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Cheese supplementation with five species of edible seaweeds: Effect on proteolysis, lipolysis and volatile compounds

Ana del Olmo, Olga López-Pérez, Antonia Picon, Pilar Gaya, Manuel Nuñez*

Departamento de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain

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ABSTRACT

The effect on the biochemical characteristics of Ibérico semi-hard cheese supplemented with *Himanthalia elongata* (HE), *Laminaria ochroleuca* (LO), *Porphyra umbilicalis* (PU), *Ulva lactuca* (UL) or *Undaria pinnatifida* (UP) seaweeds was investigated. Addition of 1% dehydrated seaweed to curd increased total free amino acids in LO cheese and decreased them in HE and UP cheeses at 60 days with respect to control cheese. Lipolysis was markedly enhanced in UL cheese, which showed on day 60 a 6.0-fold higher concentration of total free fatty acids than control cheese. Seventy-six volatile compounds were detected in cheeses with seaweeds and 54 in control cheese. Aldehydes and alcohols reached higher levels in the control cheese, esters and ketones in PU cheese, acids and sulphur compounds in UL cheese, hydrocarbons in HE and PU cheeses, and furans in HE and UP cheeses.

1. Introduction

Fermented milks and dairy products have beneficial health properties that allow their consideration as functional foods (Nachay, 2015; Shiby & Mishra, 2013). Moreover, because of their composition and microstructure, which includes hydrophilic (aqueous) and hydrophobic (lipid) phases, they show great potential as carriers for nutrients from different sources that might promote their health-related properties (Calligaris, Ignat, Biasutti, Innocente, & Nicoli, 2015; Giroux et al., 2013; Sawale, Patil, Singh, Arvind, & Ghule, 2012). Thus, fortification of fluid milk with proteins, vitamins, omega-3 fatty acids, phytosterols, fibre or iron (Lin, Kelly, O'Mahony, & Guinee, 2016; Nagarajappa & Battula, 2017; Yeh, Barbano, & Drake, 2017) and of fermented milks with proteins, folate or vitamin D (Hanson & Metzger, 2010; Karam, Gaiani, Hosri, Burgain, & Scher, 2013; Rad, Khosroushahi, Khalili, & Jafarzadeh, 2016) has been investigated. Cheese fortification with omega-3 fatty acids, vitamins B_{12} or D_3, zinc and other compounds (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Galante et al., 2017; Giroux et al., 2013; Tippetts, Martini, Brothersen, & McMahon, 2012) has also attracted the interest of researchers.

Edible seaweeds are a rich source of antioxidants, dietary fibres, essential amino acids, vitamins, phytochemicals, polyunsaturated fatty acids and minerals (Ibañez & Cifuentes, 2013; Wells et al., 2017). They are of great relevance in the diet of Asian countries but still remain of minor importance in the diet of Western countries (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007). Meat, fish, bakery and other food products have been enriched with seaweeds or their extracts with the aim of enhancing their quality and health-related properties, either by increasing the concentration of certain beneficial nutrients or by lowering calories and saturated fatty acids (Cofrades, Benedí, Garcimartin, Sánchez-Muniz, & Jimenez-Colmenero, 2017; Gupta & Abu-Ghannam, 2011; Moroney, O' Grady, O' Doherty, & Kerry, 2013; Roohinejad et al., 2017).

Fortification with micronutrients generally improves health-related properties with no significant effect on other food characteristics (Galante et al., 2017; Hanson & Metzger, 2010). However, food enrichment with seaweeds or seaweed extracts may influence their technological and sensory characteristics, as shown for some meat and dairy products (Jiménez-Colmenero et al., 2010; O'Sullivan et al., 2016). Fermented milks and certain acid-curd cheeses can be enriched in nutrients once their conventional manufacturing process is ended (Nuñez & Picon, 2017; Yeh et al., 2017). However, rennet-curd cheeses must be enriched during manufacture by addition of nutrients to milk or curd (Bermúdez-Aguirre & Barbosa-Cánovas, 2012; Giroux et al., 2013). It has been shown that seaweed addition to curd influences cheese physicochemical parameters and enzymatic activities (del Olmo, Picon, & Nuñez, 2018). Consequently, the biochemical reactions taking

* Corresponding author. Tel.: + 34 913476799.
E-mail address: nunez@inia.es (M. Nuñez).

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place during cheese ripening, including those involved in proteolysis and lipolysis, may be affected. Furthermore, the cheese volatile fraction may be altered by seaweed addition to curd since many of the volatile compounds and their precursors are generated through proteolysis and lipolysis (Collins, McSweeney, & Wilkinson, 2003; Liu, Holland, & Crow, 2004; Yvon & Rijnen, 2001) and also because some of the volatile compounds in seaweeds (López-Pérez, Picon, & Nuñez, 2017) may be retained in the cheese.

In a previous study we reported the influence of adding five dehydrated edible seaweeds (*Himanthalia elongata*, *Laminaria ochroleuca*, Porphyra *umbilicalis*, *Ulva lactuca*, or *Undaria pinnatifida*) to curd on the microbiota, antioxidant activity, colour, texture and sensory characteristics of Ibérico cheese, a semi-hard variety produced in Spain from a mixture of cow, ewe and goat milk (del Olmo et al., 2018). The objective of the present research was to investigate the effect of the addition of those five seaweeds to curd on proteolysis, lipolysis and volatile compounds produced during the ripening process of Ibérico cheese. To our knowledge, there is no published information on how seaweed addition to curd affects the cheese ripening process, as evaluated through the determination of free amino acids, free fatty acids and volatile compounds.

2. Materials and methods

2.1. Cheese manufacture and seaweeds

The manufacturing process of Ibérico cheese from a mixture of cow, ewe and goat milk (40%, 40% and 20%, respectively) on a pilot plant scale and the main characteristics of dehydrated seaweeds *H. elongata* (HE; sea spaghetti), *L. ochroleuca* (LO; kombu, kelp), *P. umbilicalis* (PU; nori, laver), *U. lactuca* (UL; sea lettuce) and *U. pinnatifida* (UP; wakame) added to curd (10 g per kg of fresh curd) were described in a previous paper (del Olmo et al., 2018); cheeses were designated HE, LO, PU, UL and UP, respectively. Briefly, duplicate cheesemaking trials were carried out on different days, each consisting of six 40 L vats (one control vat and five vats with added seaweeds) of pasteurised milk to which 40 mL of a 100 g L^{-1} $CaCl_2$ solution and 400 mL of a lactic starter culture (CHOOZIT™ MA 16, Danisco A/S, Copenhagen, Denmark) grown in UHT milk for 16 h at 25 °C were added. Liquid lamb rennet extract (8 mL per vat, of 1:15,000 strength, Laboratorios Arroyo, Santander, Spain) was added to the milk, which coagulated at 32 °C in 40 min. Four cheeses, of approximately 2 kg in weight, were obtained from each vat. Cheeses were sampled, after eliminating the rind, on days 1, 20, 40 and 60 of ripening, for the analytical determinations reported in a previous study (del Olmo et al., 2018) and to investigate the production of free amino acids, free fatty acids and volatile compounds during cheese ripening.

2.2. Aminopeptidase and lipase activity of seaweeds

Aminopeptidase activity of dehydrated seaweeds, with Leu-*p*-nitroanilide, Lys-*p*-nitroanilide and Pro-*p*-nitroanilide as substrates, was determined in triplicate using a method previously described for cheese (Garde, Tomillo, Gaya, Medina, & Nuñez, 2002b), which was adapted for seaweeds. The enzymatic reaction took place in 100 mM sodium phosphate buffer, pH 7.0. Blanks without substrate were incubated for each of the seaweeds. Absorbance was read at 410 nm after incubation at 30 °C for a maximum of 2 h and the aminopeptidase activity expressed as nmol of Leu-, Lys- or Pro-*p*-nitroaniline per min and g of dehydrated seaweed.

Lipase activity of dehydrated seaweeds was determined in triplicate on a butter-in-water emulsion (2.5 g butter in 50 mL Milli-Q water) as substrate to which 1.0 g of each dehydrated seaweed was separately added. A control emulsion without added seaweeds was also prepared. After incubation of the mixture in an orbital shaker for 5 h at 25 °C followed by 1 h at 37 °C, samples were taken and frozen at −40 °C until analysis. Individual free fatty acids (FFAs) were determined by gas chromatography (GC) using a flame ionisation detector (FID) as previously described (Fernández-García, Carbonell, Calzada, & Nuñez, 2006). Lipase activity was expressed as mg of C4 to C18:2 FFAs released per g of dehydrated seaweed. For this purpose, the sum of the concentrations of C4 to C18:2 FFAs after incubation of control emulsion was subtracted from the sum of the concentrations of C4 to C18:2 FFAs after incubation of each of seaweed-containing emulsions. To verify that microorganisms from seaweeds were not responsible for lipolysis, aerobic microbial counts were determined before and after incubation of the emulsions on plate count agar (PCA), marine agar and MRS agar, as previously described (del Olmo et al., 2018).

