PAPER

EVALUATION OF LIPOLYSIS AND VOLATILE COMPOUNDS PRODUCED BY THREE *PENICILLIUM ROQUEFORTI* COMMERCIAL CULTURES IN A BLUE-TYPE CHEESE MADE FROM OVINE MILK

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ABSTRACT

The aim of this work was to compare the effect of three different *Penicillium roqueforti* commercial cultures (named PS1, PS2 and PS3) on proteolysis, lipolysis and volatile flavour profile of a blue cheese made from ovine milk and lamb paste rennet. Proteolytic parameters were not significantly affected by the *Penicillium roqueforti* culture, while cheeses manufactured using PS2 and PS3 cultures showed the higher amount of free fatty acids (FFA) and volatile FFA when compared with PS1 culture after 30 days of ripening. This study can provide important information for obtaining the desired extent of lipolysis in this type of blue cheese.

- Keywords: lipolysis, ovine blue cheese, *Penicillium roqueforti*, volatile compounds -

INTRODUCTION

Blue cheeses represent a cheese variety characterised by the presence of blue or blue-green veins, caused by induced growth of the mould *Penicillium roqueforti* within the cheese matrix. This category includes, among others, PDO (protected designation of Origin) or PGI (protected geographical indication) cheeses made from bovine (Gorgonzola, Italy; Danablu, Denmark; Stilton, United Kingdom), and ovine (Roquefort, France) milk. The manufacture process of blue mould cheeses has been well described previously (ARDÖ, 2011), but it can vary depending on country or region where cheese is produced. In particular in Sardinia island (Italy), a small production of ovine blue cheese is manufactured on industrial scale. This cheese is characterized by the use of lamb paste rennet for its production, differently from most of blue cheeses, where the milk coagulation is usually induced by the action of liquid rennet. Cheeses produced with paste rennet are characterized, at late ripening stages, by high amounts of free fatty acids due to the presence of lipolytic enzymes (lipases) in the rennet extract (ADDIS *et al.*, 2005; VIRTO *et al.*, 2003).

The ovine blue cheese is made following the process described herein. Thermised whole ovine milk is inoculated with a *Penicillium roqueforti* culture and a mesophilic starter at 36°C. Milk is coagulated using a water solution of lamb paste rennet, and the coagulum is cut into small granules (about 4 mm in size), drained, moved into moulds, dry salted and ripened for 30 days at 10°C and 85% of relative humidity. Cheeses are pierced using a stainless steel needle 7 days after production. At the end of ripening (30 days) cheeses are cylindrical in shape (height and diameter around 100 and 200 mm, respectively) and weigh between 2.5 and 3.0 kg.

The growth of *Penicillium roqueforti* within the cheese matrix results in a high production of its extracellular enzymes, and consequently in an extensive secondary proteolysis and lipolysis of blue cheeses during ripening (CALZADA *et al.*, 2013; CONTARINI and TOPPINO, 1995; PRIETO *et al.*, 1999; 2000).

Furthermore, blue cheeses are characterised by an high level of flavour compounds produced by lipid, lactose and protein catabolism (ARDÖ, 2011); in particular a large amount of methyl ketones is produced by the β-oxidation of free fatty acids followed by a decarboxylation reaction (QIAN *et al.*, 2002; VOIGT *et al.*, 2010).

The aim of this work was to compare the effect of three different *Penicillium roqueforti* commercial cultures on proteolysis, lipolysis and volatile flavour profile of Sardinian ovine blue cheese after 30 days of ripening, in order to provide useful information to cheese makers about the biochemical effects produced by each culture during ripening of this cheese.

MATERIALS AND METHODS

Mould cultures

Three commercially available *P. roqueforti* cultures, named PS1 (PRB 6 HYP 5 D, Danisco Deutschland GmbH, Niebull, Germany), PS2 (PR4, Chr. Hansen, Hørsholm, Denmark) and PS3 (PV LYO 10 D, Danisco Deutschland GmbH, Niebull, Germany), were separately used to produce blue-type cheeses. More details about the specific properties of each mould culture can be found in the respective product description documents provided by the Supplier.

Mould cultures were dissolved in water and added to 50 L of milk before renneting at a final concentration of 5.0E+6 CFU per L of milk.

