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Fresh cheese as a vehicle for polyunsaturated fatty acids integration: effect on physico-chemical, microbiological and sensory characteristics

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ABSTRACT

Five different vegetable oils were used in the production of fresh cheese to increase the concentration of polyunsaturated fatty acids (PUFAs), particularly α -linolenic acid (ALA), the most important omega-3 fatty acid of vegetable origin. Physico-chemical and microbiological characteristics of functionalized cheeses were evaluated after 1 and 3 days of ripening at 4°C while the consumer appreciation was evaluated in the final product at 3 days of ripening. After 3 days, the cheeses with *Camelina sativa* and *Echium plantagineum* oils added exhibited the highest retention of PUFAs (mostly ALA) compared to those with flaxseed, raspberry and blackcurrant oils. The addition of oil showed little effects on physico-chemical characteristics and also consumers' evaluation highlighted that all of the fresh cheeses were considered acceptable although those with flaxseed and raspberry oils were the most appreciated.

ARTICLE HISTORY

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KEYWORDS

Fortified cheese; vegetable oil; omega-3; healthy benefits; consumer acceptance

Introduction

In recent years, the attention of consumers has increasingly shifted to the so-called functional foods, which in addition to their normal functions, can provide health benefits and prevent various diseases (Ganesan et al. 2014).

The market trend toward functional foods is continuously growing, pushing companies to invest in these foods to meet the needs of the consumers. This broad category of functional foods includes the foods fortified with omega-3 polyunsaturated fatty acids (PUFAs), long-chain fatty acids that cannot be synthesized by human metabolic processes but must be provided by the diet (Iafelice et al. 2008).

The beneficial effects of omega-3 PUFAs on human health are due to the ability of omega-3 PUFAs to prevent and treat cardiovascular diseases, to exhibit anti-inflammatory and anti-allergic effects, to promote the development and function of the brain, retinas and nervous systems and to protect against certain types of cancer (De Deckere et al. 1998; Singh et al. 2011).

For these reasons, the increased consumption of omega-3 PUFAs has been suggested by health

authorities in the United States, Canada and the United Kingdom as well as in Europe, where a daily intake of approximately 200–400 mg of omega-3 PUFAs has been recommended (De Deckere et al. 1998; Simopoulos et al. 1999).

The best sources of omega-3 PUFAs are fish and their derivatives which, although often characterised by an unpleasant odour and taste, contain large amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ganesan et al. 2014).

Some vegetable products, such as nuts, seeds and vegetable oils (flaxseed, canola and soybean) may also provide suitable amounts of omega-3 PUFAs (Bermúdez-Aguirre & Barbosa-Cánovas 2011; Lane et al. 2014) mostly α -linolenic acid (ALA C18:3 n-3) as an equally useful source of omega-3 PUFAs (Iafelice et al. 2008; Ritter-Gooder et al. 2008).

Previous research has suggested that increasing the ALA consumption to a dietary intake of 1% (other than minimizing the background intake of linoleic acid, the most predominant omega-6 PUFA in the daily human diet) is important to maximize the conversion of ALA to EPA (Emken et al. 1994) and help to reduce the risk of cardiovascular disease (Metcalf et al. 2003; Das 2006).

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B Supplemental data for this article can be accessed here.

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Considering the interesting health benefits associated with omega-3 consumption (Welch et al. 2010) and that the use of plant oils as sources of omega-3 is more sustainable than using fish oils, vegetable oils have been added to different foods, such as infant formula, dairy and meat products (Kuratko et al. 2013; Dal Bello et al. 2015) bakery products and juices (Ganesan et al. 2014) to obtain an increase in the omega-3 content.

Hence, the objective of this research study was to produce fresh cheeses with a high content of omega-3 PUFAs, particularly ALA, by adding different vegetable oils evaluating the physico-chemical and the microbiological effects of this addition during ripening time as well as the consumer acceptance of the products.

Material and methods

Omega-3 sources

Vegetable oils originating from cold pressing of flax (FS, 71% ALA), *Camelina sativa* (CAM, 36% ALA), raspberry (RAS, 29% ALA), blackcurrant (BC, 14% ALA) and *Echium plantagineum* (EC, 33% ALA) seeds were provided by AVG s.r.l, (Milan, Italy). Modified potato starch (Prodotti Gianni, Milan, Italy) was used $(2 \text{ g kg}^{-1} \text{ of curd})$ to adsorb oils and to increase their retention into the cheeses.

Manufacture of fresh cheese

Raw milk coming from cows (protein 3.5%, w/w, fat 3.6%, w/w, lactose 5.1%, w/w) was provided from a local farm, pasteurized at 72 °C for 15s and calcium chloride (0.1 g L^{-1}) was added to the final volume of 100 L of milk. Coagulation was performed at 38-40 °C with 50 mL of cow liquid rennet (chymosin:pepsin 25:75; Clerici, Milan, Italy). After 30-40 min of resting, the curd was cut twice and left to stand for 10 min at 37 °C. The curd obtained was subdivided into six separate batches, then poured into moulds and allowed to drain. At this point, the resulting mixture of oil and starch was added and manually mixed. The percentage of oil addition was defined according to its omega-3 PUFA content to obtain a cheese with at least 200-400 mg per 100 g of product. Considering this, the addition of the different vegetable sources was 10 g kg^{-1} for FS oil, 15 g kg^{-1} for EC and RAS oils, 20 g kg^{-1} for CAM oil and 40 g kg^{-1} for BC oil. For the fresh cheese used as a Control, only starch was added.

The cheeses of approximately 250 g each were stored at $4 \degree \text{C}$ for 3 days in a ripening room with

relative humidity of approximately 85%. Two batches were prepared with each vegetable oil.

Chemical analyses

Protein, fats, moisture and ash were determined after three days of ripening according to D.M. 21/04/1986n° 229 (Official Methods of Cheese Analysis 1986) while the pH was recorded with a portable pH meter (PH 25, Crison, Milan, Italy). All the analyses were carried out in duplicate after 3 days of ripening.

