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The impact of some parameters on volatile compounds in hard type cheeses

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Summary

Volatile compounds (VCs) analysis was performed by solid-phase microextraction-gas chromatography-mass spectrometry. Main groups that define typical cheese flavour formed as a result of the addition of a starter culture (SC). The cheesemaking environment, the type of milk (cow, ewe, goat), and the heat treatment of milk were observed. The SC had influenced the total amount of some fatty acids and ketones. Compared to cow and ewe cheeses, goat cheese had higher values of hexanoic and octanoic acids, however, two alcohols, 1-hexanol, 2-ethyl- and hexanol, were only present in cow cheeses. We confirmed that the cheesemaking environment is also an important parameter influencing VC profiles of cheese. Higher amount of esters and the absence of 2-phenylethanol were observed in raw milk cheese, compared to thermised milk cheese where δ-octalactone was present.

Keywords: cow, goat, ewe hard type cheeses; volatile compounds

Introduction

Cheese is a fermented dairy product and its quality - chemical, technological and microbiological – depends mainly on the characteristics of milk and the cheesemaking process (Stefanon and Procida, 2004; Amenu and Deeth, 2007). One of the key cheese quality components is its flavour (Ross et al., 2000; Delgado et al., 2010)

The flavour of cheese is defined by the complex environment of volatile and non-volatile compounds (Delgado et al., 2010). Analysis of VCs in foods, including dairy products, is one of the most common methods for quality determination. VCs in cheeses are of different chemical classes, such as fatty acids, alcohols, ketones, esters, aldehydes, lactones, etc. (Milosavljević et al., 2012).

Glycolysis, lipolysis and proteolysis are principal pathways of VCs formation (McSweeney and Sousa, 2000), but within these processes numerous reactions and some conversion pathways, which are still unclear, may occur (Ayad et al., 2000). VCs found in cheese are important constituents of cheese aroma which is unique and dependent on a cheese variety, and is affected by many factors such as the animal diet, cheese ripening conditions, technological processes, indigenous microbiota of raw milk, type of milk (cow, ewe, goat), rennet, season, environment (Centeno et al., 2004; Stefanon and Procida, 2004; Gioacchini et al., 2010), activity of different enzymes (lipases, proteinases, peptidases), and finally the type of a SC used in cheesemaking (Ozcan and Kurdal, 2012). The same VC (e.g. butanoic acid) could be of different origin or pathways (Alewijn, 2006), but also the same pathway of VCs formation (e.g. lipolysis) may be influenced by different factors (McSweeney and Sousa, 2000).

SCs (starter cultures) are microbial preparations generally consisting of lactic acid bacteria (LAB), which are also normally present in milk as indigenous microbiota. Several physiological functions of a SC are of great importance in the cheese production and maturation due to their influence on the final organoleptic properties of cheese (Leroy and De Vuyst, 2004). Both starter and non-starter LAB (which are part of the environment) have the potential to produce aroma compounds (Kieronczyk et al., 2003) and could thus contribute to the final sensory properties of cheese (Båth et al., 2012).

It has already been shown that the addition of a SC affects cheese flavour profile to a great extent (Randazzo et al., 2007) and its enhancement (Ortigosa et al., 1999), especially during cheese ripening (Leroy and De Vuyst, 2004), and

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therefore greatly contributes to the final cheese quality (Wouters et al., 2002). However, it is known, that SCs promote the production of various aromatic compounds through different pathways. Some SCs elevate production of lactic acid, while the others are more prone to transform lactic acid into other substances (Ayad et al., 2000; Settanni and Moschetti, 2010). SC can also transform citrate, proteins and lipids into flavour compounds and so they define the final cheese flavour (Broome et al., 2002).

Environment, which is closely linked to the endogenous microbiota in raw milk, also affects the presence of certain VCs in cheese (Toso et al., 2002). During cheesemaking, due to their activities, microbiota from raw milk and the cheesemaking environment contribute not only to the final VCs profile, but also to the cheese flavour (Irlinger and Mounier, 2009). Many bacterial species present in raw milk produce enzymes (proteases, lipases, phospholipases) whose activities are directly linked to the formation of cheese flavour (Munsch-Alatossava and Alatossava, 2006; Deetae et al., 2009).

Type of milk (cow, ewe or goat) also significantly influences the formation of VCs profiles and hence the final cheese flavour (Pappa et al., 2013). In the previous studies it has already been shown that the flavour characteristic of different type of milk cheese is mostly due to the presence of specific VCs (Delgado et al., 2010) and their levels (Massouras et al., 2006).

The research of Buchin et al., (1998) demonstrated that the differences in VCs profile can also be caused by the milk heat treatment and milk composition. Heating of milk at a thermisation regime (63-65 °C, 30 min) slightly modifies the characteristics of milk and microbiota (Desmazeaud, 2000), as well as inactivates enzymes which lead to certain changes in the biochemical and microbiological processes during ripening. All of these changes also influence on the VC and the flavour of cheese (Ozcan and Kurdal, 2012).

