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Foeniculum vulgare Mill. as natural conservation enhancer and health promoter by incorporation in cottage cheese

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Abstract

Food industry is focused on the development of novel functional foods containing health promoting natural ingredients, avoiding the potential harm of synthetic food additives. In the present work, the antioxidant and antimicrobial potential of *Foeniculum vulgare* Mill. (fennel) decoction (phenolic-enriched extract) was evaluated; after chemical characterization of the extract by HPLC-DAD-ESI/MS, it was used as natural ingredient in cottage cheese samples for two purposes: increase shelf life and bring bioactive properties. The incorporation of fennel-based ingredients did not altered significantly the nutritional characteristics of control cottage cheese (without fennel-based ingredients), but avoided the increase in yellowness (after 7 days of storage), and the decrease in lactose content (after 14 days of storage) observed in control samples. Control samples after 14 days of storage, were the only ones showing signs of degradation. Furthermore, the incorporation of the fennel decoction improved the antioxidant properties of cottage cheese, up to 14 days of storage. Overall, fennel decoction can be used as a natural conservation enhancer in cottage cheese, while bringing antioxidant properties to the final product.

Keywords: *Foeniculum vulgare*; cottage cheese; functional foods; natural preservers.

1. Introduction

The demand for foods with high nutritional and bioactive value (functional foods), and longer shelf life, is a mandatory challenge for food science and industry (Carocho, Barreiro, Morales & Ferreira, 2014). Current interests of the food industry are focused on avoiding potential harmfulness of synthetic food additives and developing novel functional foods containing health promoting ingredients. Natural matrices/compounds with antioxidant and antimicrobial properties could serve both purposes.

The use of food additives comes from ancient times, where people used simple substances to increase the shelf life and assign or highlight specific characteristics of some foods (Aun, Mafra, Philippi, Kalil, Agondi & Motta, 2011). Nowadays, processed food has to be transported across large distances to reach consumers and, therefore, special requirements are needed to ensure products quality and safety, mainly in prevention of contamination and spoilage (Carocho et al., 2014). There are more than 25,000 additives used in food, from which some synthetic ones have been related with gastrointestinal, respiratory, dermatologic, and neurologic adverse reactions (Branen, Davidson, Salminen & Thorngate, 2001; Wilson & Bahna, 2005; Randhawa & Bahna, 2009). Due to those potential risks to consumer health, nowadays, there is a tendency to replace synthetic additives by natural ones (Carocho & Ferreira, 2013).

Natural ingredients with antioxidant properties could be used to replace synthetic additives, which might also have health benefits in the prevention of several diseases related to oxidative/nitrosative stress, such as cancer, cardiovascular diseases, atherosclerosis, neurological disorders, hypertension, or diabetes mellitus (Carocho & Ferreira, 2013). Likewise, antimicrobial activity of some natural ingredients could delay or inhibit the growth of pathogenic and/or toxin-producing microorganisms in food, as

also minimize the incidence of foodborne diseases caused by food spoilage bacteria and fungi (Beuchat, 2001).

Foeniculum vulgare Mill. (fennel) is a biennial plant belonging to the family Apiaceae (Umbelliferaceae), distributed in central Europe and Mediterranean region, that showed both antioxidant (Singh, Maurya, Lampasona & Catalan, 2006; Barros, Heleno, Carvalho & Ferreira, 2009) and antimicrobial (Dadalioglu & Evrendilek, 2004; Lo Cantore, Iacobellis, De Marco, Capasso & Senatore, 2004; Soylu, Yigitbas, Soylu & Kurt, 2007; Barros et al., 2009) properties. Therefore, it seemed to us a promising matrix to incorporate in cottage cheese, which is highly appreciated regarding its organoleptic properties and nutritional value, but has a very short shelf life (Díaz-Castro et al., 2012).

The present study aims to characterize and evaluate antioxidant/antimicrobial potential of *F. vulgare* decoction (phenolic-enriched extract) to be used as a natural ingredient in cottage cheese for two purposes: increase shelf life and provide bioactive properties.

2. Materials and methods

2.1. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were from Extrasynthèse (Genay, France). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, L-ascorbic acid, organic acid and sugar standards, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade,

Serbia). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Fennel-based natural ingredients: phenolic-enriched extracts

2.2.1. Preparation. Commercial samples of *Foeniculum vulgare* Mill. (fennel) were provided by Américo Duarte Paixão Lda. (Alcanede, Portugal). The dried samples were powdered (~20 mesh) and submitted to decoction in order to obtain phenolic-enriched extracts. Decoctions were performed by adding 1 g of plant material to 200 mL of distilled water, heated (heating plate, VELP scientific, Usmate, Italy), and boiled for 5 min. The mixtures were left to stand for 5 min and then filtered. The decoctions were frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

2.2.2. Chemical characterization. Phenolic compounds were determined in the decoctions, by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA), as previously described by the authors (Barros et al., 2013). A Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile, with a flow rate of 0.5 mL/min. Double online detection was carried out using diode array detector (DAD) using 280 and 370 nm as preferred wavelengths and a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. The results are expressed in mg/g of lyophilized decoction.