Fatty acid analysis

Determination of fatty acids and quantification of omega-6 and omega-3 PUFAs were carried out using gas chromatographic analysis. Lipids from the fresh cheese samples (10g) were extracted according to the Folch method (Folch et al. 1957) with slight modifications. Briefly, 10g of cheese was added with 15 mL of a chloroform-methanol solution 2:1 (v/v) (Sigma-Aldrich, Milan, Italy), shaken mechanically for 20 min and centrifuged at 10,000g for 5 min at 4 °C. The upper organic solvent fraction was carefully removed and the sediment was extracted again twice. The combined upper organic phases were added with 7 mL of a 9 g L^{-1} of NaCl solution and 3 g of Na₂SO₄. The mixture was then centrifuged at 2000 rpm for 5 min at 4°C and the lipids in the lower chloroform phase concentrated under vacuum.

The extracted fat (50 mg) was methylated as indicated by Ficarra et al. (2010) with 1 mL of hexane and $300 \,\mu\text{L}$ of $2 \,\text{mol}\,\text{L}^{-1}$ KOH in methanol (w/v) in a dark tube (Sigma-Aldrich) using C19:0 (200 mg L^{-1}) (Sigma-Aldrich) as internal standard. The extract was immediately analysed and fatty acid methyl esters (FAMEs) were determined using a GC-2010 Shimadzu gas chromatograph (Shimadzu, Milan, Italy) equipped with a flame ionisation detector, a split-splitless injector, an AOC-20i autosampler (Shimadzu, Milan, Italy) and a capillary column SP-2560, $100\,\mathrm{m}\, imes$ 0.25 mm id $\times 0.20 \mu \text{m}$ (Supelco, Milan, Italy). The oven temperature was programmed starting from 140 °C, holding for 20 min, and then ramped to 240 °C at a rate of 4 °C/min and held for 20 min. The injector and detector temperature was set at 250 °C. Each fatty acid was identified and quantified by comparing retention times with fatty acid methyl ester standards (Sigma-Aldrich) and expressed as mg fatty acid/100 g of sample calculated according to AOAC 963.22 method (Official methods of analysis of the AOAC 963.22 2000). All the analyses were carried out in duplicate on cheeses after 1 and 3 days of ripening.

Oxidation index

Cheese oxidation after 1 and 3 days of ripening at $4 \degree C$ was evaluated using three oxidation parameters: Peroxide Value (PV), Anisidine Value (p-An) and acidity. Tests were performed using the *FoodLab* Method (CDR, Florence, Italy), performing all the analyses in duplicate on cheeses.

Organic acid and sugar analysis

Organic acids (citric, pyruvic and lactic) and sugars (lactose, glucose and galactose) were determined by high performance liquid chromatography (HPLC) according to the method reported by Bertolino et al. (2011). The HPLC system (Thermo Quest, San Jose, CA) was equipped with an isocratic pump (P4000), a multiple autosampler (AS3000) fitted with a 20-µL loop, a UV detector (UV100) set at 210 and a refractive index detector (Spectra System RI-150, Thermo Electron Corporation). Data were collected using ChromQuest ver. 3.0 (Thermo Finnigan, Waltham, MA). The mobile phase was 0.013 N H₂SO₄, and the analyses were performed isocratically at 0.8 mL/min and 65 $^{\circ}\mathrm{C}$ with a 300 \times 7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a cation H⁺ micro-guard cartridge (Bio-Rad Laboratories, Hercules, CA). Identification was achieved by comparison with the retention times of authentic standards: lactose, glucose, galactose, pyruvic acid, lactic acid and citric acid purchased from Sigma-Aldrich (Milan, Italy). All the analyses were carried out in duplicate on cheeses after 1 and 3 days of ripening.

Assessment of proteolysis

The pH 4.6-insoluble extracts were prepared according to the method reported by Hayaloglu et al. Urea-polyacrylamide gel electrophoresis (2004). (Urea-PAGE) was performed on the insoluble fraction according to the method reported by Bertolino et al. (2008). After destaining, gel slabs were digitised by a scanner (Epson Perfection 1650, Seiko Epson Corporation, Nagano, Japan). Scans of the electrophoretograms were used to quantify bands using a densitometric software (Image Master TotalLab 1D Gel analysis v 1.11 software, Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK). Similar bands were recognised visually, and the relative percentage of identified caseins was determined. Two replicates for each sample were analysed after 1 and 3 days of ripening.

Microbiological analysis

Microbiological analyses were conducted after 1 and 3 days of ripening.

For the total viable count, yeast and mould counts, 10 g of cheese was suspended in 90 mL of Ringer solution (Oxoid, Milan, Italy). Serial dilutions were made and poured into Plate Count Agar for the total viable count and on malt extract agar for yeast and mould (Biolife, Milan, Italy) and incubated at $37 \degree$ C for 24–48 h.

Sensory evaluation

Consumer test

One hundred and seventy-four consumers voluntarily participated in the test (67 males, 107 females: 18–35 years: 54%, 36–55 years: 26%, >55 years: 20%). After 3 days of ripening the evaluations were conducted at a mobile stand with 15 people involved per shift. The consumers were verbally introduced to the tasting procedure and to a questionnaire. Instructions were also reported on the evaluation sheet. Because some preliminary sensory evaluations indicated that cheeses with added EC oil were clearly not acceptable, cheese with added EC oil was not included in the consumer tests to limit the contrast effect (Meilgaard et al. 2006).

Cheese samples (10 g) were served under blind conditions, in opaque white plastic cups (38 mL) sealed with a clear plastic lid and identified by random three-digit codes. The subjects were required to taste the samples according to the presentation order and to express their degree of liking each cheese on a 9point hedonic scale ranging from 'dislike extremely' (1) to 'like extremely' (9) (Peryam & Pilgrim 1957).