Small-scale cheese-making

Cheese production was performed at the dairy technology laboratories of Agris Sardegna (Olmedo, Italy). Whole ovine milk was placed in a staining steel cheese vat, batch-heated at 65°C in 10 min, and quickly cooled down to 36°C (in 5 min). After cooling, a mould culture and a milk starter culture, prepared using a freeze-dried mixed culture (Bulk Set HM M4 LYO, Danisco, Deutschland GmbH, Niebull, Germany; 1 L·100 L^{-1} of milk), were added to milk. The composition of the milk starter culture was: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar. diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*. Milk was coagulated using a water solution (30 g·100 L^{-1} of milk) of lamb paste rennet (Caglificio Manca, Thiesi, Italy). About 45 min after the addition of rennet, the coagulum was cut into small granules (about 4 mm in size), and the drained curd was moved into moulds and kept at 20-25°C under saturated humidity conditions for 18 h. Cheeses were then kept at 10°C for 24 h and 90-95% of relative humidity, dry salted and finally ripened for 30 days at 10°C and 85% of relative humidity. Cheeses were pierced using a stainless steel needle 7 days after production. Three replicates were carried out for each treatment level, for a total of nine cheese-making trials.

Cheese composition and nitrogen fractions

Samples were taken for analysis 1 day after production and after 30 days of ripening. Cheeses were analysed for pH (pH meter 420 A, Orion, Boston, USA), total solids (TS) (ISO, 2004), fat (Soxhlet method), total nitrogen (TN) (IDF, 1993), soluble nitrogen at pH 4.6 (SN), soluble nitrogen in 12% trichloroacetic acid (TCA-SN), and soluble nitrogen in 10% phosphotungstic acid (PTA-SN) (GRIPON *et al.*, 1975).

Free fatty acids analysis

The free fatty acids content was determined as previously described by ADDIS *et al.* (2005) on cheeses 1 day after production and after 30 days of ripening.

Volatile flavour profile analysis

Volatile compounds were determined by SPME (solid-phase microextraction) -GC-FID/MS on cheeses after 30 days of ripening. 0.5 g of freshly grated cheese was placed in a 10 mL vial, hermetically sealed with a seal and thin Viton septa. The vials were held at 37°C in a thermostated autosampler (8200 CX Varian, Walnut Creek, CA, USA) for 5 min to reach equilibrium between sample and above headspace prior to SPME headspace sampling. A divinylbenzene (DVB)/carboxen (CAR)/polydimethylsiloxane (PDMS) 50/30 μm fiber (Supelco Inc., Bellefonte, PA, USA) was exposed to headspace under constant stirring for 7 min in samples after 30 days of ripening. During headspace sampling, samples were maintained at 37°C, and volatile compounds adsorbed on the fiber were immediately thermally desorbed in the injector port of a 3800CX Varian GC (Walnut Creek, CA, USA) equipped with a 1077 split/splitless injector (250°C), coupled with a flame ionization detector (FID; 250°C), and a Saturn 2000 ion trap mass spectrometer system (MS detector) (Walnut Creek, Varian, CA, USA). Volatile compounds were injected in splitless mode in two identical capillary columns (DB-WAX 30 m, 0.32 mm i.d., 0.25 μm film thickness; J. & W. Scientific, Folsom, CA, USA) connected one to FID and the other to mass spectrometer. The column was operated with Helium (1 $mL \cdot min^{-1}$, constant flow), and the column temperature was held at 40°C for 3 min, then increased to 200°C at a rate of $4°C \cdot min^{-1}$, and finally held at 200°C for 5 min. MS detector was programmed in Electron Ionization (EI) mode at an ionization voltage of 70 eV in the acquisition range between 20-300 m/z, and at a scan rate of 1 scan/sec. The trap, manifold and transfer line temperature were set to 200°, 80° and 200°C respectively. Volatile compounds were identified by comparison of their mass spectral data with those of the NIST 98 library (NIST, USA), by their linear retention indexes (VAN DEN DOOL and KRATZ, 1963) and by comparison with authentic standard compounds (when available).

Statistical analysis

Statistical treatment of data was performed using the SPSS statistical package, release 11.5 (SPSS, Chicago, IL, USA). Data of chemical composition, nitrogen fractions and free fatty acids were examined using a bifactorial ANOVA model with "*P. roqueforti* culture factor" (PC) and "ripening stage factor" (R) as fixed effects, while LSD test (least significant difference test, $P < 0.05$) for multiple comparisons was used to separate treatment means. The results of volatile compounds were examined using a monofactorial ANOVA model with "*P. roqueforti* culture factor" (PC) as fixed effect.

