Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 0–0
DOI: 10.31883/pjfns/109899
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Composition and Significance of Bacterial Microbiota and Volatile Organic Compounds of Swiss-Dutch-Type Cheese as Determined by PCR-DGGE and HS-GC

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Key words: Swiss-Dutch-type cheese, microbiota, VOCs, PCR-DGGE, HS-GC, seasonal variation

This study aimed to determine seasonal differences in the composition of bacterial microbiota and volatile organic compounds (VOCs) in Swiss-Dutch-type cheese (manufactured between 2012 and 2014). Bacterial diversity and VOCs (acetaldehyde; ketones: acetone, diacetyl, acetoin; alcohols: methanol, ethanol; esters: ethyl acetate, ethyl propionate, ethyl butyrate; fatty acids: acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid) were determined by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE), and headspace gas chromatography (HS-GC), respectively. Season influenced the composition of both bacterial microbiota and VOCs in cheese. Counts of starter bacteria (*Lactococcus*, *Leuconostoc* and *Propionibacterium* – 6.51–7.14, 3.6–3.96 and 2.88–4.72 log CFU/g, respectively) were higher in the first year of the study, likewise these of the non-starter *Lactobacillus* (4.12–5.69 log CFU/g). The total VOC content was substantially lower in the summer-autumn 2012 (0.73228–3.34111 mg/g) than in the other seasons (63.28810–131.27690 mg/g). Differences in bacterial microbiota and the VOC profiles were observed between cheeses manufactured in winter-spring and summer-autumn seasons. Winter- and spring-manufactured cheeses were also characterized by a lower number of bacterial species (average 8.7–10.5 species/sample) than the cheeses produced in the summer and in the autumn (average 10–13 species/sample). The results of the study indicate that the cheese-making process has to be continuously monitored to minimize differences across manufacturing seasons.

INTRODUCTION

Ripened cheeses are manufactured with the use of microorganisms, which results in the transformation of raw material (milk) into the final product (cheese). The processes that take place during cheese making and ripening are affected mainly by the composition and activity of microorganisms. Cheeses, including those that are manufactured industrially from pasteurized milk with starter cultures, are characterized by high microbial biodiversity because starter cultures (selected species of *Lactococcus*, *Lactobacillus,* and *Propionibacterium*) are always accompanied by other bacteria, including nonstarter lactic acid bacteria (NSLAB) such as *Lactobacillus plantarum* and *L. brevis*, fecal bacteria (coliforms, enterococci), spore-forming bacteria (*Bacillus*, *Clostridium*), and others [Johnson, 2017; Ogier *et al.*, 2004; Ricciardi *et al.*, 2015; Rehfeld *et al.*, 2017; Santiago-Lopez *et al.*, 2018]. All these microorganisms synthesize a wide range of metabolites, including volatile compounds (aldehydes, ketones, esters, fatty acids) that are responsible for the flavor, aroma and consistency of cheese, and contribute to the development of desirable final product attributes [Felicio *et al.*, 2016; Franciosi *et al.*, 2009].

However, cheese production is a highly complex process; therefore, interdisciplinary methods are required to analyze the relationships between microorganisms, their metabolic activity, and changes in the physicochemical properties of cheese. Molecular biology methods and instrumental analytical techniques can be used to study these processes in greater detail. For instance, polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) is one of such methods which supports the evaluation of various microbial genera and species, their semi-quantitative composition, changes over time or the presence of viable but nonculturable (VBNC) cells and cells that are suppressed by starter bacteria [Ercolini *et al.*, 2001; Joux & Lebaron, 2000]. In turn, the metabolic activity of cheese microbiota can be determined by analyzing the content of metabolites, such as volatile organic compounds (VOCs), by using chromatographic techniques, *e.g.*, headspace gas chromatography (HS-GC) [Ayad *et al.*, 1999].

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Therefore, the aim of this study was to assess the diversity of bacterial microbiota by PCR-DGGE and to identify and quantify VOCs by HS-GC in Swiss-Dutch-type cheeses manufactured in different seasons. Seasonal differences in VOC profiles and the potential links between the identified microorganisms and the VOC profiles of the examined cheeses were determined as well.

MATERIALS AND METHODS

Cheese manufacture and sampling

Swiss-Dutch-type cheeses were manufactured and obtained from a dairy plant in the Region of Warmia and Mazury in the north-eastern Poland. The cheeses were produced from 10,000 L (each) of premium cow milk which was pasteurized (72.5°C for 15 s) and standardized (to 3.0% fat), inoculated with calcium chloride, a coloring agent, rennet (Chymax, Ch. Hansen, Cząstków Mazowiecki, Poland), and a deep-frozen cheese starter (CSK food enrichment, Toruń, Poland). The applied starter was composed of *Lactococcus lactis* spp. *lactis*, *Leuconostoc mesenteroides* spp. *cremoris* (SLAB), and *Propionibacterium freudenreichii* spp. *shermanii* (PAB). The inoculum levels were 0.7% (by volume) for SLAB and 0.007% (by volume) for PAB. Every stage of the production process was consistent with industrial standards and followed the Swiss-Dutch-type cheese technology. After brining, 5 kg cheese blocks were wrapped in FCC type (Fesco Pack, Malbork, Poland) heat shrink, oxygen barrier bags and stored (ripened) under controlled conditions at 12°C for 10 days and then at 21°C for 42 days at 85% relative humidity. Cheese samples were collected over a period of two years between June 2012 and July 2014, and two cheese pieces of 0.5 kg each were sampled at one "time-point" (month). Three samples of each "time-point" cheese were taken in accordance with ISO 707:2008 [IDF 50:2008]. The samples for testing were packed in sterile bags. Cheeses manufactured between July and September were regarded as summer samples, cheeses produced in October-December – as autumn samples, cheeses produced in January-March – as winter samples, and cheeses produced in April-June – as spring samples.

Chemical composition of cheeses

Cheeses were subjected to chemical composition analyses to determine their sodium chloride, moisture, and fat contents. Salt content was determined according to ISO 5943:2006 [IDF 88:2006], moisture content was determined by oven drying at 102°C [AOAC 2005, 926.08], and fat content was determined according to ISO 3433:2008 [IDF 222:2008].

Determination of the counts of selected bacterial groups by the culture-dependent method

The counts of bacteria of the *Lactobacillus, Lactococcus, Leuconostoc, Propionibacterium, Enterococcus, Staphylococcus, Clostridium,* and *Bacillus* genera and of coliforms were determined by the standard plate count method on the appropriate culture media which are presented in Table 1. All media, except Reinforced Clostridial Medium (RCM), were supplied by Merck (Warsaw, Poland). RCM was supplied by Oxoid (Poznań, Poland).

TABLE 1. Culture media and incubation conditions applied in the study.

Microorganism	Medium	Incubation conditions
Lactococcus	M17 agar according to Terzaghi & Sandine [1975]	30°C, 48 h
Leuconostoc	Sucrose agar: (sucrose – 50 g/L, yeast extract – 10 g/L, agar – 15 g/L; pH 7.2–7.4)	30°C, 72 h
Propionibacterium	Sodium lactate (SLA) agar [Drinan & Cogan, 1992]	30°C, 72–96 h, anaerobic
Lactobacillus	Rogosa agar	30°C, 48 h, anaerobic
Coliforms	Violet Red Bile Lactose (VRBL) agar	37°C, 24–48 h
Enterococcus	Stanetz-Bartley agar	37°C, 48 h
Staphylococcus	Rabbit plasma fibrinogen (RPF) agar	37°C, 48 h
Clostridium	Reinforced Clostridial Agar (RCM agar)	37°C, 48 h, anaerobic
Bacillus	Nutrient agar	30°C, 48 h

Anaerobic conditions were obtained with the use of Anaerocult C bags (Merck, Warszawa, Poland).