Personal data (age, gender, nationality, educational level, occupational status), frequency of consumption of fresh cheese (less than once a week, once a week, 2-3 times a week, 4-5 times a week, once a day) and perceived healthiness of fresh cheese (7-point scale; 1 = not at all healthy, 7 = extremely healthy) (Urala &Lähteenmäki 2004) were requested. Moreover, the participants were required to rate their agreement (7-point scale; 1 = strongly disagree, 7 = strongly agree) with 14 statements (1. I am very particular about the healthiness of food I eat; 2. I always follow a healthy and balanced diet; 3. It is important for me that my diet is low in fat; 4. It is important for me that my daily diet contains a lot of vitamins and minerals; 5. I eat what I like and I do not worry much about the healthiness of food; 6. I do not avoid foods, even if they may raise my cholesterol; 7. The healthiness of

Table 1. Gross composition (%, w/w) of the Control and fortified fresh cheeses after 3 days of ripening.

· -	
Ashes	рН
2.41 ± 0.01 6	5.81 ± 0.01
2.39±0.02 6	5.68 ± 0.02
2.22 ± 0.02 6	5.72 ± 0.01
2.29±0.01 6	5.74 ± 0.01
2.38±0.00 6	5.62 ± 0.03
2.22 ± 0.01 6	5.82 ± 0.01
ns r	15
	2.32 ± 0.02 6 2.22 ± 0.02 6 2.29 ± 0.01 6 2.38 ± 0.00 6 2.22 ± 0.01 6 ns r

Data are expressed as the means \pm SD (n = 4).

Means with different lowercase letters within the same column are significantly different (Duncan's test, p < .05).

Significance:

*p < .05; ns = not significant.

food has little impact on my food choices; 8. The healthiness of snacks makes no difference to me; 9. I do not believe that food should always be source of pleasure; 10. The appearance of food makes no difference to me; 11. It is important for me to eat delicious food on weekdays as well as weekends; 12. When I eat, I concentrate on enjoying the taste of food; 13. I finish my meal even when I do not like the taste of a food; 14. An essential part of my weekend is eating delicious food) related to health and interest in food (Roininen et al. 1999).

Statistical analysis

A one-way analysis of variance (ANOVA) with Duncan's test (p < .05) as a multiple range test was used to highlight the significant differences between all of the treatments in terms of physical, chemical and microbiological parameters. All of the calculations were performed with the STATISTICA for Windows statistical software package (Release 7.0; StatSoft Inc., Tulsa, OK).

The liking data (appearance, odour, taste, flavour, texture, overall liking) were independently subjected to two-way mixed ANOVA models (fixed factor: sample; random factor: subject) by performing Fisher's Least Significant Difference (LSD; p < .05). An Internal Preference Map (IPM) was obtained by applying the Principal Component Analysis (PCA) to the overall liking ratings from the 174 subjects (Unscrambler X version 10.3, Camo Software AS, Oslo, Norway). A consumer segmentation was performed by applying a K-means Cluster Analysis to coordinates of subjects for the first three principal components. Three clusters of subjects were found. The effect of interaction between the factor cluster and the product on overall liking was estimated with a two-way mixed ANOVA model (fixed factors: product, cluster; random factor: interaction product * cluster). The liking data for the three clusters were independently subjected to a two-way mixed ANOVA model, as was performed for the mean data. Differences among clusters for personal data and

frequency of consumption were analysed by Pearson's Chi-squared test, whereas the declared agreement and the perceived healthiness effects were tested through ANOVA models. All of the analyses were conducted with the SYSTAT version 13.1 software (Systat Software Inc., San Jose, CA).

Results and discussion

Gross composition

Table 1 shows the gross composition of the Control and the fortified cheeses after 3 days of ripening. The data showed that fortified cheeses were not significantly different from the corresponding Control cheese. As expected, only the fat content presented a significant difference (p < .05) among the Control and the fortified cheeses although a direct correlation with the quantity of oil added to the cheese was not found maybe due to a los of oil into the whey during the ripening.

Fatty acids

Analysis of FAMEs in Control and fortified fresh cheeses identified a total of 31 fatty acids where the higher percentage of saturated fatty acids was represented mostly by tetradecanoic, hexadecanoic and octadecanoic acids, followed by monounsaturated and polyunsaturated fatty acids (Table S1).

Table 2 shows the most important omega-6 and omega-3 fatty acids identified in the Control and fortified fresh cheeses. At day 1, the highest amounts of omega-6 and omega-3 were reached in all of the fortified products compared with the Control cheeses (p < .05). The highest quantities of omega-6 fatty acids were detected for linoleic acid (C18:2 n6) (p < .01), γ -linolenic (C18:3 n6) (p < .001) and di-homo- γ -linoleic acid (C20:3 n6) in the BC-fortified cheeses. The highest quantity of omega-3 and particularly ALA content was instead reached in fresh cheese with CAM oil (4.43 mg g⁻¹) added. The other omega-3 PUFAs showed concentrations similar to those found