2.3. Analysis of free amino acids, free fatty acids and volatile compounds in cheese

Free amino acids (FAAs) and biogenic amines were simultaneously extracted in triplicate, determined by reversed phase-high performance liquid chromatography (RP-HPLC), and quantified as previously described (Calzada, Del Olmo, Picon, Gaya, & Nuñez, 2013).

Free fatty acids (FFAs) were extracted in triplicate, analysed by GC, and quantified as previously described (Fernández-García et al., 2006). Pentanoic, nonanoic and heptadecanoic acids (200 μL of a solution containing 2, 2 and 4 mg mL^{-1} of pentanoic, nonanoic and heptadecanoic acids, respectively, added per g of cheese) were used as internal standards.

Volatile compounds present in cheese were extracted in triplicate, after grinding 5 g of cheese with 5 g of Na_2SO_4 and cyclohexanone (25 μL of a 1058 mg L^{-1} solution) as internal standard by means of solid-phase microextraction (SPME). Volatile compounds were analysed by GC coupled to mass-spectrometry (MS) and their abundance determined as previously described (López-Pérez et al., 2017).

2.4. Statistical analysis

Statistical analysis of results included two-way analysis of variance with added seaweed and ripening time as main effects, comparison of means by Tukey's test with significance assigned at $P < 0.05$, calculation of correlations, and principal component analysis (PCA) with Varimax rotation. Data of pH value, aminopeptidase activity, odour characteristics (odour quality and seaweed odour) and flavour characteristics (flavour quality, seaweed flavour, acid flavour, bitter flavour, sweet flavour, salty flavour and umami flavour) of the same cheeses determined in a previous study (del Olmo et al., 2018) were used to calculate some of the correlations with data from the present study. Also, data for odour and flavour characteristics of the same cheeses from that study were used for the PCA carried out on volatile compounds and sensory characteristics. Analysis was carried out using the SPSS Win 19.0 statistical package (SPSS Inc. Chicago, IL, USA), as previously described (del Olmo et al., 2018).

3. Results and discussion

3.1. Free amino acids in cheese and aminopeptidase activity of seaweeds

Concentration of total FAAs increased significantly ($P < 0.05$) from day 1 to day 60 (Table 1), by a factor of 5.2 for control cheese

Table 1
Levels of total free amino acids during ripening of control and experimental cheeses supplemented with five seaweed species.[a]

Days	Control cheese	Experimental cheeses				
		HE	LO	PU	UL	UP
1	2.731 ± 0.486 ^{aA}	2.569 ± 0.465 ^{aA}	2.680 ± 0.529 ^{aA}	2.636 ± 0.496 ^{aA}	2.426 ± 0.313 ^{aA}	3.038 ± 0.446 ^{aA}
20	6.809 ± 1.130 ^{abB}	5.358 ± 0.297 ^{aAB}	8.382 ± 0.452 ^{bB}	8.146 ± 1.201 ^{bB}	6.507 ± 0.603 ^{abB}	6.619 ± 0.596 ^{abB}
40	9.776 ± 1.179 ^{abB}	7.512 ± 1.078 ^{aBC}	11.037 ± 0.323 ^{bB}	11.655 ± 1.829 ^{bB}	9.604 ± 0.479 ^{abB}	8.340 ± 0.154 ^{aBC}
60	14.260 ± 1.501 ^{bC}	9.350 ± 0.467 ^{aC}	17.144 ± 1.428 ^{cC}	16.250 ± 2.110 ^{bcC}	13.937 ± 1.125 ^{bC}	10.054 ± 1.291^{aC}

[a] Levels of total free amino acids are means ± SD, expressed as mg g^{-1} cheese DM, from determinations in triplicate on two cheesemaking trials. Means in the same row followed by a different lowercase superscript letter and means in the same column followed by a different uppercase superscript letter are significantly (P < 0.05) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

and by factors ranging from 3.3 for UP to 6.4 for LO experimental cheeses. On day 60, levels of FAAs were significantly ($P < 0.05$) higher in LO cheese, and significantly lower in HE and UP cheeses, compared with the control cheese. Lower pH values found for HE and UP cheeses throughout ripening (del Olmo et al., 2018) might have hindered the activity of proteolytic enzymes such as milk plasmin, chymosin and pepsin from rennet, and proteinases and peptidases from lactic acid bacteria in those cheeses. Also, low pH values of cheeses during ripening may have been detrimental to the activity of aminopeptidases from added seaweeds. Total FAAs in the present work for control and experimental cheeses correlated with overall proteolysis values ($r = 0.981$) found for the respective cheeses from day 1 to day 60 and with the umami scores ($r = 0.684$) obtained from sensory analysis for the respective cheeses from day 20 to day 60 in a previous work (del Olmo et al., 2018). Total FAAs in 60-day control and experimental cheeses also correlated well with average aminopeptidase activities found throughout a 60-day ripening period (del Olmo et al., 2018), with r values of 0.790, 0.794 and 0.508, respectively, for the activity on Leu-*p*-nitroanilide, Leu-*p*-nitroanilide, and Pro-*p*-nitroanilide as substrates.

The most abundant FAAs detected in 60-day control cheese (Table 2) were, in decreasing order, Leu, Glu, Phe, Val, Lys and Ser while in HE cheese they were Leu, Lys, Pro, Phe, Val and Glu, in LO cheese Glu, Leu, Lys, Val, Phe and Ser, in PU cheese Glu, Leu, Lys, Val, Phe and Ser, in UL cheese Glu, Leu, Lys, Phe, Val and Ser, and in UP cheese Lys, Leu, Phe, Glu, Pro and Val. These differences point out particular patterns of release of individual FAAs in experimental cheeses, which might be related to the specific activity of peptidases coming from seaweeds. In this regard, aminopeptidase activity on Leu-*p*-nitroanilide and Lys-*p*-nitroanilide reached markedly higher values in *L. ochroleuca* than in the rest of seaweeds, while the activity on Pro-*p*-nitroanilide was considerably lower in *U. lactuca* than in the rest of seaweeds (Fig. 1A). Aminopeptidase activities of different seaweeds on Leu-*p*-nitroanilide and Lys-*p*-nitroanilide were strongly correlated among them ($r = 0.949$) but not with activity on Pro-*p*-nitroanilide (r values of 0.236 or 0.257, respectively). In the case of Pro, its concentration in 60-day cheeses with added seaweeds correlated better with the aminopeptidase activity of seaweeds on Leu-*p*-nitroanilide ($r = 0.415$) or Lys-*p*-nitroanilide ($r = 0.445$) than with the activity on Pro-*p*-nitroanilide ($r = 0.074$). The above results do not support a strong relationship among the specific aminopeptidase activity of seaweeds and the concentration of particular FAAs in experimental cheeses. Factors other than aminopeptidase activity, such as cheese pH, might influence the concentration of total FAAs at the end of their ripening period (Table 1) as shown by the correlation ($r = 0.776$) of total FAAs with the pH values of 60-day cheeses (del Olmo et al., 2018).