DNA isolation and polymerase chain reaction (PCR)

Bacterial DNA was isolated directly from cheese samples with the Genomic Mini AX FOOD Kit (A@A Biotechnology, Gdańsk, Poland) in accordance with the manufacturer's instructions. The isolated DNA was stored at a temperature of −80°C until further analysis. Amplification was carried out in the MJ Mini Gradient Thermal Cycler (Bio-Rad, Warszawa, Poland). The applied primers were U968-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGC

GAAGAACCTTAC-3') and L1401-r (5'-CGGTGTGTACAAGACCC-3') [Randazzo *et al.*, 2010] which amplify the V6-V8 region of the 16S rRNA coding gene. The Master mix (25 μL) consisted of 1×reaction PCR buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 50 μmol/L deoxyribonucleotides (dNTPs), 5 pmol/L of each primer), 1.25 U *Taq* polymerase (all reagents were supplied by Thermo Fisher Scientific, Warsaw, Poland) and 10−40 ng of the DNA template. 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 was carried out at 68°C for 7 min [Randazzo *et al.*, 2010]. The presence of PCR products was analyzed by electrophoresis on 1% agarose gel in a 0.5×Tris-borate-EDTA buffer in the MultiSub Choice system (Clever Scientific Ltd., Rugby, UK).

Denaturing gradient gel electrophoresis (DGGE)

PCR products (~450 bp) were analyzed by denaturing gradient gel electrophoresis (DGGE) with urea and formamide (Sigma, Poznań, Poland) as denaturing agents. Electrophoresis was carried out in 8% polyacrylamide gel (acrylamide:bis-acrylamide, 37.5:1) (Sigma, Poznań, Poland) where the denaturing gradient was increased from 35% to 57.5%. Electrophoresis was conducted in a 0.5×Tris-ace-

tate-EDTA buffer (Sigma, Poznań, Poland) at 60°C and 85 V for 16 h [Randazzo *et al.*, 2010] in the DCode Universal Mutation System (Bio-Rad, Warszawa, Poland). Gels were stained in SybrGreen I (1:10,000) (Sigma, Poznań, Poland) solution for 15 min and documented in G-Box (Syngen, Wrocław, Poland).

Biological diversity of cheese microbiota

DGGE band patterns of the analyzed cheeses were compared with the previously developed markers [Nalepa & Markiewicz, 2017] composed of 24 reference strains: *Propionibacterium freudenreichii* ssp. *shermanii* DSM 4902, *P. thoenii* DSM 20276, *Lactococcus lactis* ssp. *lactis* DSM 4366, *Leuconostoc mesenteroides* DSM 20346, *Lactobacillus 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, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, *E. cloacae* ATCC 13047, *Citrobacter freundii* ATCC 8090, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6051, *Clostridium butyricum* ATCC 10702, *C. tyrobutyricum* ATCC 2637, *C. perfringens* ATCC 13124, *Listeria monocytogenes* ATCC BAA-751, *Streptococcus thermophilus* ATCC 19258, *Staphylococcus xylosus* ATCC 29971, and *S. aureus* ATCC 43300. Bacterial species were identified and given a score of 0 to 4 points based on band brightness. Electrophorograms were subjected to 1D analysis in the Doc-It LS Image Analysis Software (UVP Ltd., Cambridge, UK). The results were used to calculate the Shannon-Wiener diversity index with the use of the following formula:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$ [Sienkiewicz, 2010],

where p_i is the proportion of individuals found in species *i*.

Analysis of volatile organic compounds (VOCs) by headspace gas chromatography

Selected volatile compounds, including aldehydes (acetaldehyde), ketones (acetone, diacetyl, acetoin), alcohols (methanol, ethanol), esters (ethyl acetate, ethyl propionate, ethyl butyrate), and fatty acids C_2-C_7 (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid), were identified by headspace gas chromatography (HS-GC) in the Headspace Turbomatrix 40 autosampler (Perkin Elmer, Waltham, USA) and the Clarus 500 gas chromatography system (Perkin Elmer, Waltham, USA) with a flame ionization detector (FID). The content of metabolites was expressed in mg/g. The chromatograph was calibrated for quantitative identification of selected metabolites based on external standards. Calibration curves were generated for every compound within the relevant concentration range. Equilibrium between the sample and the headspace was achieved by heating 3 g of cheese to 70°C for 40 min in tightly closed 22 mL vials. The sample was pressurized for 1 min and injected into the column (split ratio of 2:1) within 0.08 min. Needle and transfer line temperature was 100°C and 120°C, respectively. Volatile compounds were separated in an HP-INNOWAX (Agilent Technologies, Palo Alto, USA) column (60 m × 1.00 μm×0.537 mm) with the following temperature gradient: 40°C (5 min) →ΔT 10°C/min →220°C (5 min.). Injector and FID temperature was 230°C. The carrier gases were: helium (5 mL/min), synthetic air (400 mL/min), and hydrogen (40 mL/min). Column pressure was 130 kPa.

Statistical analysis

The results of chemical composition and bacterial counts were verified for normal distribution and homogeneity of variance. The significance of differences between means was analyzed by Duncan's test ($p \leq 0.05$). The interactions between factors were determined by ANOVA for the completely randomized design. The results of PCR-DGGE profile analyses were expressed as arithmetic means for each month (24 timepoints). To determine whether the presence of the identified VOCs in Swiss-Dutch-type cheeses was correlated with season, principal component analysis (PCA) was performed with VOCs as quantitative variables and seasons as qualitative variables. Before, VOCs were subjected to hierarchical cluster analysis (HCA) [Granato *et al.*, 2018] for assessment of similarity between the identified VOCs. Pearson's correlation coefficients (r) were calculated to determine whether the VOCs were associated with the microorganisms detected in cheese samples ($p \leq 0.05$). Data were processed in the Statistica v. 12.5 software (StatSoft Polska, Kraków, Poland).

RESULTS AND DISCUSSION

Chemical composition and microbiota of Swiss-Dutch-type cheese

The average water content of all cheeses was 42.65 g/100 g. Water content was the highest (43.46 g/100 g) in cheeses produced in 2012 and the lowest (42.06 g/100 g) in cheeses produced in 2013. There were no significant ($p>0.05$) differences among cheeses manufactured in different seasons or years. The fat content of the cheeses produced between 2012 and 2014 was comparable ($p>0.05$) and ranged from 25.67 g/100 g to 28.17 g/100 g. The average content of sodium chloride in cheeses produced in 2012 was 1.58 g/100 g and did not differ significantly from that noted in the cheeses produced in 2013 (1.72 g/100 g) and 2014 (1.51 g/100 g) (Table 2).

The counts of the determined bacterial groups are presented in Table 3. Starter bacteria (*Lactococcus, Leuconostoc*, and *Propionobacterium*) were detected in cheese samples in all analyzed seasons. The most prevalent bacterial group was *Lactococcus*, and its counts ranged from 5.45 to 7.14 log CFU/g of cheese. *Leuconostoc* and *Propionibacterium* counts were lower at 1.20−3.98 log CFU/g and 0.67−4.72 log CFU/g, respectively. These bacteria were more abundant in the cheese samples collected in all seasons of the first year of the experiment (Table 3). Cheese samples from all seasons contained also bacteria of the genera *Lactobacillus* (4.12 to 5.69 log CFU/g) and *Bacillus* (3.30 to 4.18 log CFU/g). The counts of the remaining bacterial groups (coliforms, *Enterococcus, Staphylococcus, Clostridium*) were considerably more varied across seasons, ranging from <1 log CFU/g (*Enterococcus* and *Staphylococcus* in the winter of 2012, *Clostridium* in the spring of 2013, coliforms in the spring of 2014)

TABLE 2. Chemical composition (g/100 g) of Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Chemical composition	2012		2013				2014	
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Water	43.92±0.48	43.00±0.40	41.72±0.37	41.59±0.31	42.97±0.67	41.95±0.72	43.29±0.43	42.78±0.33
Fat	26.50±0.50	27.67±0.29	26.00±0.50	26.17±0.58	27.50±0.50	27.33±0.29	25.67±0.29	28.17±0.29
Sodium chlorine	1.69±0.01	1.47±0.07	1.48±0.03	1.48±0.04	1.62±0.03	1.49±0.05	1.50±0.03	1.52±0.05

The presented values are means±standard deviation for $n=3$ (3 time-points) in each season.

