LWT - Food Science and Technology 79 (2017) 616–624

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Novel cheese production by incorporation of sea buckthorn berries (*Hippophae rhamnoides* L.) supported probiotic cells

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
6 November 2016
Accepted 10 November 2016
Available online 11 November 2016

Keywords:
Sea buckthorn berries
Hippophae rhamnoides L.
Lactobacillus casei
Cheese
Immobilisation

ABSTRACT

Sea buckhorn berries (SBB, *Hippophae rhamnoides* L.) were used as a novel support for immobilisation of the probiotic strain *L. casei* ATCC 393. The biocatalyst was employed for the development of a bioprocess for feta-type cheese production that leads to the improvement of quality and nutritional characteristics. Specifically, the effect of SBB supported biocatalyst on the microbiological safety and aroma profile of cheeses was compared with control samples and cheeses containing free *L. casei* cells showing superior properties with increased content of esters, alcohols and terpenes. The presence of probiotic culture, either in free or immobilised form, affected positively the physicochemical characteristics of cheeses during ripening. Cheeses with SBB had enriched aroma with terpenes and carbonyl compounds and higher probiotic cell population. The proposed bioprocess of employing SBB as immobilisation carrier shows great potential for commercialisation and application in manufacturing of probiotic functional dairy food.

1. Introduction

Worldwide food market is displaying an increased demand for functional foods that contain technologically developed novel ingredients and have extended nutritional impact with beneficial health effects (Siró, Kápolna, Kápolna, & Lugasi, 2008). The incorporation of natural compounds with advantageous effects has been used for functional dairy food production. Specifically, a variety of antioxidant supplements, either single phenolic compounds or natural plant extracts (*e.g.*, grape or green tea extract, cranberry powder etc.) have been used in cheese making process (Han et al., 2011). Novel natural preservatives (*e.g.*, pomegranate rind extract, *Thymus vulgaris* L. essential oil) (de Carvalho et al., 2015; Mahajan, Bhat, & Kumar, 2015, 2016) and spices such as black cumin (Cakir, Cakmakci, & Hayaloglu, 2016) have been widely incorporated in cheeses for improved microbiological safety and sensory characteristics.

In addition, new approaches in cheese making have been also developed to meet consumer needs for healthier, safe and high quality dairy products. Various studies have employed the use of probiotic bacteria, especially lactobacilli and *Bifidobacterium* strains, which can offer health benefits on host when present at appropriate amounts (10^6–10^7 CFU/g) (Buriti, da Rocha, Assis, & Saad, 2005; Gardiner et al., 2002; Kasımoğlu, Göncüoğlu, & Akgün, 2004; Mushtaq, Gani, Masoodi, & Ahmad, 2016). The viability of probiotic strains through processing can be improved by cell immobilisation on natural carriers or microencapsulation (De Prisco & Mauriello, 2016). Various natural supports such as apple or pear pieces (Kourkoutas, Bosnea, et al., 2006; Kourkoutas, Xolias, Kallis, Bezirtzoglou, & Kanellaki, 2005), *Pistacia terebinthus* resin (Schoina et al., 2014) and whey protein (Katechaki, Panas, Kourkoutas, Koliopoulos, & Koutinas, 2009) have been used as carriers for probiotic cell immobilisation in dairy product production.

Feta cheese, a brine curd white cheese, is one of the most popular Greek products known worldwide being characterized since 2002 as a product of protected designation of origin (PDO). It is made mainly from ewes' milk or a mixture of ewes' and up to 30% goat milk. Rennet enzyme and yogurt starter culture are added during manufacturing for lactic acid fermentation. According to relative legislation, the product is consumed after 2-month ripening for achieving the appropriate microbiological safety and organoleptic quality.

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http://dx.doi.org/10.1016/j.lwt.2016.11.021
0023-6438/

Hippophae rhamnoides L. (commonly known as sea buckthorn), an ancient plant widely found in Asia and Europe, has received the increasing attention of scientists and consumers. Its name is derived from the Greek word 'hippos' (horse) and 'phaos' (shine) consumed by Alexander the Great horses that had shiny hair. The orange soft sea buckthorn berries (*i.e.*, SBB) contain numerous bioactive compounds (phenolics, vitamins, fatty acids, sterols, carotenoids) with antioxidant, antimicrobial and potential medicinal properties (Ma et al., 2016; Teleszko & Wojdyło, 2015). Sea buckthorn is considered by the nutritionists as "superfood" and is already used in food market for beverage, jam and dairy product production, as well as in cosmetics and food supplements (Bal, Meda, Naik, & Satya, 2011).

In this study, feta-type cheeses enriched with sea buckthorn berries with immobilised cells of the probiotic strain *Lactobacillus casei* ATCC 393 are produced. The aim of the work is to study the combination of beneficial effects of both SBB and probiotic strain of *L. casei* as well as the assessment of the effect of the incorporated SBB biocatalysts on the microbiological safety and aroma profile of the products.

2. Materials and methods

2.1. Microbial starter cultures

The probiotic strain *Lactobacillus casei* ATCC 393 (DSMZ, Braunschweig, Germany) was used for the immobilisation process on SBB. *Lactobacillus casei* was grown at 37 °C in de Man-Rogosa-Sharpe (MRS) liquid medium (LabM, UK) for 72 h. Wet biomass was harvested by centrifugation (Sigma 3K12, Bioblock Scientific, France) at 5000 rpm for 10 min and stored at 5 °C. All media were autoclaved at 120 °C at 1–1.5 atm for 15 min prior to use. The commercial starter culture used for this experiment was the classic yogurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:1 w/w).