2.2.3. Evaluation of antioxidant properties. To obtain stock solutions of 5 mg/mL, the lyophilized decoctions were re-dissolved in water. The mentioned stock solutions were successively diluted until determination of EC_{50} values (sample concentration providing a value of 50% in the DPPH, β-carotene bleaching and TBARS assays or 0.5 absorbance in the reducing power assay).

DPPH radical-scavenging activity was evaluated using an ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated by the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) was evaluated by the lipid peroxidation inhibition in porcine brain homogenates where the color intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A-B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively. Trolox was used as positive control in all the assays.

2.2.4. Evaluation of antimicrobial properties. Antibacterial activity was evaluated against Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter*

cloacae (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), following the procedure previously described by the authors (Soković, Glamočlija, Marin, Brkić & van Griensven, 2010). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined following the same reference. Streptomycin and ampicillin were used as positive controls. Antifungal activity was evaluated against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061), and *Penicillium verrucosum* var. *cyclopium* (food isolate), following the procedure previously described by the authors (Soković & van Griensven, 2006). The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations were determined following the same reference. Bionazole and ketokonazole were used as positive controls.

2.3. Incorporation of the fennel-based natural ingredients in cottage cheese

2.3.1 Preparation of the cottage cheese samples. All the samples of cottage cheese were prepared by "Queijos Casa Matias Lda." (one of the main producer companies of "Serra da Estrela" cheese, the most famous Portuguese cheese). Three groups of samples, each one with nine ewe's cottage cheeses (250 g), were prepared: control sample (cottage cheese without the fennel-based natural ingredient); sample with the fennel decoction (it was incorporated in cottage cheese at the EC_{25} value previously determined by DPPH assay: 0.35 mg/mL, corresponding to 1.05 g for each 250 g cottage cheese sample); and sample with the fennel powder (it was incorporated at 1.75 mg/mL, considering the decoction yield of 20%, corresponding to 5.25 g for each 250 g cottage cheese sample).

The samples (three different cottage cheeses for each storage time) were submitted to an evaluation of color, nutritional composition, and antioxidant activity, immediately after preparation and after seven and fourteen days of storage at 4 ºC.

2.3.2. Evaluation of color, nutritional composition and antioxidant activity of control and incorporated cottage cheese samples along storage time

The color of the samples was measured in a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Japan), using the illuminant C and diaphragm aperture of 8 mm; the CIE *L*a*b** color space values were registered using the data software "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Fernandes, Antonio, Barreira, Oliveira, Martins & Ferreira, 2012). The color was measured with 3 readings on the top and bottom part, for each sample.

The samples were also analyzed for proximate composition (moisture, protein, fat, carbohydrates and ash) using the AOAC procedures. The crude protein content (N × 6.38) of the samples was estimated by Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. Total energy was calculated according to the following equations: Energy (kcal) = 4 × (g protein +g carbohydrate) + 9 × (g lipid).

Fatty acids were determined in the soxhlet extract by gas-chromatography coupled to flame ionization detector (GC-FID), according to the procedure previously described by the authors (Barros et al., 2013). The identification was made by comparing the relative retention times of fatty acid methyl esters from samples with standards. The results were expressed in relative percentage of each fatty acid.

Free sugars were determined in defatted samples by HPLC coupled to a refraction index (RI) detector, according to the procedure previously described by the authors (Barros et al., 2013). The compounds were identified by chromatographic comparisons with authentic standards, and quantification was performed using the internal standard (melezitose) method. Sugars content was expressed in g/100 g of cottage cheese.

Organic acids were determined in defatted samples by HPLC coupled to a photodiode array detector (PDA), according to the procedure previously described by the authors (Barros et al., 2013). Detection was carried out using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g/100 g of cottage cheese.

For evaluation of antioxidant activity, the samples were submitted to DPPH and reducing power assays, previously described in section 2.4.

2.4. Statistical analysis

In each group, three different samples were prepared and analysed in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 22.0 program (IBM Corp., Armonk, NY: USA).

3. Results and discussion

3.1. Chemical characterization, antioxidant and antimicrobial properties of fennel phenolic-enriched extracts

The chromatographic profile of the fennel decoction can be observed in **Figure 1**. Up to seventeen phenolic compounds were identified, including twelve phenolic acids and derivatives, and five flavonoids (**Table 1**).

p-Hydroxybenzoic acid (compound 4), 5-*O*-caffeolylquinic acid (compound 3), caffeic acid (compound 5), epicatechin (compound 7), *p*-coumaric acid (compound 9), quercetin-3-*O*-rutinoside (compound 10) and quecetin-3-*O*-glucoside (compound 12) were positively identified by comparison of their retention time, mass and UV-vis characteristics with commercial standards. Most of these compounds have been previously described in fennel samples by different authors (Parejo, Jauregui, Sánchez-Rabaneda, Viladomat, Bastida & Codina, 2004a; Parejo, Viladomat, Jaume & Codina, 2004b; Križman, Baričevič & Prošek, 2007; Faudale, Viladomat, Bastida, Poli & Codina, 2008; Rather, Dar, Sofi, Bhat & Qurishi, 2012; Roby, Sarhana, Selima & Khalel, 2013).