TABLE 3. Microbial counts (log CFU/g) in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Genera/group of microorganisms	2012		2013				2014	
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Lactococcus	6.51 ± 0.39^{ab}	7.01 ± 0.13^{a}	7.14 ± 0.14^{a}	6.79 ± 0.70^{ab}	5.51 ± 0.55^{ab}	6.28 ± 0.34^{ab}	5.45 ± 0.29^{a}	6.31 ± 0.39^{ab}
Leuconostoc	3.62 ± 0.49^{ab}	3.60 ± 0.19^{ab}	3.98 ± 0.36^{bc}	3.96 ± 0.66^{abd}	1.58 ± 0.65^{cd}	3.02 ± 0.40^{abcd}	1.20 ± 0.64^{c}	3.26 ± 0.73^{acd}
Propionibacterium	2.88 ± 0.54^{ab}	2.75 ± 0.85^{ab}	4.72 ± 0.25^{c}	3.70 ± 0.23^{bc}	1.47 ± 0.54^{a}	2.08 ± 0.52^{ab}	0.67 ± 0.50^{a}	2.86 ± 0.52^{ab}
Lactobacillus	5.08 ± 0.41^{abc}	5.64 ± 0.21^{c}	5.69 ± 0.23^{bc}	4.12 ± 0.52^{ab}	4.35 ± 0.45^{abc}	4.98 ± 0.13^{abc}	4.48 ± 0.17^{a}	4.79 ± 0.49^{abc}
Coliforms	1.31 ± 0.76^{ab}	3.00 ± 0.57^{abc}	3.15 ± 0.71^{ab}	3.71 ± 0.29^{bc}	3.18 ± 0.58^{ab}	3.13 ± 0.11^{ab}	1.50 ± 0.95^{abc}	$<1.00\pm0.00^{c}$
Enterococcus	2.77 ± 0.67^{a}	3.04 ± 0.80^{a}	$<1.00\pm0.00^{a}$	1.41 ± 0.76^{a}	2.25 ± 0.60^{a}	2.76 ± 0.70^{a}	2.13 ± 0.8^{a}	0.72 ± 0.50^{a}
Staphylococcus	1.00 ± 0.63^{a}	0.75 ± 0.62^{a}	$<1.00\pm0.00^{a}$	1.32 ± 0.74^{a}	2.69 ± 0.61^{a}	3.65 ± 0.37^{a}	0.95 ± 0.67^{a}	0.85 ± 0.74^{a}
Clostridium	2.22 ± 0.80^{ab}	4.02 ± 0.68^{a}	3.74 ± 0.83^{ab}	$<1.00\pm0.69^{b}$	2.77 ± 0.74^{ab}	3.70 ± 0.65^{a}	3.92 ± 0.67^{a}	3.03 ± 0.27^{a}
Bacillus	3.74 ± 0.20^{a}	3.69 ± 0.06^{a}	3.88 ± 0.18^{a}	3.54 ± 0.26^{a}	3.80 ± 0.19^{a}	3.89 ± 0.26^{a}	3.30 ± 0.19^{a}	4.18 ± 0.37^{a}

The presented values are means±standard deviation for $n=3$ in seasons. Mean values in rows with different superscript letters are significantly different ($p\leq0.05$). Limit of detection=1 log CFU/g.

to 4.02 log CFU/g (*Clostridium* in the autumn of 2012) (Table 3). Similar *Enterobacteriaceae* and *Staphylococcus* counts in cheese were reported by Frece *et al.* [2016].

Bacterial species were identified based on DGGE bands which were obtained with the use of the developed markers [Nalepa & Markiewicz, 2017] and given a score of 0 to 4 based on their brightness (Figure 1). PCR-DGGE is a semi-quantitative method where the quantity of target DNA corresponding to the number of cells from which it was isolated is positively correlated with the number of amplicons and the brightness of DGGE bands. The band brightness of starter cultures (*L. lactis, L. mesenteroides*, and *P. freudenreichii*) scored 0 to 3 points in the first year (summer 2012 – spring 2013), and 1 to 4 points in the second year (summer 2013 – spring 2014) of the experiment (Figure 1). The most prevalent nonstarter bacteria (lactic acid bacteria and propionic acid bacteria) were *L. brevis* and *L. fermentum*, but they showed in the cheese samples randomly. *P. thoenii* and *L. acidophilus* were found mainly in the samples collected between the summer of 2013 and the spring of 2014. *L. delbrueckii*, *L. helveticus* and *L. casei* were not observed in winter and spring samples, regardless of the experimental year. Among spore-forming bacteria, the most predominant microorganisms were *B. subtilis* and *C. tyrobutyricum*, where *B. subtilis* was more prevalent and was identified in all samples collected between the summer of 2013 and the spring of 2014. *E. coli* and *E. faecalis* were more prevalent in the samples collected between the summer of 2013 and the spring of 2014 than in the remaining months of the study (Figure 1).

In the group of the 24 analyzed species, 6 to 17 bacterial species were detected in the analyzed cheese samples (Table 4). A smaller number of bacterial species was identified in the first year: 11 in the summer of 2012, 11.3 in the autumn of 2012, 8.7 in the winter of 2013, and 10.5 in the spring of 2013 on average. In the second year of the study, the number of identified species was higher reaching 13 in the summer-autumn of 2013 and 10 in the winter of 2013 – spring of 2014 on average. Considerable variations in the number of species were observed between the months/seasons of both years. The number of bacterial species was higher in the samples collected in summer-autumn than in winter-spring periods. Starter bacteria were identified in all samples, excluding the sample from the autumn of 2012 where *L. lactis* was not identified (Figure 1). The most prevalent other bacteria were *L. brevis* and *B. subtilis* which were present in 17 samples (70.8%) (Table 4), followed by *C. tyrobutyricum* which was found in 15 samples (62.5%). *L. fermentum, E. coli*, and *S. thermophilus/S. xylosus* were detected in 14 samples (58.3%), *P. thoenii* and *L. acidophilus* were noted in 12 samples (50.0%), and *L. casei* and *E. faecalis* were found in 11 samples (45.8%).

DGGE band patterns were also used in 1D analysis and to determine the Shannon-Wiener diversity index (H').