2.2. Cell immobilisation

Dry sea buckthorn berries (SBB), obtained by a local market were used as immobilisation carrier for the probiotic strain of *L. casei*. The immobilisation process was performed by mixing 10 g of SBB, various amounts wet *L. casei* biomass (0.5, 1.0, 2.0, 3.0 g) and 500 mL of MRS broth. After that the system was allowed to ferment without agitation at 37 °C for 24 h. Then, the liquid was decanted and the immobilised biocatalyst was washed twice with sterile Ringer solution (1/4 strength) and used for cheese production. Each of the above four combinations was performed in duplicate.

2.3. Enumeration of immobilised cells

For the enumeration of the immobilised *L. casei* cells, 10 g of the immobilised biocatalyst were blended with 90 mL of sterile Ringer's solution (1/4 strength). The suspension was serially diluted (ten-fold), plated on MRS agar and incubated at 37 °C for 72 h. The biocatalyst that contained the highest population of *L. casei* immobilised cells was chosen for the cheese making process.

2.4. Bioprocess development for feta-type cheeses

Pasteurized and standardized ewes' milk and less than 30% goat milk (6% fat, pH 6.5, casein-to-fat ratio: 0.8) was obtained by a local cheese factory (Chelmos S.A., Achaia, Greece). It was heated at 65 °C for 30 min and then cooled down at 37 °C. Starter culture was added at a level of 1.0% (v/v) and incubated for 20–30 min before the addition of rennet. Subsequently, the coagulum is cut into cubes (lower than 1 cm diameter) and at this point the adjunct culture is added either free or immobilised form, mixed well and left undisturbed for 20 min. Then, the enriched curd is transferred gradually into circular molds and stirred periodically to facilitate whey drainage. The curd is then removed from the molds, left undisturbed for 10 min and finally placed in 12% brine for ripening. Four lots of 500 g blocks were made for each batch and each lot was enhanced with a different type of starter culture, namely: *Cheese 1 (S1)* – feta-type cheese with commercial starter culture (control), *Cheese 2 (S2)* – feta-type cheese with free *L. casei* cells (10^9 CFU/g), *Cheese 3 (S3)* - feta-type cheese with immobilised biocatalyst (2.0 g wet biomass/10 g biocatalyst/Kg cheese). Ripening of cheeses was studied at 4 °C and 22 °C for up to 100 consecutive days. All cheese samples were stored at initial brine content of 12% (w/v) at 22 °C and when pH value dropped at 4.6, the brine was changed to 6% w/v and samples were stored at 4 °C for further ripening.

2.5. Microbiological analysis of cheese products

Ten-gram portions of cheese from interior of each sample were blended with 90 mL of sterilized 2.0% tri-sodium citrate solution and submitted to serial dilutions. Microbial enumerations were performed during maturation using plate counting on appropriate solid media (LabM, UK): (i) total mesophilic flora (Plate Count Agar-PCA, 30 °C for 48 h), (ii) yeasts and molds (Potato Dextrose Agar - PDA, 30 °C, 48 h), (iii) lactococci (gram positive, catalase negative) (M-17 agar, 37 °C, 48 h), (iv) lactobacilli (MRS agar, 37 °C, 48 h), (v) coliforms (Violet Red Bile Agar-VRBA, 30 °C, 24 h), and (vi) enterobacteria (Violet Red Bile Glucose Agar-VRBGA, 37 °C, 24 h). The selective enumeration of *L. casei* cells in cheeses was performed by plating on MRS broth containing the antibiotic vancomycin (MRS-V) 1.0% (Fluka, Buchs, Switzerland) and incubation at 37 °C for 72 h (Tharmaraj & Shah, 2003). Microbiological analysis was performed in duplicate using duplicate cheese samples. The original count in the sample was expressed as log CFU per gram of cheese.

2.6. Physicochemical analysis

Cheese samples (20 g each) were macerated with warm water (40 °C) to produce a total volume of 210 mL. Each sample was then filtered and the filtrate was used for sugar and organic acid determination. Cheese pH was measured using a digital pH meter (HI 99161, Hanna Inc.). Total acidity was determined according to the official method by AOAC International (1995) and expressed as lactic acid content.

High performance liquid chromatography (HPLC) was used for sugar and organic acid quantification. Lactose and galactose were determined on a Shimadzu chromatograph with a Nucleogel Ion 300 OA column, a LC-9A pump, a CTO-10A oven at 40 °C and a RID-6A refractive index detector. The mobile phase used was 0.008 N H_2SO_4 using a flow rate of 0.5 mL/min and 1-propanol was used as an internal standard. The samples were filtered with a disposable cellulose acetate filters (Chromafil) with 0.20 nm pore size and then 60 μL of the final solution were injected directly to the column. Sugar concentrations were calculated using standard curves. Lactic acid was analyzed on a Jasco LC-2000 Series hplc system (Jasco Inc., Japan) equipped with a size-exclusion organic acid analysis column (Aminex HPX-87H, 300 × 7.8 mm i.d., 9 μm particle size, Bio-rad, France) fitted in a CO-2060 Plus column oven, a PU-2089 pump, a AS 2050 Plus autosampler and a MD-2018 Photodiode array detector operated at 210 nm. Isocratic separation at 50 °C was performed with 0.008 N H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. The detector signals were recorded and analyzed by ChromNav software. Aliquots of the samples were filtered through 0.2 μm nylon filters. For quantitative analysis, standard solutions of acids

(Sigma-Aldrich Ltd, St Louis, US) in pure water were prepared at various concentrations.

