Safranal transference from ewe's milk to cheese and whey and antifungal properties of fortified whey

Celia Librán, Carmen Licón, Jéssica Serrano-Díaz, Manuel Carmona, María Berruga

Dairy Sci. & Technol. (2014) 94:83–89
DOI 10.1007/s13594-013-0145-2

NOTE

Safranal transference from ewe's milk to cheese and whey and antifungal properties of fortified whey

Celia M. Librán · Carmen C. Licón · Jéssica Serrano-Díaz · Manuel Carmona · María I. Berruga

Received: 9 April 2013 / Revised: 24 June 2013 / Accepted: 25 June 2013 /
Published online: 19 July 2013

Abstract Growth of undesirable moulds is one of the major causes of defects in modern cheese production, and it is necessary to discover natural antifungal substances that are non-toxic to humans. Saffron spice has been successfully used as a new ingredient in some Italian or Spanish cheeses, but transference of safranal (the main compound responsible for saffron aroma) during cheesemaking has not yet been studied. In this study, the distribution of safranal in cheese, whey and *requesón* (Spanish whey cheese) and the antifungal potential of whey fractions against the mould *Penicillium verrucosum* were studied. To this end, a duplicate fabrication of cheeses was performed with raw ewe's milk with the addition of 40 μg safranal.kg^{-1} or control milk (no safranal addition). Safranal was more concentrated in cheese and *requesón* (78.91 and 76.95 μg.kg^{-1}, respectively) than in whey and *requesón* whey (35.19 and 34.64 μg.kg^{-1}, respectively). These liquid fractions showed good antifungal properties and inhibited mould with a safranal concentration of around 35 μg.kg^{-1}, while a solution of 80 μg.kg^{-1} was required for a standard safranal solution to obtain a similar inhibition. These results reveal that whey has a synergistic effect with safranal in preventing cheese spoilage by moulds, which encourages making full use of this problematic by-product.

Celia M. Librán and Carmen C. Licón contributed equally to this work.

C. M. Librán · C. C. Licón · M. I. Berruga (✉)
Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain
e-mail: mariaisabel.berruga@uclm.es

J. Serrano-Díaz · M. Carmona
Cátedra de Química Agrícola, ETSIA, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain

J. Serrano-Díaz
Departamento de Tecnología de los Alimentos, Nutrición y Bromatología, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", Universidad de Murcia, CIBERobn, ISCIII, 30100 Murcia, Spain

M. Carmona
Parque Científico y Tecnológico de Albacete, Campus Universitario, 02071 Albacete, Spain

Keywords Safranal · Cheese · Whey · *Penicillium verrucosum*

1 Introduction

Most defects in modern cheese production are due to undesirable mould growth (Neaves and Williams 2012), i.e. curd liquefaction, unpleasant odour and flavour development or texture discoloration (Sarić and Dizdarević 2011). The presence of moulds on cheeses can also have severe consequences for human health, such as the case of human strikes due to mycotoxin production (Belitz et al. 2009). The most commonly isolated mould genera from contaminated cheeses are *Penicillium* and *Aspergillus* (Montagna et al. 2004; Kure and Skaar 2000); among them, *Penicillium verrucosum* has been isolated and found to be one of the commonest in dairy industries (Hayaloglu and Kirbag 2007). The traditional way to prevent such spoilage is by using cheese covers, sometimes supplemented with antifungals, which are not always legally allowed (Sørhaug 2011). Hence, there is a need to find antifungal substances that are non-toxic for humans.

In the last past years, the antifungal activity of whey proteins and fatty acids has been demonstrated in vitro (González-Chávez et al. 2009; Clément et al. 2008). Thus, whey can be used as a natural alternative to control cheese spoilage by moulds. The antifungal potential of whey can be improved with the addition of bioactive compounds such as saffron spice (*Crocus sativus* L. dried stigmas), which has been recently employed as an ingredient in the production of Spanish or Italian ewe's milk cheeses and offers adequate colour transference to cheeses (Carpino et al. 2008; Licón et al. 2012a; Licón et al. 2012b). Nevertheless, the transference of saffron compounds related to aroma, such as safranal, during the cheesemaking process is still to be quantitatively determined. Safranal has numerous biological activities, such as antioxidant, anxiolytic, anticonvulsant, antidepressant or antinociceptive (Karimi et al. 2010; Licón et al. 2010; Hosseinzadeh and Noraei 2009). Pintado et al. (2011) tested safranal against *Salmonella enterica*, but its antifungal activity remains to be studied.

