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Seasonal variances in bacterial microbiota and volatile organic compounds in raw milk

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ABSTRACT

The aim of this study was to define the composition of microbiota and the volatile organic compounds (VOCs) in samples of raw milk collected for 22 months between 2012 and 2014 originated from north-eastern region of Poland. The results revealed that the VOCs profile changed with respect to the season of milk collection, and milk collected in autumn was characterized by a higher content of acetic acid (C_2), propionic acid (C_3) and valeric acid (C_5), whereas spring was characterized by a frequent presence of acetone (Ac), ethanol (Et) and ethyl acetate (EtAc). Bacterial species composition changed considerably within the tested period and some bacterial species/groups occurred seasonally, e.g. L. *helveticus* (summer), *L. casei* (winter). The results show usefulness of the applied techniques (PCR-DGGE and HS-GC) and data analysis (PCA, correlation coefficients) methods in characterizing the raw milk quality intended for dairy production.

1. Introduction

The aim of all technological treatments applied in food production is to make sure that the customer is safe and to obtain products of the highest quality. In the dairy industry, the microbiological quality of a final product is determined by the quality of its main material - raw milk. The hygienic requirements (Commission Regulation (EC) No 1662/, 2006) define only an acceptable number of bacteria in 1 cm^3 of raw milk, not specifying the qualitative content of its microbiota, while those are some biochemical groups or microorganism species that play a crucial role in determining the quality and shelf life of milk products. Lactic acid bacteria (LAB), such as *Lactococcus*, *Lactobacillus*, *Enterococcus* may cause the sourness of milk (Jarosińska et al., 2014) or may function as non-starter lactic acid bacteria (NSLAB) (Settanni and Moschetti, 2010). Coliforms cause organoleptic changes and early cheese blowing (Jarosińska et al., 2014). Psychrotrophic bacteria (*Pseudomonas*, *Aeromonas*, *Bacillus*) and the enzymes they produce lead to unfavourable physicochemical and organoleptic changes. By causing protein decomposition, proteolytic enzymes alter milk texture (thickening, gelification) and change taste and smell (e.g., putrid, bitter). By hydrolysing milk fat, lipolytic enzymes cause a rancid, soap-like, tallowy taste and smell of milk (Adamiak et al., 2015; Hantsis-Zacharov and Halpern, 2007). By surviving thermal processing, spore-forming bacteria (*Bacillus*, *Clostridium*) may cause milk spoilage, including rotting, a bitter aftertaste, a rancid taste, or late cheese blowing (Klijn et al., 1995). Thus, the microorganisms that occur in milk are of decisive significance in determining properties of a final product. However, performing the examinations aimed at identifying all bacterial groups and species in the material with standard methods is relatively laborious and time-consuming. Therefore, it is becoming more frequent to apply the tools of molecular biology combined with modern instrumental methods. The molecular methods (e.g., PCR-DGGE) enable a precise identification of species diversity for the microorganisms in a studied sample (Franciosi et al., 2009; Nalepa and Markiewicz, 2017) with no cultivation needed. The instrumental techniques, e.g., Head-Space - Gas Chromatography (HS-GC) (Ayad et al., 1999) make it possible to trace metabolic changes performed by those microorganisms, e.g., identifying the content of volatile organic compounds (VOCs) produced. The presence of bacteria in raw milk and the VOCs they produce determines the taste and smell of a final product, especially ripened cheeses. Therefore, the aim this study was to identify microbiota and the VOCs in the pooled raw milk.

Differences and some potential links between those data have been determined in order to evaluate the quality of raw milk intended for dairy production.

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2. Materials and methods

2.1. Raw milk samples

The pooled raw milk samples from a dairy plant in the Region of Warmia and Mazury in north-eastern Poland were studied. The samples were collected into sterile vessels on a monthly basis for the period of twenty-two months: from September 2012 to July 2014. A period from July to September was consider summer, months from October to December were autumn, January to March were winter and April to June were consider spring.

2.2. DNA isolation

The total DNA from the raw milk samples was obtained using the Genomic Mini AX FOOD kit (A@A Biotechnology, Gdańsk, Poland) in accordance with the manufacturer's instructions. The isolated DNA was stored at − 80 °C for further analyses.

2.3. Polymerase chain reaction (PCR)

The reaction mixture (25 μL) contained: 20 mM of *Tris*-HCl (pH 8.4), 50 mM of KCl, 3 mM of $MgCl_2$, 50 μM of dNTPs, 5 pM of every primer, 1.25 U of Taq polymerase (Fermentas), and 20–40 ng of the DNA. The following primers were used: U968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGGG AAC GCG AAG AAC CTT AC-3′) and L1401-r (5′-CGG TGT GTA CAA GAC CC-3′) amplifying a fragment of region V6-V8. Amplification was carried out in the MJ Mini Gradient Thermal Cycler (Bio—Rad, Poland). The PCR profile was as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 68 °C for 40 s. Final extension at 68 °C for 7 min. (Randazzo et al., 2010). The PCR product with the estimated size of 450 bp was analyzed by electrophoresis on 1% agarose gel in 0.5 × TBE buffer in the MultiSub Choice system (Clever Scientific Ltd., UK).