Generally, VCs in cheese are determined by employing a gas chromatography (GC) - mass spectrometry (MS) (Salles et al., 2002). The improvement of GC - MS analysis took place during the last twenty years by the introduction and development of new sampling technique named solid phase micro extraction (SPME) (Careri et al., 1994; Chin, Bernhard and Rosenberg, 1996; Milosavljević et al., 2012). It was efficiently implicated in the cheese aroma studies during the last decade (Delgado et al., 2010).

The aim of our work was to indicate the possible effect of different parameters, such as addition of a commercial SC, use of different milk types, cheesemaking environment and milk heat-treatment on VC profiles in cheese samples. Having studied the huge amount of papers related to the aroma of hard cheeses, we noticed the need to compare the several parameters, including the importance of two border regions on cheese making: the Primorska region (country Slovenia) with Nanos cheese, and the Friuli-Venezia Giulia region (country Italy) with Montasio cheese, and several other parameters that are studied for the first time together in order to find some cross-linked information.

Materials and methods

Material

Cheeses were sampled and further analysed for VC profiles after two months of ripening. The raw goats' and four ewes' cheeses were collected from cheesemakers in the western part of Slovenia, while four cow milk cheeses were provided by small dairy plants; one cheese from dairy plant in Primorska region, Slovenia (Nanos cheese), and three cheeses from 3 different dairy plants in Friuli-Venezia Giulia region, Italy (Montasio cheese).

Experimental design

Our experiment was divided into four parts. In Part I, we compared the effect of the SC on VCs profile. For this study, samples of ewe's cheeses with and without addition of a SC were collected. In Part II the effect of the environment on VCs profile was studied in cow's Montasio cheeses (C-dM1-no, C-dM2-no, C-dM3-no). Milk for Montasio cheese was inoculated with 1% of natural milk culture, which was prepared by incubating thermized milk (63 °C for 20 min) at 44 °C for 18-20 h. The effect of the milk type on the VCs profile in cheese was studied in the part III, where cow's, ewe's and goat's raw milk cheeses, without addition of a SC, were sampled. Heat treatment effect was evaluated in Part IV, comparing the VC profiles of raw milk Montasio cheese (C-dM1-no, C-dM2-no, C-dM3-no), and thermised milk Nanos cheese (C-dV-STLH). Cheese samples, names, type of milk and cheesemakers are shown in Table 1, while information about SCs used are shown in Table 2. Cheeses made without the addition of a SC served as a control sample.

Table 1. Characteristics of cheese samples

Cheese sample	Type of milk	Cheese producer	Starter culture (SC	Heat treatment
E-cM-no	E	cM - cheesemaker M	no	raw milk
E-cM-L82LD			L82 (1/3) + LD (2/3)	raw milk
E-cO-no		cO - cheesemaker O	no	raw milk
E-cO-L62			L62	raw milk
G-cX-no	G	cX - cheesemaker X	no	raw milk
C-dM1-no	C	dM1-dairy plant M1	no	raw milk
C-dM2-no		dM2-dairy plant M2	no	raw milk
C-dM3-no		dM3-dairy plant M3	no	raw milk
C-dV-STLH		dV- dairy plant V	ST-M-05, LHB-02	thermised milk

Legend: E=ewe, G=goat, C=cow, no = no starter culture added

Table 2. Characteristic of a starter culture

Starter culture	Producer	Bacteria
L62 (Lyofast MS 062 CM)	Clerici-Sacco	*Str. thermophilus*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *lactis* biovar *diacetylactis*
L82 (Lyofast MOTC 082)	Clerici-Sacco	*Str. thermophilus*, *Lc. lactis* ssp. *lactis*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. casei*
LD (Lyobac D)	Alce International S.r.l.	*Str. thermophilus*
ST-M-05	Chr. Hansen	*Str. thermophilus*
LHB-02	Chr. Hansen	*Lb. helveticus*

Analysis of volatiles compounds in cheese samples

Cheeses were sampled by cutting the cheese wheel with a conical cheese borer from the lateral surface of the cheese to the centre. The 10 mm of the outer surface of cheese samples was not used, while the rest was ground in a blender and the amount of 4 g was transferred into a 20 mL headspace vial. Each cheese and milk sample was performed in triplicates. VCs present in cheeses were analysed using a solid phase micro-extraction – SPME kit (Supelco, Bellefonte, PA, USA). On the SPME device, the 20 mm 50/30 μm DVB/CAR/PDMS fibre (divinylbenzene / carboxen / polydimethylsiloxane) was mounted. Samples of cheese were exposed for 24 h extraction at 25 ± 1 °C to the SPME fibre. Afterwards the device was introduced in a gas chromatograph with a mass selective detector (GC-MS - Agilent 6890 Series GC System with Agilent 5973 Mass Selective Detector) in the splitless injector at 270 °C for 10 min. On a daily basis, prior to analysis, the fibre was conditioned and activated by inserting it into the GC injector at 270 °C for 30 min. Volatiles were separated on Rtx-20 column (60 m, 0.25 mmID, 1μm, Restek, USA). The temperature program was as follows: initial temperature 50 °C (2 min) - 10 °C min^{-1} - 150 °C (for 3 min) - 10 °C min^{-1} - 250 °C (for 5 min). Total run time was 30 min. The mass spectrometer was operated in the electron ionisation mode at a voltage of 70 eV, the temperature of the MS Quad was set at 150 °C and the ion source at 230 °C. Compounds were identified on the basis of their retention times using the searchable EI-MS spectra library (NIST02). The peak area for quantification was measured in a TIC chromatogram.