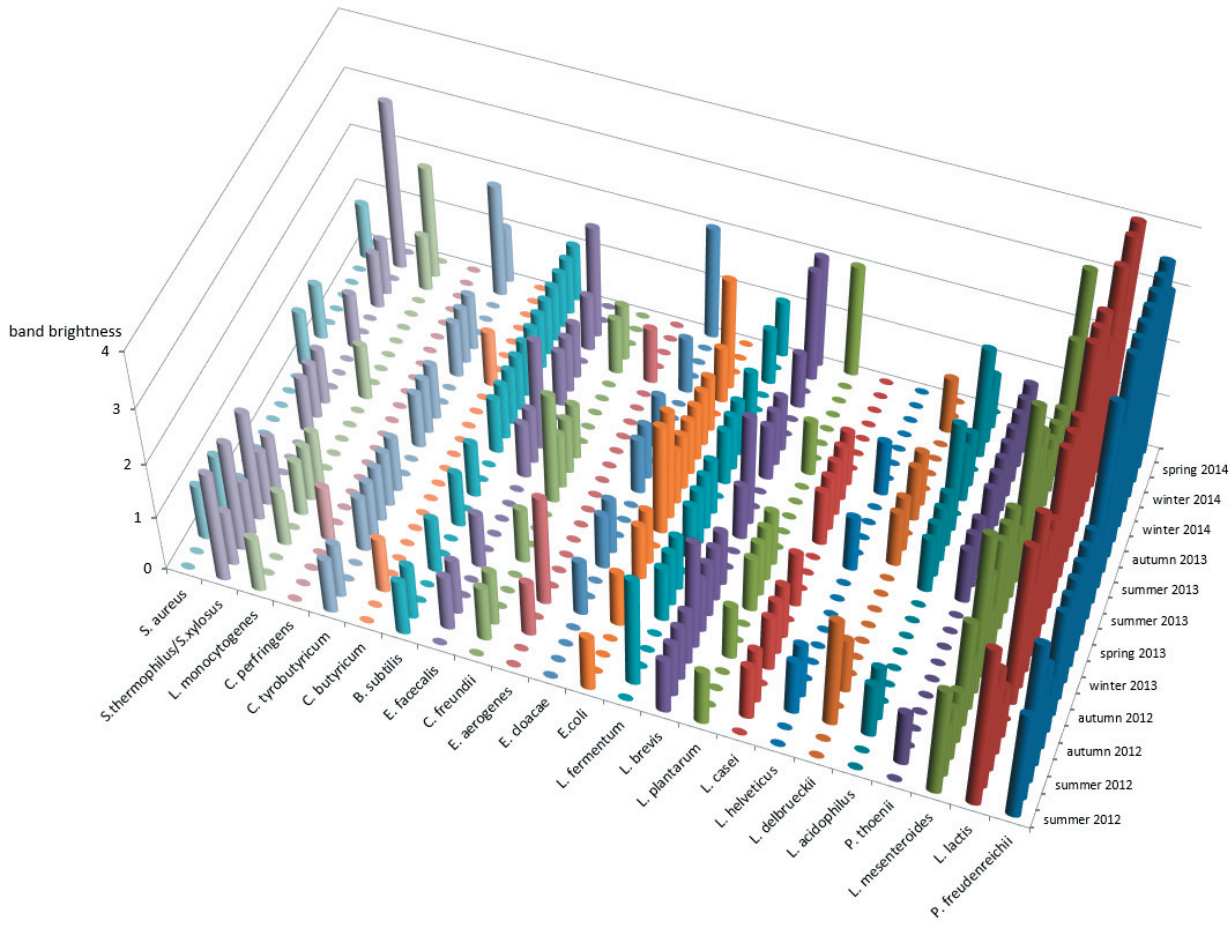

FIGURE 1. The identified microorganisms and relative band brightness on a scale of 0 to 4, determined in the PCR-DGGE assay of Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

The values of H' ranged from 1.6704 in cheeses with 6 bacterial species to 2.9779 in cheeses with 17 bacterial species (data not shown). The evaluated cheeses were characterized by high bacterial diversity and contained from 3 to 14 bacterial species in addition to starter cultures (*P. freudenreichii, L. lactis, L. mesenteroides*).

The sensory attributes of ripened cheeses, produced both traditionally and industrially, are determined by numerous factors, in particular by the qualitative and quantitative composition of microbiota, its physiological status and metabolic activity [Smit *et al.*, 2005]. For this reason, the microbiome of dairy raw materials and cheeses has to be monitored throughout the production process. Molecular biology methods, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) or next-generation sequencing (NGS), have been used by many authors to evaluate microbial communities in raw milk and cheeses [Alegría *et al.*, 2009; Duru *et al.*, 2018; Ercolini *et al.*, 2001; Gala *et al.*, 2008; Mangia *et al.*, 2016; Porcellato & Skeie, 2016; Randazzo *et al.*, 2006; Skelin *et al.*, 2012]. Most of these studies analyzed the microbiome composition of traditional cheeses produced in the Mediterranean region from unpasteurized milk and without starter cultures. Thermophilic species of lactic acid bacteria, such as *S. thermophilus, S. macedonicus, L. helveticus* and *L. delbrueckii*, are more frequently encountered in warmer regions of the southern Europe [Alegría *et al.*, 2009; Franciosi *et al.*, 2009]. In our study, *L. helveticus* and *L. delbrueckii* were identified only in the warmest seasons (summer–autumn) and were never observed in cold seasons (winter–spring), regardless of the year. Seasonal variations in the counts of mesophilic and thermophilic LAB were also reported in Perocino del Poro cheese [Caridi *et al.*, 2003] where mesophilic bacteria were more abundant in spring, and thermophilic bacteria – in summer. These results indicate that the manufacturing season could influence the growth of microbiota and, consequently, the quality of the final product. In other studies, ripened cheeses were found to contain mesophilic bacteria including *L. lactis, L. garvieae, L. mesenteroides, L. casei, L. paracasei, L. plantarum,* and *L. brevis*, but also enterococci (*E. faecalis, E. faecium,* and *E. hirae*), Gram-negative bacteria (*E. coli, Enterobacter* spp., and *Hafnia alvei*), and staphylococci (*S. saprophyticus* and *S. equorum*) [Abriouel *et al.*, 2008; Ercolini *et al.*, 2001; Flórez & Mayo, 2006; Marino *et al.*, 2003]. Randazzo *et al.* [2006] demonstrated that traditional cheeses made from raw milk without starter cultures were characterized by greater microbiological diversity than cheeses manufactured with starter cultures, where a predominance of starter culture species was reported. In our study,

TABLE 4. The number of bacterial species at different times of the year and the prevalence (%) of bacterial species in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Season	Number of identified species	Bacterial group	Species	Prevalence (%)
Summer 2012	10	Starter culture	*P. freudenreichii*	100.0
Summer 2012	12		*L. lactis*	95.8
Summer 2012	14		*L. mesenteroides*	100.0
Summer 2012	8		*P. thoenii*	50.0
Autumn 2012	15	Nonstarter bacteria, including nonstarter LAB	*L. acidophilus*	50.0
Autumn 2012	9		*L. delbrueckii*	29.2
Autumn 2012	10		*L. helveticus*	16.6
Winter 2013	11		*L. casei*	45.8
Winter 2013	9		*L. plantarum*	33.3
Winter 2013	6		*L. brevis*	70.8
Spring 2013	8		*L. fermentum*	58.3
Spring 2013	13	Fecal bacteria	*E. coli*	58.3
Summer 2013	16		*E. cloacae*	33.3
Summer 2013	14		*E. aerogenes*	12.5
Summer 2013	9		*C. freundii*	37.5
Autumn 2013	16		*E. faecalis*	45.8
Autumn 2013	17	Spore-forming bacteria	*B. subtilis*	70.8
Autumn 2013	10		*C. butyricum*	8.3
Winter 2014	9		*C. tyrobutyricum*	62.5
Winter 2014	10		*C. perfringens*	4.2
Winter 2014	11	Other pathogenic	*L. monocytogenes*	33.3
Spring 2014	10	bacteria	*S. aureus*	20.8
Spring 2014	11	Other non-pathogenic	*S. thermophilus*/	58.3
Spring 2014	9	bacteria	*S. xylosus*	

starter culture bacteria (*L. lactis, L. mesenteroides, P. freudenreichii*) were also most abundant in the analyzed cheeses. In DGGE profiles, the brightest bands corresponded to starter culture species, whereas the bands corresponding to other bacteria were less bright. The results of quantitative analyses revealed that starter culture bacteria were more prevalent than other bacteria (Figure 2 and Table 3). Duru *et al.* [2018] relied on next-generation sequencing (NGS) to demonstrate a predominance of *Lactococcus*, *Lactobacillus*, and *Propionibacterium* starter cultures which accounted for 80–82% of all reads in industrially manufactured Swiss-type cheese. In industrially produced Dutch-type cheese, starter bacteria (*Lactococcus*, *Leuconostoc*, and *Lactobacillus*) also accounted for more than 99% of the bacterial community in the NGS assay conducted by Porcellato & Skeie [2016]. However, the production of cheese from pasteurized milk with the addition of starter cultures does not guarantee the growth of starter bacteria only, but NSLAB may also grow and be active. Lindberg *et al.* [1996] and Jordan & Cogan [1993] identified *Lactobacillus paracasei*, *L. casei*, and *L. plantarum* nonstarter bacteria in Swedish and Norwegian cheeses produced with the addition of *Lactococcus* and *Leuconostoc* starter cultures. In our study, the most prevalent NSLAB were *L. brevis* and *L. fermentum*, whereas *L. casei*/*L. paracasei* and thermophilic *L. delbrueckii* and *L. helveticus* were identified only in the summer and autumn. The analyzed cheeses also frequently contained spore-forming bacteria *B. subtilis* and *C. tyrobutyricum*. Klijn *et al.* [1995] demonstrated that the late-blowing defect in cheese was caused mainly by *C. tyrobutyricum* and butyric acid, its main fermentation product, in quantities greater than 100 mg/kg. The natural environment and silage are the main sources of spore-forming bacteria that can lead to spoilage when transferred to raw milk and the cheese matrix.