Therefore, the aim of this work was to test the antifungal properties of whey obtained from cheese and *requesón* production with safranal-fortified milk. Besides, safranal distribution among different dairy products was studied, which contributes knowledge about how safranal is transferred during cheesemaking.

2 Materials and methods

2.1 Safranal

The safranal used for cheesemaking was obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with a purity of 88%.

2.2 Milk

Manchega breed ewe's raw milk from a commercial farm (Albacete, Spain) was used for cheese fabrication. This milk had the following compositional values (in grams per 100 grams): dry matter of 16.30±0.10; fat content of 5.21±0.18; and protein content of 5.42±1.10.

2.3 Fungal strain

The mould strain *P. verrucosum* CECT 2906 was provided by the Spanish Type Culture Collection (CECT) (Burjassot, Spain).

2.4 Cheese and *requesón* fabrication

For each fabrication type (control and safranal), two milk vats (2 kg each) were used in duplicate. After safranal addition (40 $\mu g.kg^{-1}$), milk was heated in a water bath (30 °C) and commercial rennet was added (0.015 $g.L^{-1}$). After 30 min, the curd was cut into 8–10 mm cubes and heated to 37 °C for 20 min before whey separation. Curd was placed in perforated plastic moulds (6×6×7 cm) weighing approximately 100 g and was pressed by gravity for 2 h. Cheese whey was stirred at 1,500 rpm and heated to reach a temperature of 75–80 °C. Then, stirring was stopped and cheese whey was heated to 90 °C to allow for the flocculation of whey proteins. Proteins were separated from whey with a perforated spoon to obtain the traditional Spanish whey cheese called *requesón*, which was placed in the same moulds as those used for cheese to cool down in for 2 h. All the samples were kept in aliquots at 2 °C for up to 18 h until the analyses were performed. Cheese yields were 20.04±0.25% for cheese (CH) and 3.02±0.55% for *requesón* (R). The CH and R compositions were obtained using a Foss FoodScan analyzer (FoodScan Lab, FOSS, Hillerød, Denmark), while the compositional whey (W) and *requesón* whey (RW) values were acquired in the MilkoScan Minor Type 78110 equipment (FOSS, Hillerød, Denmark). Products' composition was analysed twice.

2.5 Safranal determination

Safranal was analysed in all the fractions obtained from the cheesemaking process (CH, W, R and RW). Safranal isolation was done by the headspace sorptive extraction (HSSE) technique, and it was analysed by thermal desorption coupled to gas chromatography/mass spectrometry (TD/GC/MS) according to Licón et al. (2012b). For the liquid samples, weight and the extraction time were modified to 10 g of sample and to 2 h, respectively, using 1-cm length sorptive bars. Safranal was identified by comparing retention time and spectra with the real standard. For its quantification, calibration curves were prepared in ewe's milk and curd by spiking a standard solution of safranal at five different concentration levels within the 10- to 160-$\mu g.kg^{-1}$ range. Ethyl octanoate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as an internal standard (1 $mg.kg^{-1}$). The results were expressed as micrograms per kilogram.

2.6 Antifungal activity determination

P. verrucosum inocula were obtained by subculturing weekly on potato dextrose agar (Merck, Darmstadt, Germany) at 25 °C and in the darkness. Conidia were harvested according to Baratta et al. (1998) and spore suspension was adjusted (λ=530 nm) to an optical density of 0.5, the equivalent to 10^7 spores.mL^{-1}.

