



## SHORT COMMUNICATION

# Proteolytic volatile compounds in milk and cheese of cows fed dried olive pomace supplementation

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**ABSTRACT.** The aim of the study was to evaluate the effect of dietary inclusion of dried olive pomace (DOP) on proteolytic volatile compounds in raw milk and pasteurised milk cheese. Two groups of lactating cows (20 animals in each) were randomly assigned to isoenergetic and isoproteic treatments provided as control mixed ration (CON) and as CON with addition of 2 kg of DOP/day/animal (EXP). DOP partially replaced maize silage, alfalfa hay, sunflower and wheat. After 60 days of feeding, raw bulk milk was collected, sampled, pasteurised and used to produce cheese that was sampled at days 1, 7 and 30 of maturation. Samples were analysed by solid phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS) and data were expressed considering the relative peak area. In raw milk the phenylacetaldehyde and 2-phenylethyl alcohol, the only detected phenylalanine catabolites, were increased by dietary treatment by 61.9 and 235%, respectively ( $P < 0.01$ ). In CON and EXP cheeses the compounds derived from phenylalanine, leucine and methionine catabolism were present being generally higher in EXP cheese during ripening time. In particular, among the volatile compounds, the contents of which were higher, were: the phenylacetaldehyde – 32.6% and 34% higher at days 7 and 30 ( $P < 0.01$  and  $P < 0.05$ , respectively); the 2-phenylethyl alcohol – 229% higher at day 7 ( $P < 0.001$ ), the 3-methyl-1-butanol – 299% and 59.1% higher at days 7 and 30 ( $P < 0.001$  and  $P < 0.05$ , respectively), the isoamyl butyrate – 58.2% higher at day 30 ( $P < 0.05$ ) and the isoamyl isopentanoate – 290% and 60.1% higher at days 7 and 30 ( $P < 0.01$  and  $P < 0.05$ , respectively). Dietary DOP addition may affect aroma of dairy products, however the sensory evaluation panel test is needed to complete the feedback supporting industry choices.

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## Introduction

Flavour is a sensory characteristic that may affect dairy products quality and acceptability. Several factors such as species, diet, management and environment strongly influence the aromatic profile of raw milk (Moio et al., 1993a; Bergamaschi et al., 2015) and related cheese (Povolo et al., 2007). In addition, cheese flavour depends on milk treatment (i.e., pasteurisation and homogenisation), starter

bacteria, cheese manufacture and ripening length and conditions (Murtaza et al., 2014).

Conversion of lactose and citrate, lipolysis and proteolysis are the main chemical and biochemical processes involved in the development of aroma in dairy products. Among these processes, proteolysis of caseins is an important biochemical pathway responsible for the formation of flavour and texture in hard- and semi hard-type cheeses (van Kranenburg et al., 2002). During ripening, proteolysis is performed

by rennet, native milk enzymes, starter bacteria and non-starter bacteria (McSweeney and Sousa, 2000). Branched chain amino acids (leucine, isoleucine, valine), aromatic amino acids (phenylalanine, tyrosine, tryptophan) and methionine are thought to be the precursors of important volatile compounds in dairy products (Yvon and Rijnen, 2001).

Olive pomace, a by-product of olive oil production, may be used in feeding operations and its positive effect on quality of dairy products is reported in various studies (Vargas-Bello-Pérez et al., 2013; Zilio et al., 2014). Although some studies on the effect of olive pomace on pooled volatile compounds developed in dairy products were performed (Caputo et al., 2015; Castellani et al., 2017), none information is available about single volatiles produced by proteolytic processes.

Thus, the goal of the present study was to investigate the possible effect of dried olive pomace (DOP) inclusion into dairy cow diet on the development of volatile compounds derived from proteolysis in raw milk and pasteurised milk cheese at different time of ripening.

## Material and methods

The study was conducted according to the Directive 2010/63/EU of the European Parliament (European Union, 2010) and the Directive 86/609/EEC (European Economic Community, 1986) regarding the protection of animals used for experimentation or other scientific purposes.