Compounds 1 and 2 showed the same pseudomolecular ion as compound 3 and were identified based on their fragmentation pattern according to the clues described by Clifford et al. (2003, 2005). Compound 1 was assigned as 3-*O*-caffeoylquinic acid based on the MS^2 base peak at *m/z* 191 (deprotonated quinic acid) and the second major ion at *m/z* 179 $[\text{caffeic acid-H}]^-$, with an intensity >60% of base peak, similar to those reported by Clifford and coworkers for 3-acylchlorogenic acids. Compound 2 showed a base peak at *m/z* 173 $[\text{quinic acid-H-H}_2\text{O}]^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 89% abundance of base peak, consistent with 4-*O*-caffeoylquinic acid (Clifford et al., 2003, 2005). The presence of 3-, 4- and 5-*O*-caffeoylquinic acids in fennel samples was already reported by other authors (Parejo et al., 2004a,b; Križman et al., 2007; Faudale et al., 2008; Rather, 2012). Compound 8 was tentatively identified as 5-*O*-feruloylquinic acid taking into account its pseudomolecular

ion and fragment ions with relative abundance similar to those of 5-*O*-caffeoylquinic acid. This compound was identified by Parejo et al. (2004a) in fennel wastes. Four compounds (peaks 6, 13, 14 and 17) showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 515, corresponding to dicaffeoylquinic acids; they were also tentatively assigned based on their fragmentation patterns according to Clifford et al. (2005) as 1,3-; 1,4-; 1,5- and 3,4-*O*-dicaffeoylquinic acids, respectively. With the exception of 3,4-*O*-dicaffeoylquinic acid, all the other dicaffeoylquinic derivatives were previously reported in fennel (Parejo et al., 2004a,b; Križman et al., 2007; Faudale et al., 2008; Rather et al., 2012). Compound 15 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 601, 86 mu higher than a di-*O*-caffeoylquinic acids and with similar MS^2 fragmentation pattern similar as those compounds; the mass difference can be related to a malonyl moiety, so that it was tentatively identified as malonyl di-*O*-caffeoylquinic acid. To the best of our knowledge such a compound has not been previously reported in fennel.

Compound 11 ($[M-H]^-$ at *m/z* 477) presented a UV spectrum with λ_{max} around 350 nm and a unique MS^2 product ion at *m/z* 301; it was identified as quercetin 3-*O*-glucuronide as confirmed by comparison with a standard obtained and characterized in our laboratory (Dueñas, Chronet, Pérez-Alonso, Paola-Naranjo, González-Paramás & Santos-Buelga, 2008). This compound has been reported in fennel (Parejo et al., 2004a,b; Faudale et al., 2008; Rather et al., 2012). Compound 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 491, 14 mu higher than compound 11, and fragment ions at *m/z* 315 and 300, from the consecutive losses of 176 mu (glucuronide moiety) and 15 mu (methyl group), which allowed assigning it as a methylquercetin-*O*-glucuronide. It was tentatively identified isorhamnetin 3-*O*-glucuronide owing the previous identification of that compound in fennel by Parejo et al (2004a). Quercetin-3-*O*-glucuronide was the most abundant compound in the studied fennel sample, whereas

5-*O*-caffeoylquinic acid was the most abundant phenolic acid. The quantitative results obtained in our study for fennel decoction (**Table 1**) cannot be directly compared with those given by other authors (Parejo et al., 2004b; Križman et al., 2007; Faudale et al., 2008; Roby et al., 2013) which expressed them regarding dry plant material.

Four different *in vitro* assays were applied to evaluate the antioxidant activity of the phenolic compounds-enriched extract of *F. vulgare* (fennel) prepared by decoction, and the results are shown in **Table 2**. The high antioxidant activity of fennel has been previously reported for methanol (Barros et al., 2009) and boiling aqueous (Mata, Proença, Ferreira, Serralheiro, Nogueira & Araújo, 2007) extracts. However, the decoction prepared in this study showed lower DPPH scavenging activity, reducing power and lipid peroxidation inhibition than the ones reported in the mentioned studies. A recent investigation described the evaluation of antioxidant properties of different fennel extracts (methanol, ethanol, diethyl ether and hexane), concluding that methanol and ethanol gave more efficient extracts than the other less polar solvents (Roby et al., 2013). Nevertheless, water was not used in that study and the mentioned solvents exhibit some toxicity; therefore, the decoction used in the present work is more suitable for incorporation in food matrices.