						Echium		
		Control	Raspberry	Flaxseed	Camelina sativa	plantagineum	Blackcurrant	
	Time days	[mg·g ^{−1}]	(RAS) $[mg \cdot g^{-1}]$	(FS) [mg·g ^{−1}]	(CAM) [mg·g ⁻¹]	(EC) $[mg \cdot g^{-1}]$	(BC) [mg·g ^{−1}]	Significance
Omega-6								
Linoleic C18:2 n-6	1	$3.88 \pm 0.20^{a,A}$	7.71 ± 2.28 ^{bc}	$5.64 \pm 0.12^{ab,B}$	6.14 ± 0.23^{ab}	5.72 ± 0.14^{ab}	10.28 # 2.15 ^c	**
	3	$4.98 \pm 0.20^{a,B}$	7.53 ± 0.59 ^b	$4.58 \pm 0.28^{a,A}$	5.24 ± 0.71^{a}	5.07 ± 0.36^{a}	9.16 # 1.18 ^c	***
	Significance	*	ns	*	ns	ns	ns	
γ-Linolenic C18:2 n-6	1	0.08 ± 0.02^{a}	0.19 ± 0.08^{a}	0.18 ± 0.04^{a}	0.12 ± 0.01^{a}	0.88 ± 0.02^{b}	1.74 # 0.52 ^c	***
	3	0.08 ± 0.01^{a}	0.11 ± 0.02^{a}	0.07 ± 0.01^{a}	0.14 ± 0.01^{a}	0.74 ± 0.04^{b}	1.62 # 0.20 ^c	***
	Significance	ns	ns	ns	ns	ns	ns	
Di homo-γ-Linoleic acid C20:3 n-6 (DGLA)	1	0.16 ± 0.01	0.20 ± 0.06	0.20 ± 0.00	0.20 ± 0.02	0.18±0.01	0.19 # 0.00	ns
	3	0.20 ± 0.02	0.21 ± 0.02	0.17 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	0.15 # 0.02	ns
	Significance	ns	ns	ns	ns	ns	ns	
Omega-3								
Linolenic C18:3 n-3 (ALA)	1	1.01 ± 0.54^{a}	2.41 ± 0.67 ^b	$4.02 \pm 0.03^{d,B}$	4.21 ± 0.33 ^d	3.54 ± 0.26 ^{cd}	2.53 # 0.65 ^{bc}	***
	3	0.68 ± 0.07^{a}	2.23 ± 0.20 ^b	2.11 ± 0.19 ^{b,A}	3.47 ± 0.54 ^d	2.89 ± 0.21 ^{cd}	2.29 # 0.28 ^{bc}	***
	Significance	ns	ns	**	ns	ns	ns	
Eicosatrienoic C20:3 n-3 (E	TE)							
	1	0.05 ± 0.01	0.08 ± 0.05	0.04 ± 0.00	0.02 ± 0.00	0.10 ± 0.08	0.02 # 0.02	ns
	3	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}	0.01 ± 0.01^{a}	0.02 ± 0.02^{b}	0.02 ± 0.00^{a}	0.00 # 0.01 ^a	***
	Significance	ns	ns	ns	ns	ns	ns	
Eicosapentaenoic C20:5 n-2	3 (EPA)							
	1	0.06 ± 0.03	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 # 0.00	ns
	3	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.03 # 0.00	ns
	Significance	ns	ns	ns	ns	ns	ns	
Omega-6	1	4.12	8.09	6.02	6.46	6.78	12.21	
	3	5.27	7.85	4.82	5.54	5.99	10.94	
Omega-3	1	0.77	2.54	4.10	4.43	3.68	2.74	
	3	0.75	2.30	2.17	3.68	2.95	2.33	
Omega-6/Omega-3	1	5	3	2	2	2	5	
omegu o, omegu o	3	7	3	2	2	2	5	
	1	60	54	52	52		40	
∑JFA [%]	1	00	54	52	23	22 50	49	
	3	29 77	23	01	27	29	58 26	
	1	27	33 27	38 21	3D 21	33 21	30 20	
	3 1	30	3/ 11	3 ا 10	31 11	3 I 11	28 15	
∑PUFA [%]	1	5	11	10	11	11	15	
	3	6	10	/	9	10	14	

Table 2. Concentration of omega-6 and omega-3 (mg g^{-1}) in fresh cheeses fortified with vegetable oils and the Control after 1 and 3 days of ripening.

Data are expressed as the means ± SD (n = 4). Means with different lowercase letters within the same row are significantly different (Duncan's test, p < .05); means with different uppercase letters within the same column are significantly different (Duncan's test, p < .05). Significance: *p < .05; **p < .01; ***p < .001; ns = not significant.

in the Control cheeses. After 3 days of ripening, no significant differences among the Control and fortified cheeses in terms of both the omega-6 and omega-3 concentrations were observed with the exception of the fresh cheeses with FS oil added, which showed a significant decrease in ALA content (p < .01). Moreover, the addition of vegetable oils rich in omega-3 led to an increase in the ratio omega-6/ omega-3 compared with the Control.

Considering the easy way to incorporate the omega-3, the results obtained are surprising. In previous work other authors indicated an increase in retention as well as the stability of omega-3 using microencapsulated oil high-pressure homogenization and ultrasonication (Calligaris et al. 2013, 2015) or by monoglyceride-based self-assembly structures (Frankel 2005). Although these treatments seem to increase the retention as well as the oxidative stability of omega-3, the native structure of milk (e.g. proteins, milk fat

globules) and then the final cheese quality attributes were modified.

In this research, the decreasing in omega-3 concentration naturally occurs in the matrix due to the high level of PUFA. Therefore, the decreasing in ALA concentration observed can be reasonably attributed to the manually homogenization of the mixture oil in starch used for the integration. Even if the decreasing in ALA concentration was observed in all the fortified cheeses, the level of fortification used was sufficient to achieve an omega-3 PUFA content on the order of $2-4 \text{ mg g}^{-1}$ of product covering abundantly 10% of recommended daily intake level of 2 g of ALA (Regulation EU n°432/2012).

Oxidation tests

Table 3 shows the results regarding the oxidation rate in the Control and fortified fresh cheeses. No

Table 3. Oxidation and hydrolysis rate of fresh cheeses fortified with vegetable oils and the Control after 1 and 3 days of ripening.