Statistical analysis

One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's *post-hoc* test) were used to evaluate the significant difference of the data at *p*-value 0.05. Data were expressed as means with a relative standard deviation (RSD).

Results and discussion

SPME-GC-MS analysis revealed a vast number of VCs present in cheese samples. Within 55 volatiles, a range of different fatty acids, esters, ketones, alcohols, aldehydes and others was identified (Table 3). Both qualitatively and quantitatively, the use of a SC, the cheesemaking environment, type of milk and milk heat treatment significantly influenced the analysed volatile fractions of cheeses.

Table 3. Total volatile compounds in cheese samples

	1		2		3		4		5		6		7		8		9	
Samples	**E-cO-no**		**E-cM-no**		**E-cO-L62**		**E-cM-L82LD**		**C-dM1-no**		**C-dM2-no**		**C-dM3-no**		**G-cX-no**		**C-dV-STLH**	
FATTY ACIDS	MEAN	*RSD*	MEAN	*RSD*	MEAN	*RSD*	MEAN	*RSD*	MEAN		MEAN	*RSD*	MEAN	*RSD*	MEAN	*RSD*	MEAN	*RSD*
1. Acetic acid	$8.39x10^{6\ a}$	*0.08*	$5.71x10^{6\ a}$	*0.37*	$9.27x10^{6\ a}$	*0.30*	$5.97x10^{6\ a}$	*0.10*	$5.42x10^{6\ a}$	*0.23*	$1.63x10^{7\ b}$	*0.09*	$2.00x10^{7\ c}$	*0.05*	$9.60x10^{6\ a}$	*0.37*	$1.73x10^{7\ bc}$	*0.14*
2. Isobutryc acid	$6.60x10^{6\ a}$	*0.51*	*nd* ^{b}		$9.42x10^{5\ c}$	*0.10*	*nd* ^{b}		*nd* ^{b}		*nd* ^{a}		*nd* ^{a}		*nd* ^{b}		*nd* ^{b}	
3. Butanoic acid	$2.20x10^{7\ a}$	*0.26*	$4.25x10^{7\ a}$	*0.40*	$9.36x10^{7\ b}$	*0.71*	$5.36x10^{7\ c}$	*0.19*	$1.85x10^{8\ e}$	*0.12*	$1.41x10^{8\ d}$	*0.15*	$1.21x10^{8\ d}$	*0.16*	$1.23x10^{8\ d}$	*0.07*	$1.28x10^{8\ d}$	*0.11*
4. Isovaleric acid	$5.75x10^{7\ a}$	*0.32*	$6.62x10^{6\ bc}$	*0.10*	$1.20x10^{7\ bc}$	*0.17*	$1.80x10^{7\ b}$	*0.19*	$7.13x10^{6\ bc}$	*0.09*	$6.49x10^{6\ c}$	*0.21*	$1.04x10^{7\ bc}$	*0.13*	$2.29x10^{6\ c}$	*0.29*	$4.24x10^{6\ c}$	*0.15*
5. Hexanoic acid	$5.51x10^{7\ a}$	*0.31*	$7.13x10^{7\ ab}$	*0.16*	$1.10x10^{8\ bc}$	*0.20*	$2.57x10^{8\ d}$	*0.02*	$1.20x10^{8\ bc}$	*0.09*	$1.40x10^{8\ c}$	*0.03*	$1.25x10^{8\ c}$	*0.02*	$3.48x10^{8\ e}$	*0.21*	$1.44x10^{8\ c}$	*0.12*
6. Heptanoic acid	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$3.91x10^{6\ b}$	*0.11*	*nd* ^{a}	
7. Octanoic Acid	$1.95x10^{7\ ab}$	*0.35*	$2.23x10^{7\ ab}$	*0.25*	$2.92x10^{7\ b}$	*0.20*	$5.96x10^{7\ c}$	*0.07*	$1.59x10^{7\ a}$	*0.24*	$2.16x10^{7\ ab}$	*0.16*	$2.17x10^{7\ ab}$	*0.04*	$1.54x10^{8\ d}$	*0.08*	$6.39x10^{7\ c}$	*0.04*
8. Benzoic Acid	$9.11x10^{6\ a}$	*0.08*	$2.52x10^{7\ b}$	*0.09*	$1.73x10^{7\ c}$	*0.24*	*nd* ^{d}		*nd* ^{d}		*nd* ^{d}		*nd* ^{d}		$9.14x10^{6\ a}$	*0.03*	*nd* ^{d}	
9. Nonanoic acid	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.96x10^{6\ b}$	*0.01*	*nd* ^{a}	
10. Decanoic acid	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$9.76x10^{7\ b}$	*0.03*	$1.87x10^{7\ c}$	*0.08*
11. Butanoic acid, 2-methyl-	$2.20x10^{7a}$	*0.30*	$1.57x10^{6\ b}$	*0.31*	$3.17x10^{6\ b}$	*0.17*	$5.50x10^{6\ b}$	*0.16*	*nd* ^{d}		*nd* ^{d}		*nd* ^{d}		$3.92\ x10^{5\ c}$	*0.39*	$1.32x10^{6\ b}$	*0.17*
ESTERS																		
12. Ethyl Acetate	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$2.39x10^{6\ b}$	*0.09*	$4.71x10^{6\ b}$	*0.18*	$1.31x10^{8\ c}$	*0.08*	$5.65x10^{5\ d}$	*0.11*	$1.30x10^{6\ b}$	*0.12*
13. Butanoic acid, ethyl ester	$5.33x10^{7\ a}$	*1.32*	$3.45x10^{6\ b}$	*0.02*	$5.50x10^{6\ b}$	*0.21*	$2.79x10^{7\ ab}$	*0.25*	$1.37x10^{8\ c}$	*0.14*	$5.52x10^{7\ a}$	*0.20*	$3.93x10^{7\ ab}$	*0.17*	$4.99x10^{6\ b}$	*0.06*	$8.15x10^{6\ b}$	*0.14*
14. Isovaleric acid, ethyl ester	$7.88x10^{5\ a}$	*0.37*	*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}	