2.4. Denaturing gradient gel electrophoresis (DGGE)

The PCR products were analyzed using the electrophoresis in a gradient of denaturing agents. The electrophoresis was conducted in the 8% polyacrylamide gel (acrylamide:bisacrylamide 37.5:1) with the gradient of denaturing agent (urea) ranging from 35% to 57.5%. The electrophoresis was carried out in the 0.5 × TAE buffer at the temperature of 60 °C under the voltage of 85 V for 16 h in the DCode Universal Mutation System (BioRad, Poland) (Randazzo et al., 2010). On each gel a marker set was run to enable the identification of DGGE bands (Nalepa and Markiewicz, 2017). The gels were stained in the SybrGreen I (1,10,000) solution for 15 min and then they were archived using G-Box (Syngen, Poland).

2.5. Detecting volatile organic compounds using gas chromatography

Detection of selected volatile compounds: aldehydes (acetaldehyde), ketones (acetone, diacetyl, acetoin), alcohols (methanol, ethanol), esters (ethyl acetate, ethyl propionate, ethyl butyrate), fatty acids C_2 - C_7 (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid) was performed in duplicate using headspace gas chromatography (HS-GC) with the Headspace Turbomatrix 40 autosampler (Perkin Elmer, USA) and the Clarus 500 gas chromatograph (Perkin Elmer, USA) with a flame ionization detector (HS-GC-FID). The chromatograph was calibrated for a quantitative detection [μg/cm^3] of the selected metabolites using some external standards. Calibration curves were made for every compound in the respective concentration scope. Equilibrium between the sample and the headspace was obtained as a result of thermostating of 5 cm^3 of milk at 70 °C for 40 min in 22-mm tightly closed vials. Then, the sample was pressurized for 1 min and injected into the column (split ratio of 2:1) in 0.08 min. The temperatures of the needle and the transfer line were 100 °C and 120 °C, respectively. The volatile compound separation was performed on the HP-INNOWAX column (60 m × 1.00 μm × 0.537 mm) by Agilent Technologies, USA, applying the following thermal gradient: 40 °C (5 min) → ΔT 10 °C/min → 220 °C (5 min). The temperature of the injector and the FID was 230 °C. The following gases were applied for the analysis: helium (5 cm^3/min), synthetic air (400 cm^3/min), and hydrogen (40 cm^3/min) with the pressure of 130 kPa in the column (Mikš-Krajnik, 2013; internal study).

2.6. Raw milk microbiota biodiversity

The DGGE profiles for the raw milk samples were compared with the markers that had been constructed in previous research (Nalepa and Markiewicz, 2017) and they included 24 referential strains: *P. freudenreichii* ssp. *shermanii* DSM 4902, *L. lactis* ssp. *lactis* DSM 4366, *L. mesenteroides* DSM 20346, *P. thoenii* DSM 20276, *L. acidophilus* DSM 9126, *L. plantarum* ATCC 8014, *L. brevis* DSM 1267, *L. casei* ATCC 334, *L. delbrueckii* DSM 20080, *L. fermentum* DSM 200052, *L. helveticus* DSM 20075, *E. coli* ATCC 8739, *E. aerogenes* ATCC 13048, *E. cloacae* ATCC 13047, *C. freundii* ATCC 8090, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6051, *C. butyricum* ATCC 10702, *C. tyrobutyricum* ATCC 2637, *C. perfringens* ATCC 13124, *L. monocytogenes* ATCC BAA-751, *S. thermophilus* ATCC 19258, *S. xylosus* ATTC 29971, and *S. aureus* ATCC 43300. Basing on that, the presence of particular bacterial species was detected and assigned scores from 0 to 4 depending on their band brightness. Also, the 1D analysis of electrophorograms was performed using the Doc-It LS Image Analysis application (UVP Ltd., UK). The data obtained in that way were used to calculate the Shannon–Wiener index that defines biodiversity and is expressed by the following formula: (Sienkiewicz, 2010),

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where p_i is the proportion of individuals in species i to the total number of individuals in the community.

2.7. Statistical analysis

Seasonal variations in the VOC profiles of raw milk and associations between the VOC profiles and the microbial compositions of the collected samples were determined in the principal component analysis (PCA). The direction and strength of these correlations were determined basing on the values of the Pearson correlation coefficient (r) at the significance level of $p < 0.05$. The data were processed in Statistica v. 12.5 (StatSoft Inc., Tulsa, USA).

3. Results

3.1. Raw milk microbiota

Upon comparing the DGGE profiles of the raw milk samples collected from September 2012 to July 2014, 3 to 12 bacterial species out of the 24 identified ones were detected (Table 1). The presence of 3 species was detected in 6 milk samples, 4 species were detected in 1 sample, 5 - in 2 samples, 6 - in 3 samples, 7 - in 5 samples, and 9 - in 2 samples. 8, 10, and 12 bacterial species were identified in the remaining 3 samples. The values of the Shannon-Wiener index (H′) (Table 1) correspond to the biological diversity of the collected raw milk samples. In 54.5% milk samples, the H′ indexes ranged from 1.3693 to 1.9782 (presence of 5–8 species) regardless of the analyzed season. In six (27%) milk samples the biological diversity was very low