Time days	Control	Raspberry (RAS)	Flaxseed (FS)	Camelina sativa (CAM)	Echium plantagineum (EC)	Blackcurrant (BC)	Significance
Peroxide (mE	$qO_2 kg^{-1}$						
1	0.21 ± 0.08^{aA}	0.37 ± 0.06^{ab}	0.53 ± 1.34 ^b	0.24 ± 0.05^{a}	1.06 ± 0.02^{cA}	0.58 ± 0.02^{bA}	***
3	0.91 ± 0.04^{aB}	1.19 ± 0.38^{a}	0.98 ± 0.04^{a}	1.33 ± 0.48^{a}	1.38 ± 0.18^{bB}	1.03 ± 0.01^{aB}	*
Significance	**	ns	ns	ns	**	***	
Acidity (% ol	eic acid)						
1	0.15 ± 0.01^{A}	0.15 ± 0.01	0.13 ± 0.09	0.16 ± 0.01^{A}	0.15 ± 0.01^{A}	0.16 ± 0.02	ns
3	0.20 ± 0.00^{bB}	0.17 ± 0.01^{a}	0.20 ± 0.01^{b}	0.30 ± 0.01^{cB}	0.21 ± 0.00^{bB}	0.17 ± 0.01^{a}	***
Significance	**	ns	ns	**	*	ns	
p-Anisidine (p-An)						
. 1	0.60 ± 0.14	0.75 ± 0.35	0.50 ± 0.78	0.55 ± 0.07	0.90 ± 0.57	0.50 ± 0.01^{A}	ns
3	0.50 ± 0.00^{a}	$0.95 \pm 0.07^{\circ}$	0.60 ± 0.14^{ab}	$1.15 \pm 0.21^{\circ}$	$1.20 \pm 0.28^{\circ}$	1.35 ± 0.07 ^{cB}	**
Significance	ns	ns	ns	ns	ns	**	

Data are expressed as the means \pm SD (n = 4).

Means with different lowercase letters within the same row are significantly different (Duncan's test, p < .05); means with different uppercase letters within the same column are significantly different (Duncan's test, p < .05).

**p* < .05;

***p < .01;
****p < .001; ns = not significant.</pre>

significant differences were observed among the samples at day 1. After 3 days of ripening, significant increases were observed for some of the oxidation parameters evaluated. In particular, a significant increase in the Peroxide Value (PV) of the Control (from 0.21 to 0.91 mEq $O_2 kg^{-1}$) (p < .01), EC (from 1.06 to 1.98 mEq $O_2 kg^{-1}$) (p < .01) and BC (from 0.58 to 1.03 mEq $O_2 kg^{-1}$) (p < .001) were observed. The acidity value increased slightly for the Control and CAM (p < .01) as well as EC (p < .05). For p-Anisidine value (p-An), only the BC reached the highest value (p < .01). Overall, the PV, acidity and p-An values remained lower than the acceptable levels (Gracey et al. 1999; Frankel 2005).

Organic acid and sugar profiles

Table S2 shows the sugar and organic acid concentrations of the Control and fortified fresh cheeses. Among the sugars, only lactose, and among organic acids, only citric acid, were detected. For both compounds, statistically significant differences in their concentrations were observed among the samples at any sampling time. The highest concentration of lactose at 1 day of ripening was observed in the Control and EC samples with a mean value of 52.70 ± 2.11 and $51.93 \pm 0.38 \text{ g kg}^{-1}$ of cheese, respectively. The other samples showed a lower concentration compared to the Control with a mean value of $47.94 \pm 1.38 \,\mathrm{g \, kg^{-1}}$. After 3 days of ripening, the lactose concentration decreased in all of the samples because the lactose was metabolized by bacteria. The highest concentration was still observed for the EC samples (49.24 ± 2) . $30\,\mathrm{g\,kg^{-1}}$) and the lowest for FS and BC samples

with a mean concentration of $40.35 \pm 0.73 \, \text{g kg}^{-1}$. The decreased in lactose concentration during the ripening was 5% for the EC samples as the lowest percentage and 18% for the CAM samples as the highest.

The highest concentration of citric acid at 1 day of ripening was observed in the Control samples with a mean value of $3.44 \pm 0.16 \text{ g kg}^{-1}$ of cheese. The BC samples showed the lowest concentration of citric acid with a mean concentration of $3.00 \pm 0.06 \text{ g kg}^{-1}$. After three days of ripening, the citric acid concentration decreased in all of the samples. The highest concentration was still observed for the Control samples $(2.97 \pm 0.04 \text{ g kg}^{-1})$ but also for EC samples $(2.94 \pm 0.10 \text{ m g kg}^{-1})$. The lowest concentration was observed for FS, RAS and BC samples with a mean concentration of $2.62 \pm 0.04 \text{ m g kg}^{-1}$.

Proteolysis analysis

The present contribution of the major caseins in cheese samples during ripening is reported in Table 4. The residual coagulant and milk enzymes in curd caused the degradation of caseins with a higher action on β -caseins and a less extensive action on α_{s1} -caseins. With respect to γ -caseins, the polypeptides produced by the action of the plasmin on β -caseins, the γ_2 -casein [β -casein (f106-209)] were the most commonly present in all of the samples at each stage of ripening. At one day of ripening, the percentage of contribution of γ_2 -casein to the general proteolysis differs among the fortified cheeses and the Control. The BC fortified cheeses showed the lowest concentration, possibly due to the high concentration of oil that can physically

Significance:

Time days	Control (CTR)	Raspberry (RAS)	Flaxseed (FS)	Camelina sativa (CAM)	Echium plantagineum (EC)	Blackcurrant (BC)	Significance
γ_2 -casein (β -CN	f 106-209)						
1	$7.03 \pm 0.69^{a,b}$	$7.36 \pm 0.08^{a,b}$	7.53 ± 0.01 ^{b,c,A}	$7.47 \pm 0.07^{b,c}$	$8.21 \pm 0.05^{\circ}$	$6.65 \pm 0.15^{a,A}$	**
3	10.04 ± 3.97	7.78 ± 0.21	8.84 ± 0.36^{B}	7.97 ± 0.22	8.89 ± 0.62	7.72 ± 0.23^{B}	ns
Significance	ns	ns	**	ns	ns	**	
γ_1 -casein (β -CN	f 29-209)						
1	3.88 ± 0.46	4.04 ± 0.22	4.18 ± 0.19^{B}	4.2 ± 0.23	4.03 ± 0.1	3.73 ± 0.05	ns
3	3.84 ± 0.13	3.88 ± 0.21	3.51 ± 0.04 ^A	3.81 ± 0.15	3.93 ± 0.31	3.69 ± 0.03	ns
Significance	ns	ns	**	ns	ns	ns	
γ_3 -casein (β -CN	f 108-209)						
1	3.66 ± 0.13	3.5 ± 0.06	3.63 ± 0.43	3.24 ± 0.07^{A}	3.67 ± 0.22	3.3 ± 0.07^{A}	ns
3	3.87 ± 0.09 ^b	$3.69 \pm 0.06^{a,b}$	4.53 ± 0.08 ^d	4.26 ± 0.14 ^{c,B}	$4.33 \pm 0.16^{c,d}$	3.6 ± 0.01 ^{a,B}	***
Significance	ns	ns	ns	**	ns	**	
β-casein							
1	36.05 ± 1.19	36.44 ± 1.54	37.34 ± 0.59 ^B	35.91 ± 1.73	34.66 ± 2.6	37.07 ± 2.74	ns
3	34.53 ± 0.08	35.69 ± 2.08	35.05 ± 0.16 ^A	33.82 ± 0.95	30.93 ± 3.84	35.66 ± 2.53	ns
Significance	ns	ns	**	ns	ns	ns	
β-I-casein (β-CN	V f 1-192)						
1	2.96 ± 0.96^{b}	1.71 ± 0.18^{a}	$1.95 \pm 0.03^{a,b,A}$	$1.84 \pm 0.35^{a,b}$	$2.32 \pm 0.28^{a,b,A}$	$1.26 \pm 0.06^{a,A}$	*
3	2.93 ± 0.98 ^{b,c}	$1.9 \pm 0.2^{a,b}$	$2.57 \pm 0.04^{a,b,B}$	2.1 ± 0.07 ^{a,b}	$3.74 \pm 0.03^{c,B}$	$1.63 \pm 0.03^{a,b,B}$	**
Significance	ns	ns	**	ns	**	ns	
α_{s1} -casein							
1	42.36 ± 1.87	44.04 ± 1.23	40.88 ± 0.52	43.1 ± 2.4	43.09 ± 2.75	45.00 ± 2.3	ns
3	40.46 ± 3.10	43.38 ± 1.68	40.52 ± 0.38	43.23 ± 1.13	42.74 ± 3.04	43.76 ± 2.31	ns
Significance	ns	ns	ns	ns	ns	ns	
α_{s1} -casein (α_{s1} -	CN f 102-199)						
1	$2.01 \pm 0.37^{b,c}$	1.39 ± 0.04^{a}	$2.38 \pm 0.01^{\circ}$	$2.22 \pm 0.21^{b,c}$	$2.23 \pm 0.04^{b,c}$	$1.7 \pm 0.38^{a,b}$	**
3	$2.03 \pm 0.19^{a,b}$	1.79 ± 0.13^{a}	$2.53 \pm 0.06^{a,b}$	2.59 ± 0.61^{b}	2.81 ± 0.24^{b}	$2.24 \pm 0.25^{a,b}$	*
Significance	ns	ns	ns	ns	ns	ns	
α_{s1} -casein (α_{s1} -	CN f 24-199)						
1	$2.06 \pm 0.01^{b,c,A}$	$1.51 \pm 0.01^{a,b}$	$2.12 \pm 0.27^{\circ}_{1.12}$	$2.01 \pm 0.02^{b,c}$	1.8 ± 0.03^{b}	1.3 ± 0.14^{a}	**
3	2.31 ± 0.20 ^{b,c,B}	$1.9 \pm 0.14^{a,b}$	2.45 ± 0.09 ^{b,c}	$2.23 \pm 0.27^{a,b,c}$	$2.63 \pm 0.49^{b,c}$	1.7 ± 0.25^{a}	*
Significance	*	ns	ns	ns	ns	ns	

Table 4. Relative percentage of identified casein fractions of the Control and fortified fresh cheeses calculated from densitometry analysis after 1 and 3 day of ripening.

Data are expressed as the means \pm SD (n = 4).

Means with different lowercase letters within the same row are significantly different (Duncan's test, p < .05); means with different uppercase letters within the same column are significantly different (Duncan's test, p < .05).

Significance:

*p<.05; **p<.01;

***p < .001; ns = not significant.

interfere with the action of the plasmin. However, there is no direct correlation between the oil concentration and proteolysis because the cheeses fortified with the other oils were characterised by a higher concentration of this casein with respect to the Control cheese. The γ_3 - [β -casein (f108-209)] showed a significant difference among the oils used in this study, especially at 3 days of ripening (p < .01), and the trends observed were the same as those found for γ_2 -casein.

Statistically significant differences for β -I-casein [β -casein (f1-192)], the peptide produced by the action of the chymosin on β -caseins, were observed among the fortified cheeses at both days of ripening. At 1 day of ripening, all of the fortified cheeses showed a lower concentration with respect to the Control. This trend was also observed at 3 days of ripening, except that the EC fortified cheese showed a higher concentration.

More statistically significant differences were observed in the products of α_{s1} -caseins. The oil that affects the degradation of α_{s1} -caseins the most was the

additional RAS oil. In particular, the RAS oil seems to interfere with the chymosin action, possibly due to the lower pH of the cheeses.

Total viable count, yeasts and moulds

The microbial analysis showed no significant differences in bacteria and mould growth among cheeses (data not shown). During storage, in the total viable count had grown from approximately $2 \log \operatorname{cfu} g^{-1}$ at day 1 to $4 \log \operatorname{cfu} g^{-1}$ at day 3. The yeast and mould counts remained in a range of 2–3 log cfu g⁻¹. However, after 3 days of storage, the counts had grown with a similar trend without important differences between all the cheeses.