The VOC content of Swiss-Dutch-type cheese

The content of selected volatile compounds (aldehydes, alcohols, esters, ketones, and fatty acids C_2-C_7) in the examined cheeses is presented in Table 5. Considerable differences

TABLE 5. The content of volatile organic compounds (VOCs) (mg/g) in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Season	Aldehydes	Alcohols	Esters	Ketones	Fatty acids	Total
Summer 2012	0.00048	0.36485	–	0.13640	0.23643	0.73817
Summer 2012	0.00138	0.00842	–	0.17972	0.54277	0.73228
Summer 2012	0.00060	0.17595	0.000025	0.10961	1.79419	2.08038
Summer 2012	0.00021	0.10131	–	0.09144	1.42441	1.61738
Autumn 2012	0.00055	0.09510	–	0.23241	2.39689	2.72495
Autumn 2012	0.00069	0.12584	–	0.15029	3.06430	3.34111
Autumn 2012	0.00019	0.05991	–	0.07645	2.47629	2.61284
Winter 2013	0.04798	2.56398	0.09433	0.00348	74.81968	77.52943
Winter 2013	0.00025	2.37653	0.10095	0.00733	7.37763	9.86268
Winter 2013	0.05265	2.38913	0.21640	0.00375	74.23943	76.90135
Spring 2013	0.04455	2.92663	0.15565	0.00310	60.15818	63.28810
Spring 2013	0.05028	2.58768	0.21038	0.00458	120.03033	122.88323
Summer 2013	0.05385	2.42525	0.24041	0.00536	111.08645	113.81133
Summer 2013	0.05588	3.03558	0.24185	0.00860	127.93500	131.27690
Summer 2013	0.00045	4.65645	0.20115	0.00775	21.41735	26.28315
Autumn 2013	0.04420	–	0.00080	–	89.18433	89.22933
Autumn 2013	0.04820	2.37845	0.09713	0.00588	75.83090	78.36055
Autumn 2013	0.05385	2.39680	0.09740	0.00603	65.49263	68.04670
Winter 2014	0.04351	0.63736	0.09884	0.00464	95.75041	96.53476
Winter 2014	0.03669	1.78571	0.09324	0.00415	89.19380	91.11359
Winter 2014	0.03069	1.73941	0.10704	0.00345	84.79109	86.67168
Spring 2014	0.02041	1.32240	0.11075	0.00293	86.83456	88.29105
Spring 2014	0.02810	1.81126	0.17619	0.00141	84.25300	86.26996
Spring 2014	0.04028	1.22004	0.08508	0.01268	72.45116	73.80923

Aldehydes: acetaldehyde; Alcohols: methanol, ethanol; Esters: ethyl acetate, ethyl propionate, ethyl butyrate; Ketones: acetone, diacetyl, acetoin; Fatty acids: acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid.

in VOC content were observed between seasons. In the summer-autumn of 2012, the total VOC content of the evaluated samples ranged from 0.73228 to 3.34111 mg/g of cheese and was substantially lower than in the winter of 2013–spring of 2014 when it ranged from 63.28810 to 131.27690 mg/g. Two exceptions were noted in the second year of the study (26.28315 mg/g in the summer of 2013 and 9.86267 mg/g in the winter of 2013) when VOC concentrations were lower than in the remaining samples from the corresponding period, but were still several times higher than in the summer-autumn of 2012 (Table 5). The predominant VOCs in all samples were fatty acids whose content ranged from 0.23643 to 127.93500 mg/g and was characterized by similar seasonal fluctuations as total VOC levels. In 2013, alcohol concentrations in the examined samples were determined at 2.37652 to 4.65645 mg/g and were significantly higher than in 2014 (0.63736–1.81126 mg/g) and 2012 (0.00841–0.36485 mg/g). Very low levels of total VOCs and fatty acids C_2–C_7 were noted in 2012. The contents of aldehydes and esters were also very low in 2012. Aldehyde levels reached 0.00059 mg/g in 2012 on average, and they were nearly 65-fold higher in the remaining seasons at 0.03834 mg/g on average. Even greater variations were observed in the ester content of cheeses. Ester contents were negligible in 2012, whereas in 2013–2014, they were determined in the range of 0.08507 to 0.24185 mg/g. An opposite trend was noted in ketone levels which were substantially higher in 2012 than in the remaining years. On average, ketone contents were determined at 0.1395 mg/g in 2012 and at 0.00500 mg/g in 2013–2014 (Table 5).

The unique flavor of different cheeses can be attributed to both starter and nonstarter bacteria. The sensory attributes of cheese, including flavor, aroma and consistency, are developed during complex interactions between bacteria. The synthesized VOCs play a very important role in this process [Pérès *et al.*, 2001; Mondello *et al.*, 2005; Januszkiewicz *et al.*, 2008]. Volatile organic compounds are produced *via* three

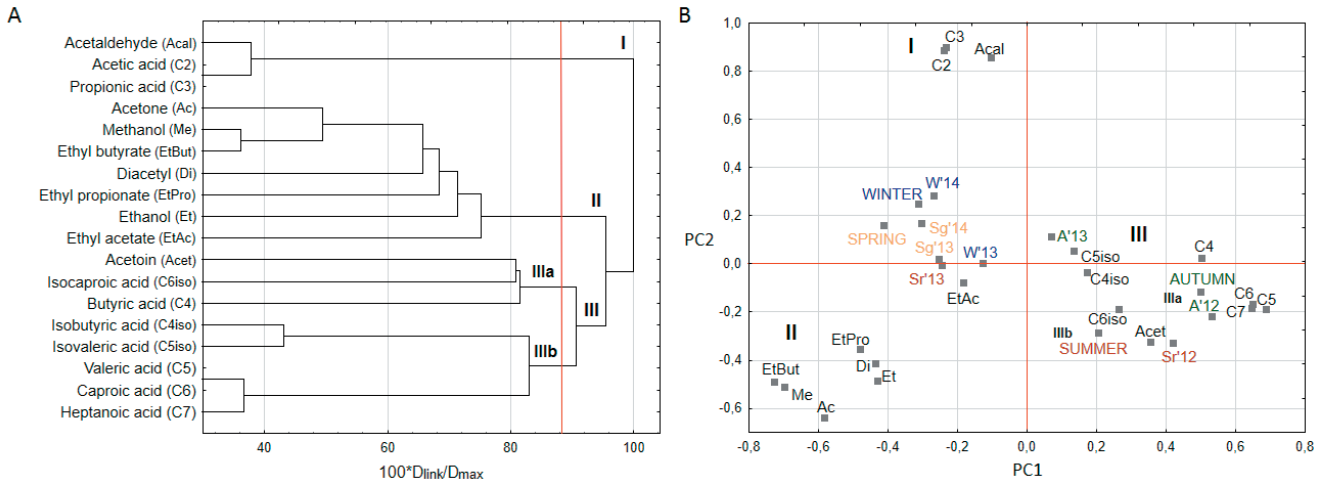

FIGURE 2. A – The dendrogram of a dissimilarity measure between the volatile compounds; B – The Principal Component Analysis *biplot* of seasonal variables and volatile compounds related to component 1 (*PC1*) and component 2 (*PC2*).

Acal – acetaldehyde; Ketones: Ac – acetone, Di – diacetyl, Acet – acetoin; Alcohols: Me – methanol; Et – ethanol; Esters: EtAc – ethyl acetate, EtPro – ethyl propionate, EtBut – ethyl butyrate; Fatty acids: C_2 – acetic acid, C_3 – propionic acid, C_{4iso} – isobutyric acid, C_4 – butyric acid, C_{5iso} – isovaleric acid, C_5 – valeric acid, C_{6iso} – isocaproic acid, C_6 – caproic acid; C_7 – heptanoic acid.