First, the antifungal activity of the safranal solutions at the same concentration as found in milk (Saf-40) (40 μg.kg^{-1}) and at the double concentration (Saf-80) (80 μg.kg^{-1}) was assayed by the broth dilution method, which was adapted from Baratta et al. (1998). In a 50-mL Erlenmeyer flask, 10 mL of safranal solution in yeast extract sucrose broth (BD, Le Pont de Claix, France) was inoculated with the mould to obtain 10^5 cfu.mL^{-1} of spore suspension, which were incubated at 25 °C for 1 week in the OM10E shaking incubator (150 rpm) (Ovan, Badalona, Spain). Then, the weight of the filtered dried mycelium (105 °C overnight) was obtained and the level of inhibition was calculated as:

$$\%\,\text{inhibition} = \left[(C - T) \big/ C \right] \times 100 \tag{1}$$

where T is the weight of the mycelium from the test flasks and C is the weight of the mycelium from the control ones.

Afterwards, the antifungal activity of the liquid fractions (W and RW) from the safranal-fortified cheesemaking (Section 2.4) was determined by following the same methodology as that explained above and by utilising the correspondent liquid fractions of the control cheesemaking as a control. Flasks were prepared in duplicate and the experiment was performed twice.

2.7 Statistical analysis

An analysis of variance (p<0.05) was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Tukey's test at a level of p<0.05 was used to determine the differences between the control and safranal compositional values for the different dairy products obtained and to evaluate safranal antifungal activity.

3 Results and discussion

3.1 Safranal transference

Table 1 shows the average safranal concentration (in micrograms per kilogram) and its total content (in micrograms) obtained in the different fractions. Solid fractions displayed higher safranal concentrations (78.91 μg.kg^{-1} in CH and 76.95 μg.kg^{-1} in R) than the liquid ones (35.19 μg.kg^{-1} in W and 34.64 μg.kg^{-1} in RW), although this trend changed when safranal was expressed as total content due to the greater yields reached for the liquid fractions. Table 2 summarises the compositional values of the dairy products from both the control and safranal fabrications, while CH and R are characterised by their high total solid content as compared to the liquid ones. The major compounds in CH and R (caseins and lipids or whey proteins, respectively) can interact with aromatic molecules by hydrophobic or covalent interactions and may therefore retain compounds like safranal more effectively (Kühn et al. 2007). However, the higher safranal contents found in the liquid fractions can be associated with the solubility in

Table 1 Safranal concentration and safranal total content in different dairy products analysed by headspace sorptive extraction/thermal desorption/gas chromatography/mass spectrometry (HSSE/TD/GC/MS)

Safranal		
Product	Concentration (μg.kg^{-1})	Total content (μg)
Milk	33.82±0.43	74.42±0.95
Cheese	78.91±5.21	34.79±2.30
Whey	35.19±4.07	55.22±6.39
Requesón	76.95±5.80	3.83±2.58
Requesón whey	34.64±1.74	44.06±2.22

Data are expressed as mean ±standard deviation (n=4)

water of safranal as both these matrices presented a larger aqueous fraction (more than 90 g.100 g^{-1}) than the solid ones (Table 2).

Heat treatment studies have not only evidenced the binding properties of flavour compounds with whey proteins, but have also revealed that heat treatment affects flavour binding to the extent that higher temperatures 0and longer times can lead to less binding properties of proteins (Kühn et al. 2008; Kühn et al. 2007).

All these feasible interactions deserve further study in order to acquire knowledge about safranal and milk components with a view to optimising the addition of safranal to dairy products.