The antimicrobial and antifungal activity of the fennel extract was examined against a panel of eight microorganisms selected on the basis of their relevance to public health. The data were expressed as minimum inhibitory concentration (MIC), bactericidal concentration (MBC) and fungicidal concentration (MFC) and reported in **Table 2**. *Salmonella typhimurium* and *Bacillus cereus* were the most sensitive bacteria, while *Aspergillus niger*, *Aspergillus versicolor* and *Penicillium funiculosum* were the most susceptible fungi, showing the lowest MIC and MBC values. Moreover, the fennel extract showed bactericide and fungicide effects against the tested microorganism.

Some previous studies reported *F. vulgare* as a good source for the preparation of new therapeutic and antimicrobial agents (Lo Cantore et al., 2004; Singh et al., 2006). As for the antioxidant activity, Roby et al. (2013) described the antimicrobial activity of methanol and ethanol extracts prepared from fennel seeds showing much lower MICs (0.010-0.015 mg/mL) than those obtained in the present study.

3.2. Incorporation of fennel-based natural ingredients in cottage cheese

3.2.1. Effects on color and nutritional parameters

Considering the high antioxidant properties obtained in the present study for fennel decoction, this preparation was incorporated in cottage cheese samples. In addition to the incorporation of fennel decoction, fennel powder was also directly incorporated in other cottage cheese samples to compare effects and evaluate if the preparation of decoction would be worthy. Color parameters, nutritional value, fatty acids composition and antioxidant activity of the prepared cottage cheese samples were evaluated along shelf life (0 days, 7 days and 14 days). Pictures of the cheese throughout that period are shown in **Figure 2A**.

The results of color evaluation are collected in **Table 3**. The L^* parameter indicates lightness so, higher values result in clearer objects; a^* value indicates the redness-greeness tendency and b^* value indicates the blueness-yellowness tendency. A statistical significant increase of b^* value (yellow component) of the control cottage cheese, was observed after 7 days of storage; after 14 days, all the parameters changed. The samples with fennel powder and decoction only showed increased b^* values after 14 days, maintaining L^* parameter (**Table 3; Figure 2A**). Some authors related the increase in these values with the occurrence of proteolysis, which decreases the luminosity due to the production of browning compounds (Lucas, Rock, Agabriel,

Chilliard & Coulon, 2008). The values obtained for the different parameters are in the same order of magnitude in all the sample groups. A study with ricotta cheese reported similar L^* (93.63-94.31) but lower b^* values (7.65-8.13) (Pizzilo, Claps, Cifuni, Fedele & Rubino, 2005) as those obtained in the present work. Another study with "coalho" cheese made from cow's and goat's milk and their mixture, found similar L^* and b^* values, as also an increase in b^* values along 28 days (Queiroga et al., 2013). No statistical significant differences were observed in a^* parameter; moreover, its value is close to zero and, therefore, the contribution to total color value is minimum.

All the cottage cheese samples are characterized by high moisture, protein and fat contents (**Table 3**). The samples complied with the standard of identity for cottage cheese, which states that they should have a minimum fat free dry matter content of 18% (Codex standard, 2010). The moisture contents were similar to the ones reported by Queiroga et al. (2013), which also described a slight decrease in moisture along shelf life (28 days). Otherwise, Silva, Ramos, Moreno & Moraes (2010) found lower moisture contents (45.5-51.5 g/100 g) in "Coalho" cheese made from cow's milk. In the present study, as expected, a decrease in moisture levels was observed along the storage. In our investigation, the amounts of protein and fat increased over time, however, it is necessary to take into account that moisture decreased and, therefore, a relative increase in nutrients concentration is expected. Nevertheless, the results obtained when expressed in dry matter basis showed maintenance of the protein content along shelf life, indicating that the total amount of protein does not change. A study with ripened cheeses described a decrease in protein content during storage (Pappa, Kandarakis, Anifantakis, Zerfiridis, Anifantakis & Sotirakoglou, 2006). Protein loss during ripening is related with protein hydrolysis and production of water-soluble nitrogen compounds, which are released in the brine (Pintado, Pinho, Ferreira, Pintado, Gomes & Malcata,

2008). This does not apply in our study, as cottage cheese do not suffer relevant ripening.

The energy values also increased along shelf life, being in the same magnitude in all the samples (**Table 3**); this variation is also due to the moisture decrease and increasing concentration of nutrients as previously explained. A study with fresh soft cheese described energetic values varying between 174 and 197 kcal/100 g (Krbavcic and Baric, 2004), lower than those obtained in our samples.

Lactose was the free sugar identified and quantified in the samples; fennel-based ingredients seem to protect lactose in the prepared cheeses, avoiding its decrease after 14 days of storage, which was observed in the control sample (**Table 3**).