major catabolic pathways that are involved in flavor development: fermentation (lactose and citrates), lipolysis (milk fat), and proteolysis (casein) [Gobbetti *et al.*, 2015]. During these processes, lactose is converted to diacetyl, acetoin, acetaldehyde, and acetic acid. Fatty acids are converted to ketones, secondary alcohols, esters, and lactones; and casein – to alcohols, aldehydes, acids, esters, and sulfur compounds [Smit *et al.*, 2005]. In this study, HS-GC revealed the presence of all VOC groups in Swiss-Dutch-type cheeses. The presence of alcohols, esters, ketones, short-chain and medium-chain fatty acids was also determined in Serbian Pirotski kačkavalj cheese [Milosavljević *et al.*, 2012] and French Cantal cheese [De Freitas *et al.*, 2007]. According to Pastink *et al.* [2008], considerable differences in the flavor-forming ability of LAB exist not only between species, but also between strains. The cited authors demonstrated that "wild" LAB strains produced much greater quantities of methyl aldehydes and methyl alcohols than industrial strains. In our study, cheese samples from all seasons of 2013 were abundant in alcohols, in particular methyl alcohol. The prevalence of NSLAB was particularly high in 2013, especially in the summer and autumn. In the cited study concerning Cantal cheese [De Freitas *et al.*, 2007], ethanol was the major VOC. Ethanol is a metabolite of complex processes involving yeast, *Leuconostoc, Lactococcus*, and thermophilic LAB. Like other alcohols synthesized in cheese, it is not directly responsible for the flavor of cheese, but it determines the production of ethyl esters which are flavor-forming compounds [Thierry *et al.*, 2006]. In our study, ester contents were also higher in cheese samples characterized by a higher alcohol content than in the samples where alcohol levels were low or where alcohol was not detected. Lactate fermentation, amino acid catabolism, and fat lipolysis are sources of free fatty acids in ripened cheese [McSweeney & Sousa, 2000]. Dolci *et al.* [2008] demonstrated moderate levels of lipolytic activity in Castelmango cheese containing short-chain fatty acids such as formic acid, acetic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid. The cited authors attributed their findings to the presence of enterococci, staphylococci, and yeast whose counts were high in all stages of the cheese production process. In another study, the presence of short-chain fatty acids was attributed not only to fecal streptococci, but to all secondary microorganisms [Fox *et al.*, 2004]. In our study, fatty acid contents were several dozen times higher in cheese samples characterized by a higher activity and/or prevalence of both starter and nonstarter bacteria, *i.e.* in the samples collected between the winter of 2013 and the spring of 2014.

Statistical analysis of VOC profiles

The seasonal variations in the VOC profiles of Swiss-Dutch-type cheeses produced between 2012 and 2014 were determined by principal component analysis (PCA) which was preceded by hierarchical cluster analysis (HCA) (Figure 2). An HCA dendrogram showed that VOCs were organized in three clusters: I, II and III, with the last one comprising two sub-clusters (IIIa and IIIb), which showed a dissimilarity >80 (Figure 2A) and was largely consistent with the results of the PCA (Figure 2B). However, the first (PC 1) and second (PC 2) principal component explained 46.63% of the total variation. Acetaldehyde (Acal), acetic acid (C_2), and propionic acid (C_3) were grouped together in cluster I, since they were variables with the highest negative loading in PC 2 (Figure 2 and Table 6). Cluster II was comprised primarily of acetone (Ac), methanol (Me), and ethyl butyrate (EtBut), since they were variables with moderate negative loading in PC 1 and PC 2. They were also accompanied by weak negative loadings of diacetyl (Di), ethanol (Et), ethyl propionate (EtPro), and ethyl acetate (EtAc). Cluster III was comprised primarily of valeric acid (C_5), caproic acid (C_6), and heptanoic acid (C_7), since they were variables with moderate positive loading in PC 1 and they accompanied by isobutyric acid (C_{4iso}), isovaleric acid (C_{5iso}) (cluster IIIa) as well as acetoin (Acet), isocaproic acid (C_{6iso}), and butyric acid (C_4) (cluster IIIb). As demonstrated by Figure 2, the resulting three

TABLE 6. The factor loadings after the varimax rotation of the volatile organic compounds (VOCs) data and the correlation coefficients (*r*) between VOCs and the microorganisms detected in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

		VOCs	PC1	PC2	Microorganisms (*r*)				
Cluster I		C_3	-0.23	-0.90***	*P. thoenii* (**0.70**[a])	*L. lactis* (**0.48**)	*B. subtilis* (**0.43**)	*L. acidophilus* (**0.43**)	*P. freudenreichii* (**0.42**)
		C_2	-0.24	-0.89***	*P. thoenii* (**0.65**)	*L. lactis* (**0.45**)	*P. freudenreichii* (0.39)	*B. subtilis* (0.39)	*L. acidophilus* (0.35)
		Acal	-0.10	-0.86***	*P. thoenii* (**0.52**)	*L. lactis* (**0.42**)	*P. freudenreichii* (0.37)	*B. subtilis* (0.39)	*L. acidophilus* (0.22)
Cluster II		Ac	-0.58**	-0.63**	*E. coli* (**0.46**)	*C. freundii* (0.35)	*L. fermentum* (0.24)	*L. mesenteroides* (0.08)	*L. helveticus* (0.03)
		Me	-0.70**	-0.50**	*E. coli* (**0.47**)	*L. lactis* (0.32)	*B. subtilis* (0.22)	*L. mesenteroides* (0.19)	*P. freudenreichii* (0.17)
		EtBut	-0.73**	-0.48*	*L. lactis* (0.35)	*E. coli* (0.30)	*P. freudenreichii* (0.29)	*B. subtilis* (0.28)	*P. thoenii* (0.17)
		Di	-0.44*	-0.41*	*E. cloacae* (**0.56**)	*L. plantarum* (**0.44**)	*B. subtilis* (0.35)	*L. lactis* (0.25)	*L. mesenteroides* (0.22)
		EtPro	-0.48*	-0.35*	*C. freundii* (0.34)	*E. coli* (0.25)	*P. thoenii* (0.24)	*L. acidophilus* (0.16)	*C. tyrobutyricum* (0.15)
		Et	-0.43*	-0.48*	*L. brevis* (0.35)	*P. freudenreichii* (0.26)	*L. delbrueckii* (0.25)	*E. cloacae* (0.23)	*L. casei* (0.23)
		EtAc	-0.18	-0.07	*E. faecalis* (**0.45**)	*P. thoenii* (0.35)	*L .monocytogenes* (0.28)	*E. cloacae* (0.26)	*B. subtilis* (0.26)
Cluster III	IIIa	C_5	0.68**	-0.18	*L. casei* (**0.45**)	*C. perfringens* (0.29)	*E. aerogenes* (0.25)	*L. brevis* (0.24)	*L. delbrueckii* (0.20)
		C_6	0.64**	-0.16	*L. casei* (0.35)	*C. perfringens* (0.28)	*E. aerogenes* (0.28)	*L. delbrueckii* (0.30)	*L. brevis* (0.21)
		C_7	0.64**	-0.18	*L. casei* (**0.51**)	*C. perfringens* (0.33)	*E. aerogenes* (0.28)	*L. brevis* (0.27)	*L. delbrueckii* (0.16)
		C_{4iso}	0.17	-0.03	*L. casei* (0.29)	*E. cloacae* (0.17)	*C. tyrobutyricum* (0.16)	*L. plantarum* (0.16)	*L. brevis* (0.04)
		C_{5iso}	0.13	0.05	*L. casei* (0.21)	*E. cloacae* (0.19)	*L. plantarum* (0.19)	*C. tyrobutyricum* (0.06)	*E. coli* (0.03)
		Acet	0.35*	-0.32*	*C. perfringens* (**0.58**)	*E. aerogenes* (**0.53**)	*S. thermophilus/S. xylosus* (**0.50**)	*L. casei* (0.37)	*L. brevis* (0.32)
	IIIb	C_{6iso}	0.26	-0.18	*S. thermophilus/S. xylosus* (0.29)	*C. tyrobutyricum* (0.25)	*L. fermentum* (0.22)	*B. subtilis* (0.16)	*L. brevis* (0.16)
		C_4	0.50*	0.02	*L. fermentum* (0.27)	*L. plantarum* (0.20)	*C. perfringens* (0.17)	*L .monocytogenes* (0.15)	*L. mesenteroides* (0.11)

* 0.30–0.50 (weak); **0.50–0.75 (moderate); ***>0.75 (strong loading).

[a] The r values in bold indicated significant ($p \leq 0.05$) differences.

