequent chaotropic solid phase extraction using DNeasy Tissue kit (Qiagen) (Pangallo et al., 2014). Bacterial 16S rRNA gene, eukaryotic 28S rRNA gene and ITS fragment were amplified in two steps, a portion of the PCR product of the first step being used for the construction of clone libraries, and another portion in the second amplification step, a semi-nested PCR facilitating fingerprint analysis based on denaturing gradient gel electrophoresis (DGGE). The first step involved PCR with primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3‘; Lane, 1991) and LAC2 (5′-ATT YCA CCG CTA CAC ATG-3’; Walter et al., 2001) targeting 16S rRNA gene. The 28S rRNA gene was amplified by the primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman & Robnett, 1998). For amplification of ITS region of yeasts and fungi, primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used (White, Bruns, Lee, & Taylor, 1990). The PCR mixture (25 μl) contained 50 pmol of each primer, 200 μmol l^{-1} of dNTP (Life Technologies, Gaithersburg, Maryland, USA), 1.5 U SuperHot-*Taq* DNA polymerase (Bioron, Ludwigshafen, Germany) and 1 × PCR buffer. Three μl of the extracted DNA were used as a template in the first amplification. The temperature programme for 16S rRNA and ITS consisted of initial denaturation at 94 °C for 5 min, 30 cycles (94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min) and a final polymerization step at 72 °C for 10 min. The 28S rRNA PCR programme included an initial denaturation step at 95 °C for 5 min, 35 cycles (95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min) and a final polymerization at 72 °C for 8 min. For each DNA target (16S rRNA, 28S rRNA and ITS), four reactions of 25 μl (100 μl altogether) were done. Products of the four reactions of each DNA target were pooled, and specificity of amplification was checked by agarose gel electrophoresis.

2.4. Semi-nested PCR and DGGE fingerprint analysis

The PCR product of the first step (2 μl) was used as a template in the second amplification, a semi-nested PCR for each DNA target. The 16S rDNA was re-amplified with primers LAC1 (5′-AGC AGT AGG GAA TCT TCC A-3‘) and LAC2-GC (5‘-CGC CCG GGG CGC GCC CCG GGC GGC CCG GGG GCA CCG GGG GAT TYC ACC GCT ACA CAT G-3’) (Walter et al., 2001). The semi-nested PCR for 28S rRNA utilized the primers NL1-CG (5‘-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGC ATA TCA ATA AGC GGA GGA AAA G -3′) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin, Aggio, Manzano, Cantoni, & Comi, 2002). Primers ITS1f-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCA CGG GGG GTC CGT AGG TGA ACC TGC GG-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) were used for the semi-nested amplification of ITS fragment (Pangallo et al., 2014). The PCR conditions were the same as stated in the previous paragraph. Four semi-nested PCR products (4 reactions) for each DNA target were pooled, checked by electrophoresis in agarose gel, and precipitated with 96% ethanol, then resuspended in 20 μl H_2O and the precipitate (10 μl) was analysed by DGGE in 8% polyacrylamide gel (acrylamide-bisacrylamide 37.5:1) with the denaturation gradient of 25%–55% for separation of 16S rRNA amplicons, and 20%–50% for separation of 28S rRNA as well as ITS amplicons (100% denaturant contained 7 mol l^{-1} urea and 40% (v/v) formamide). DGGE was run on DCode System (Bio-Rad) in 0.5 × TAE (20 mmol l^{-1} Tris, 10 mmol l^{-1} acetate, 0.5 mmol l^{-1} Na_2 EDTA; pH 8.0) at 200 V and 60 °C for 3 h for bacteria, or for 5 h for fungi.

2.5. Construction of clone libraries and sequencing

The rest of the PCR products from the first amplifications were used for the construction of bacterial 16S rRNA and eukaryotic 28S rRNA and ITS clone libraries. Briefly, the PCR products were purified by QIAquick PCR purification kit (Qiagen), ligated to pGEM-T Easy vector (Promega, Madison, Wisconsin, USA), transformed to *E. coli* XLI-Blue, and spread to LB plates with ampicillin (100 μg ml^{-1}), X-Gal (0.1 mmol l^{-1}) and IPTG (0.2 mmol l^{-1}). A number of about 60 white colonies from each clone library was checked by vector-specific PCR with primers SP6 (5′-ATT TAG GTG ACA CTA TAG AAT AC-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′). Positive clones of each library were analysed by DGGE at conditions described above, using bacterial primers LAC1 and LAC2-GC, fungal primers