Essential fatty acids and their derivatives represent an important nutritional role and have a high dietetic significance in dairy products. In all the samples, saturated fatty acid (SFA) predominated, followed by monounsaturated (MUFA) and then polyunsaturated (PUFA) fatty acids (**Table 3**). Studies with ricotta cheese showed similar fatty acid distribution as observed in the present study (Pizzillo et al., 2005). Palmitic acid (C16:0) and oleic acid (C18:1n9) were the predominant fatty acids in all the cheese samples (**Table 3**). The next most predominant fatty acid in the control cottage cheese was stearic acid (C18:0), while for the samples with fennel-based ingredients was capric acid (C10:0). Queiroga et al. (2013) reported the same predominant fatty acids in Coalho cheeses made from different types of milk. Otherwise, C6, C8, C10 and C12 fatty acids were described as the main fatty acids in cheeses made from goat's milk (Lucas et al., 2008; Ceballos, Morales, Adarve, Castro, Martínez & Sampelayo, 2009).

3.2.2. Effects on antioxidant parameters

As expected, cottage cheese did not show relevant antioxidant properties; the reducing power observed after 7 and 14 days is probably related with the formation of reducing substances after lipid peroxidation process. The incorporation of fennel improved the antioxidant activity of cottage cheese (**Table 4**). Samples incorporated with plant powder revealed higher antioxidant properties than samples incorporated with decoction, either in 0 or 7 days of storage. A decrease in the antioxidant potential of the cottage cheese with both fennel preparations was observed along the shelf life. Nevertheless, it is important to highlight that the samples, after 14 days, still display antioxidant properties. At that time, cottage cheese incorporated with fennel decoction gave somewhat better DPPH scavenging activity than the one with fennel powder.

Food industry is focused on the development of novel functional foods containing health promoting natural ingredients. There are other available studies that also describe improvements in the nutritional characteristics, antioxidant and antimicrobial properties of cheese incorporated with different natural ingredients, such as *Agaricus bohusii* mushroom extract (Reis et al., 2012) or lupin milk (Elsamani, Habbani, Babiker & Ahmed, 2014).

4. Conclusions

Overall, the incorporation of fennel-based ingredients did not altered significantly the nutritional characteristics of control cottage cheese, but seems to avoid the increase in *b** color parameter (yellowness) after 7 days of storage (**Figure 2A**), and the decrease in lactose content observed after 14 days of storage in control samples. Furthermore, control samples of cottage cheese (without fennel-based ingredients) after 14 days of storage, were the only ones showing signs of degradation (**Figure 2B**). The incorporation of a fennel phenolic-enriched extract (decoction) improved the

antioxidant properties of cottage cheese, up to 14 days of storage. To preserve the antioxidant activity along the shelf life, microencapsulation techniques could be applied to fennel decoction. The development of new functionalized dairy products is important for consumers that demand for valuable health effects while enjoying a highly appreciated product.

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in fennel decoction.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*) (% base peak)	Tentative identification	Quantification (mg/g)
1	5.1	328	353	191(100),179(62),173(5),161(5),135(45)	3-*O*-Caffeolyquinic acid	1.12 ± 0.02
2	7.2	326	353	191(60),179(89),173(100),161(7),135(31)	4-*O*-Caffeolyquinic acid	2.25 ± 0.09
3	7.8	326	353	191(100),179(20),173(14),161(5),135(14)	5-*O*-Caffeolyquinic acid	4.54 ± 0.15
4	9.5	256	137	109(100)	*p*-Hydroxybenzoic acid	0.04 ± 0.01
5	11.1	324	179	135(100)	Caffeic acid	0.31 ± 0.01
6	12.0	328	515	353(95),191(85),179(79),173(3),161(3),135(41)	1,3-Di-*O*-caffeoylquinic acid	0.76 ± 0.04
7	12.5	274	289	175(100),159(40),147(60),131(37),115(41)	Epicatechin	0.43 ± 0.01
8	14.8	324	367	193(11),191(100),173(12),134(5)	5-*O*-Feruloylquinic acid	0.32 ± 0.01
9	16.9	310	163	119(100)	*p*-Coumaric acid	tr
10	18.9	358	609	301(100)	Quercetin-3-*O*-rutinoside	0.28 ± 0.01
11	19.4	356	477	301(100)	Quercetin-3-*O*-glucuronide	8.81 ± 0.07
12	20.3	358	463	301(100)	Quercetin-3-*O*-glucoside	0.57 ± 0.02
13	20.6	326	515	353(87),191(20),179(62),173(100),161(4),135(18)	1,4-Di-*O*-caffeoylquinic acid	1.15 ± 0.07
14	22.5	328	515	353(77),191(100),179(24),173(4),161(15),135(7)	1,5-Di-*O*-caffeoylquinic acid	3.84 ± 0.08
15	23.5	330	601	557(20),515(13),353(12),233(100),191(8),179(9),173(21),161(4),135(3)	Malonyl di-*O*-caffeoylquinic acid	2.48 ± 0.14
16	25.1	354	491	315(100),300(42)	Isorhamnetin-3-*O*-glucuronide	1.43 ± 0.01
17	25.2	332	515	353(95),191(24),179(65),173(100),161(4),135(25)	3,4-Di-*O*-caffeoylquinic acid	1.43 ± 0.04
					Total phenolic acids	18.25 ± 0.62

Table 2. Antioxidant and antimicrobial activity of fennel phenolic-enriched extracts obtained by decoction.