No biogenic amines were detected in the present study throughout the ripening period of control and experimental cheeses. This may be the result of using pasteurised milk and the low counts of non-starter lactic acid bacteria in all cheeses (del Olmo et al., 2018). For raw milk cheeses manufactured using procedures similar to those of Ibérico cheese, 90.3 mg kg^{-1} total

Table 2
Levels of individual free amino acids (FAAs) in control and experimental cheeses supplemented with five seaweed species after 60 days of ripening.[a]

FAA	Control cheese	Experimental cheeses				
		HE	LO	PU	UL	UP
Asp	0.292 ± 0.062^{a}	0.246 ± 0.072^{a}	0.377 ± 0.071^{a}	0.286 ± 0.088^{a}	0.302 ± 0.065^{a}	0.249 ± 0.067^{a}
Ser	1.005 ± 0.218 ^{ab}	0.571 ± 0.159^{a}	1.138 ± 0.213 ^{ab}	1.149 ± 0.233^{b}	1.045 ± 0.168 ^{ab}	0.566 ± 0.106^{a}
Glu	1.875 ± 0.520 ^{ab}	0.750 ± 0.165^{a}	2.589 ± 0.613^{b}	2.489 ± 0.545^{b}	2.065 ± 0.352^{b}	0.982 ± 0.219^{a}
Gly	0.277 ± 0.072^{a}	0.153 ± 0.039^{a}	0.365 ± 0.127^{a}	0.318 ± 0.151^{a}	0.233 ± 0.049^{a}	0.164 ± 0.043^{a}
His	0.605 ± 0.146^{a}	0.378 ± 0.059^{a}	0.766 ± 0.181^{a}	0.739 ± 0.253^{a}	0.599 ± 0.155^{a}	0.449 ± 0.163^{a}
Arg	0.245 ± 0.032^{a}	0.294 ± 0.044 ^{ab}	0.341 ± 0.068^{b}	0.351 ± 0.005^{b}	0.285 ± 0.037 ^{ab}	0.295 ± 0.028 ^{ab}
Thr	0.151 ± 0.039 ^{ab}	0.139 ± 0.024^{a}	0.243 ± 0.032 ^{bc}	0.283 ± 0.036^{c}	0.244 ± 0.025 ^{bc}	0.170 ± 0.002 ^{ab}
Ala	0.376 ± 0.061 ^{ab}	0.225 ± 0.009^{a}	0.461 ± 0.070^{b}	0.473 ± 0.095^{b}	0.432 ± 0.002^{b}	0.261 ± 0.045 ^{ab}
Pro	0.712 ± 0.032^{a}	1.035 ± 0.129^{b}	0.986 ± 0.152 ^{ab}	0.760 ± 0.071 ^{ab}	0.859 ± 0.137 ^{ab}	0.912 ± 0.078 ^{ab}
Tyr	0.770 ± 0.147 ^{ab}	0.440 ± 0.086^{a}	0.827 ± 0.161^{b}	0.818 ± 0.146^{b}	0.557 ± 0.078^{a}	0.462 ± 0.086^{a}
Val	1.583 ± 0.310 ^{ab}	0.798 ± 0.037^{a}	1.743 ± 0.313^{b}	1.651 ± 0.392^{b}	1.328 ± 0.098 ^{ab}	0.885 ± 0.212^{a}
Met	0.472 ± 0.125 ^{ab}	0.242 ± 0.040^{a}	0.523 ± 0.142^{b}	0.516 ± 0.164^{b}	0.384 ± 0.066 ^{ab}	0.278 ± 0.076^{a}
Lys	1.388 ± 0.307^{a}	1.368 ± 0.284^{a}	1.775 ± 0.263^{a}	1.877 ± 0.345^{a}	1.694 ± 0.210^{a}	1.517 ± 0.191^{a}
Ile	0.539 ± 0.154 ^{ab}	0.277 ± 0.029^{a}	0.682 ± 0.135^{b}	0.641 ± 0.194^{b}	0.510 ± 0.045 ^{ab}	0.386 ± 0.074^{a}
Leu	2.299 ± 0.552 ^{ab}	1.506 ± 0.190^{a}	2.560 ± 0.380^{b}	2.303 ± 0.569 ^{ab}	2.010 ± 0.092 ^{ab}	1.482 ± 0.327^{a}
Phe	1.670 ± 0.391^{b}	0.928 ± 0.036^{a}	1.727 ± 0.357^{b}	1.600 ± 0.420^{b}	1.392 ± 0.125 ^{ab}	0.998 ± 0.246^{a}

[a] Levels of individual free amino acids are means ± SD, expressed as mg g^{-1} cheese DM, from determinations in triplicate on two cheesemaking trials. Means in the same row followed by a different lowercase superscript letter are significantly (P < 0.05) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

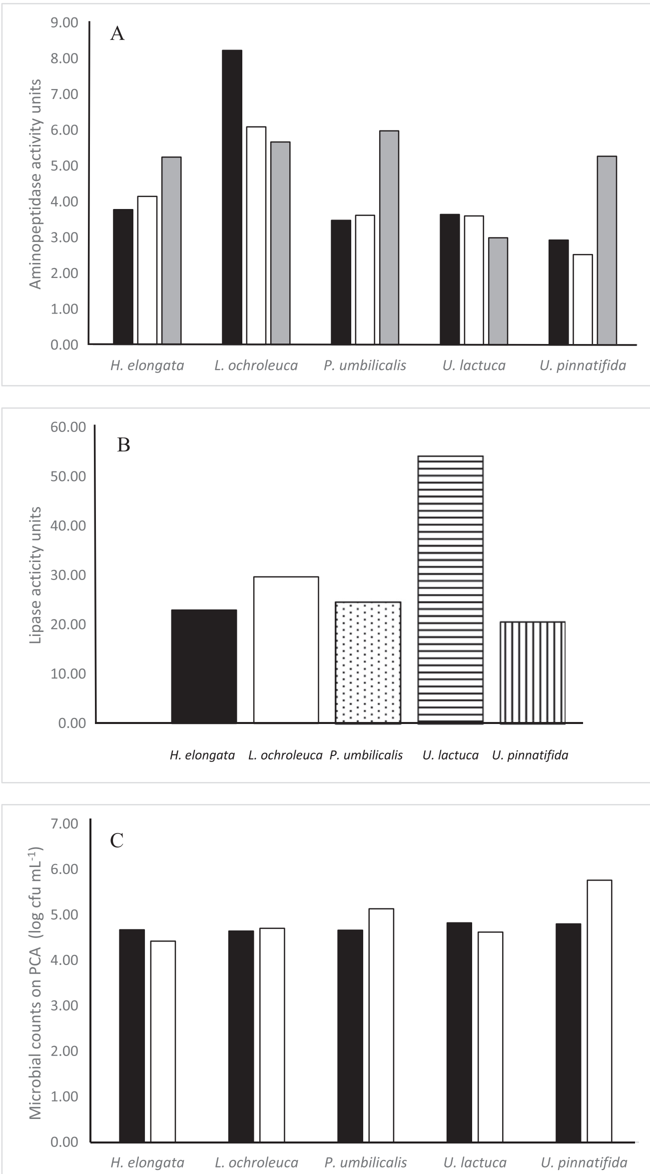

Fig. 1. Aminopeptidase activity of seaweeds (A) on Leu- (■), Lys- (□) or Pro- (■) *p*-nitroanilide as substrates, expressed as nmol of Leu-, Lys- or Pro-*p*-nitroaniline min g^{-1} dehydrated seaweed, (B) lipase activity of dehydrated seaweeds (■, *H. elongata*; □, *L. ochroleuca*; ⬚, *P. umbilicalis*; ▤, *U. lactuca*; ▥, *U. pinnatifida*) on a butter-in-water emulsion after 6 h of incubation, expressed as mg FFA released g^{-1} of seaweed and (C) microbial counts (log cfu mL^{-1}) on plate count agar in the butter-in-water emulsion after 0 h (■) and 6 h (□) incubation.

biogenic amines were found in 60-day Hispánico cheese (Fernández-García, Tomillo, & Nuñez, 2000) and 554.5 mg kg^{-1} in 60-day Arzúa cheese (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2015), while no biogenic amines were detected in 60-day pasteurised milk cheeses, like Brie, in spite of extensive proteolysis (Calzada, del Olmo, Picon, Gaya, & Nuñez, 2014a).

3.2. Free fatty acids in cheese and lipase activity of seaweeds

The concentration of total FFAs (from C4 to C18:3) increased from day 1 to day 60 by a factor of 2.2 in control cheese and by factors ranging from 1.9 for PU to 14.3 for UL experimental cheeses, with significantly ($P < 0.05$) higher levels of total FFAs in UL cheese than in the rest of cheeses throughout ripening (Table 3). Total FFAs (from C4 to C18:3) reached 5.946 mg g^{-1} cheese dry matter in control cheese on day 60 and ranged from 5.313 mg g^{-1} cheese dry matter in PU to 35.716 mg g^{-1} cheese dry matter in UL experimental cheeses (Table 3). Saturated and unsaturated FFAs (Table 4), as well as short-, medium- and long-chain FFAs, reached their maximum values in UL cheese at the end of the ripening period. Acetic acid, produced by lactic acid bacteria from lactose, citrate and amino acids (Fox & Wallace, 1997), was highest in control cheese and lowest in HE and UL cheeses on day 60 (Table 4). The most abundant FFAs (C4 to C18:3) in 60-day control and experimental cheeses were, in decreasing order, C18:1, C16:0, C14:0 followed by C18:0, C10:0 and C18:2, in variable order (Table 4). The pattern of preferential release of individual FFAs was similar in control and experimental cheeses, but the quantitative aspects of release differed markedly between the UL cheese and the rest.