### Animals, dietary treatments and samples collection

The experimental design and dietary treatment have been previously reported in Castellani et al. (2017). Briefly, twenty Holstein Friesian dairy cows were selected to form two groups (ten animals in each) homogeneous for milk yield ( $26.96 \text{ kg} \pm 2.10$  vs  $27.73 \text{ kg} \pm 3.51$  for CON and EXP, respectively), parity ( $2.10 \pm 0.88$  vs  $2.30 \pm 0.82$  for CON and EXP, respectively) and days in milk (DIM;  $118 \pm 34$  vs  $121 \pm 42$  for CON and EXP, respectively). Each group was randomly allocated to one of the following two diets: a control diet (CON) and a control diet supplemented with 2 kg DM/animal/day of DOP (EXP). The olive pomace derived from pressing mill for olive oil production was dried in a heated chamber at  $35^\circ\text{C}$  and stored at room temperature until use.

Diets were offered as total mixed ration (TMR, 20 kg of DM per animal/day) and were composed of maize silage and alfalfa hay as forage and maize,

soybean, sunflower and wheat as meal. DOP partially replaced maize silage, alfalfa hay, sunflower and wheat, resulting in a forage:concentrate ratio of 66:34 and 54:46 in the CON and EXP diets, respectively. Diets were isoenergetic ( $0.92$  vs  $0.90$  milk UFL/kg of DM for CON and EXP groups, respectively) and isoproteic ( $15.31$  vs  $15.29\%$  for CON and EXP groups, respectively).

The trial lasted 74 days, with the first 14 days of adaptation to the feeding regimen and the following 60 days of treatment. At the day 60 of the treatment, three samples of raw bulk milk for each group were taken and stored at  $-20^\circ\text{C}$  until analysis. The remain bulk milk was pasteurised ( $72^\circ\text{C}$  for 15 s) and used for the manufacture of cheese. Mesophilic (*Lactococcus lactis* subsp. *lactis* and *diacetylactis*) and thermophilic (*Streptococcus thermophilus*) starter bacteria (3 g/100 kg milk for each, CSL Sacco System, Lodi, Italy) and liquid calf rennet (15 ml/100 kg of milk as 75% of chymosin and 25% of pepsin; 1:18000 strength; Clerici, Cadorago, Italy) were used. Cheeses were brined in a 25% NaCl water solution for 2 h and stored at  $10^\circ\text{C}$  and 85% relative humidity during maturation.

Three cheeses for group were sampled at days 1, 7 and 30 of ripening ( $T_1$ ,  $T_7$  and  $T_{30}$ , respectively).

### Volatile compounds extraction and GC-MS analysis

The extraction of volatile compounds from milk and cheese was performed by a solid-phase micro-extraction (SPME) and then analysed using a gas-chromatograph coupled with a mass spectrometry (GC-MS). Milk (10 ml) or grated cheese (5 g) were mixed with a saturated NaCl solution and then 4-methyl-2-heptanone was added as internal standard solution. The SPME was performed by exposing a  $50/30 \mu\text{m}$  of divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB/CAR/PDMS Supelco, Bellefonte, PA, USA) into the headspace of a capped vials with a PTFE septum for 30 min for milk and 1 h for cheese at  $60^\circ\text{C}$  and in stirring conditions.

Desorption of volatile compounds was obtained into the splitless injector of the GC system set at  $250^\circ\text{C}$  for 1 min. The gas-chromatograph (Clarus 580; Perkin Elmer, Waltham, MA, USA) was coupled with a mass spectrometer (SQ8S; Perkin Elmer, Waltham, MA, USA) and equipped with a PE-ELITE-5MS  $30 \times 0.25 \text{ mm}$ ,  $0.25 \mu\text{m}$  column (Perkin Elmer, Waltham, MA, USA). The oven temperature was  $50^\circ\text{C}$  for 1 min, then was increased to  $200^\circ\text{C}$  at  $3^\circ\text{C}/\text{min}$  for 1 min and to  $250^\circ\text{C}$  at  $15^\circ\text{C}/\text{min}$ , hold for 15 min. The carrier gas was helium at

1 ml/min. Source and interface temperature were held at 250 °C. The mass detector operated in electronic impact mode (70 eV) and data were acquired in full scan mode (range 35–350 m/z, dwell time 0.2 s/scan). The volatile compounds were identified by comparing their mass spectra with those of the National Institute of Standards and Technology library (NIST, Gaithersburg, MD, USA) and comparing the eluting order with Kovats retention indexes. The isoamyl butyrate and isoamyl isobutyrate were identified comparing the mass spectra and the retention time of authentic standard compounds (Sigma Aldrich, St. Louis, MO, USA). Samples were analysed in triplicate and quantification was carried out considering the relative peak area expressed as Arbitrary Unit (AU, target ion area  $\times 10^{-3}$ ).