2.7. Analysis of SBB and cheese samples by solid phase microextraction (SPME) gas chromatography–mass spectrometry (GC–MS)

Volatile compounds of SSB and the produced cheese samples after 15 and 60 days ripening were determined by solid-phase micro-extraction (SPME) gas chromatography/mass spectrometry (GC/MS). Prior to SPME, 7.0 g dry berries were placed into the headspace vials with 10 mL methanol 20% (Merck, Darmstadt, Germany) for volatile compound extraction at 25 °C for 24 h.

For the analysis, grated cheese samples (7.0 g each) or the aforementioned SBB sample with methanol were placed in a 20 mL headspace vial was fitted with a Teflon-lined septum through which the SPME syringe needle bearing a 2-cm fibre coated with 50/30 mm Divinylbenzene/Carboxen (Supelco, Bellefonte, PA, USA) was inserted. Each sample was thermostated at 60 °C for 3 min prior the fibre exposure to headspace for 45 min at the same temperature. A Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer with a capillary Supelco CO Wax-10 column (60 m, 0.32 mm i.d., 0.25 μm film thickness) was used with helium as carrier gas (1.8 mL/min). The temperature programs and internal standards for cheese and SBB analysis were used as described previously (Gialleli, Bekatorou, Kanellaki, Nigam, and Koutinas (2016); Kourkoutas, Bosnea, et al. (2006)). Molecular identification were carried out by comparing the mass spectra obtained from NIST107, NIST21 and SZTERP libraries, and by determining kovats' retention indexes and comparing with those reported in the literature.

2.8. Electron microscopy

Pieces of dry sea buckthorn berries were studied after and prior to immobilisation. The samples were frozen to −45 °C at a cooling rate of 3 °C/min and then were freeze-dried for 48 h at 5×10^{-3} mbar and at −45 °C in a Freeze Drying System, Freezone 4.5 (Labconco, Kansas City, Missouri, USA). No cryoprotectant medium was used during freeze-drying. The samples were coated with gold in a Balzers SCD 004 Sputter coater (Bal-Tec, Schalksmühle, Germany) for 2 min and examined in a JSM-6300 scanning electron microscope (Jeol, Tokyo, Japan), operated at an accelerating voltage of 20 kV.

2.9. Preliminary taste tests

Cheese samples ripened for 60 days were cut into pieces (5 × 5 cm) and served at room temperature (22 °C). Sensory evaluation was carried out by 10 laboratory members using locally approved protocols. The coded samples were served in a randomized order and the panel was asked to mark the products on a 0–7 scale (0 = unacceptable, 7 = exceptional) based on aroma, taste and flavour.

2.10. Experimental design and statistical analysis

All fermentations and analyses were carried out in triplicate and the results are presented as mean values ± standard deviation. The significance of differences in the means of various groups was checked by One-way Analysis of Variance (ANOVA) at the 5% level of significance.

3. Results and discussion

3.1. Rationale

Hippophae rhamnoides L. is a plant containing high concentration of antioxidants (Teleszko & Wojdyło, 2015), showed prebiotic and antimicrobial properties (Gunenc et al., 2016; Ma et al., 2016). Cell immobilisation has shown higher viability of cells and promotion of the fermentation rate and therefore ripening of fermented foods (Kourkoutas, Kanellaki, Koutinas, & Tzia, 2006). Feta cheese is a well known fermented dairy product and its production through the proposed bioprocess will provide the above described assets. This research has been organized in order to study the effect of SBB supported biocatalysts on cheese quality and ripening through the development of a bioprocess based on immobilised *L. casei* cells on SBB. Specifically, the effect on feta cheese chemical composition and microbial flora during ripening will be examined, using immobilised cells in comparison with free cells of *L. casei* and with a commercial cheese product. For chemical composition emphasis is given to lactic acid formation and pH value, because these parameters combine improvement of self-life and taste. Likewise, volatile compounds formation was analyzed with GC-MS, related with cheese aroma and therefore its quality. Moreover, microbiological analyses of pathogens and *L. casei* cell viabilities through of *Lactobacillus* survival have been also performed.

3.2. Cell immobilisation

The probiotic L. *casei* ATCC 393 employed in the experiments was selected according to its *in vitro* and *in vivo* microbial survival in GI tract, adhesion to the intestine and modulation of the intestinal microflora in rats as previously reported (Schoina et al., 2014; Sidira et al., 2010). Immobilised L. *casei* cells on SBB were added after the filtration stage and the biocatalyst was distributed uniformly in the curd mass. For comparison reasons, cheese with free *L. casei* cells and cheese with commercial culture were also produced accordingly. The enumeration of immobilised cells showed that the biocatalyst prepared by mixing 2.0 g wet *L. casei* biomass with 10 g SBB, yielded the highest population of immobilised cells ($>10^9$ cfu/g). Further increase of the biomass did not result in higher numbers of immobilised cells (data not shown). Likewise, the immobilised biocatalyst obtained from 2.0 g of *L. casei* biomass was chosen for the experiments of cheese making.