Consumer acceptance

Usual fresh cheese consumers were mainly involved in the study, with 64% of the subjects reporting a

Total (<i>n</i> = 174)	Control	Blackcurrant (BC)	Flaxseed (FS)	Camelina sativa (CAM)	Raspberry (RAS)	F	р
Appearance	6.23 ± 0.13	6.05 ± 0.13	6.10±0.13	6.11 ± 0.13	6.06 ± 0.13	1.022	ns
Odour	5.73 ± 0.13^{a}	5.26 ± 0.13^{bc}	5.44 ± 0.13^{b}	5.24 ± 0.14^{bc}	$5.08 \pm 0.14^{\circ}$	6.444	<.001
Taste	5.41 ± 0.14^{a}	$4.75 \pm 0.13^{\circ}$	5.13 ± 0.13^{b}	$4.83 \pm 0.15^{\circ}$	$4.78 \pm 0.14^{\circ}$	7.814	<.001
Flavour	5.32 ± 0.13^{a}	4.68 ± 0.13^{b}	5.09 ± 0.14^{a}	4.66 ± 0.15^{b}	4.79 ± 0.14^{b}	9.003	<.001
Texture	5.67 ± 0.14^{a}	5.27 ± 0.14^{b}	5.56 ± 0.14^{a}	5.29 ± 0.15^{b}	5.44 ± 0.14^{ab}	3.952	.004
Overall liking	5.49 ± 0.13^{a}	$4.76 \pm 0.13^{\circ}$	5.15 ± 0.13^{ab}	4.92 ± 0.15^{bc}	$4.89 \pm 0.14^{\circ}$	10.000	<.001
Cl1 $(n = 64)$							
Appearance	6.42 ± 0.19	6.27 ± 0.21	6.56 ± 0.19	6.42 ± 0.19	6.42 ± 0.21	0.943	ns
Odour	5.77 ± 0.20^{a}	5.17 ± 0.20^{b}	5.92 ± 0.19^{a}	5.58 ± 0.21^{ab}	5.53 ± 0.22^{ab}	3.561	.008
Taste	5.16 ± 0.23 ^{bc}	$4.52 \pm 0.22^{\circ}$	5.92 ± 0.18^{a}	5.16 ± 0.23^{b}	5.53 ± 0.22^{ab}	9.990	<.001
Flavour	5.33 ± 0.21 ^b	$4.61 \pm 0.21^{\circ}$	5.95 ± 0.18^{a}	5.14 ± 0.22^{b}	5.53 ± 0.19^{ab}	10.296	<.001
Texture	5.66 ± 0.23 ^{bc}	$5.33 \pm 0.24^{\circ}$	5.98 ± 0.22^{ab}	5.70 ± 0.23^{bc}	6.14 ± 0.21^{a}	5.103	.001
Overall liking	5.25 ± 0.20^{b}	$4.42 \pm 0.20^{\circ}$	6.00 ± 0.17^{a}	5.30 ± 0.21^{b}	5.80 ± 0.19^{a}	22.001	<.001
Cl2 $(n = 65)^{-1}$							
Appearance	6.00 ± 0.22	5.80 ± 0.21	5.92 ± 0.23	5.66 ± 0.23	5.65 ± 0.21	1.806	ns
Odour	5.72 ± 0.21^{a}	5.25 ± 0.22^{b}	5.46 ± 0.22^{ab}	$4.42 \pm 0.24^{\circ}$	$4.57 \pm 0.22^{\circ}$	13.217	<.001
Taste	5.48 ± 0.23^{a}	4.75 ± 0.19 ^b	4.94 ± 0.22^{b}	$3.89 \pm 0.21^{\circ}$	$3.92 \pm 0.21^{\circ}$	22.419	<.001
Flavour	5.17 ± 0.21^{a}	4.63 ± 0.20^{b}	4.78 ± 0.21^{ab}	$3.62 \pm 0.20^{\circ}$	$4.00 \pm 0.21^{\circ}$	19.220	<.001
Texture	5.63 ± 0.22^{a}	5.11 ± 0.20 ^b	5.51 ± 0.21^{a}	$4.45 \pm 0.23^{\circ}$	$4.63 \pm 0.22^{\circ}$	14.620	<.001
Overall liking	5.66 ± 0.22^{a}	4.89 ± 0.20^{b}	5.05 ± 0.21^{b}	$3.85 \pm 0.22^{\circ}$	$3.89 \pm 0.20^{\circ}$	37.885	<.001
Cl3 (n = 45)							
Appearance	6.29 ± 0.26^{a}	6.09 ± 0.25^{ab}	5.71 ± 0.29 ^b	6.33 ± 0.23^{a}	6.16 ± 0.23^{a}	2.679	ns
Odour	5.69 ± 0.25^{ab}	5.40 ± 0.24^{b}	$4.73 \pm 0.22^{\circ}$	5.96 ± 0.27^{a}	5.18 ± 0.25^{bc}	6.286	<.001
Taste	5.69 ± 0.30^{a}	5.09 ± 0.28^{b}	$4.27 \pm 0.28^{\circ}$	5.73 ± 0.27^{a}	4.96 ± 0.28^{b}	11.258	<.001
Flavour	5.53 ± 0.27^{a}	4.84 ± 0.27^{b}	$4.31 \pm 0.28^{\circ}$	5.49 ± 0.28^{a}	4.89 ± 0.26^{b}	8.706	<.001
Texture	5.76 ± 0.28^{ab}	5.42 ± 0.26^{bc}	$5.02 \pm 0.26^{\circ}$	5.93 ± 0.30^{a}	5.60 ± 0.29^{ab}	5.372	<.001
Overall liking	5.60 ± 0.27^{a}	5.07 ± 0.26^{b}	$4.09 \pm 0.26^{\circ}$	5.93 ± 0.23^{a}	5.04 ± 0.30^{b}	23.352	<.001

Table 5. Results of two-way mixed ANOVA models (fixed factor: sample; random factor: subject) separately conducted on the liking ratings of all of the subjects, Cl1, and Cl2.

Mean values, standard errors of mean and Fisher's Least Significant Difference are reported for liking based on appearance, odour, taste, flavour and texture as well as overall liking.

Data are expressed as the means \pm SD (n = 4).