	Antioxidant activity			
	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS inhibition
Fennel (EC_{50}, mg/mL)	0.75 ± 0.01	0.42 ± 0.06	0.17 ± 0.01	0.37 ± 0.01
Trolox (EC_{50}, μg/mL)	41.43 ± 1.27	41.68 ± 0.28	18.21 ± 1.12	22.84 ± 0.74

	Antibacterial activity							
	Staphylococcus aureus	*Bacillus cereus*	*Micrococcus flavus*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Enterobacter cloacae*	*Salmonella typhimurium*
Fennel								
MIC	0.20 ± 0.01	0.02 ± 0.01	1.00 ± 0.02	0.75 ± 0.1	0.20 ± 0.09	1.00 ± 0.02	0.75 ± 0.03	0.035 ± 0.009
MBC	0.75 ± 0.03	0.05 ± 0.005	1.50 ± 0.1	1.50 ± 0.1	0.75 ± 0.03	1.50 ± 0.2	3.0 ± 0.1	0.05 ± 0.01
Streptomycin								
MIC	0.04 ± 0.002	0.10 ± 0.003	0.20 ± 0.01	0.20± 0.001	0.20 ± 0.006	0.20 ± 0.003	0.20 ± 0.003	0.25 ± 0.007
MBC	0.10 ± 0.003	0.20 ± 0.06	0.30 ± 0.000	0.30 ± 0.02	0.30 ± 0.003	0.30 ± 0.03	0.30 ± 0.000	0.50 ± 0.003
Ampicillin								
MIC	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.02	0.40 ± 0.03	0.75 ± 0.1	0.40 ± 0.02	0.25 ± 0.01	0.40 ± 0.01
MBC	0.40 ± 0.01	0.40 ± 0.00	0.40 ± 0.005	0.50 ± 0.003	1.20 ± 0.5	0.50 ± 0.05	0.50 ± 0.003	0.75 ± 0.1

	Antifungal activity							
	Aspergillus fumigatus	*Aspergillus versicolor*	*Aspergillus ochraceus*	*Aspergillus niger*	*Trichoderma viride*	*Penicillium funiculosum*	*Penicillium ochrochloron*	*Penicillium verrucosum*
Fennel								
MIC	3.00 ± 0.2	0.40 ± 0.09	0.75 ± 0.2	0.20 ± 0.03	0.75 ± 0.1	0.40 ± 0.09	1.50 ± 0.1	3.00 ± 0.1
MFC	6.00 ± 0.3	6.00 ± 0.6	6.00 ± 0.9	6.00 ± 0.75	1.50 ± 0.2	1.50 ± 0.1	3.00 ± 0.2	6.00 ± 0.3
Bifonazole								
MIC	0.15 ± 0.01	0.10 ± 0.02	0.15 ± 0.002	0.15 ± 0.003	0.15 ± 0.002	0.20 ± 0.06	0.20 ± 0.02	0.10 ± 0.01
MFC	0.20 ± 0.02	0.20 ± 0.007	0.20 ± 0.000	0.20 ± 0.03	0.20 ± 0.000	0.25 ± 0.02	0.25 ± 0.03	0.20 ± 0.003
Ketoconazole								
MIC	0.20 ± 0.000	0.20 ± 0.01	1.50 ± 0.1	0.20 ± 0.003	1.00 ± 0.07	0.20 ± 0.003	2.50 ± 0.3	0.20 ± 0.000
MFC	0.50 ± 0.01	0.50 ± 0.03	2.00 ± 0.02	0.50 ± 0.007	1.00 ± 0.01	0.50 ± 0.002	3.50 ± 0.2	0.30 ± 0.007

The antioxidant activity was expressed as EC_{50} values (Mean ± SD, n = 9), what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Minimum inhibitory concentration (MIC), bactericidal concentration (MBC) and fungicidal concentration (MFC).

Table 3. Color parameters, nutritional value and fatty acids composition of the cottage cheese samples along shelf life.