3.3. Effect of SBB biocatalyst on physicochemical characteristics of feta-type cheeses

An important key point for successful brine cheese manufacturing is the achievement of high acidification rate exerted by the starter culture and the consequent decrease of pH values during coagulation and draining. At this point, the adjunct probiotic culture (immobilised or free) was added for the reinforcement of starter culture and subsequent promotion of pH decrease. During ripening at different temperatures, sugar concentration, pH values and acidity were determined for all samples and the results are presented in Table 1. Cheeses with adjunct immobilised probiotic strain (S3) presented lower pH values at the end of ripening were compared to S1 and S2 samples. In addition, SBB may also affect the acidification rate as mentioned in the previous study where yogurts that contained SBB as adjunct, presented significantly lower pH values and higher acidity than control samples (Gunenc et al., 2016). During ripening, in all samples, lactose concentration decreases and subsequently lactic acid content increases due to fermentation by lactic acid bacteria in S2 and S3 cheese sample. Lactic acid content increased from 1 to 30 days in S2 and S3,

Table 1
Physicochemical characteristics of feta-type cheeses prepared using commercial starter culture (S1), free (S2) or immobilised (S3) *L. casei* cells on SBB, during ripening at various temperatures.

Cheese type	Ripening temperature (°C)	Ripening duration (Days)	Lactose (g/100 g cheese)	Galactose (g/100 g cheese)	pH	% acidity (g lactic acid/100 g cheese)	Lactic acid (g/100 g cheese)
S1	22	1	4.04	Nd	5.88	0.11	0.15
		5	3.84	Nd	5.75	0.41	0.16
		10	2.78	Nd	5.54	0.56	0.17
		20	1.88	Nd	5.19	0.68	0.15
		25	1.81	Nd	4.72	0.92	0.11
	4	**30**	**1.74**	**Nd**	**4.60**	**1.02**	**0.10**
		40	1.33	Nd	4.56	1.06	0.14
		50	1.11	Nd	4.55	1.07	0.13
		60	0.89	Nd	4.56	1.07	0.15
		70	0.09	Nd	4.52	1.10	0.10
		80	Tr	Nd	4.52	1.10	0.10
		90	Tr	Nd	4.47	1.21	0.10
		100	nd	Nd	4.47	1.21	nd
S2	22	1	2.70	0.65	5.73	0.16	0.41
		5	2.45	Nd	5.55	0.41	0.48
		10	1.84	Nd	5.41	0.58	0.45
		20	1.54	Nd	5.04	0.67	0.46
		25	1.32	Nd	4.83	1.02	0.48
	4	**30**	**1.08**	**Nd**	**4.57**	**1.10**	**0.51**
		40	0.83	Nd	4.55	1.11	0.54
		50	0.57	Nd	4.54	1.12	0.57
		60	0.07	Nd	4.54	1.12	0.61
		70	Tr	Nd	4.50	1.15	0.59
		80	Tr	Nd	4.48	1.19	0.60
		90	Tr	Nd	4.48	1.19	0.57
		100	Tr	Nd	4.47	1.21	0.58
S3	22	1	2.68	0.62	5.62	0.18	0.44
		5	2.66	Nd	5.30	0.42	0.52
		10	1.58	Nd	5.26	0.56	0.62
		20	1.06	Nd	5.19	0.68	0.66
		25	0.32	Nd	4.84	0.92	0.67
	4	**30**	**0.21**	**Nd**	**4.55**	**1.01**	**0.72**
		40	0.18	Nd	4.55	1.06	0.74
		50	0.16	Nd	4.55	1.07	0.78
		60	Tr	Nd	4.54	1.07	0.83
		70	Tr	Nd	4.54	1.11	0.82
		80	Tr	Nd	4.52	1.11	0.84
		90	Tr	Nd	4.48	1.19	0.85
		100	nd	Nd	4.45	1.26	0.87

Nd: not detected, Tr: traces.

because of the lactic acid bacteria high activity at the relatively high temperature (22 °C) of the ripening room. The acidification of the curd is essential for assuring the good quality and the proper ripening process of feta cheese (Abd El-Salam & Alichanidis, 2004).

3.4. Microbiological characteristics of cheese samples during ripening at different temperatures

Given the fact that foods are dynamic systems, the concept of creation of a novel cheese product with conditions that would prevent the growth of pathogens or spoilage microorganisms was the aim of this work. Cheese samples were analyzed by plate counting for microbial population of total mesophilic flora, yeast and molds, enterobacteria and coliforms as well as lactobacilli and lactococci through ripening (Table 2). The addition of probiotic strain, either free or immobilised, affected significantly ($P < 0.05$) the total mesophilic flora and yeast/mould counts compared to control samples. Yeast and mould population, probably derived by equipment and brine, decreased during ripening especially in the case of *L. casei* presence. This decrease is due may be to a combination of unfavorable conditions for yeast growth (low pH value, low temperature, sugar depletion) and proliferation of *L. casei* cells. In all samples, enterobacteria and coliform counts showed a sharp decrease during ripening (data not shown) and were not detected in the samples at the end of the process. This phenomenon is also reported in other studies using immobilised biocatalysts and probiotic strains in cheese-making (Dimitrellou, Kandylis, Sidira, Koutinas, & Kourkoutas, 2014; Masoumikia & Ganbarov, 2015). Scientific research has drawn the conclusion that lactic acid bacteria (LAB) exhibit antimicrobial activity due to the production of acids, antifungal peptides and bacteriocins (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013; Yang, Fan, Jiang, Doucette, & Fillmore, 2012). Likewise, the combination of LAB with hippophae berries which also show antimicrobial properties (Michel, Destandau, Le Floch, Lucchesi, & Elfakir, 2012) reinsures microbiological safety of the products.