15. Hexanoic acid, ethyl ester	$3.30x10^{6\ a}$	*0.25*	$2.80x10^{6\ a}$	*0.11*	$4.89x10^{6\ a}$	*0.20*	$4.76x10^{7\ b}$	*0.02*	$1.89x10^{8\ c}$	*0.05*	$1.67x10^{8\ d}$	*0.00*	$1.61x10^{8\ d}$	*0.04*	$8.66x10^{6\ a}$	*0.25*	$9.19x10^{6\ a}$	*0.17*
16. Octanoic acid, ethyl ester	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.48x10^{7\ b}$	*0.25*	$1.78x10^{7\ b}$	*0.22*	$1.99\ x10^{7\ b}$	*0.08*	$8.45x10^{6\ c}$	*0.11*	$4.74x10^{6\ c}$	*0.03*
17. Decanoic acid, ethyl ester	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.98x10^{6\ b}$	*0.33*	$2.96x10^{6\ bc}$	*0.24*	$3.09x10^{6\ c}$	*0.08*	$1.52x10^{7\ d}$	*0.18*	$2.57x10^{6\ bc}$	*0.12*
18. Propanoic acid, ethyl ester	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$3.99x10^{6\ b}$	*0.05*	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}	
19. Lactic acid, ethyl ester	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$7.17x10^{6\ b}$	*0.16*	$3.15x10^{6\ c}$	*0.04*	$5.52x10^{6\ bc}$	*0.19*	*nd* ^{a}		*nd* ^{a}	
20. Heptanoic acid, ethyl ester	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$2.97x10^{6\ b}$	*0.21*	$3.50x10^{6\ b}$	*0.08*	$4.44x10^{6\ b}$	*0.03*	*nd* ^{a}		*nd* ^{a}	
KETONES																		
21. Diacetyl	$1.03x10^{7\ a}$	*0.07*	*nd* ^{b}		$1.17x10^{7\ a}$	*0.20*	*nd* ^{b}		$1.90x10^{6\ c}$	*0.05*	$1.06x10^{6\ c}$	*0.04*	*nd* ^{b}		$1.88x10^{6\ c}$	*0.13*	$1.37x10^{6\ c}$	*0.27*
22. 2-Butanone	$4.61x10^{6\ a}$	*0.38*	$2.12x10^{7\ b}$	*0.29*	*nd* ^{c}		$2.79x10^{6\ a}$	*0.81*	$4.76\ x10^{6\ a}$	*0.08*	$1.37x10^{5\ a}$	*0.23*	$2.70x10^{7\ b}$	*0.41*	*nd* ^{c}		$1.12x10^{6\ a}$	*0.20*
23. 2-Pentanone	$8.76x10^{6\ a}$	*0.06*	$1.43x10^{7\ b}$	*0.27*	$3.64x10^{7\ b}$	*0.22*	$7.27x10^{7\ c}$	*0.41*	$3.51x10^{7\ b}$	*0.13*	*nd* ^{d}		*nd* ^{d}		$2.21x10^{7\ b}$	*0.27*	$1.67x10^{7\ b}$	*0.17*
24. 2-Hydroxy-3-pentanone	*nd* ^{a}		*nd* ^{a}		$1.35x10^{6\ b}$	*0.20*	*nd* ^{a}		$1.02x10^{6\ b}$	*0.20*	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}	
25. Acetoin	$1.36x10^{7\ a}$	*0.19*	*nd* ^{b}		$3.16\ x10^{7\ a}$	*0.12*	$2.65x10^{7\ a}$	*0.28*	$1.39x10^{7\ a}$	*0.15*	$2.97x10^{6\ c}$	*0.22*	$9.65x10^{6\ d}$	*0.10*	$2.28x10^{6\ c}$	*0.13*	$1.47x10^{6\ c}$	*0.30*
26. 2-Heptanone	*nd* ^{a}		*nd* ^{a}		$1.45\ x10^{8\ b}$	*0.34*	$3.67x10^{8\ b}$	*0.03*	$2.58x10^{8\ b}$	*0.23*	$1.63x10^{7\ b}$	*0.09*	$2.00x10^{7\ c}$	*0.05*	$9.60x10^{6\ a}$	*0.37*	$1.73x10^{7\ bc}$	*0.14*
27. 2-Octanone	$9.22x10^{5\ a}$	*0.08*	*nd* ^{b}		$3.77x10^{6\ c}$	*0.19*	$8.06x10^{6\ d}$	*0.11*	$3.37x10^{6\ c}$		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}	
28. Undecane	$7.43x10^{5\ a}$	*0.11*	*nd* ^{b}		$1.46x10^{6\ c}$	*0.12*	*nd* ^{b}		*nd* ^{b}	*0.12*	$1.41x10^{8\ d}$	*0.15*	$1.21x10^{8\ d}$	*0.16*	$1.23x10^{8\ d}$	*0.07*	$1.28x10^{8\ d}$	*0.11*
29. 2-Nonanone	$1.45x10^{7\ ab}$	*0.52*	$8.55x10^{6\ a}$	*0.12*	$4.69x10^{7\ bc}$	*0.18*	$1.44x10^{8\ e}$	*0.02*	$3.91x10^{7\ bc}$	*0.09*	$6.49x10^{6\ c}$	*0.21*	$1.04x10^{7\ bc}$	*0.13*	$2.29x10^{6\ c}$	*0.29*	$4.24x10^{6\ c}$	*0.15*
30. Acetophenone	$2.01x10^{6\ a}$	*0.05*	*nd* ^{b}		*nd* ^{b}		*nd* ^{b}		*nd* ^{b}	*0.09*	$1.40x10^{8\ c}$	*0.03*	$1.25x10^{8\ c}$	*0.02*	$3.48x10^{8\ e}$	*0.21*	$1.44x10^{8\ c}$	*0.12*
31. 2-Undecanone	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$3.91x10^{6\ b}$	*0.11*	*nd* ^{a}	
ALCOHOLS																		
32. 2-Butanol	*nd* ^{a}		$2.54\ x10^{6\ b}$	*0.06*	*nd* ^{a}		*nd* ^{a}		$5.84x10^{6\ c}$	*0.14*	$5.10x10^{6\ c}$	*0.44*	$5.45x10^{7\ d}$	*0.14*	*nd* ^{a}		*nd* ^{a}	
33. 2-Pentanol	*nd* ^{a}		$1.91x10^{6\ b}$	*0.07*	*nd* ^{a}		$4.14x10^{6\ c}$	*0.20*	*nd* ^{a}		$4.91x10^{6\ c}$	*0.23*	$1.03x10^{7\ d}$	*0.16*	*nd* ^{a}		$1.15x10^{6\ b}$	*0.33*