3.2 Antifungal activity of liquid fractions

The obtained antifungal activity results are provided in Fig. 1. Pure safranal solutions, as well as fortified whey with safranal, were able to inhibit over 15% of mould growth

Table 2 Compositional values (in grams per 100 grams) of cheese, *requesón*, whey and *requesón* whey from the fabrication of the control cheese (control) and the preparation of cheese fortified with safranal (safranal)

	Product	Control	Safranal	ANOVA*
Dry matter	Cheese	43.43±1.57	43.25±1.30	NS
	Whey	8.35±0.08	8.04±0.33	NS
	Requesón	27.40±0.20	25.26±0.70	NS
	Requesón whey	7.65±0.11	7.25±0.54	NS
Fat	Cheese	19.04±1.22	20.05±0.35	NS
	Whey	0.43±0.29	0.30±0.13	NS
	Requesón	9.30±0.35	9.80±0.35	NS
	Requesón whey	0.06±0.08	0.05±0.01	NS
Protein	Cheese	20.94±0.42	20.44±0.70	NS
	Whey	1.07±0.12	1.15±0.05	NS
	Requesón	10.49±0.09	10.79±0.25	NS
	Requesón whey	0.36±0.02	0.42±0.12	NS

Data are expressed as mean±standard deviation (n=4)

*Significant differences were not found between control and safranal products (p<0.05)

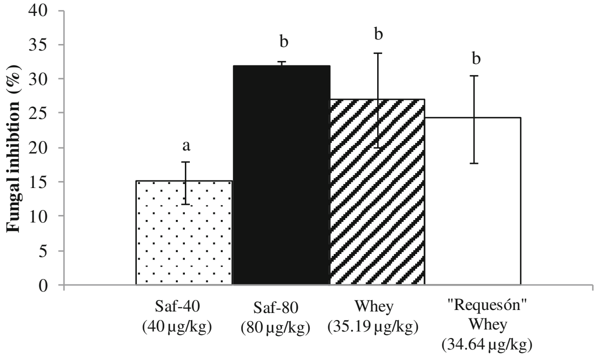

Fig. 1 The fungal inhibition reached with whey and *requesón* whey obtained from cheesemaking with safranal addition and solutions of 40 and 80 μg of safranal per kilogram (mean; %±SE) (n=4). The different letters *a, b* among the results indicate significant differences (p<0.05)

if compared to their corresponding control flasks. In decreasing order of inhibition, the samples were: Saf-80 > W > RW > Saf-40. As seen, both the food matrices assayed offered a better antifungal potential than Saf-40 (40 μg.kg^{-1}of safranal), even though their safranal concentration was lower (34.64 and 35.19 μg.kg^{-1}, respectively) (Table 1). These outstanding antifungal results can be explained by the possible synergistic inhibitory effect of safranal and some whey compounds against *P. verrucosum*. Previous studies have suggested that some fatty acids and lactoferrin from bovine whey can have antifungal properties (González-Chávez et al. 2009; Clément et al. 2008). Very few studies have been conducted on the bioactivity of ovine whey peptides and it is believed that some of them, which remain unidentified, from α-lactalbumin and β-lactoglobulin hydrolysis have antimicrobial properties (Hernández-Ledesma et al. 2011). The results obtained in this work motivate future research in this field.

The antimicrobial activity of safranal can be attributed to its chemical structure because it is an oxygenated monoterpene. Terpenes undergo biochemical modifications which add oxygen molecules and move or remove methyl groups (Hyldgaard et al. 2012). These compounds are recognised for its antimicrobial activity (Bakkali et al. 2008). Nevertheless, safranal has not been extensively assayed because it has been tested only against bacteria and by in vitro tests (Pintado et al. 2011). Therefore, the present work is relevant for this field because it proves that W and RW added with safranal have antifungal properties, which are even better than the compound itself.

4 Conclusions

Whey and *requesón* whey obtained from cheesemaking with safranal addition can be a helpful natural means to prevent cheese spoilage by moulds. These results encourage the use of the whey fractions resulting from cheesemaking with saffron addition to prevent mould growth, which encourages making full use of this problematic dairy by-product.

Acknowledgments This work has been financially supported by the Consejería de Educación y Ciencia of the JCCM with Research Project PBI08-0245-8453 and funding for C. C. Licón. The authors wish to thank G. Alonso for his valuable advice while conducting the study and Julián Molero for his technical support.