As also shown in Table 2, the microbial counts of lactococci, which are considered indigenous milk microflora, did not significantly differ among all samples during ripening while the presence of starters affected less the population of the immobilised *L. casei* cells (S3) compared to the free ones (S2). On the other hand, the addition of SBB significantly increased the population of lactobacilli ($P < 0.05$) in the case of immobilised biocatalyst compared to free cells and control sample. Both cheese samples (S2, S3) retained the probiotic stain at the appropriate amounts (10^6–10^7 CFU/g) (Buriti et al., 2005; Gardiner et al., 2002) throughout 100 storage days. More specifically, *L. casei* count levels in S3 laid within the range of 9.45–7.82 log CFU/g whereas in the case of S2 its levels ranged within 8.56–6.17 log CFU/g. These findings are in accordance with a previous study which reported that SBB used as adjunct in yogurt

Table 2
Microbial counts of cheeses produced by commercial starter culture (S1), free (S2) or immobilised (S3) *L. casei* cells on SBB, during ripening at various temperatures.

Ripening temperature (°C)	Ripening duration (Days)	Microbial counts (log CFU/g)												*L. casei*	
		Total mesophilic flora			Yeasts and molds			Lactococci			Lactobacilli				
		S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S2	S3
	1	5.74	7.17	7.30	3.84	4.36	5.50	7.48	7.10	7.35	6.00	10.02	10.38	8.17	8.23
22	5	6.52	9.05	9.33	4.38	4.99	5.06	7.69	7.01	7.18	6.18	9.33	9.57	8.42	8.57
	10	6.18	8.28	9.02	4.17	4.17	5.03	7.00	6.69	6.90	6.79	8.56	9.83	8.27	8.76
	20	6.00	8.24	8.72	4.47	3.81	4.96	7.89	5.72	6.04	6.94	8.88	10.00	8.24	9.14
	25	6.97	8.66	8.19	4.37	3.92	4.33	7.77	6.00	6.63	6.90	9.01	10.16	8.26	9.16
4	30	6.86	8.32	8.82	3.79	4.05	3.76	6.78	7.25	7.49	5.83	8.41	9.88	8.32	9.19
	40	6.80	8.51	8.51	3.09	3.73	3.22	6.07	6.80	7.12	5.57	8.90	9.98	8.37	9.21
	50	6.34	8.30	8.49	2.67	3.14	2.80	6.00	6.98	6.36	5.80	9.05	9.47	8.46	9.29
	60	6.11	7.87	8.35	2.28	2.81	2.04	6.90	6.88	6.02	5.07	9.22	9.56	8.52	9.45
4	70	6.60	7.26	8.40	2.10	2.21	1.28	7.69	7.20	6.50	5.05	8.57	8.92	8.56	8.40
	80	6.58	7.56	8.38	1.90	1.83	1.16	7.11	6.72	6.20	4.61	8.29	8.98	8.26	8.38
	90	6.24	7.01	8.31	1.57	1.45	1.09	6.02	6.63	6.02	3.89	8.23	8.76	7.71	8.31
	100	5.82	6.17	7.42	1.30	1.12	1.00	5.60	6.60	5.87	2.63	7.51	8.05	6.17	7.82

can serve as a novel prebiotic source while it increased the viability of both starter culture and added free probiotic culture compared to control samples after storage (28 days) at 4 °C (Gunenc et al., 2016).

3.5. Effect of SBB on the aroma profile of manufactured cheeses

Various esters, organic acids, terpenes and carbonyl compounds frame the aroma of all feta-type cheeses analyzed by SPME GC/MS. Ripening is crucial for the development of the desirable cheese flavour that defines consumers' acceptance. The majority of volatiles especially ones that define a characteristic aroma to maturated cheese products, are formed after 2 months of ripening in brine. Semi-quantitative results of the detected volatile compounds are presented in Table 3.

In total, 65 compounds were detected in S3 cheese containing *L. casei* cells immobilised on SBB and 40 compounds in S2 cheese containing free *L. casei* cells and 40 compounds in control cheese S1 respectively after 60 days of ripening. After two months of ripening when feta cheese is ready for consumption, most volatile compounds grouped in chemical families have reached their highest level while the content of volatiles, determined by semi-quantitative GC/MS analysis, ranged according to the series S3 > S2 > S1. The concentration of organic acids detected in manufactured cheeses, originating from milk fat lipolysis or amino acid metabolism, is higher after 60 days of ripening for all samples (Kourkoutas, Kanellaki, et al., 2006). Alcohols, derived from oxidative deamination and decarboxylation of amino acids were increased at the end of ripening after 60 days for all samples (Katechaki et al., 2009). Also, phenylethanol was detected in all samples, confers a pleasant rosy, floral aroma to the products (Dimitrellou, Kandylis, Sidira, et al. 2014; Esteban-Fernández, Rocha-Alcubilla, Muñoz-González, Moreno-Arribas, & Pozo-Bayón, 2016).

Most of the esters detected in cheeses have a fruity aroma attribute and their content increased during ripening. However, some esters, mainly methyl and 3-methylbutyl esters that offer fruity, floral and brandy-like aroma (Leung & Marriott, 2016), are present only in S3 sample originating from sea buckthorn berries used as immobilisation carrier. The main contribution of SBB biocatalyst in cheese aroma is the enrichment with many terpenes and carbonyl compounds. Terpenes have an important, positive organoleptic effect in combination with antioxidant and antimicrobial properties against pathogenic, fungi and spoilage bacteria (Solís, Becerra, Flores, Robledo, & Silva, 2004; Zengin & Baysal, 2014). Specifically, compounds such as caryophyllene and copaene are sesquiterpenes that comprises the essential oil of sea buckthorn berry. Carbonyl compounds as L-fenchone, isomenthone and cuminyl aldehyde are detected only in S3 samples. Feta-type cheeses produced with SBB biocatalyst present the highest terpene and carbonyl compound concentration compared to other samples, indicating their higher nutritional value.