Acal – acetaldehyde; Ketones: Ac – acetone, Di – diacetyl, Acet – acetoin; Alcohols: Me – methanol; Et – ethanol; Esters: EtAc – ethyl acetate, EtPro – ethyl propionate, EtBut – ethyl butyrate; Fatty acids: C_2 – acetic acid, C_3 – propionic acid, C_{4iso} – isobutyric acid, C_4 – butyric acid, C_{5iso} – isovaleric acid, C_5 – valeric acid, C_{6iso} – isocaproic acid, C_6 – caproic acid; C_7 – heptanoic acid.

clusters represented a collection of cheeses produced in different years and even seasons. The cheeses manufactured in 2012 were largely characterized by VOCs from cluster III, which were discriminated from the samples of 2013/2014 seasons. Moreover, cluster III contained sub-clusters a and b, which represented collections of samples from the autumn and summer, respectively. In turn, cheeses produced mainly in winter-spring 2013/14 were characterized either by VOCs from cluster I or VOCs from cluster II. Importantly, significant coefficients of correlation (r) were calculated for the relationships between C_3 *vs. P. thoenii* (0.70), *L. lactis* (0.48), *L. acidophilus* (0.43), *B. subtilis* (0.43), and *P. freudenreichii* (0.42); C_2 *vs*. *P. thoenii* (0.65) and *L. lactis* (0.45); as well as acetaldehyde (Acal) *vs*. *P. thoenii* (0.52) and *L. lactis* (0.42). The above indicates that these microorganisms contributed to the synthesis of acetaldehyde, C_2, and C_3 in cheese (Table 6). The prevailing *E. coli*, *E. cloacae*, and *E. faecalis* correlated with Ac/Me, Di, and EtAc from cluster II, respectively. Among VOCs from cluster III, C_5 and C_7 exhibited the highest correlation with *L. casei* and the Acet with *C. perfringens*, *E. aerogenes*, and *S. thermophilus/S. xylosus*, indicating that they could be considered as important contributors to these VOCs. An analysis of the correlations between VOCs and bacteria revealed that the sensory attributes of Swiss-Dutch-type cheeses were formed primarily by starter bacteria (*L. lactis, L. mesenteroides*, and *P. freudenreichii*), whereas nonstarter bacteria exerted strong flavor-forming effects after 52 days of ripening.

The two-year study yielded massive amounts of data (DGGE profiles, VOC profiles, bacterial counts) which were difficult to interpret. Advanced computational methods, such as HCA, PCA or partial least squares (PLS) regression, are increasingly often used in studies of this type. Milosavljević *et al.* [2012] relied on AHC and PCA to compare contents of VOC determined by different gas chromatography methods. Based on an analysis of GC data, they concluded that the results of AHC and PCA should be analyzed in combination to produce a more comprehensive VOC profile of ripened cheeses. In turn, Mauriello *et al.* [2003] were able to identify the region of origin of mozzarella cheese based on the results of PCA, whereas Duthoit *et al.* [2005] also relied on PCA and PLS regression analysis to determine changes in the sensory and microbiological properties of Salers cheese and reported correlations between sensory properties and bacterial cultures. Similarly to our study, they observed that nonstarter bacteria, such as *Enterobacteriaceae* and *Bacillus*, were responsible for the texture, flavor, and aroma of cheese.

A combination of culture-dependent and culture-independent methods supported more accurate analyses of the cheese microbiota and its role in the formation of volatile compounds, as well as the identification of bacterial species synthesizing compounds that affect the sensory quality of ripened cheese. Thus, the results of this study indicate that the diversity of bacterial communities and profiles of volatile compounds are highly dependent upon the seasonality of cheese production. For cheese manufacturers, the biodiversity of cheese batches, despite being carried out according to the same technological scheme, is a very important observation. Awareness of these dependencies may contribute to a better understanding of phenomena occurring during the cheese production process, giving the opportunity to prevent technological fluctuations and obtain the final product of excellent quality.

CONCLUSIONS

The study revealed differences in the composition of microbiota and the content of VOCs in Swiss-Dutch-type cheeses manufactured in different seasons of the year. Therefore, cheese samples were divided into two groups. The first group was composed of cheeses produced in winter-spring (clusters I and II). These samples were characterized by the prevalence of *P. thoenii, L. lactis, L. acidophilus*, and *B. subtilis* which were correlated with the contents of acetaldehyde and fatty acids C_2 and C_3, whereas *E. coli, E. cloacae*, and *L. plantarum* increased contents of methanol, acetone, and diacetyl. The second group was composed of cheeses produced in summer-autumn (cluster III). It was characterized by the prevalence of NSLAB (including *L. casei, L. fermentum*, and *L. brevis*) which increased the content of fatty acids C_4-C_7, as well as *C. perfringens, E. aerogenes*, and *S. thermophilus/S. xylosus* which probably increased the content of acetoin. The observations made in this study do not exhaust the possibilities of this approach, but indicate that the described methods can be effectively used to control the cheese production process and to monitor microbiological contamination.

ACKNOWLEDGEMENTS

We are grateful to Dr. Marta Mikš for calibration of the gas chromatograph for quantitative identification volatile compounds of microbiological origin in samples of milk and ripened chees.

RESEARCH FUNDING

This study was supported by the National Science Center, Poland (grant no. N N312 484140) and by University of Warmia and Mazury in Olsztyn (17.610.015–300).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Abriouel, H., Martin-Platero, A., Maqueda, M., Valdivia, E., Martinez-Bueno, M. (2008). Biodiversity of the microbial community in Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology, 127*(3), 200−208.
2. Alegría, A., Alvarez-Martin, P., Sacristan, N., Fernandez, E., Delgado, S., Mayo, B. (2009). Diversity and evolution of the microbial populations during manufacture and ripening of Casin, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology, 136*(1), 44−51.
3. AOAC International 2005. Method 926.08. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD, USA: AOAC International; 2005.