References

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils—a review. Food Chem Toxicol 46:446–475

Baratta MT, Dorman HJD, Deans SG, Figueiredo AC, Barroso JG (1998) Antimicrobial and antioxidant properties of some commercial essential oils. Flavour Fragance J 13:235–244

Belitz HD, Grosch W, Schieberle P (2009) Food contamination. In: Food chemistry, 4th edn. Springer, Berlin, pp. 472–475

Carpino S, Rapisarda T, Belvedere G, Licitra G (2008) Volatile fingerprint of Piacentinu cheese produced with different tools and type of saffron. Small Rum Res 79:16–21

Clément M, Tremblay J, Lange M, Thibodeau J, Belhumeur P (2008) Purification and identification of bovine cheese whey fatty acids exhibiting in vitro antifungal activity. J Dairy Sci 91:25–35

González-Chávez SA, Arévalo-Gallegos S, Rascón-Cruz Q (2009) Lactoferrin: structure, function and applications. Int J Antimicrobial Agents 33:301–308

Hayaloglu AA, Kirbag S (2007) Microbial quality and presence of moulds in Kuflu cheese. Int J Food Microbiol 115:376–380

Hernández-Ledesma B, Ramos M, Gómez-Ruiz JA (2011) Bioactive components of ovine and caprine cheese whey. Small Rum Res 101:196–204

Hosseinzadeh H, Noraei NB (2009) Anxiolytic and hypnotic effect of *Crocus sativus* aqueous extract and its constituents, crocin and safranal, in mice. Phytother Res 23:768–774

Hyldgaard M, Mygind T, Meyer RL (2012) Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. Front Microbiol 3:1–24

Karimi E, Oskoueian E, Hendra R, Jaafar HZE (2010) Evaluation of *Crocus sativus* L. stigma phenolic and flavonoid compounds and its antioxidant activity. Mol 15:6244–6256

Kühn J, Zhu XQ, Considine T, Singh H (2007) Binding of 2-nonanone and milk proteins in aqueous model systems. J Agri Food Chem 55:3599–3604

Kühn J, Considine T, Singh H (2008) Binding of flavor compounds and whey protein isolate as affected by heat and high pressure treatments. J Agri Food Chem 56:10218–10224

Kure CF, Skaar I (2000) Mould growth on the Norwegian semi-hard cheeses Norvegia and Jarlsberg. Int J Food Microbiol 62:133–137

Licón CC, Carmona M, Llorens S, Berruga MI, Alonso GL (2010) Potential healthy effects of saffron spice (*Crocus sativus* L. stigmas) consumption. Functional Plant Sci Biotechnol 4:64–73

Licón CC, Carmona M, Rubio R, Molina A, Berruga MI (2012a) Preliminary study of saffron (*Crocus sativus* L. stigmas) color extraction in a dairy matrix. Dyes Pigments 92:1355–1360

Licón CC, Hurtado De Mendoza J, Maggi L, Berruga MI, Martín Aranda RM, Carmona M (2012b) Optimization of headspace sorptive extraction for the analysis of volatiles in pressed ewes' milk cheese. Int Dairy J 23:53–61

Montagna MT, Santacroce MP, Spilotros G, Napoli C, Minervini F, Papa A, Dragoni I (2004) Investigation of fungal contamination in sheep and goat cheese in southern Italy. Mycopathologia 158:245–249

Neaves P, Williams AP (2012) Microbiological surveillance and control in cheese manufacture. In: Law BA, Tamine AY (eds) Technology of cheesemaking. Wiley, Oxford, pp 384–412

Pintado C, De Miguel A, Acevedo O, Nozal L, Novella JL, Rotger R (2011) Bactericidal effect of saffron (*Crocus sativus* L.) on *Salmonella enterica* during storage. Food Control 22:638–642

Sarić ZO, Dizdarević TA (2011) Occurrence of yeast and moulds in cheese and measures for growth control. Prehrambena industrija-Mleko i Mlečni Proizvodi 1:8–11

Sørhaug T (2011) Yeasts and molds. Spoilage molds. In: Fuquay JW (ed) Encyclopedia of dairy sciences 2nd edn. Academic Press, San Diego, pp 780–784