	Control cottage cheese			Cottage cheese with fennel powder			Cottage cheese with fennel decoction		
Storage days	**0**	**7**	**14**	**0**	**7**	**14**	**0**	**7**	**14**
Color parameters									
*L**	92.94±0.75^{a}	90.46±1.5a	86.03±4.71^{b}	82.59±3.92^{a}	80.62±3.15^{ab}	76.47±5.49^{b}	88.14±1.49^{a}	84.38±5.22^{a}	84.59±2.32^{a}
*a**	-2.44±0.39^{a}	-2.34±0.25^{a}	-2.59±0.51^{a}	-1.47±0.36^{a}	-1.45±0.20^{a}	-1.22±0.48^{a}	-1.45±0.21^{a}	-1.55±0.40^{a}	-1.65±0.29^{a}
*b**	10.40 ±0.51^{c}	11.24 ±0.63^{b}	14.91±1.51^{a}	15.10±1.61^{b}	15.46±0.64^{b}	17.59±1.82^{a}	14.77±1.70^{b}	15.51±0.33^{b}	17.42±0.61^{a}
Nutritional value									
Moisture (g/100 g)	64.5±1.33^{a}	63.8±0.90^{a}	60.10±0.58^{b}	64.80±0.81^{a}	63.43±1.32^{b}	60.54±0.26^{c}	66.26±0.79^{a}	63.37±1.02^{a}	59.20±1.01^{b}
Protein (g/100 g)	11.01±0.02^{c}	12.08±0.09^{b}	13.25±0.07^{a}	11.70±0.11^{c}	12.30±0.08^{b}	13.34±0.24^{a}	11.30±0.36^{c}	12.09 ±0.04^{b}	13.62±0.12^{a}
Ash (g/100 g)	2.35±0.04^{a}	2.15±0.05^{c}	2.24±0.01^{b}	2.03±0.08^{b}	2.17±0.02^{a}	2.20±0.03^{a}	2.01±0.01^{c}	2.25 ±0.03^{b}	2.46±0.03^{a}
Fat (g/100 g)	18.98±0.33^{c}	19.97±0.09^{b}	22.33±0.20^{a}	19.45±0.44^{b}	19.38±0.04^{b}	20.40±0.02^{a}	18.06±0.51^{c}	19.10 ±0.12^{b}	22.55±0.01^{a}
Carbohydrates (g/100g)	3.16±1.00^{a}	2.00±0.14^{a}	2.08±0.21^{a}	2.02±1.07^{a}	2.72±0.07^{a}	3.52±0.05^{a}	2.38±0.35^{b}	3.17 ±0.09^{a}	2.18±0.02^{c}
Lactose (g/100 g)	2.00±0.06^{a}	1.95±0.03^{a}	1.77±0.04^{b}	1.71±0.01^{b}	1.83±0.04^{a}	1.75±0.07^{ab}	2.04±0.02^{a}	2.02 ±0.02^{a}	2.07±0.07^{a}
Energy (kcal/100 g)	227.47±6.98^{b}	236.08±0.24^{b}	262.30±0.98^{a}	229.96±0.73^{c}	234.48±0.13^{b}	251.07±0.01^{a}	220.03±0.95^{c}	233.00 ±0.71^{b}	266.09±0.19^{a}
Fatty acids									
C4:0	7.84±0.52^{a}	7.42±0.85^{ab}	6.57±0.55^{b}	6.86±0.75^{a}	5.12±0.16^{b}	6.72±0.06^{a}	6.74±0.32^{a}	6.27±0.01^{ab}	5.74±0.85^{b}
C6:0	7.09±0.68^{a}	6.79±0.63^{a}	6.28±0.30^{a}	6.41±0.03^{b}	5.52±0.49^{c}	6.70±0.37^{a}	6.22±0.31^{b}	5.97±0.35^{b}	6.93±0.15^{a}
C8:0	4.77±0.27^{a}	4.94±0.12^{a}	4.39±0.52^{a}	4.79±0.23^{b}	4.82±0.08^{b}	5.79±0.09^{a}	4.74±0.17^{b}	4.80±0.38^{ab}	5.39±0.47^{a}
C10:0	9.95±0.21^{a}	10.20±0.17^{a}	10.29±0.85^{a}	10.82±0.81^{b}	10.46±0.75^{b}	11.55±0.20^{a}	10.95±0.20^{b}	11.42±0.64^{b}	12.66±0.45^{a}
C12:0	4.48±0.02^{b}	4.49±0.01^{b}	5.02±0.32^{a}	5.20±0.38a^{b}	4.73±0.54^{b}	5.49±0.04^{a}	5.32±0.03^{a}	5.49±0.10^{a}	5.57±0.34^{a}
C14:0	8.74±0.30^{b}	8.79±0.07^{b}	9.85±0.12^{a}	9.95±0.50^{a}	9.29±0.46^{a}	9.48±0.07^{a}	10.11±0.11^{a}	10.34±0.11^{a}	10.18±0.37^{a}
C15:0	1.03±0.07^{b}	1.05±0.01^{b}	1.15±0.05^{a}	1.16±0.04^{a}	1.02±0.13^{b}	1.01±0.02^{b}	1.20±0.02^{a}	1.19±0.03^{a}	1.12±0.01^{b}