4. Ayad, E.H.E., Verheul, A., De Jong, C., Wouters, J.T.M., Smit, G. (1999). Flavour forming abilities and amino acid requirements of *Lactococcus lactis* strains isolated from artisanal and non-dairy origin. *International Dairy Journal, 9*, 725–735.
5. Caridi, A., Micari, P., Caparra, P., Cufari, A., Sarullo, V. (2003). Ripening and seasonal changes in microbial groups and in physico-chemical properties of the ewes' cheese Pecorino del Poro. *International Dairy Journal, 13*, 191−200.
6. De Freitas, I., Pinon, N., Thierry, A., Lopez, C., Maubois, J.-L., Lortal, S. (2007). In depth dynamic characterization of French POD Cantal cheese made from raw milk. *Le Lait, 87*, 97−117.
7. Dolci, P., Alessandria, V., Rantsiou, K., Rolle, L., Zeppa, G., Cocolin, L. (2008). Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. *International Journal of Food Microbiology, 122*(3), 302−311.
8. Drinan, F.D., Cogan, T.M. (1992). Detection of propionibacteria in cheese. *Journal of Dairy Research, 59*(1), 65–69.
9. Duru, I.C., Laine, P., Andreevskaya, M., Paulin, L., Kananen, S., Tynkkynen, S., Auvinen, P., Smolander O.-P. (2018). Metagenomic and metatranscriptomic analysis of the microbial community in Swiss-type Maasdam cheese during ripening. *International Journal of Food Microbiology, 281*, 10–22.
10. Duthoit, F., Callon, C., Tessier, L., Montel, M.-C. (2005). Relationships between sensorial characteristics and microbial dynamics in "Registered Designation of Origin" Salers cheese. *International Journal of Food Microbiology, 103*(3), 259−270.
11. Ercolini, D., Moschetti, G., Blagiotta, G., Coppola, S. (2001). The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo Mozzarella cheese production: bias of culture-dependent and culture-independent analyses. *Systematic and Applied Microbiology, 24*(4), 610–617.
12. Felicio T.L., Esmerino E.A., Vidal V.A.S., Cappato L.P., Garcia R.K.A., Cavalcanti R.N., Freitas M.Q., Conte Juniord C.A., Padilha M.C., Silva M.C., Raices R.S.L., Arellano D.B., Bollini H.M.A., Pollonio M.A.R., Cruz A.G. (2016). Physico-chemical changes during storage and sensory acceptance of low sodium probiotic Minas cheese added with arginine. *Food Chemistry, 196*, 628–637.
13. Flórez, A.B., Mayo, B. (2006). PCR–DGGE as a tool for characterizing dominant microbial populations in the Spanish blue-veined Cabrales cheese. *International Dairy Journal, 16*, 1205–1210.
14. Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (2004). *Cheese: Chemistry, Physics and Microbiology*. 3nd edition, Elsevier Ltd., London, UK.
15. Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E. (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *International Dairy Journal, 19*, 3−11.
16. Frece, J., Vrdoljak, M., Filipčić, M., Jelić, M., Čanak, I., Jakopović, Ž., Pleadin, J., Gobin, I., Landeka Dragičević, T., Markov, K. (2016). Microbiological quality and variability of natural microbiota in Croatian cheese maturing in lambskin sacks. *Food Technology and Biotechnology, 54*(2), 129–134.
17. Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A., Giudici, P. (2008). Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. *International Journal of Food Microbiology, 125*(3), 347−351.
18. Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L., Fox, P.F. (2015). Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends in Food Science and Technology, 45*(2), 167−178.
19. Granato D., Putnik P., Bursać Kovačević D., Sousa Santos J., Calado V., Silva Rocha R., Gomes Da Cruz A., Jarvis B., Ye Rodionova O., Pomerantsev A. (2018). Trends in chemometrics: Food authentication, microbiology, and effects of processing. *Comprehensive Review in Food Science and Food Safety, 17*(3), 663–677.
20. IDF 222: 2008. ISO 3433:2008. Cheese – determination of fat content – Van Gulik method. International Standard Organization; 2008.
21. IDF 88:2006. ISO 5943:2006. Cheese and processed cheese products – Determination of chloride content -- Potentiometric titration method. International Standard Organization; 2006.
22. IDF 50:2008. ISO 707:2008. Milk and milk products — Guidance on sampling; International Standard Organization; 2008.
23. Januszkiewicz, J., Sabik, H., Azarnia, S., Lee, B. (2008). Optimization of headspace solid-phase microextraction for the analysis of specific flavors in enzyme modified and natural cheddar cheese using factorial design and response surface methodology. *Journal of Chromatography A, 1195*(1−2), 16–24.
24. Johnson, M.E. (2017). A 100-Year Review: Cheese production and quality. *Journal of Dairy Science, 100*(12), 9952–9965.
25. Jordan, K.N., Cogan, T.M. (1993). Identification and growth of non-starter lactic acid bacteria in Irish cheddar cheese. *Irish Journal of Agricultural and Food Research, 32*, 47−55.
26. Joux, F., Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection, 2*(12), 1523−1535.
27. Klijn, N., Nieuwenhof, F.F.J., Hoolwerf, J.D., van der Waals, C.B., Weerkamp, A.H. (1995). Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Applied and Environmental Microbiology, 61*, 2919–2924.
28. Lindberg, A.-M., Christiansson, A., Rukke, E.-O., Eklund, T., Molin, G. (1996). Bacterial flora of Norwegian and Swedish semi-hard cheese after ripening, with special reference to *Lactobacillus*. *Netherlands Milk and Dairy Journal, 50*, 563−572.
29. Mangia, N.P., Fancello, F., Deiana, P. (2016). Microbiological characterization using combined culture dependent and independent approaches of Casizolu pasta filata cheese. *Journal of Applied Microbiology, 120*, 329–345.
30. Marino, M., Maifreni, M., Rondinini, G. (2003). Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. *FEMS Microbiology Letters, 229*, 133−140.
31. Mauriello, G., Moio, L., Genovese, A., Ercolini, D. (2003). Relationships between flavoring capabilities, bacterial composition, and geographical origin of natural whey cultures used for traditional water-buffalo Mozzarella cheese manufacture. *Journal Dairy Science, 86*, 486–497.
32. McSweeney, P.L.H., Sousa, M.J. (2000). Biochemical pathways for the production for flavour compounds in cheese in during ripening: a review. *Le Lait, 80*(3), 293−324.
33. Milosavljević, N.P., Blagojević, P.D., Savić, D.S., Radulović, N.S. (2012). Application of HS-SPME–GC-MS-derived variables for monitoring ripening-induced changes in cheese volatile compounds. *Dairy Science Technology, 92*(4), 321–333.

34. Mondello, L., Costa, R., Tranchid, P.Q., Chiofalo, B., Zumbo, A., Dugo, P., Dugo, G. (2005) Determination of flavour components in Sicilian goat cheese by automated HS-SPME-GC. *Flavour and Fragrance Journal, 20*(6), 659–665.
35. Nalepa, B., Markiewicz, L.H. (2017). PCR-DGGE markers for qualitative profiling of microbiota in raw milk and ripened cheeses. *LWT – Food Science and Technology, 84*, 168−174.
36. Ogier, J.-C., Lafarge, V., Girard, V., Rault, A., Maladen, V., Gruss, A., Leveau, J.-Y., Delacroix-Buchet, A. (2004). Molecular fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 70*(9), 5628−5643.
37. Pastink, M.I., Sieuwerts, S., de Bok, F.A.M., Janssen, P.W.M., Teusink, B., van Hylckman Vlieg, J.E.T., Hugenholtz, J. (2008). Genomics and high-throughput screening approaches for optimal flavor production in dairy fermentation. *International of Dairy Journal, 18*(8), 781−789.
38. Pérès, C., Viallon, C., Berdague, J.-L. (2001). Solid-phase microextraction-mass spectrometry: a new approach to the rapid characterization of cheeses. *Annals of Chemistry, 73*, 1030–1036.
39. Porcellato, D., Skeie, S.B. (2016). Bacterial dynamics and functional analysis of microbial metagenomes during ripening of Dutch-type cheese. *International Dairy Journal, 61*, 182−188.
40. Randazzo, C.L., Pitino, I., Ribbera, A., Caggia. C. (2010). Pecorino Crotonese cheese: Study of bacterial population and flavour compounds. *Food Microbiology, 27*(3), 363−374.
41. Randazzo, C.L., Vaughan, E.E., Caggia, C. (2006). Artisanal and experimental Pecorino Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR-DGGE analyses. *International Journal of Food Microbiology, 109*(1–2), 1−8.
42. Rehfeld I.S., Fraiha A.L.S., Matos A.C.D., Guedes M.I.M.C., Costa E.A., de Souza M.R., Cavalcante L.F.L., Lobato Z.I.P. (2017). Survival of *Vaccinia* virus in inoculated cheeses during 60-day ripening. *Journal of Dairy Science 100*(9), 7051–7054.
43. Ricciardi, A., Guidone, A., Ianniello, R.G., Cioffi, S., Aponte, M., Pavlidis, D., Tsakalidou, E., Zotta, T., Parente, E. (2015). A survey of non-starter lactic acid bacteria in traditional cheeses: Culture dependent identification and survival to simulated gastrointestinal transit. *International Dairy Journal, 43*, 42−50.
44. Santiago-López, L., Aguilar-Toalá, J.E., Hernández-Mendoza, A., Vallejo-Cordoba, B., Liceaga, A.M., González-Córdova, A.F. (2018). Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science, 101*(5), 3742–3757.
45. Sienkiewicz, J. (2010). Concepts of biodiversity – their dimensions and measurements in the light of the subject literature. *Ochrona Środowiska i Zasobów Naturalnych, 45*, 7−29 (in Polish).
46. Skelin, A., Mrkonjić Fuka, M., Čanžek Majhenič, A., Redžepović, S., Samaržija, D., Bogovič Matijašić, B. (2012). Phenotypic and genotypic characterization of indigenous Lactobacillus community from traditional Istrian ewe's cheese. *Food Technology and Biotechnology, 50*(3), 362–370.
47. Smit, G., Smit, B.A., Engel, W.J.M. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*, 591–610.
48. Terzaghi, B.E., Sandine, W.E. (1975). Improved medium for lactic streptococci and their bacteriophages. *Applied Microbiology, 29*, 807–813.
49. Thierry, A., Maillard, M.B., Richoux, R., Lortal, S. (2006). Ethyl ester formation is enhanced by ethanol addition in mini Swiss cheese with and without added propionibacteria. *Journal of Agricultural and Food Chemistry, 54*(18), 6819−6824.

Submitted: 13 February 2019. Revised: 15 May 2019. Accepted: 6 June 2019. Published on-line: 3 July 2019.