RESULTS AND DISCUSSION

Chemical composition and nitrogen fractions

The chemical composition of cheeses 1 day after production and after 30 days of ripening is reported in Table 1. Gross composition was not significantly affected by the *Penicillium roqueforti* culture at 1 day and after 30 days, whereas it changed significantly ($P < 0.05$) during the ripening (with the exception of protein content). The values of moisture, fat and protein to total solids ratio after 30 days of ripening were in agreement with data reported by LAWLOR *et al.* (2003) for other blue-type cheeses. pH values increased of around 1.4 units for all treatments from day 1 to day 30, probably as consequence of the consumption of lactate and the oxidative formation of NH_3 from amino acids operated by moulds during ripening (CANTOR *et al.*, 2004). It has been seen, for example, that the pH may increase of around 2 units in Danablu during the first 5 weeks of ripening (ARDÖ, 2011).

The data reported in Table 1 indicate that all proteolytic parameters increased significantly throughout ripening ($P < 0.05$). The level of pH 4.6-SN (expressed as a percentage of total nitrogen) ranged from 31.03 to 33.97% after 30 days of ripening (Table 1), in some agreement with results published for a number of different blue-type cheeses with longer ripening times (from 34% up to 72% of pH 4.6-SN; FERNÁNDEZ-SALGUERO *et al.*, 1989; LAWLOR *et al.*, 2003; VOIGT *et al.*, 2010). The level of secondary proteolysis is higher in blue cheeses compared to other cheese varieties (CANTOR *et al.*, 2004); therefore, the raised values at 30 days reported here for TCA-SN and PTA-SN (both expressed as a percentage of total nitrogen; Table 1), compared with the values of pH 4.6-SN, highlighted that the most of the soluble fraction included non-protein substances (FERNANDEZ-SALGUERO *et al.*, 1989).

Free fatty acids analysis

Table 2 summarises the extent of lipolysis (expressed as mmol of free fatty acids per kg of cheese) of cheeses made with each of the three *Penicillium roqueforti* cultures both 1 day and 30 days after production.

The type of *Penicillium roqueforti* culture significantly influenced ($P < 0.05$) the amount of short-chain (C4:0-C8:0), medium-chain (C10:0-C14:0), and long-chain (C16:0-C18:3) FFA. Overall, all individual FFA increased significantly ($P <$

Table 1 - Composition and nitrogen fractions of cheeses (PS1, PS2, PS3) 1 day after production and after 30 days of ripening.

	1 day			30 days			SEM[e]	F test[f]	
	PS1	PS2	PS3	PS1	PS2	PS3		PC[g]	R[h]
pH	4.85	4.82	4.83	6.35	6.38	6.05	0.18	NS	*
Moisture (g.100g^{-1})	49.26	49.63	50.04	42.28	42.97	42.30	0.89	NS	*
Fata	55.02	54.93	54.88	56.15	56.63	55.60	0.26	NS	*
Protein[a]	38.25	38.02	38.19	37.50	37.67	37.42	0.16	NS	NS
pH 4.6-SN[b]	8.92	8.84	9.25	31.03	33.97	32.10	2.89	NS	*
TCA-SN[c]	5.40	5.60	5.65	28.28	30.86	29.42	2.93	NS	*
PTA-SN[d]	1.11	1.23	1.49	12.92	13.05	11.85	1.42	NS	*

[a]Expressed as a percentage of total solids (% w/w).
[b,c,d]Expressed as a percentage of total nitrogen (% w/w); [b]Soluble nitrogen at pH 4.6; [c]Soluble nitrogen in 12% trichloroacetic acid (TCA); [d]soluble nitrogen in 10% phosphotungstic acid (PTA).
[e]Standard error mean.
[f]Significant differences: * $P < 0.05$; NS, no significant differences.
[g]*P. roqueforti* culture factor.
[h]Ripening stage factor.

Table 2 - Free fatty acids content (mmol.kg^{-1} of cheese) in cheeses (PS1, PS2, PS3) 1 day after production and after 30 days of ripening.

	1 day			30 days			SEM[b]	F test[c]	
	PS1	PS2	PS3	PS1	PS2	PS3		PC[d]	R[e]
C4:0a	0.47	0.57	0.74	16.07	28.28	31.65	3.37	*	*
C6:0	0.11	0.14	0.17	3.89	9.46	11.55	1.19	*	*
C8:0	0.20	0.20	0.20	2.74	5.50	6.85	0.68	*	*
C10:0	0.15	0.17	0.21	5.83	10.93	13.81	1.39	*	*
C12:0	0.11	0.11	0.13	2.56	4.64	5.96	0.59	*	*
C14:0	0.21	0.22	0.23	6.53	9.11	12.68	1.25	*	*
C16:0	0.46	0.45	0.47	11.88	15.20	22.90	2.24	*	*
C18:0	0.12	0.11	0.11	2.71	3.51	4.98	0.49	*	*
C18:1	0.34	0.32	0.31	17.09	27.38	32.14	3.31	*	*
C18:2	0.07	0.05	0.02	2.62	3.97	4.51	0.47	*	*
C18:3	0.02	0.02	0.02	2.08	2.85	3.88	0.39	*	*
TFFAs	2.26	2.41	2.61	74.00	120.83	150.90	15.16	*	*

[a]C4:0, butyric acid; C6:0, caproic acid; C8:0, caprylic acid; C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; TFFAs, total free fatty acids.
[b]Standard error mean.
[c]Significant differences: * $P < 0.05$.
[d]*P. roqueforti* culture factor.
[e]Ripening stage factor.