The results of SPME GC/MS analysis indicate the significant contribution of SBB to the aroma profile of produced feta-type cheeses as the content of esters and mainly terpenes and carbonyl compounds was upgraded.

3.6. Preliminary sensory evaluation

The sensory characteristics of feta-type cheeses produced by free and immobilised *L. casei* cells on SBB were compared to those of control cheese. The samples were taken on 60, 90 and 100th day of ripening in brine at 4 °C (Table 4). The color of brined cheeses is pure white (porcelain white, marble white, or snow white) when they are made from ewes' and goat's milk. Control sample S1 and sample with free probiotic culture S2 were both characterized by pure snow white color when the third sample with SBB (S3) was characterized by the orange supplement of sea buckthorn berries (Fig. 1). Regarding the overall score of the sensory evaluation no significant differences ($P > 0.05$) were detected among the samples. Probiotic cheeses S2 and S3 had soft, pleasant taste while the sour flavour of sea buckthorn berries was masked in the environment of feta-type cheese.

3.7. Scientific and technological considerations

In 2013, cheese production in the ten top countries was about 49,950 × 10^3 Tons/year with a consumption of approximately 94.3 kg per person yearly. Also, the worldwide production of cheese increases about 3% per year (FAOStat Online Database, 2015). Following the trend of fortified foods development, cheese supplemented with exogenous compounds like vitamins A, C and D were reported (Sweeney & Ashoor, 1989). A recent approach in fortified food production was made by adding ginger extract to soft cheese which is beneficial since it increases *L. lactis* populations (El-Aziz et al., 2012). Another strategy involving the augmentation of *Lactobacillus* spp. populations in dairy products by immobilisation of starters on different matrices such as resin, cellulose, whey protein, and fruit pieces were successfully found (Dimitrellou, Kandylis, & Kourkoutas, 2014; Gialleli et al., 2016; Kourkoutas et al., 2005; Schoina et al., 2014). In the present work, SBB was

Table 3
Volatile compounds (μg/kg cheese) isolated from sea buckthorn berries (SBB) and Feta-type cheeses produced by commercial starter culture (S1), free (S2) or immobilised (S3) *L. casei* cells after ripening for 15 days and 60 days.