To ascertain the cause of the markedly higher FFA concentration in UL cheese, the lipase activity of the different seaweeds in a butter-in-water emulsion was determined. Lipase activity, expressed as mg of total FAAs (C4 to C18:3) released per g of dehydrated seaweed in the butter-in-water emulsion after 6 h of incubation, was 22.98, 29.70, 24.65, 54.11 and 20.61 mg FFAs for *H. elongata*, *L. ochroleuca*, *P. umbilicalis*, *U. lactuca* and *U. pinnatifida*, respectively (Fig. 1B). The higher lipase activity of *U. lactuca* in comparison with the rest of seaweeds, a fact which had not been previously reported, appears the most plausible explanation for the high FFA concentration in UL cheese, supported by the strong correlation ($r = 0.982$) between lipase activity of seaweeds and total FFAs in cheeses with added seaweeds. The microbiota present in the different seaweeds did not seem to be responsible for the differences in lipase activity found in the butter-in-water emulsions, since the initial counts on plate count agar of all emulsions were below 4.9 log cfu mL^{-1} and the final counts, after 6 h of incubation, were below 5.8 log cfu mL^{-1} (Fig. 1C). Counts of all samples on Marine agar and MRS agar were slightly lower than on plate count agar. Microbial populations coming from seaweeds, which remained below 6 log cfu mL^{-1} along the 6 h incubation, together with the low incidence of lipolytic microorganisms in seaweeds (Martin et al., 2016), do not seem capable of significantly influencing the release of FFAs.

The only free omega-6 and omega-3 fatty acids found at detectable levels in cheeses were C18:2 (*n*-6) and C18:3 (*n*-3), respectively linoleic and linolenic acids (Table 4). Relative levels of total C18:2 (*n*-6) and C18:3 (*n*-3) fatty acids in control and experimental cheeses may be estimated by assuming that the same proportions of C18:0, C18:1, C18:2 and C18:3 fatty acids on the total content of each of those C18 fatty acids were released from triglycerides in all cheeses. The percentage of free C18:2 on free C18:0 + free C18:1 + free C18:2 + free C18:3 in 60-day cheeses was 15.22% for control cheese and 15.02, 16.41, 16.30, 16.17 and 15.88% for HE, LO, PU, UL and UP cheeses, respectively, with small differences among cheeses. This suggests that the contents of total C18:2 were similar in all cheeses. In contrast, the percentage of free C18:3 on free C18:0 + free C18:1 + free C18:2 + free C18:3 in 60-day cheeses was 3.30% for control cheese and 5.87, 4.13, 3.06, 5.52 and 9.13% for HE, LO, PU, UL and UP cheeses, respectively, with more marked differences among cheeses, which points to higher C18:3 contents in HE, UL and UP cheeses. High contents of C18:3 fatty acid were reported for *H. elongata* (Cofrades et al., 2010), *U. pinnatifida* (Dawczynski, Schubert, & Jahreis, 2007) and *U. lactuca* (Pereira et al., 2012) and a low content for *P. umbilicalis* (Cofrades et al., 2010). The free C18:2/free C18:3 ratio in 60-day cheeses was 4.61 for control cheese and 2.56, 3.97, 5.32, 2.93 and 1.74 for HE, LO, PU, UL and UP cheeses, respectively. This indicates that HE, UL and UP cheeses had the lowest total C18:2/total C18:3 ratio. A low value of

Table 3
Levels of total free fatty acids during ripening of control and experimental cheeses supplemented with five seaweed species.[a]

Days	Control cheese	Experimental cheeses				
		HE	LO	PU	UL	UP
1	2.711 ± 0.326 [aA]	2.578 ± 0.325 [aA]	2.824 ± 0.260 [aA]	2.735 ± 0.198 [aA]	2.501 ± 0.320 [aA]	2.649 ± 0.332 [aA]
20	3.782 ± 0.545 [aAB]	3.496 ± 0.370 [aAB]	4.400 ± 0.298 [aAB]	3.614 ± 0.283 [aAB]	18.582 ± 1.703 [bB]	3.950 ± 0.287 [aAB]
40	5.083 ± 0.401 [abBC]	4.509 ± 0.326 [aBC]	6.245 ± 0.447 [bBC]	4.682 ± 0.471 [aBC]	33.908 ± 3.126 [cC]	4.972 ± 0.460 [aBC]
60	5.946 ± 0.148 [abC]	5.372 ± 0.229 [aC]	7.797 ± 0.493 [bC]	5.313 ± 0.546 [aC]	35.716 ± 3.088 [cC]	6.014 ± 0.545 [abC]

[a] Levels of total free fatty acids (C4 to C18:3) are means ± SD, expressed as mg g^{-1} cheese DM, from determinations in triplicate on two cheesemaking trials. Means in the same row followed by a different lowercase superscript letter and means in the same column followed by a different uppercase superscript letter are significantly ($P < 0.05$) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

Table 4
Levels of acetic acid and individual free fatty acids (FFAs) in control and experimental cheeses supplemented with five seaweed species after 60 days of ripening.[a]

FFA	Control cheese	Experimental cheeses				
		HE	LO	PU	UL	UP
C2:0	0.968 ± 0.128[b]	0.698 ± 0.135[a]	0.833 ± 0.059 [ab]	0.853 ± 0.126 [ab]	0.682 ± 0.070[a]	0.758 ± 0.047[a]
C4:0	0.324 ± 0.057[b]	0.108 ± 0.008[a]	0.231 ± 0.026 [ab]	0.277 ± 0.063 [ab]	1.003 ± 0.190[c]	0.169 ± 0.052 [ab]
C6:0	0.139 ± 0.009[a]	0.088 ± 0.008[a]	0.183 ± 0.031[a]	0.129 ± 0.026[a]	0.982 ± 0.149[b]	0.114 ± 0.029[a]
C8:0	0.139 ± 0.020[a]	0.112 ± 0.013[a]	0.179 ± 0.031[a]	0.136 ± 0.020[a]	0.854 ± 0.115[b]	0.119 ± 0.018[a]
C10:0	0.419 ± 0.023[a]	0.370 ± 0.024[a]	0.503 ± 0.038[a]	0.390 ± 0.030[a]	2.046 ± 0.220[b]	0.375 ± 0.037[a]
C12:0	0.285 ± 0.011[a]	0.268 ± 0.013[a]	0.329 ± 0.016[a]	0.251 ± 0.011[a]	1.430 ± 0.144[b]	0.267 ± 0.023[a]
C14:0	0.702 ± 0.025[a]	0.678 ± 0.037[a]	0.895 ± 0.056[a]	0.607 ± 0.027[a]	4.425 ± 0.532[b]	0.674 ± 0.058[a]
C16:0	1.242 ± 0.131[a]	1.191 ± 0.146[a]	1.626 ± 0.139[a]	1.172 ± 0.105[a]	8.394 ± 0.865[b]	1.271 ± 0.163[a]
C18:0	0.482 ± 0.060 [ab]	0.455 ± 0.038 [ab]	0.628 ± 0.049[a]	0.410 ± 0.057[a]	2.365 ± 0.229[c]	0.471 ± 0.047 [ab]
C18:1	1.713 ± 0.184 [ab]	1.552 ± 0.124[a]	2.432 ± 0.275[b]	1.485 ± 0.203[a]	10.619 ± 1.103[c]	1.795 ± 0.268 [ab]
C18:2	0.410 ± 0.046 [ab]	0.381 ± 0.052[a]	0.632 ± 0.067[b]	0.383 ± 0.050[a]	2.682 ± 0.289[c]	0.480 ± 0.061 [ab]
C18:3	0.089 ± 0.016[a]	0.149 ± 0.038[a]	0.159 ± 0.023[a]	0.072 ± 0.014[a]	0.916 ± 0.108[c]	0.276 ± 0.027[b]
Saturated	3.732 ± 0.109[a]	3.290 ± 0.096[a]	4.573 ± 0.318[a]	3.373 ± 0.345[a]	21.500 ± 1.982[b]	3.461 ± 0.342[a]
Unsaturated	2.212 ± 0.236 [ab]	2.082 ± 0.200[a]	3.223 ± 0.344[b]	1.940 ± 0.258[a]	14.217 ± 1.467[c]	2.552 ± 0.319 [ab]

[a] Levels of acetic acid and individual free fatty acids are means ± SD, expressed as mg g^{-1} cheese DM, from determinations in triplicate on two cheesemaking trials. Saturated FFAs do not include acetic acid. Means in the same row followed by a different lowercase superscript letter are significantly ($P < 0.05$) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

the omega 6/omega 3 ratio, preferably below 4, is recommended for the prevention of cardiovascular diseases (Simopoulos, 2008).