0.05) during ripening, and cheeses manufactured with the cultures named PS2 and PS3 showed the higher amount of each FFA after 30 days of ripening when compared with PS1 culture. Three free fatty acids (C4:0, C16:0, C18:1) represented around 60% of total free fatty acids for all treatments both 1 day and 30 days after production. After 30 days of ripening, the content of C4:0, C16:0, and C18:1 ranged on average (depending on the culture tested) from 16.07 to 31.65 mmol.kg^{-1}, from 11.88 to 22.90 mmol.kg^{-1}, and from 17.09 to 32.14 mmol.kg^{-1} of cheese, respectively. In general, the action of *Penicillium roqueforti* lipases releases higher concentrations of long-chain FFA than short- and medium-chain FFA (ARDÖ, 2011). A recent study reported that palmitic and oleic acids reached the highest levels of long-chain FFA in a blue cheese (CALZADA *et al.*, 2013). On the contrary, the values of butyric acid presented in the present study were higher than those found by other authors in different blue cheese varieties (CALZADA *et al.*, 2013; WOO *et al.*, 1984). In particular, PS1, PS2 and PS3 cheeses after 30 days of ripening showed about 1.8-3.6 times higher values of C4:0 when compared with results of CALZADA *et al.* (2013), who reported that the content of butyric acid in a blue cheese after 90 days of ripening reached a value of 1.32 mg.g^{-1} (14.98 mmol.kg^{-1}) of cheese dry matter. The raised values of C4:0 and, in general, of short-chain FFA observed in the present study can be ascribed to the use of lamb paste

rennet for milk coagulation. Lamb paste rennet contains a pregastric lipase, which preferentially hydrolyzes short chain fatty acids (KIM HA and LINDSAY, 1993). Furthermore, it is important to point out that even the lipolytic system of *Penicillium roqueforti* can exhibit a selectivity similar to that of the pregastric lipase (KIM HA and LINDSAY, 1993).

SPME analysis

Cheeses (PS1, PS2 and PS3) after 30 days of ripening were subjected to volatile flavour profile analysis by SPME-GC-FID/MS (Table 3). A total of 34 volatile compounds were identified in cheese samples, and among them only some volatile FFA (butyric, pentanoic and hexanoic acids) and some alcohols (2-pentanol, 1-pentanol and phenyl ethyl alcohol) were significantly affected by *Penicillium roqueforti* culture ($P < 0.05$). Ketones and acids represented almost the totality of volatile fraction and resulted as more abundant in all samples (about 70 and 27%, respectively). Among ketones, 2-heptanone and 2-nonanone presented the highest values of FID Peak Area (Table 3). These results were in agreement with those reported in literature relating to concentration of ketones in this category of cheeses (ARDÖ, 2011; CANTOR *et al.*, 2004). The presence of ketones is correlated to the typical flavour of blue cheeses, and they are produced by the β-oxidation of free fatty acids followed by a decarboxylation reaction.

Table 3 - Volatile compounds (FID Peak Area) in cheeses (PS1, PS2, PS3) after 30 days of ripening.