Means with different lowercase letters within the same row are significantly different (Fisher's LSD, p < .05). Hedonic scale from 1 (extremely dislike) to 9 (extremely like).

self-declared fresh cheese consumption of at least 2 times a week.

Table 5 shows the average liking ratings (appearance, odour, taste, flavour, texture, overall liking) calculated for the totality of 174 subjects. The enrichment with omega-3 significantly affected the liking of all of the sensory modalities, with the exception of appearance, which resulted in 'slightly liked' for all of the samples. The Control and FS samples reported comparable average values for overall liking, flavour and texture, resulting in the most appreciated samples. All of the fortified samples (BC, FS, CAM and RAS) were judged acceptable considering odour and texture, while for taste, a flavour tended to be slightly below acceptability, except for FS. Overall, FS showed the best hedonic performance.

In Figure 1, the IPM is shown. The first two dimensions accounted for 55% of the total explained variance. Even if consumers generally seemed to prefer the Control cheeses, a quite uniform distribution of subjects was observed on the map revealing that a clear preference for specific fortified samples was not evident. From the consumer segmentation performed by applying the K-means Cluster to the coordinates of subjects on the first three principal components (80% of the explained variance), three clusters (Cl) were

found: Cl1 (n = 64; male = 25), Cl2 (n = 65; male = 24)and Cl3 (n = 45; male = 18). All the clusters were constituted mainly of young consumers, with the percentages of subjects aged from 18 to 35 in Cl1, Cl2 and Cl3 equal to 56, 48 and 64, respectively. The results from the groups did not differ significantly, considering either the personal data or the frequency of consumption of fresh cheese (p > .05). For the two-way mixed ANOVA model performed considering product and cluster factors, a significant effect of product on liking of clusters was found (F = 4.31; p = .02), as well as of the cluster (F = 13.82, p < .001) and of the interaction cluster * product (F = 12.51; p < .001), indicating that clusters had different preferences. Table 5 shows the average liking ratings (appearance, odour, taste, flavour, texture, overall liking) obtained from each cluster. Cl1 significantly preferred FS and RAS samples. Those samples had the best hedonic performances considering all the sensory modalities, whereas the BC sample was the lowest performing sample for Cl1. Moreover, the samples were not discriminated for liking of appearance by this group. Cl2 preferred the Control cheeses. That cluster provided the lowest liking scores, with the fortified samples clearly resulting in dislike, particularly considering RAS and CAM. Cl3 preferred the Control and CAM, with no significant



Figure 1- Internal preference map of 174 consumers who rated the overall liking (1 = extremely dislike; 9 = extremely dislike) for the Control (CTR) and four fortified fresh cheeses (Blackcurrant BC, Flaxseed FS, *Camelina sativa* CAM, Raspberry RAS).

differences between them for all of the sensory modalities. The FS sample was the least appreciated sample by Cl3. Considering the questionnaire, few significant differences were found among the clusters and only in terms of items related to the health attitude (p < .05). In particular, Cl2 was significantly more in agreement with the statement '*It is important for me that my daily diet contains many vitamins and minerals*' than Cl1. Moreover, for Cl2, the statement '*I always follow a healthy and balanced diet*' resulted in a significantly more important issue than for Cl3.

Developing omega-3 PUFA fortified products could be a challenging issue considering consumer satisfaction. In particular, the enrichment of cheese with omega-3 PUFA could contribute a 'fishy' off-flavour when adding encapsulated fish oil in high amounts (Gracey et al. 1999). This sensory perception affected the acceptability negatively (Iafelice et al. 2008). Therefore, evaluating the effect of omega-3 fortification on consumer liking appears compelling, especially if considering that "fishy-off" flavours can be detected at very low level of 10 g kg^{-1} (Ye et al., 2009). The study of Ye and colleagues (2009) showed a better sensory performance of encapsulated omega-3 PUFA compared to the directly added omega-3 PUFA.

In the current study, the omega-3 enrichment provided satisfactory sensory results overall, and three segments with opposite preferences were found. This liking variability among samples suggested a high discrimination of samples in terms of sensory properties. Interestingly, the enrichment did not negatively affect the appearance, except for one cluster. This result is positive, considering that appearance modifications in newly developed products could worsen the acceptability (Lavelli et al. 2014; Torri et al. 2015).

Similarly, in a recent study on queso fresco, mozzarella and cheddar cheese fortified with omega-3 from flaxseed oil (FS oil) and from microencapsulated fish oil (MFO) showed that the addition had only a minimal effect on whether consumers liked the appearance (Bermúdez-Aguirre & Barbosa-Canovas, 2012). The same study observed a significant effect of the omega-3 source (either FS or MFO oils) on whether consumers liked the odour.

In general, the present study confirms that the fortification of dairy products with omega-3 is possible, as previously found by Kolanowski and Weißbrodt (2007).

Moreover, this result consolidates the finding that vegetable omega-3 seemed particularly suited for cheese fortification considering the hedonic performance compared with the animal-extracted omega-3 (Bermúdez-Aguirre & Barbosa-Canovas, 2012).

Interestingly, due to a strong effect of the vegetable source on acceptability, a further sensory characterisation of prototypes could be beneficial to the exploration of the sensory drivers of liking associated with each vegetable source and potentially responsible for the different clusters of preferences.

Conclusions

This study highlighted the simple and successful possibility to improve the nutritional value of fresh cheeses by addition in the curd of vegetable oils naturally rich in omega-3 PUFA. High retention of omega-3 was achieved in all the cheeses produced, reaching an ALA content of more than $2 g k g^{-1}$ of product. The fortified fresh cheese with the highest omega-3 content was that produced with CAM oil whereas most appreciated by the consumer that with FS oil. Considering the importance and benefits of omega-3 PUFA in the daily intake, in this study an excellent amount of these healthy fatty acids was fully achieved. For this reason, the validation of the proposed approach could be taken into consideration as natural and easy way to incorporate omega-3 into the cheese.

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Disclosure statement

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