3.3. *Volatile compounds*

A total of 76 volatile compounds were identified during the ripening period of cheeses, including 10 aldehydes, 8 ketones, 14 alcohols, 11 acids, 6 esters, 18 hydrocarbons, 3 sulphur compounds, 4 terpenes and 2 furans. Of these 76 compounds, only 54 were found in control cheese (8 aldehydes, 8 ketones, 12 alcohols, 11 acids, 5 esters, 3 hydrocarbons, 1 sulphur compound, 4 terpenes and 2 furans), which indicates 22 volatile compounds are coming from seaweeds. This proportion was highest in the case of hydrocarbons, most of which (15 out of 18) appeared to be derived from seaweeds. This confirms the hypothesis that part of the volatile compounds present in seaweeds (López-Pérez et al., 2017) might be retained in the cheese.

The development of the nine groups of volatile compounds during ripening differed considerably among cheeses (Table 5). Significant variations during ripening were recorded (i) for aldehydes, which increased ($P < 0.05$) in control, UL and UP cheeses, (ii) for ketones, which decreased ($P < 0.05$) in control cheese and increased ($P < 0.05$) in PU cheese, (iii) for alcohols, which decreased ($P < 0.05$) in control, LO, PU and UL cheeses, (iv) for acids, which increased ($P < 0.05$) in control, HE, PU and UL cheeses, (v) for esters, which increased ($P < 0.05$) in control, HE and PU cheeses, (vi) for hydrocarbons, which increased ($P < 0.05$) in HE and PU cheeses, (vii) for sulphur compounds, which increased ($P < 0.05$) in HE and UL cheeses, and (viii) for furans, which increased ($P < 0.05$) in control, HE and PU cheeses. Terpenes did not vary during ripening in any of the cheeses. At the beginning of the ripening period, the high level of sulphur compounds in UL cheese was noteworthy. At the end of the ripening period, the highest levels of total aldehydes, ketones, alcohols, acids, esters, hydrocarbons, sulphur compounds and furans were respectively found in control, PU, control, UL, PU, HE, UL and HE cheeses (Table 5).

The levels of the main individual volatile compounds, with mean abundances above 10^6 considering as a whole control and experimental cheeses, are shown in Table 6. Those 25 volatile compounds included 2 aldehydes, 6 ketones, 3 alcohols, 7 acids, 2 esters, 3 hydrocarbons, 1 sulphur compound and 1 terpene.

Ethanal reached higher level ($P < 0.05$) on day 60 in control cheese than in experimental cheeses (Table 6), a difference which was also found in 20-day and 40-day cheeses (data not shown). Ethanal had been detected in the five dehydrated seaweeds used in the present study, with the maximum levels being found for *P. umbilicalis* (López-Pérez et al., 2017). However, addition of seaweeds to curd during the manufacture of experimental cheeses either inhibited ethanal formation by microorganisms or enhanced its reduction to ethanol through the antioxidant activity of seaweeds. In the case of 3-methylbutanal, which was detected in all cheeses except LO cheese, differences between control cheese and experimental cheeses were less marked than in the case of ethanal (Table 6). This aldehyde had also been found in the five dehydrated seaweeds used in the present study, with maximum values for *U. lactuca* (López-Pérez et al., 2017).

Regarding ketones, the maximum levels of 2-propanone, 2-butanone, 2-pentanone, 3-hydroxy-2-butanone and 2,3-butanedione were found in PU cheese (Table 6), a result which is difficult to explain based on differences in microbial metabolism or

Table 5
Levels of the main chemical groups of volatile compounds during ripening of control and experimental cheeses supplemented with five seaweed species.[a]