### Statistical analysis

Volatile compounds in raw milk and pasteurised milk cheeses were analysed by one-way analysis of variance (ANOVA). Diet was considered as fixed factor in the model. Data of cheeses at days 1, 7 and 30 of ripening were independently performed, ripening effect was not tested and three datasets were separately processed. Means separation was assessed by Tukey's test and differences were declared significant at  $P < 0.05$  using SAS software (version 9.0; SAS Institute Inc., Cary, NY, USA).

### Results and discussion

Volatile compounds were detected in raw milk and pasteurised cheeses from both groups (CON and EXP). Two volatile compounds derived from phenylalanine catabolism were observed in raw milk, while those derived from phenylalanine (2), leucine

(5) and methionine (1) catabolisms were identified in pasteurised milk cheeses.

Data indicated an effect of DOP on the development of proteolytic volatile compounds in raw milk and pasteurised milk cheeses. Various volatiles were significantly higher in EXP dairy products. This result may be partially related to the higher level of protein observed in EXP raw milk (Castellani et al., 2017).

Phenylacetaldehyde (42 vs 68 AU for CON and EXP, respectively) and 2-phenylethyl alcohol (20 vs 67 AU for CON and EXP, respectively), catabolites derived from metabolism of phenylalanine, were higher in EXP raw milk than in CON one ( $P < 0.01$  for both compounds). As indicated by McSweeney and Sousa (2000) the phenylacetaldehyde metabolism in dairy products may occur by non-enzymatic Strecker degradation of phenylalanine or by enzymatic transamination of phenylalanine as imide that is subsequently degraded to aldehyde. The presence of 2-phenylethyl alcohol in EXP raw milk was due to the phenylacetaldehyde reduction. Further studies are needed to well understand the relationship between olive pomace supplementation and the pattern of free amino acids in raw milk as well as the mechanisms involved in the production of proteolytic compounds.

Volatile compounds detected in cheese at different times of ripening are shown in Table 1. Regarding the content of phenylalanine metabolites, no statistical changes were observed in  $T_1$  samples, whereas at day 7 of ripening both phenylalanine catabolites were higher in EXP cheese ( $P < 0.01$  for phenyl acetaldehyde and  $P < 0.001$  for 2-phenylethyl alcohol). Later, at  $T_{30}$  (commercial time of cheese maturation) the phenylacetaldehyde was still higher in EXP cheese ( $P < 0.05$ ) while no differences were detected for the 2-phenylethyl alcohol ( $P > 0.05$ ).

**Table 1.** Proteolytic volatile compounds in cheese obtained from milk produced by cows fed control diet (CON) and control diet supplemented with 2 kg (as DM) of dried olive pomace (EXP)

Volatile compounds	T.I.	Ripening time <sup>1</sup>											
		$T_1$				$T_7$				$T_{30}$			
		CON	EXP	SEM	P-value	CON	EXP	SEM	P-value	CON	EXP	SEM	P-value
<b>Phenylalanine</b>													
phenylacetaldehyde	91	419	1112	212	ns	4141	5491	204	**	2868	3843	205	*
2-phenylethyl alcohol	91	2573	11267	3907	ns	4028	13245	526	***	28642	18841	6922	ns
<b>Leucine</b>													
$\alpha$ -ketoisocaproic acid	85	59	1145	167	**	325	1040	44	***	360	1115	93	**
methyl $\alpha$ -hydroxy isocaproate	87	361	2331	342	*	669	3142	103	***	743	3524	91	***
3-methyl-1-butanol	71	2816	7372	1394	ns	5411	21610	777	***	13551	21563	1722	*
isoamyl butyrate	71	nd	nd	–	–	nd	nd	–	–	421	666	43	*
isoamyl isopentanoate	71	nd	nd	–	–	188	734	72	**	2326	3724	159	*
<b>Methionine</b>													
3-methylthiopropional (methional)	48	564	565	280	ns	1625	1435	315	ns	1474	2248	233	ns

