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

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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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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