34. 2-Heptanol	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$5.26x10^{6\ b}$	*0.18*	$1.99x10^{7\ c}$	*0.04*	$1.36x10^{7\ c}$	*0.13*	$1.44\ x10^{7\ c}$	0.13	$2.39x10^{5\ d}$	*0.80*	$8.17x10^{6\ b}$	*0.14*
35. 2-Phenylethanol	$1.68x10^{7\ a}$	*0.19*	$1.66x10^{7\ a}$	*0.12*	$5.64x10^{6\ b}$	*0.21*	$2.30x10^{7\ a}$	*0.05*	$1.56x10^{7\ a}$	*1.16*	$3.62x10^{6\ b}$	*0.10*	$3.05x10^{6\ b}$	*0.30*	*nd* ^{c}		*nd* ^{c}	
36. 1-Butanol, 3-methyl-	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$6.68x10^{6\ b}$	*0.22*	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}	
37. 2,3-Butanediol	*nd* ^{a}		*nd* ^{a}		$4.57x10^{6\ b}$	*0.19*	$4.29x10^{6\ b}$	*0.16*	$1.19x10^{7\ c}$	*0.10*	$5.90x10^{6\ b}$	*0.21*	$1.74x10^{6\ d}$	*0.04*	$3.11x10^{6\ b}$	*0.19*	$6.13x10^{5\ b}$	*0.06*
38. 1-Hexanol, 2-ethyl-	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.43x10^{6\ b}$	*0.05*	$2.27x10^{6\ b}$	*0.10*	$1.37x10^{6\ b}$	*0.14*	*nd* ^{a}		$8.06x10^{5\ c}$	*0.10*
39. Hexanol	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.03x10^{7\ b}$	*0.03*	$6.94x10^{6\ c}$	*0.06*	$8.81x10^{6\ c}$	*0.08*	*nd* ^{a}		$5.85x10^{5\ d}$	*0.12*
40. 2-Hexanol	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.06x10^{6\ b}$	*0.15*	*nd* ^{a}		*nd* ^{a}	
41. 2-Nonanol	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$4.38x10^{5\ b}$	*0.38*	$1.73x10^{6\ c}$	*0.20*
42. Ethanol, 2-butoxy-	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$8.83x10^{6\ b}$	*0.10*	$1.55x10^{6\ c}$	*0.34*	*nd* ^{a}		*nd* ^{a}	
43. 1-Butanol	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$8.84x10^{5\ b}$	*0.24*
ALDEHYDES																		
44. Nonanal	$2.06x10^{6\ ab}$	*0.11*	$1.48x10^{6\ b}$	*0.47*	$2.07x10^{6\ ab}$	*0.09*	$2.99x10^{6\ c}$	*0.19*	$1.48x10^{6\ b}$	*0.25*	$2.17x10^{6\ a}$	*0.06*	$1.38x10^{6\ b}$	*0.22*	$6.07x10^{5\ d}$	*0.09*	$2.48x10^{6\ ac}$	*0.09*
45. Benzeneacetaldehyde	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$9.06x10^{5\ b}$	*0.10*	$2.60x10^{6\ c}$	*0.07*	*nd* ^{a}		$6.60x10^{5\ d}$	*0.19*	$6.54x10^{6\ d}$	*0.08*
46. Octanal	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.04x10^{7\ b}$	*0.15*	$1.60x10^{7\ b}$	*0.15*	$1.60\ x10^{7\ b}$	*0.07*	$1.97x10^{6\ b}$	*0.17*	$1.01x10^{7\ b}$	*0.10*
47. Butanal, 3-methyl-	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$3.98x10^{7\ b}$	*0.16*	$3.03x10^{6\ c}$	*0.26*	$7.39x10^{6\ c}$	*0.03*	*nd* ^{a}		*nd* ^{a}	
48. Heptanal	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.40x10^{6\ b}$	*0.08*	$7.98x10^{5\ c}$	*0.15*	$8.69x10^{5\ c}$	*0.14*	*nd* ^{a}		$5.92x10^{5\ c}$	*0.08*
MISCELLANEOUS COMPOUNDS																		
49. Ethylbenzene	$1.39x10^{6\ a}$	*0.02*	*nd* ^{b}		$2.35x10^{6\ a}$	*0.22*	*nd* ^{b}		$7.17x10^{6\ c}$	*0.10*	$4.24x10^{6\ d}$	*0.10*	$6.75x10^{6\ c}$	*0.34*	*nd* ^{b}		$6.53x10^{5\ e}$	*0.28*
50. α-Pinene	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$2.87x10^{6\ b}$	*0.06*	*nd* ^{a}		$1.58x10^{6\ b}$	*0.10*	*nd* ^{a}		$1.11x10^{6\ b}$	*0.15*
51. Limonene	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$3.14x10^{6\ b}$	*0.10*	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$2.25x10^{6\ b}$	*0.11*
52. p-Cymene	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.15x10^{6\ b}$	*0.06*	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.05x10^{6\ b}$	*0.12*
53. δ-Decalactone	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$1.29x10^{6\ b}$	*0.29*	$1.83x10^{6\ b}$	*0.23*	$1.73x10^{6\ b}$	*0.03*	*nd* ^{a}		$3.76x10^{6\ b}$	*0.19*
54. δ-Octalactone	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		$2.89x10^{6\ b}$	*0.17*
55. Caryophyllene	*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}		*nd* ^{a}				*nd* ^{a}		$9.05x10^{5\ b}$	*0.34*