Chemical group	Days	Control cheese	Experimental cheeses				
			HE	LO	PU	UL	UP
Aldehydes	1	24.1 ± 5.1 ^{aA}	40.5 ± 14.3 ^{aA}	46.4 ± 14.2 ^{aA}	28.9 ± 7.2 ^{aA}	22.8 ± 11.2 ^{aA}	39.9 ± 8.5 ^{aA}
	20	158.2 ± 68.1 ^{bAB}	43.5 ± 21.4 ^{aA}	79.0 ± 41.1 ^{abA}	46.9 ± 12.1 ^{aA}	32.7 ± 5.4 ^{aA}	51.5 ± 8.0 ^{aAB}
	40	284.7 ± 125.6 ^{bB}	66.7 ± 22.6 ^{aA}	73.7 ± 27.9 ^{aA}	72.5 ± 35.5 ^{aA}	50.5 ± 2.6 ^{aB}	63.7 ± 8.6 ^{aB}
	60	241.9 ± 134.1 ^{bB}	73.4 ± 14.8 ^{aA}	66.9 ± 35.7 ^{aA}	80.3 ± 16.9 ^{aA}	48.1 ± 3.2 ^{aB}	53.7 ± 8.5 ^{aAB}
Ketones	1	568.4 ± 109.4 ^{abB}	246.2 ± 183.8 ^{aA}	385.9 ± 118.6 ^{abA}	413.5 ± 52.8 ^{bA}	342.4 ± 94.1 ^{abA}	460.8 ± 131.3 ^{abA}
	20	313.9 ± 20.2 ^{abA}	210.7 ± 37.2 ^{aA}	247.0 ± 106.9 ^{abA}	358.1 ± 29.9 ^{bA}	337.2 ± 80.6 ^{abA}	324.8 ± 41.0 ^{abA}
	40	304.9 ± 25.1 ^{abA}	244.5 ± 26.0 ^{aA}	300.6 ± 94.1 ^{abA}	429.0 ± 36.4 ^{bA}	284.0 ± 77.5 ^{abA}	369.1 ± 154.7 ^{abA}
	60	334.2 ± 38.3 ^{abA}	271.1 ± 36.5 ^{abA}	207.5 ± 20.0 ^{aA}	619.9 ± 65.1 ^{cB}	281.6 ± 32.9 ^{abA}	348.1 ± 100.5 ^{bA}
Alcohols	1	955.8 ± 99.0 ^{aB}	583.6 ± 43.9 ^{aA}	878.3 ± 361.9 ^{aB}	930.6 ± 216.2 ^{aB}	477.4 ± 87.7 ^{aB}	869.4 ± 347.1 ^{aA}
	20	765.5 ± 59.5 ^{bcAB}	522.4 ± 117.8^{abA}	676.8 ± 204.8 ^{abcAB}	672.5 ± 163.8 ^{abcA}	425.7 ± 28.1 ^{aAB}	856.7 ± 126.7 ^{cA}
	40	663.7 ± 115.6 ^{bA}	565.1 ± 154.4^{abA}	544.3 ± 158.0 ^{abAB}	607.7 ± 83.2 ^{abA}	276.3 ± 28.8 ^{aAB}	575.4 ± 133.4 ^{abA}
	60	698.7 ± 96.5 ^{bA}	436.4 ± 33.0 ^{abA}	299.5 ± 71.4 ^{aA}	609.9 ± 20.9 ^{abA}	347.9 ± 60.3 ^{aA}	540.4 ± 325.7 ^{abA}
Acids	1	288.4 ± 43.8 ^{aA}	298.2 ± 19.0 ^{aA}	284.2 ± 92.4^{aA}	320.9 ± 89.5 ^{aA}	813.0 ± 312.2^{bA}	565.4 ± 239.5 ^{abA}
	20	438.4 ± 26.5 ^{aAB}	409.1 ± 82.8 ^{aA}	489.8 ± 98.9 ^{aA}	574.3 ± 67.0 ^{abAB}	901.0 ± 383.8 ^{bAB}	525.3 ± 26.3 ^{abA}
	40	809.5 ± 161.4 ^{aBC}	679.1 ± 58.1 ^{aB}	723.3 ± 209.2 ^{aA}	966.3 ± 390.6 ^{aB}	1918.2 ± 468.7 ^{bB}	789.7 ± 62.9 ^{aA}
	60	1042.0 ± 356.9 ^{aC}	807.0 ± 69.7 ^{aB}	592.7 ± 62.3 ^{aA}	920.3 ± 396.1 ^{aB}	2787.4 ± 338.6 ^{bB}	794.0 ± 292.5 ^{aA}
Esters	1	20.0 ± 2.3 ^{aA}	7.0 ± 0.7 ^{aA}	17.6 ± 3.1 ^{aA}	74.1 ± 12.7 ^{bA}	25.6 ± 15.7 ^{aA}	18.8 ± 4.8 ^{aA}
	20	19.6 ± 1.7 ^{aA}	10.6 ± 3.5 ^{aAB}	32.4 ± 5.7 ^{abA}	101.4 ± 54.2 ^{bAB}	31.1 ± 10.9 ^{abA}	16.1 ± 4.2 ^{aA}
	40	24.1 ± 2.4 ^{aA}	17.7 ± 3.8 ^{aB}	29.6 ± 11.3 ^{aA}	290.0 ± 86.0 ^{bB}	32.8 ± 4.9 ^{aA}	19.3 ± 3.5 ^{aA}
	60	32.7 ± 2.7 ^{aB}	16.3 ± 3.8 ^{aB}	28.4 ± 19.2 ^{aA}	298.7 ± 113.3 ^{bB}	46.5 ± 7.5 ^{aA}	16.9 ± 4.0 ^{aA}
Hydrocarbons	1	10.4 ± 2.9 ^{aA}	48.5 ± 9.5 ^{aA}	15.7 ± 6.6 ^{aA}	14.9 ± 2.2 ^{aA}	154.9 ± 87.2 ^{bA}	76.3 ± 50.9 ^{abA}
	20	14.7 ± 6.8 ^{aA}	1138.2 ± 487.6^{bAB}	20.8 ± 3.6 ^{aA}	1814.6 ± 537.2 ^{bB}	378.6 ± 214.9^{aA}	63.3 ± 27.3 ^{aA}
	40	46.1 ± 23.6 ^{aB}	964.8 ± 432.6 ^{bAB}	34.9 ± 7.8 ^{aA}	2869.3 ± 1050.1 ^{cB}	204.1 ± 146.2^{aA}	168.3 ± 71.2 ^{aA}
	60	16.5 ± 2.6 ^{aA}	2795.3 ± 1714.1 ^{bB}	45.7 ± 28.5 ^{aA}	1631.2 ± 861.9 ^{abB}	278.5 ± 214.4^{aA}	212.4 ± 99.3 ^{aA}
Sulphur compounds	1	4.8 ± 0.6 ^{aA}	4.2 ± 1.4 ^{aA}	4.7 ± 1.2 ^{aA}	7.0 ± 2.2 ^{aA}	116.1 ± 45.7 ^{bA}	7.1 ± 2.1 ^{aA}
	20	5.6 ± 0.5 ^{aA}	5.3 ± 1.4 ^{aAB}	5.0 ± 1.1 ^{aA}	6.0 ± 1.2 ^{aA}	154.9 ± 37.9 ^{bA}	6.0 ± 1.4 ^{aA}
	40	5.5 ± 2.4 ^{aA}	6.3 ± 0.3 ^{aAB}	6.0 ± 3.4 ^{aA}	6.6 ± 1.8 ^{aA}	189.7 ± 49.4 ^{bAB}	5.9 ± 1.1 ^{aA}
	60	6.0 ± 0.9 ^{aA}	6.4 ± 0.6 ^{aB}	3.5 ± 0.3 ^{aA}	6.0 ± 1.3 ^{aA}	276.8 ± 46.7 ^{bB}	5.3 ± 0.9 ^{aA}
Terpenes	1	47.6 ± 31.6 ^{aA}	40.7 ± 25.0 ^{aA}	53.6 ± 38.6 ^{aA}	46.2 ± 29.1 ^{aA}	32.3 ± 24.9 ^{aA}	45.1 ± 25.6 ^{aA}
	20	45.4 ± 29.1 ^{aA}	51.8 ± 38.2 ^{aA}	38.4 ± 16.7 ^{aA}	48.6 ± 33.1 ^{aA}	43.1 ± 27.0 ^{aA}	44.9 ± 25.5 ^{aA}
	40	52.5 ± 34.3 ^{aA}	55.9 ± 31.6 ^{aA}	46.6 ± 28.0 ^{aA}	44.3 ± 24.4 ^{aA}	41.5 ± 25.6 ^{aA}	42.2 ± 25.6 ^{aA}
	60	47.4 ± 31.2 ^{aA}	59.5 ± 39.0 ^{aA}	34.1 ± 19.4 ^{aA}	47.5 ± 29.5 ^{aA}	53.2 ± 22.0 ^{aA}	45.6 ± 28.6 ^{aA}
Furans	1	1.3 ± 0.1 ^{aA}	1.1 ± 0.3 ^{aA}	1.3 ± 0.4 ^{aA}	1.1 ± 0.2 ^{aA}	1.5 ± 0.6 ^{aA}	2.6 ± 1.9 ^{aA}
	20	2.8 ± 0.5 ^{abB}	3.1 ± 2.1 ^{abA}	1.2 ± 0.2 ^{aA}	3.5 ± 0.1 ^{bA}	1.3 ± 0.4 ^{aA}	3.8 ± 0.9 ^{bA}
	40	3.8 ± 1.2 ^{aB}	14.6 ± 5.7 ^{bB}	1.8 ± 0.6 ^{aA}	7.2 ± 2.8 ^{abB}	1.4 ± 0.3 ^{aA}	13.6 ± 7.2 ^{bB}
	60	4.1 ± 0.3 ^{abB}	13.3 ± 6.4 ^{cB}	1.3 ± 0.2 ^{aA}	7.6 ± 3.5 ^{abcB}	1.7 ± 0.4 ^{abA}	10.6 ± 6.1 ^{bcB}

[a] Levels of groups of chemical compounds are means ± SD, expressed as sums of abundances of individual compounds multiplied by 10^{-5}, from determinations in triplicate on two cheesemaking trials. Means in the same row followed by a different lowercase superscript letter and means in the same column for the same group of volatile compounds followed by a different uppercase superscript letter are significantly ($P < 0.05$) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

seaweed composition. Those ketones were detected in all the dehydrated seaweeds used in the present study, with highest values for 2-propanone in *H.* elongata, 2-butanone in *U. pinnatifida*, 2-pentanone in *P. umbilicalis*, 3-hydroxy-2-butanone in *H. elongata* and 2,3-butanedione in *L. ochroleuca* and *U. lactuca* (López-Pérez et al., 2017).

For alcohols, ethanol was found at a maximum level in control cheese followed by PU and UP cheeses, 1-pentanol in HE cheese followed by UP cheese, and 2,3-butanediol in control cheese followed by PU and HE cheeses (Table 6). Ethanol was below detection level in all the dehydrated seaweeds while 1-pentanol was detected in the five dehydrated seaweeds, with maximum levels in *P. umbilicalis* followed by *U. lactuca* and *H. elongata*, and 2,3-butanediol was only detected in *U. pinnatifida* (López-Pérez et al., 2017). There was no apparent relationship among the alcohols present in seaweeds and those found in experimental cheeses.

Regarding volatile acids, acetic acid reached its maximum level in control cheese followed by UP and HE cheeses (Table 6) while its highest levels in dehydrated seaweeds were found in *U. pinnatifida*, *P. umbilicalis* and *L. ochroleuca* (López-Pérez et al., 2017). Butanoic, hexanoic, octanoic and decanoic were detected at markedly higher levels in UL cheese than in the rest of cheeses (Table 6), in accordance with the data obtained for individual FFAs (Table 4) and with the high lipase activity of *U. lactuca* (Fig. 1B). In contrast, heptanoic acid reached its maximum level in PU cheese in agreement with the high level in dehydrated *P. umbilicalis* (López-Pérez et al., 2017), pointing to a formation route, probably through lipid degradation, different from that of even-number FFAs. Nonanoic acid, which was below detection level in all the dehydrated seaweeds used in the present study (López-Pérez et al., 2017), showed its maximum level in LO cheese (Table 6). Regarding esters, the maximum levels of ethyl butanoate and ethyl hexanoate were in PU cheese. Those esters were the only ones previously detected in Hispánico cheese (Garde, Carbonell, Fernández-García, Medina, & Nuñez, 2002a). However, they were not found in any of the five dehydrated seaweeds used in the present study (López-Pérez et al., 2017), what excludes seaweed origin in cheese.