<sup>1</sup> 1, 7 and 30 days of ripening ( $T_1$ ,  $T_7$  and  $T_{30}$ , respectively); T.I. – Target Ion; SEM – standard error of mean; nd – not detected; ns – not significant; \* –  $P < 0.05$ ; \*\* –  $P < 0.01$ ; \*\*\* –  $P < 0.001$ . Results were expressed as Arbitrary Unit (AU, target ion area  $\times 10^{-3}$ )

Aromatic compounds from phenylalanine have an impact on the aroma of cheese. Phenylacetaldehyde for instance is related to floral, rosy and violet-like odours (Thierry and Maillard, 2002) and represents the major active odour compound in Mozzarella cheese (Moio et al., 1993b). Whereas phenylethyl alcohol is generally associated with a rose flower odour (Suriyaphan et al., 2001) and it is among the most odorous aromatic compounds.

The first leucine catabolites were higher in EXP cheese at all different times of sampling during ripening ( $P < 0.01$ ,  $P < 0.001$  and  $P < 0.01$  at  $T_1$ ,  $T_7$  and  $T_{30}$ , respectively for  $\alpha$ -ketoisocaproic acid;  $P < 0.05$ ,  $P < 0.001$  and  $P < 0.001$  at  $T_1$ ,  $T_7$  and  $T_{30}$ , respectively for methyl  $\alpha$ -hydroxy isocaproate). After an extracellular enzymatic degradation of casein by starter bacteria protease, leucine is converted to the corresponding  $\alpha$ -keto acid ( $\alpha$ -ketoisocaproic acid) by an intracellular transamination (Smit et al., 2005).

The  $\alpha$ -ketoisocaproic acid is the central intermediate in the leucine metabolism that can be converted to  $\alpha$ -hydroxy acids, aldehydes or CoA-esters (Smit et al., 2004). The corresponding methyl  $\alpha$ -hydroxy isocaproate production, derived from the  $\alpha$ -hydroxy isocaproic acid by the hydrogenation of the  $\alpha$ -ketoisocaproic acid, depends on the availability of methanol. However, these first leucine metabolites are not considered to be important for the development of the cheese flavour (McSweeney, 2004).

The content of 3-methyl-1-butanol, derived from the hydrogenation of the corresponding aldehyde (3-methylbutanal), was higher in EXP cheese at  $T_7$  ( $P < 0.001$ ) and  $T_{30}$  ( $P < 0.05$ ) but not at  $T_1$  ( $P > 0.05$ ). It was identified in bovine mozzarella as a minor odorant that confers a fresh cheese aroma (Moio et al., 1993b) It is responsible for alcoholic and fruity odours in Swiss-type cheese (Thierry and Maillard, 2002). The isoamyl isopentanoate was not detected at  $T_1$  in both groups while it was higher in EXP cheese at  $T_7$  ( $P < 0.01$ ) and  $T_{30}$  ( $P < 0.05$ ). The isoamyl butyrate was detected only at  $T_{30}$  in both cheeses and it was higher in EXP cheese in comparison with the CON one ( $P < 0.05$ ).

Finally, any difference was observed for 3-methylthiopropional, originated from methionine catabolism.

## Conclusions

In dairy cows, dietary supplementation of dried olive pomace may affect volatile compounds in raw milk and pasteurised milk cheese. In particular, the dietary treatment increased the proteolytic volatiles (phenyl acetaldehyde, phenylethyl alcohol

and 3-methyl-1-butanol) associated with fresh, floral and fruity aromas in cheese. Furthermore, the valorisation of by-products from agro-industry in the diet of ruminants, instead of their difficult disposal, may represent a potential strategy to increase sustainability of agro-systems. Nevertheless, a consumer test would be necessary to investigate if these aromatic changes are perceivable by taste and appreciated. This information is important to support dairy industry choices.

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