The same letter in the row indicates no significant differences (Ducan's test, $p< 0.05$), RSD - relative standard deviation, *nd* - not detected

Part I: The effect of addition of different starter cultures

Results showing the effect of a SC are presented in Table 3, columns 1-4. For this purpose ripened ewe cheeses of two cheesemakers were analysed. Cheesemaker M provided us with ewe cheese produced with the two commercial SC Lyofast MOTC 082 and Lyobac D (E-cM-L82LD) mixed in ratio 1: 3, respectively. Ewe cheese, made without the addition of a starter culture (E-cM-no) served as a negative control. Similarly, cheesemaker O provided us with ewe cheese produced with the use of the SC Lyofast MS 062 CM (E-cO-L62) and starter-free cheese (E-cO-no).

When VC profiles of cheeses made with a SC (E-cM-L82LD and E-cO-L62) were compared to VC profiles of starter-free cheeses (E-cM-no and E-cO-no), the increase in the total amount of VCs, especially of fatty acids and ketones, was observed. Among fatty acids the increased quantities were noted for butanoic, hexanoic and octanoic acid. Our observation is in accordance with the previous study of Settanni and Moschetti (2010), confirming that cheeses made with a SC lead to a significantly higher level of free fatty acids in comparison to cheeses made without an adjunct culture. It is known that bacteria added as a SC could have lipolitic activity (Curioni and Bosset, 2002). Fatty acids are important constituents of cheese aroma. Besides, they are precursors of methyl ketones, alcohols, lactones, and esters (Ayad et al., 2000).

In cheese samples E-cO-L62 and E-cM-L82LD, the content of three ketones undoubtedly increased (2-pentanone, 2-octanone, 2-nonanone). Moreover, 2-heptanone was the only volatile identified in cheeses made with a SC, compared to starter-free cheese samples E-cO-no and E-cM-no, although a potential precursor,octanoicacid, was present in all samples. It is known that methyl ketones (C2n-1) are the product of fatty acids (C2n) (Alewijn 2006) via β-oxidation of a long-chain fatty acid, which can be also performed by the enzymes derived from bacteria (Wang, Zhang and Li, 2012). It was found that not only mould *Penicillium roqueforti* (McSweeney and Sousa, 2000), but even some starter bacteria strain properties may influence the formation of methyl ketones (Collins, McSweeney and Wilkinson, 2003).