Hydrocarbons, octane, 2,4-dimethylheptane and 1,3-pentadiene reached maximum levels in HE cheese (Table 6). Octane and 2,4-dimethylheptane were found at high levels in dehydrated *H. elongata*, only second to *U. pinnatifida* among the five dehydrated seaweeds used in the present study, but 1,3-pentadiene was not detected in any of the five dehydrated seaweeds (López-Pérez et al., 2017). Octane, 2,4-dimethylheptane and 1,3-pentadiene have been reported in ewe milk cheeses and may derive from lipid oxidation (Carbonell, Nuñez, & Fernández-García, 2002; Fernández-García,

Table 6
Levels of the most abundant volatile compounds in control and experimental cheeses supplemented with five seaweed species after 60 days of ripening.[a]

Compound	Control cheese	Experimental cheeses				
		HE	LO	PU	UL	UP
Ethanal	186.8 ± 105.0^{b}	8.6 ± 4.0^{a}	13.2 ± 8.8^{a}	27.3 ± 2.6^{a}	13.5 ± 1.4^{a}	14.0 ± 3.9^{a}
3-Methylbutanal	17.4 ± 8.3^{ab}	27.9 ± 5.6^{b}	ND^{a}	22.5 ± 10.3^{b}	10.9 ± 3.6^{ab}	14.1 ± 8.7^{ab}
2-Propanone	76.4 ± 24.0^{ab}	55.4 ± 13.6^{ab}	38.6 ± 12.0^{a}	127.7 ± 54.7^{b}	82.4 ± 13.4^{ab}	77.4 ± 49.1^{ab}
2-Butanone	16.8 ± 4.1^{bc}	13.2 ± 1.3^{ab}	6.8 ± 1.2^{a}	23.3 ± 9.1^{c}	17.1 ± 1.8^{bc}	12.0 ± 3.1^{ab}
2-Pentanone	31.2 ± 6.9^{a}	39.4 ± 15.0^{a}	32.2 ± 21.1^{a}	84.7 ± 36.9^{b}	60.6 ± 29.7^{ab}	37.5 ± 16.7^{a}
2-Heptanone	19.2 ± 2.5^{a}	43.4 ± 12.4^{b}	19.3 ± 4.4^{a}	19.0 ± 3.3^{a}	30.2 ± 4.1^{ab}	18.9 ± 6.6^{a}
3-Hydroxy-2-butanone	163.5 ± 40.6^{b}	90.5 ± 14.9^{a}	88.7 ± 24.4^{a}	312.9 ± 44.6^{c}	63.4 ± 2.0^{a}	167.9 ± 38.8^{b}
2.3-Butanedione	20.6 ± 3.7^{a}	14.2 ± 2.5^{a}	15.9 ± 7.3^{a}	44.3 ± 12.1^{b}	19.5 ± 6.8^{a}	27.2 ± 11.1^{ab}
Ethanol	570.7 ± 81.6^{b}	283.6 ± 36.4^{ab}	210.7 ± 57.3^{a}	450.2 ± 44.4^{ab}	252.6 ± 55.6^{ab}	423.3 ± 186.6^{ab}
1-Pentanol	7.3 ± 0.8^{ab}	18.5 ± 8.6^{c}	6.7 ± 1.2^{a}	13.6 ± 3.5^{abc}	11.1 ± 1.3^{abc}	16.1 ± 1.8^{bc}
2,3-Butanediol	101.5 ± 18.6^{b}	87.2 ± 4.5^{ab}	55.1 ± 9.9^{a}	99.6 ± 24.8^{b}	60.0 ± 9.0^{a}	68.8 ± 16.3^{ab}
Acetic acid	213.4 ± 53.7^{b}	198.4 ± 16.6^{b}	88.0 ± 14.6^{a}	140.1 ± 25.6^{ab}	104.7 ± 12.7^{a}	205.6 ± 48.2^{b}
Butanoic acid	568.7 ± 210.1^{a}	286.8 ± 28.7^{a}	220.3 ± 18.7^{a}	448.8 ± 165.1^{a}	1159.5 ± 157.5^{b}	309.4 ± 173.9^{a}
Hexanoic acid	168.6 ± 30.0^{a}	193.6 ± 23.6^{a}	168.4 ± 11.7^{a}	228.2 ± 118.5^{a}	1139.0 ± 167.6^{b}	158.5 ± 65.6^{a}
Heptanoic acid	2.3 ± 1.4^{a}	2.8 ± 0.4^{a}	8.1 ± 4.5^{a}	24.3 ± 13.0^{b}	12.3 ± 4.5^{ab}	12.4 ± 4.2^{ab}
Octanoic acid	63.5 ± 9.7^{ab}	79.2 ± 7.3^{b}	53.4 ± 16.7^{ab}	35.6 ± 12.6^{a}	289.3 ± 26.3^{c}	58.2 ± 5.7^{ab}
Nonanoic acid	5.2 ± 3.1^{a}	5.3 ± 1.2^{a}	37.8 ± 12.0^{b}	23.5 ± 7.6^{ab}	18.8 ± 9.8^{ab}	31.5 ± 0.8^{b}
Decanoic acid	20.5 ± 2.9^{ab}	39.0 ± 4.8^{b}	11.1 ± 6.6^{a}	7.3 ± 1.8^{a}	60.9 ± 21.9^{c}	8.5 ± 1.9^{a}
Ethyl butanoate	18.1 ± 2.3^{ab}	7.6 ± 3.0^{a}	10.4 ± 1.0^{a}	41.4 ± 17.2^{c}	29.8 ± 3.9^{bc}	7.8 ± 2.0^{a}
Ethyl hexanoate	2.6 ± 0.6^{a}	2.4 ± 0.4^{a}	2.8 ± 0.5^{a}	243.4 ± 96.5^{b}	8.0 ± 1.3^{a}	1.3 ± 0.6^{a}
Octane	10.9 ± 3.9^{a}	52.2 ± 31.9^{b}	7.6 ± 0.4^{a}	9.2 ± 1.7^{a}	8.8 ± 2.3^{a}	8.3 ± 1.9^{a}
2,4-Dimethylheptane	4.4 ± 3.2^{a}	70.7 ± 37.9^{b}	10.6 ± 6.0^{a}	5.2 ± 3.1^{a}	15.2 ± 9.5^{a}	3.5 ± 2.0^{a}
1,3-Pentadiene	1.3 ± 1.2^{a}	2523.7 ± 1671.0^{b}	ND^{a}	1613.4 ± 1357.0^{ab}	247.5 ± 193.3^{a}	191.5 ± 102.5^{a}
Dimethylsulphide	ND^{a}	ND^{a}	ND^{a}	ND^{a}	265.5 ± 46.1^{b}	ND^{a}
α-Pinene	34.3 ± 24.9^{a}	42.3 ± 30.0^{a}	23.0 ± 14.3^{a}	34.7 ± 22.8^{a}	37.9 ± 16.6^{a}	32.8 ± 22.5^{a}

[a] Levels of individual volatile compounds are means ± SD, expressed as abundances multiplied by 10^{-5}, from determinations in triplicate on two cheesemaking trials. ND, below the detection level. Means on the same row followed by a different lowercase superscript letter are significantly ($P < 0.05$) different. Experimental cheeses HE, LO, PU, UL and UP were supplemented with Himanthalia elongata, Laminaria ochroleuca, Porphyra umbilicalis, Ulva lactuca and Undaria pinnatifida, respectively.

Carbonell, & Nuñez, 2002). Dimethylsulphide was only detected in UL cheese (Table 6), which compares well with the more than 100-fold higher level of this compound in *U. lactuca* than in the rest of dehydrated seaweeds (López-Pérez et al., 2017). Also, the presence of dimethylsulphide has been reported in pasteurised milk cheeses such as Hispánico (Alonso et al., 2011) and Brie (Calzada, del Olmo, Picon, & Nuñez, 2014b).

The main terpene found in the present study, α-pinene, not detected in the dehydrated seaweeds (López-Pérez et al., 2017), was present at similar levels in control and experimental cheeses (Table 6). Cheese terpenes commonly originate from plants present in natural pastures, from which they are transferred to the milk of grazing animals and afterwards to cheese (Curioni & Bosset, 2002).