KI	Compound name	ID[c]	Cheese type[a]						SBB[b]
			$S1_{15}$	$S2_{15}$	$S3_{15}$	$S1_{60}$	$S2_{60}$	$S3_{60}$	
	Esters								
1038	Ethyl butanoate	MS,KI	0.11	0.12	0.16	3.42	5.18	6.13	nd
1132	3-methylbutyl acetate	MS,KI	0.10	5.32	3.28	Tr	Tr	Tr	nd
1148	Ethyl pentanoate	MS,KI	nd	nd	nd	Tr	Tr	Tr	nd
1250	Ethyl hexanoate	MS,KI	0.11	0.19	0.21	4.12	10.18	13.16	+
1259	Iso-amyl 2-methyl butyrate	MS,KI	nd	nd	nd	nd	nd	0.04	+
1273	Iso-amyl isovalerate	MS,KI	nd	nd	nd	nd	nd	0.46	+
1284	Hexyl acetate	MS,KI	Tr	Tr	Tr	nd	nd	nd	nd
1369	Heptyl acetate	MS,KI	Tr	Tr	Tr	nd	nd	nd	nd
1380	Methyl octanoate	MS,KI	0.15	0.36	0.72	Tr	Tr	0.11	+
1419	3methyl butyl hexanoate	MS,KI	nd	nd	nd	nd	nd	0.05	+
1421	Ethyl octanoate	MS,KI	2.11	1.23	1.36	3.28	6.15	11.18	+
1454	Methyl nonanate	MS,KI	nd	nd	nd	nd	nd	0.07	+
1562	methyl decanoate	MS,KI	nd	nd	nd	nd	nd	0.04	+
1633	3-methylbutyl octanoate	MS,KI	nd	nd	nd	nd	nd	0.07	+
1634	Ethyl decanoate	MS,KI	0.86	1.56	3.36	7.11	10.38	18.31	nd
1776	Methyl salicylate	MS,KI	nd	nd	nd	nd	nd	0.01	+
1828	2-Phenylethyl acetate	MS,KI	0.18	1.13	0.72	2.18	7.13	4.90	nd
1844	Ethyl nonadecanoate	MS	Tr	Tr	Tr	Tr	nd	nd	nd
1864	Ethyl- 4-decenoate	MS,KI	nd	nd	nd	nd	nd	0.31	+
1873	Hexyl butanoate	MS	nd	nd	nd	nd	nd	0.01	+
1898	2-phenylethyl propanoate	MS	Tr	Tr	Tr	Tr	Tr	Tr	nd
1929	3-methylbutyl benzoate	MS,KI	nd	nd	nd	nd	nd	2.16	+
	Total conc.		*3.62*	*9.91*	*9.81*	*20.11*	*39.02*	*57.01*	
	Organic acids								
950	Oxalic acid	MS	Tr	Tr	Tr	Tr	nd	nd	nd
1515	Acetic acid	MS,KI	0.15	0.11	0.21	1.76	1.28	1.12	nd
1642	Butanoic acid	MS,KI	1.45	1.15	1.86	2.03	1.84	1.46	nd
1762	Oleic acid	MS	0.18	0.06	0.12	nd	0.22	0.38	nd
1851	Hexanoic acid	MS,KI	3.61	4.16	5.88	8.32	10.19	30.16	nd
1970	Heptanoic acid	MS	Tr	Tr	Tr	0.08	0.05	0.66	nd
2064	Octanoic acid	MS,KI	5.19	6.23	11.42	10.33	12.18	30.86	+
2211	Nonanoic acid	MS,KI	2.38	1.79	2.36	13.11	12.46	13.58	nd
2301	n-Decanoic acid	MS,KI	14.66	17.02	50.28	148.1	163.2	216.1	nd
2485	Dodecanoic acid	MS,KI	61.44	58.11	72.56	128.3	182.1	228.0	nd
	Total conc.		*89.06*	*88.63*	*144.69*	*312.0*	*383.5*	*522.3*	
	Alcohols								
950	Ethanol	MS,KI	>10.000	"	"	"	"	"	nd
1026	2-Butanol	MS,KI	Tr	Tr	Tr	0.12	2.16	0.26	nd
1037	3-methyl-2-butanol	MS	Tr	Tr	Tr	nd	nd	nd	nd
1327	2- heptanol	MS	Tr	Tr	Tr	0.18	2.15	1.05	nd
1332	3-Methyl- 2-buten-1-ol	MS,KI	Tr	Tr	Tr	nd	nd	nd	nd
1363	1-Hexanol	MS,KI	Tr	Tr	Tr	0.11	0.06	Tr	nd
1441	1- heptanol	MS,KI	Tr	Tr	Tr	Tr	Tr	Tr	nd
1457	1-octen-3-ol	MS,KI	Tr	Tr	Tr	nd	nd	nd	nd
1502	2- nonanol	MS	Tr	Tr	Tr	Tr	Tr	Tr	nd
1569	2,3- butanediol	MS,KI	Tr	Tr	Tr	1.18	3.16	5.23	nd
1612	Menthol	MS,KI	nd	nd	0.88	nd	nd	1.02	+
1739	1,6- dideoxy-1-mannitol	MS	Tr	Tr	Tr	nd	nd	nd	nd
1750	5-ethyl-2-heptanol	MS	Tr	Tr	Tr	Tr	Tr	Tr	nd
1923	Phenyl ethanol	MS,KI	1.05	2.18	1.15	12.41	12.63	38.44	nd
	Total conc.		*1.05*	*2.18*	*2.03*	*14.00*	*20.16*	*46.00*	
	Carbonyl compounds								
887	4-hydroxy-2-butanone	MS	Tr	Tr	Tr	Tr	Tr	nd	nd
1066	2 methyl 3 pentanone	MS	Tr	Tr	nd	Tr	Tr	nd	nd
1088	Hexanal	MS,KI	0.35	1.05	3.09	0.16	0.18	2.19	nd
1254	4-heptenal	MS	Tr	Tr	Tr	nd	nd	nd	nd
1301	Octanal	MS,KI	0.15	0.48	1.02	0.16	0.45	1.39	nd
1334	2-heptenal	MS	0.05	0.08	nd	0.02	0.07	nd	nd
1356	L-fenchone	MS,KI	nd	nd	0.11	nd	nd	0.13	+
1383	Nonanone	MS	Tr	Tr	Tr	Tr	Tr	Tr	nd
1395	Nonanal	MS,KI	2.44	0.28	0.05	6.05	2.15	1.18	+
1310	3-Hydroxy 2-butanone	MS,KI	Tr	Tr	Tr	0.13	2.15	8.38	nd
1451	Isomenthone	MS,KI	nd	nd	0.05	nd	nd	0.05	+
1461	Decanal	MS,KI	nd	nd	Tr	nd	nd	Tr	+
1528	Benzaldehyde	MS,KI	nd	Tr	0.07	0.36	0.45	0.21	+
1781	Cuminyl aldehyde	MS,KI	nd	nd	0.11	nd	nd	0.11	+
Total conc.			*2.99*	*1.89*	*4.5*	*6.88*	*5.45*	*13.64*	
	Terpenes								
1212	Limonene	MS,KI	0.21	0.23	0.67	0.05	0.18	0.41	+
1246	*p*-cymene	MS,KI	nd	nd	Tr	nd	nd	Tr	+

(*continued on next page*)

Table 3 (*continued*)

KI	Compound name	ID[c]	Cheese type[a]						SBB[b]
			$S1_{15}$	$S2_{15}$	$S3_{15}$	$S1_{60}$	$S2_{60}$	$S3_{60}$	
1440	Copaene	MS,KI	nd	nd	Tr	nd	nd	Tr	+
1513	Linalool	MS,KI	nd	nd	0.47	nd	nd	0.52	+
1558	b-caryophyllene	MS,KI	nd	nd	0.08	nd	nd	0.07	+
1573	Terpinen-4-ol	MS,KI	nd	nd	0.48	nd	nd	0.56	+
1701	Cedrene	MS,KI	nd	nd	0.11	nd	nd	0.09	+
1765	a-curcumene	MS,KI	nd	nd	0.23	nd	nd	0.27	+
1831	Anethole	MS,KI	nd	nd	1.27	nd	nd	1.16	+
2238	Carvacrol	MS,KI	nd	nd	Tr	nd	nd	Tr	+
	Total conc.		*0.21*	*0.23*	*3.31*	*0.05*	*0.18*	*3.08*	
	Other compounds								
975	n-decane	MS,KI	nd	nd	0.07	nd	nd	0.27	+
1041	Toluene	MS,KI	0.11	0.08	0.12	0.42	0.56	0.43	nd
1100	Undecane	MS,KI	nd	nd	Tr	nd	nd	Tr	+
1170	Dodecane	MS,KI	nd	nd	0.42	nd	nd	0.37	+
1271	Styrene	MS,KI	0.42	0.58	0.27	0.06	0.02	0.07	nd
1649	Estragole	MS,KI	nd	nd	0.19	nd	nd	0.22	+
1907	Nonadecane	MS	nd	nd	0.34	nd	nd	0.36	+
	Total conc.		*0.53*	*0.66*	*1.41*	*0.48*	*0.58*	*1.72*	
Total volatile compounds conc.			***97.5***	***103.5***	***165.8***	***353.6***	***450.9***	***643.8***	