LRI[a]		PS1	PS2	PS3	SEM[b]	PC[c]
839	2-propanone	275,452	196,359	179,488	70,535	NS
926	2-butanone	4,681	3,307	3,295	671	NS
1,004	2-pentanone	949,455	706,739	742,756	108,431	NS
1,107	2-hexanone	22,208	12,493	17,289	2,347	NS
1,211	2-heptanone	1,791,884	1,299,484	1,624,590	146,423	NS
1,315	2-octanone	28,394	19,954	30,148	4,275	NS
1,333	3-hydroxy 2-butanone	7,969	8,587	5,481	1,753	NS
1,422	2-nonanone	1,047,296	678,913	1,204,917	187,792	NS
1,481	8-nonen 2-one	78,175	55,547	87,753	12,625	NS
1,631	2-undecanone	13,624	3,499	11,418	3,510	NS
1,690	acetophenone	362	183	295	95	NS
	Ketones	4,219,501	2,985,068	3,907,431	493,937	NS
1,581	propanoic acid	1,805	2,052	2,763	435	NS
1,609	2-methyl propanoic acid	951	1,141	1,400	139	NS
1,671	butyric acid	407,334	1,137,832	957,216	136,292	*
1,712	3-methyl butyric acid	6,539	3,390	4,240	912	NS
1,787	pentanoic acid	2,476	8,945	6,831	1,142	*
1,889	hexanoic acid	314,878	673,581	540,975	63,090	*
1,990	heptanoic acid	1,313	2,965	2,445	363	NS
2,103	octanoic acid	62,326	102,518	81,564	8,519	NS
2,316	decanoic acid	8,299	17,473	11,960	1,935	NS
	Acids	805,921	1,949,897	1,609,395	208,018	*
910	ethyl acetate	293	176	125	36	NS
1,057	ethyl butyrate	5,024	6,800	3,500	1,703	NS
1,256	ethyl hexanoate	9,744	15,343	10,222	2,315	NS
1,459	ethyl octanoate	11,645	28,596	26,947	4,415	NS
	Esters	26,511	50,914	40,794	7,354	NS
945	2-propanol	10,390	2,791	7,434	2,096	NS
1,137	2-pentanol	75,497	24,527	45,705	9,397	*
1,270	1-pentanol	2,115	281	0	368	*
1,344	2-heptanol	90,554	34,925	52,055	14,579	NS
1,538	2-nonanol	8,074	2,898	8,414	2,048	NS
1,575	2,3-butanediol	1,402	2,296	2,664	264	NS
1,950	phenyl ethyl alcohol	236	0	0	30	*
2,047	phenol	323	322	341	31	NS
	Alcohols	188,591	68,368	117,699	26,439	NS
1,301	styrene	2,537	5,351	2,784	940	NS
	Hydrocarbons	2,537	5,351	2,784	940	NS
940	3-methylbutanal	229	403	245	66	NS
	Aldehydes	229	403	245	66	NS
	Totals	5,243,291	5,060,001	5,678,349	398,884	NS

[a]Linear Retention Indexes using a DB-WAX column.
[b]Standard error mean.
[c]*P. roqueforti* culture factor, significant differences: * $P < 0.05$; NS, no significant differences.

The reaction is catalysed by enzymes contained both in spores and mycelium of *Penicillium spp.* (QIAN *et al.*, 2002; VOIGT *et al.*, 2010).

Table 3 also highlights that PS1 presented significantly lower values of acids than other cheeses. This was consistent with what discussed above concerning the higher lipolytic activity of PS2 and PS3 compared to PS1 (Table 2), and was also probably due to the greater aptitude of PS1 in converting FFA to 2-alkanones.

Among volatile FFA, butyric acid was the most abundant (50, 58 and 59% of total FFA in PS1, PS2 and PS3, respectively) followed by hexanoic (39, 35 and 34% of total FFA in PS1, PS2 and PS3, respectively) and octanoic (8, 5 and 5% of total FFA in PS1, PS2 and PS3, respectively) acids. The origin of raised values of butyric acid has been discussed in the previous section, while hexanoic and octanoic acids are important flavor compounds of blue cheeses (ARDÖ, 2011).

Esters are produced by free fatty acids esterification with primary alcohols, and may attenuate the typical pungent flavour of blue cheeses due to the methyl ketones (MOIO *et al.*, 2000). They represented only 1% on average of totals volatile compounds (Table 3); PS2 and PS3 showed higher FID Peak Area (but without any statistical significance) of esters as a consequence of their higher content of FFA compared to PS1.

The strong reducing environment present in ripened cheese favoured the production of 2-alkanols from corresponding 2-alkanones. PS1 showed significantly higher values of 1- and 2-pentanol when compared with other samples ($P < 0.05$), and tended to have the highest levels both of 2-alkanones and 2-alkanols. The parallel evolution of these volatile compounds was previously observed in other blue cheeses (GONZÁLES DE LLANO *et al.*, 1990).

CONCLUSIONS

The results indicated that the ovine blue cheese made in Sardinia was more subjected to lipolysis and presented higher amounts of short chain fatty acids when compared to the most known blue cheese varieties. This evolution of lipolysis in the product was also due to the use of lamb paste rennet. Two cultures (PS2 and PS3) were characterised by the highest values of total free fatty acids. In contrast, proteolytic parameters and the most volatile compounds did not vary significantly depending on the culture tested.

In conclusion, this study may provide valid information about the use of the appropriate culture for managing the ripening process (with particular regard to lipolysis) also in blue cheeses different from that studied here.

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