Furthermore, we also compared VC profiles of cheeses made with a different SC. The use of the SC L82 and LD, which included more species of thermophilic bacteria than the SC L62, resulted in a higher amount of total fatty acids and ketones in cheese sample E-cM-L82LD. Similar results were presented by Pappa et al. (2013) in their study, where they compared formation of VCs in Teleme cheese manufactured with a thermophilic and mesophilic SC; the use of thermophilic starters resulted in a higher total mean level of ketones and acids.

Isobutyric acid, which has origin in proteins and fat (Alewijn, 2006), was only identified in cheese samples from cheesmaker O (cO). The 2-phenylethyl alcohol was present in all ewes' cheeses. Its characteristic note is typically rose floral (Ayad et al., 2000).

Acetoin can be derived from diacetyl (Corrëa Lelles Nogueira, Lubachevsky and Rankin, 2005; Quintans et al., 2008). The absence of diacetyl is in accordance with the absence of acetoin in ewe's cheese E-cM-no, and also vice versa, the increase in the concentration of diacetyl resulted in the increase of acetoin, as is the case in cheese sample E-cO-no.

Part II: The effect of the environment

The effect of the environment on VCs formation was analysed in Montasio cow cheese samples that were collected from three dairy plants, located in three different locations. For Montasio cheese production dairy plants have followed the same technological procedure using the natural milk culture that has been freshly prepared prior to cheesemaking in the dairy plant. As presented in columns 5-7 of Table 3, 40 compounds were identified in the volatile fractions of three Montasio cheese samples, C-dM1-no, C-dM2-no, C-dM3-nowhere 29 VCs were unevenly distributed among all three cheese samples and 11 VCs were found only in one or two. As we have already mentioned, flavour of different cheeses can be distinguished by the presence and levels of various VCs (Hassan, El-Gawad and Enab, 2013).

The most abundant VCs identified in all three cheese samples were butanoic and hexanoic acids, hexanoic acid, ethyl ester and 2-heptanone. The above mentioned VCs (except 2-heptanone) were also identified in the previous study of Montasio cheese reported by Innocente, Munari and Biasutti, (2013).

Our observation is strongly supported by the literature data indicating the importance of the environment on the final quality of the same type of cheese. It is known that natural milk culture, as well as raw cheese milk, contains specific strains of LAB, which are essential for producing the characteristic flavour of traditional cheese (Marino, Maifreni and Rondinini, 2003). For example, nonstarter mesophilic lactobacilli can metabolise citrate and transform it into some VCs (Skeie et al., 2008; McSweeney and Sousa 2000). Enterococci are part of the natural microbiota of many dairy products and, in some cheeses, they dominate over lactobacill and

lactococci (Suzzi et al., 2000). They can be also present in milking equipment. Findings about effect of enterococci on cheese flavour development are different: negatively (Rea et al., 2004) and positively (Giraffa, 2003; Rea et al., 2004). Rea et al.(2004) concluded in their study that enterococci have little effect on the flavour of Cheddar cheese.

Fatty acids: acetic acids, butanoic acid, isovaleric acid, hexanoic acid and octanoic acid were present in all three cheese samples (C-dM1-no, C-dM2-no, C-dM3-no), but their content varied. Among fatty acids, the most significant difference was noted in the amount of the acetic acid, which is produced in reactions by the action of lactic acid bacteria (Delgado et al., 2011). Acetic acid has a sharp vinegar sour odour (Frank, Owen and Patterson, 2004).

According to our results and those reported in the literature, the differences in VC profiles of cheese from different dairy plants were proven also in case of ketones, which have an important role in the cheese flavour (Settanni and Moschetti, 2010). Ketone 2-butanone has been detected in all three cheese samples, with the highest value in C-dM3-no. Its presence in cheese is associated with the desired flavour. Moreover, as stated in the literature, 2-butanone is usually present in starter-free cheeses (Keen, Walker and Peberdy, 1974). Diacetyl, which can also be produced by nonstarterLAB (Sgarbi et al., 2013), was present in cheese samples C-dM1-no, C-dM2-no. Diacetyl is an important aroma compound in some cheeses (McSweeney and Sousa, 2000) and contributes to the buttery flavour in dairy products (Swearingen, O'Sullivan and Warthesen, 2001). Due to their high volatility at ambient temperatures, esters are important contributors to flavour of many cheeses, even at low concentrations. They can also be produced by heterofermentative nonstarter LAB (Corrëa Lelles Nogueira et al., 2005). Among esters, hexanoic acid, ethyl ester was the most abundant in all three Montasio cheese samples (C-dM1-no, C-dM2-no, C-dM3-no). McSweeney and Sousa (2000) also reported that hexanoic acid, ethyl ester was one of the most abundant esters in hard type cheese such as Parmigiano-Reggiano. Besides hexanoic acid, ethyl ester, ethyl acetate was also mentioned as one of the significant volatile components in cheese (Singh, Drake and Cadwallader, 2003). In our case it was the most predominant in C-dM3-no.