nd: not detected, Tr = traces of volatile compounds (<0.001 μg/kg), +: detected.
[a] Cheese samples were received at the 15th and 60th day of ripening.
[b] SBB volatiles extracted in methanol.
[c] Method of identification: KI = tentative identification by Kovats retention index in accordance with literature, MS = tentative identification by mass spectra obtained from NIST107, NIST21, and SZTERP libraries.

Table 4
Sensory evaluation of ripened cheeses produced by commercial starter culture, free or immobilised *L. casei* cells on sea buckthorn berries.

Cheese type	Ripening duration (days)	Taste[a]	Odor[a]	Texture[a]	Appearance[a]	Overall score
Cheese with commercial starter culture	60	6.6	2.0	5.4	6.1	5.0
	90	6.5	6.8	6.1	7.0	6.6
	100	6.1	6.2	5.9	6.5	6.2
Cheese with free *L. casei* cells	60	6.9	2.1	5.4	6.1	5.1
	90	6.9	6.8	6.0	7.0	6.7
	100	6.2	6.7	5.8	6.5	6.3
Cheese with immobilised *L. casei* cells	60	6.8	2.2	4.5	6.1	4.9
	90	6.7	6.8	4.8	6.8	6.3
	100	6.8	6.8	5.1	6.9	6.4

[a] The flavour, aroma, texture and appearance were rated according to a scale 1–7.

effectively used as support of *L. casei* immobilisation for cheese production. However, the advantages of using SSB "matrix" is not limited only to be as biodegradable scaffold for enhancing the growth of beneficial microorganism, decreasing microbial pathogens and enhance their organoleptic properties in cheese but also the berries are the source of many healthy molecules such as

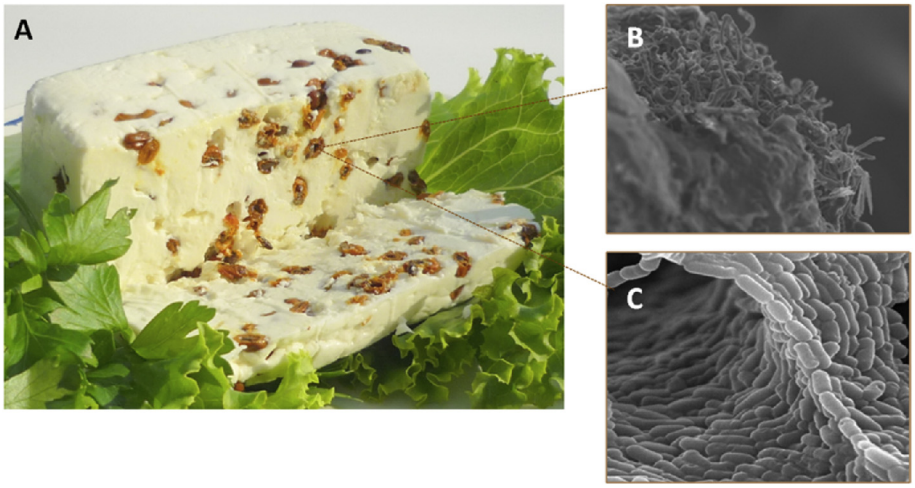

Fig. 1. (a) Feta-type cheese produced with immobilised *L. casei* cells on SBB. Scanning electron micrographs of (b) sea buckthorn berries surface (× 200) and (c) immobilised *L. casei* cells on SBB (× 5000).

unsaturated Omega-6 fatty acids (*i.e.*, linoleic and linoleic acids), carotenoids, provitamin A and vitamin E found in the seeds and berry pulp among others. Many of these healthy compounds synthesized by SBB are essential in mammalian nutrition, and also some are displaying anti-oxidant and inmunomodulatory properties (Bal et al., 2011; Suryakumar & Gupta, 2011). Additionally, *Hippophae rhamnoides* are deciduous and hard shrubs, able to grow in poor lands for traditional agriculture.

The biotechnology approach of using sea buckhorn berries as natural and biodegradable scaffolds for the development of fortified Feta cheese not only increases the nutritional value of cheeses, popular foods in the Mediterranean cultures, but also is creating a virtuous circle by using green technological methodology for making highly positive aspects in human health.

4. Conclusions

In the present study, the use of sea buckthorn berries as a novel immobilisation support for the probiotic strain *L. casei* ATCC 393 cells upgraded the manufacturing bioprocess of feta-type cheeses. Additionally, the produced cheeses with the immobilised SBB biocatalysts were characterized by improved physicochemical characteristics and aroma profile as well as microbiological safety. The produced functional cheese with SBB as immobilisation carrier for probiotic cells presents high potential for commercialisation in dairy industry as it combines the beneficial effects of both SBB and probiotic strain.

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