The first three components of a PCA carried out on the levels of seven selected groups of volatile compounds explained 81.7% of the variance. The first component, which included sulphur compounds, acids and, with negative sign, alcohols, explained 33.9% of the variance while the second component, which included ketones and esters, 26.2% and the third component, which included furans and terpenes, 21.6%. In the plane defined by the first two components of this PCA (Fig. 2A), all UL cheeses were located close to the positive X axis, with high levels of sulphur compounds and acids and low levels of alcohols, while 40-day and 60-day PU cheeses were located close to the positive Y axis, with high levels of ketones and esters. There was a clear separation between the mentioned cheeses and the rest of the cheeses, which were located in the third quadrant or close to it.

A second PCA (data not shown) was carried out on the levels of the eight individual volatile compounds showing the highest correlation with the first three components in a PCA carried out on all the individual volatile compounds. In this second PCA, the first component, which included 4-methyloctane, 2-methylnonane, 4-methyldecane and 2,4-dimethylhexane, explained 53.7% of the variance, and the second component, which included hexanoic acid, octanoic acid, dimethylsulphide and dimethylsulphoxide, 42.6%. In the plane defined by the first two components of this PCA, all HE cheeses were located near the positive X axis, with high levels of hydrocarbons, all UL cheeses near the positive Y axis, with high levels of acids and sulphur compounds, and the rest of the cheeses in the third quadrant.

The first three components of a third PCA carried out on the levels of seven selected groups of volatile compounds (alcohols, aldehydes, esters, furans, hydrocarbons, ketones, sulphur compounds) and the scores of nine selected sensory characteristics (odour quality, seaweed odour, flavour quality, seaweed flavour, acid flavour, bitter flavour, sweet flavour, salty flavour, umami flavour) evaluated in a previous study (del Olmo et al., 2018), explained 83.8% of the variance. The first component, which included seaweed flavour, seaweed odour and, with negative sign, flavour quality, aldehydes, odour quality and alcohols, explained 38.9% of the variance while the second component, which included acid flavour, salty flavour, furans and, with negative sign, sweet flavour and sulphur compounds, explained 27.4% and the third component, which included esters, ketones and hydrocarbons, explained 17.5%. In the plane defined by the first two components (Fig. 2B), HE, LO, PU and UP cheeses were located in the first quadrant or close to it while control cheeses were located in the third quadrant and UL cheeses in the fourth quadrant. In agreement with the results of this PCA, significant ($P < 0.05$) correlations were found between aldehydes and seaweed odour ($r = -0.866$), seaweed flavour ($r = -0.878$), odour quality ($r = 0.604$) or flavour quality ($r = 0.674$), between alcohols and seaweed flavour ($r = -0.470$) or flavour quality ($r = 0.472$), between sulphur compounds and odour quality ($r = -0.564$) or flavour quality ($r = -0.671$), and between esters and odour quality ($r = -0.542$).

Addition of the different seaweed species to curd resulted in distinct volatile profiles of the experimental cheeses, which could

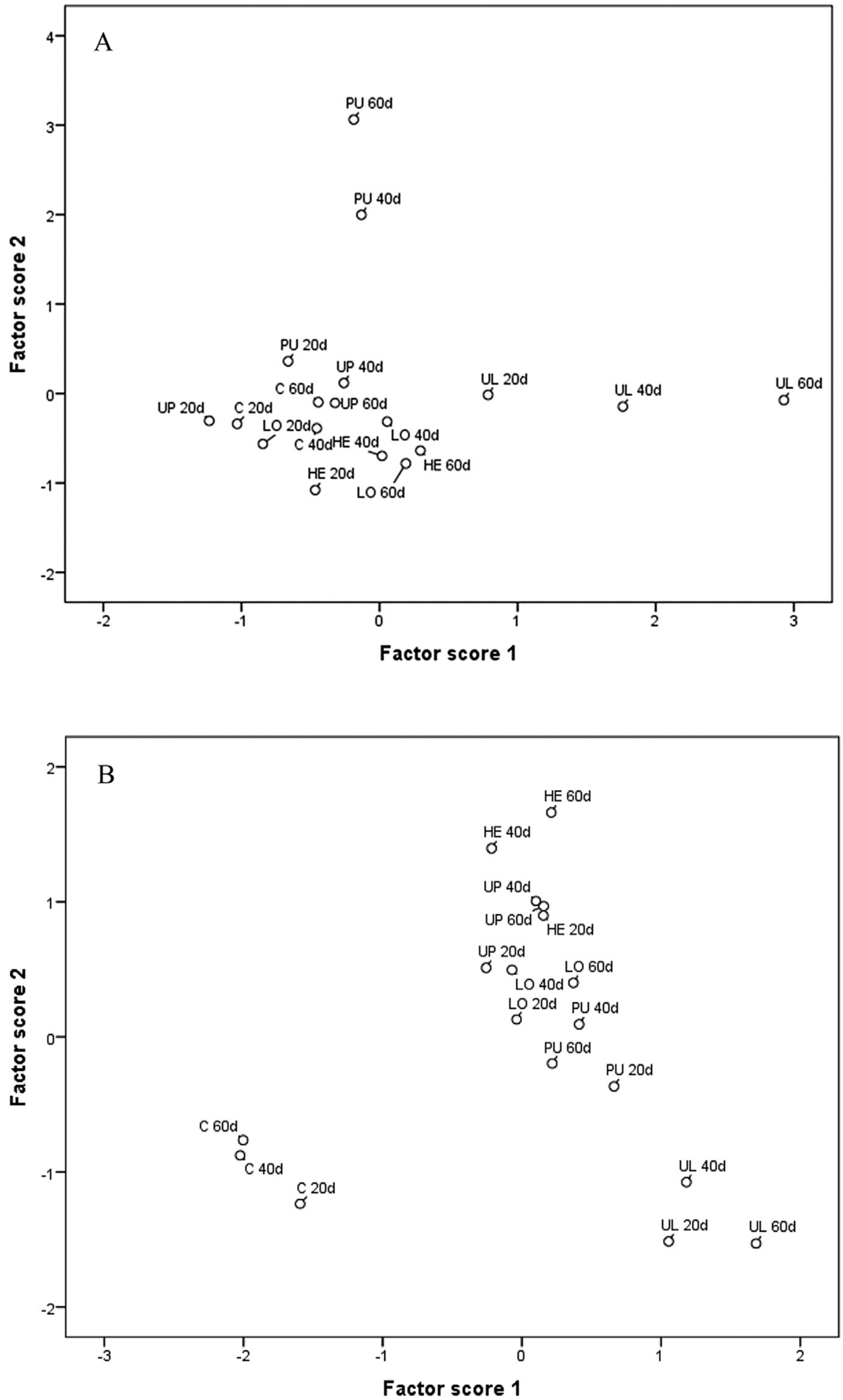

Fig. 2. Distribution of control (C) and experimental (HE, LO, PU, UL and UP) cheeses respectively supplemented with *H. elongata*, *L. ochroleuca*, *P. umbilicalis*, *U. lactuca* and *U. pinnatifida*, after 20, 40 and 60 d of ripening, in the plane defined by (A) the first two components of the PCA carried out on the levels of alcohols, aldehydes, esters, furans, hydrocarbons, ketones and sulphur compounds or by (B) the first two components of the PCA carried out on the levels of the same seven groups of volatile compounds and the scores of sensory characteristics odour quality, seaweed odour, flavour quality, seaweed flavour, acid flavour, bitter flavour, sweet flavour, salty flavour and umami flavour.

be related to the sensory characteristics of the cheeses for some groups of volatile compounds.

4. Conclusions

Cheese proteolysis was influenced by seaweed addition to curd, although the concentration of total free amino acids in experimental cheeses could not be associated with the aminopeptidase activity of dehydrated seaweeds. Factors other than seaweed aminopeptidase activity such as cheese pH apparently had a greater influence on cheese secondary proteolysis. The most intense lipolysis was recorded for the cheese with added *U. lactuca*. There was a strong correlation between the lipase activity of dehydrated seaweeds and the concentration of total free fatty acids in experimental cheeses. Cheeses supplemented with *H. elongata*, *U. pinnatifida* and *U. lactuca* showed a higher free omega 6/free omega 3 ratio than the rest. Seaweed addition to curd modified the volatile profile of cheese, by either directly supplying volatile compounds, i.e., hydrocarbons and sulphur compounds, or indirectly through the enzymatic activity of seaweeds on cheese components, i.e., *U. lactuca* lipase on triglycerides, or through the modification of cheese physicochemical parameters, i.e., pH value or antioxidant activity.

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