Octanal and 3-methyl-butanal were prevailing volatiles among aldehydes in all three cheese samples, which is in accordance with their recognition as flavour compounds in various cheeses (Marilley and Casey, 2004).

Part III: Influence of type of milk

Results presenting the effect of type of milk on VCs in cheese are given in Table 3, namely columns 1 and 2 for ewe's cheese, columns 5 to 7 for cow's cheese and column 8 for goat's cheese. We compared cheeses without the addition of a SC.

The total mean level of esters, alcohols and aldehydes was higher in cow cheeses (Table 3, columns 5, 6 and 7) compared to goat (Table 3, column 8) and ewe cheeses (Table 3, columns 1 and 2). On the other hand, analysis of goat cheese revealed the highest mean level of total fatty acids. Moreover, hexanoic and octanoic fatty acids predominated in goat cheeses, while decanoic fatty acid was only present in goat cheese. These fatty acids are typical components in goat milk and consequently in goat cheese where they significantly contribute to the formation of specific goaty flavour (Morgan and Gaborit, 2001).

Besides esters, which were identified in cheeses (Table 3), only the butanoic acid ethyl ester and hexanoic acid ethyl ester were present in all cheese samples regardless the type of milk (cow, goat, ewe) used for the cheese production. However, the butanoic acid ethyl ester contributes to fruity, apple like note (Frank et al., 2004). Only in cow milk cheeses two alcohols, 1-hexanol, 2-ethyl- and hexanol, were identified and both have their origins in milk fat (Alewijn, 2006).

Part IV: Milk heat-treatment

Heat treatment of milk was also found to affect the formation of VC profiles. VCs identified in volatile fraction of Montasio cheeses (C-dM1-no, C-dM2-no, C-dM3-no), produced from raw cow milk (Table 3, columns 5, 6 and 7), were compared to VCs identified in volatile fraction of Nanos cheese, made from thermised cow milk (Table 3, column 9). Le Quèrè and Molimard (2002) reported that the 2-phenylethanol plays an important and dominant role in raw milk cheese, which was also the case in our study. 2-phenylethanol (Table 3, line 35) was the most abundant alcohol detected and identified only in raw cow Montasio cheese. δ-decalactone was present in both cheese samples with a higher value in thermised cow milk cheese; δ-octalactone was only identified in thermised cow milk, which indicates that lactones can be formed by heating milk fat (Alewijn, 2006). δ-octalactone has coconut-like and frutty note (Curioni and Bosset, 2002).

Nevertheless, the whole group of esters was both qualitatively and quantitatively the lowest in Nanos cheese made from thermised milk, what was expected due to the natural characteristic of lower

boiling point of esters compared to other VCs. In general, it is known that there should be more esters in cheeses made from raw milk than in cheeses made from heat-treated milk (Gioacchini et al., 2010). Concentrations of other VCs were in the same range when raw milk and thermised milk cheeses are compared.

Conclusions

The impact on formation of VCs, which have effect on the cheese flavour, is difficult to determine precisely due to the complexity of the cheese system. Nevertheless, we proved the differences, in both quantity and quality of some important VCs in cheese, taking the effect of different factors into consideration, such as the addition of a SC, type of milk (cow, ewe, goat), environment and the heat-treatment of milk.

The difference in ewes' cheeses made with and without a SC, indicated that the amount of VCs is directly affected by the SC used. The addition of a SC affects the formation of a higher total amount of VCs, especially because of a higher amount of fatty acids (butanoic, hexanoic and octanoic acid) and ketones (2-pentanone, 2-octanone, 2-nonanone). We also observed the presence of the 2-heptanone only in ewe cheese sample with the addition of SC. In cheese samples with a SC which included more species of thermophilic bacteria, analysis of volatile fractions resulted in a higher amount of total fatty acids and ketones.

The part of the experiment where we examined the effect of the environment, revealed altogether 40 VCs, but their amount and presence differed between the three samples of same type of Montasio cheese made in three different dairy plants. Our results are supported by the literature data indicating the importance of the environment on the final cheese aroma.

Among volatiles identified in goat cheese the highest amount of hexanoic and octanoic acid was determined in comparison to ewe and cow cheese. Moreover, decanoic acid was only identified in goat's cheese. These fatty acids form a typical goaty flavour in cheese. Besides alcohols 1-hexanol, 2-ethyl- and hexanol were only present in cow cheeses.

The effect of the heat treatment of milk revealed the presence of 2-phenylethanol, which was identified only in Montasio cheese, whereas in thermised milk Nanos cheese was absent, but δ-octalactone was present, which has coconut-like and fruity note. The overall amount of all esters was lower in thermised milk cheese.

The results of our study highlighted the effect of different factors that greatly contribute to the formation of the VC profiles in cheeses. Therefore, in order to preserve typical characteristics of a certain type of cheese, which is particularly important for PDO cheeses, cheesemakers should follow standard manufacturing protocols and control some parameters that could influence the final cheese aroma.

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