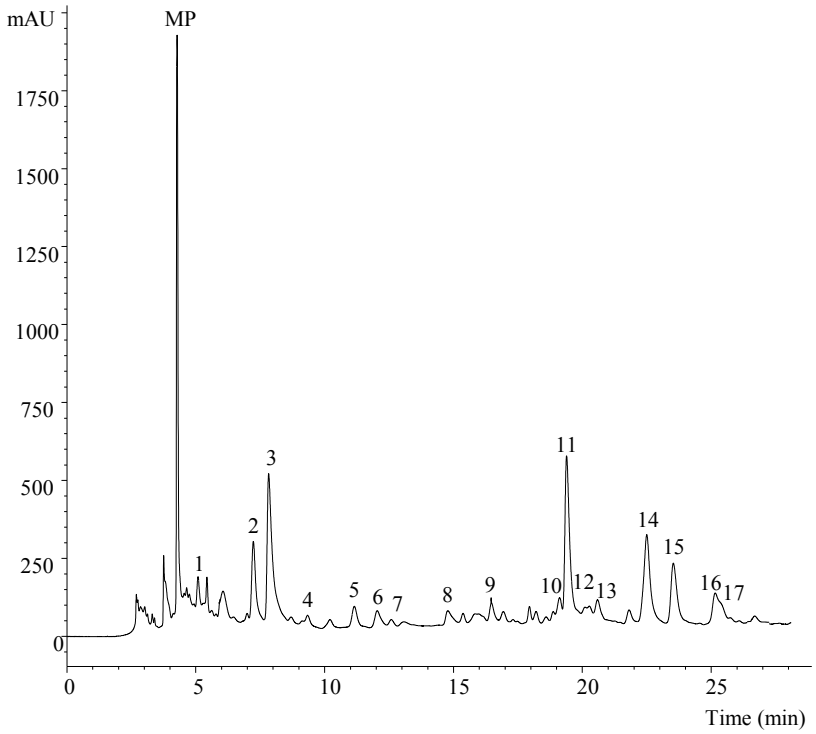
C16:0	20.70±0.56^{a}	19.96±0.27^{b}	20.59±0.90^{a}	20.66±0.24^{ab}	21.12±0.46^{a}	20.29±0.01^{b}	20.92±0.51^{ab}	21.28±0.54^{a}	20.47±0.26^{b}
C18:0	10.29±0.13^{a}	10.53±0.64^{a}	9.87±0.59^{a}	9.30±0.39^{b}	10.42±0.77^{a}	8.50±0.48^{b}	9.07±0.05^{a}	8.87±0.24^{ab}	8.47±0.42^{b}
C18:1n9	18.36±0.38^{a}	18.70±0.15^{a}	18.95±0.98^{a}	18.31±0.65^{b}	19.75±0.35^{a}	18.88±0.38^{b}	17.92±0.28^{a}	18.06±0.31^{a}	17.68±0.50^{a}
C18:2n6	2.80±0.05^{a}	2.71±0.16^{a}	2.70±0.30^{a}	2.49±0.14^{b}	2.94±0.25^{a}	2.20±0.01^{b}	2.54±0.02^{a}	2.44±0.08^{ab}	2.31±0.13^{b}
C18:3n3	1.58±0.13^{a}	1.61±0.04^{a}	1.52±0.21^{a}	1.43±0.12^{b}	1.71±0.13^{a}	1.21±0.02^{c}	1.47±0.04^{a}	1.37±0.08^{ab}	1.26±0.07^{b}
SFA (%)	76.23±0.60^{a}	75.70±0.15^{a}	75.41±1.62^{a}	76.56±0.97^{a}	74.11 ± 0.80^{b}	76.65±0.45^{a}	76.74±0.34^{b}	76.98±0.45^{ab}	77.72±0.75^{a}
MUFA (%)	19.09±0.42^{a}	19.40±0.19^{a}	19.80±1.06^{a}	19.05±0.69^{b}	20.64 ± 0.37^{a}	19.53±0.45^{b}	18.72±0.29^{a}	18.84±0.32^{a}	18.31±0.55^{a}
PUFA (%)	4.68±0.17^{a}	4.90±0.34^{a}	4.80±0.56^{a}	4.39±0.28^{b}	5.26 ± 0.43^{a}	3.81±0.01^{c}	4.54±0.04^{a}	4.18±0.14^{b}	3.96±0.21^{b}

The results are presented as mean±SD (n = 9). *L**, *a** and *b** (color parameters). Butiric acid (C4:0); Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids. The difference to 100% corresponds to other 17 less abundant fatty acids (data not shown). In each row and within each sample (Control cottage cheese, Cottage cheese with fennel powder and Cottage cheese with fennel decoction), different letters mean significant statistical differences along shelf life (p<0.05).

Table 4. Free radicals scavenging activity and reducing power (EC_{50} values, mg/mL) of cottage cheese samples along shelf life.

	Control cottage cheese			Cottage cheese with fennel powder			Cottage cheese with fennel decoction		
Storage days	**0**	**7**	**14**	**0**	**7**	**14**	**0**	**7**	**14**
DPPH assay	>200	>200	>200	30.05 ± 3.19^{b}	42.74 ± 0.35^{b}	49.42 ± 0.76^{a}	40.99 ± 0.12^{c}	44.20 ± 0.12^{b}	46.72 ± 0.09^{a}
Reducing power assay	>200	40.27 ± 0.52^{a}	14.85 ± 0.12^{b}	6.92 ± 0.13^{a}	5.75 ± 0.09^{b}	5.13 ± 0.03^{c}	8.46 ± 0.06^{a}	6.82 ± 0.10^{b}	5.50 ± 0.05^{c}

The results are presented as mean±SD (n = 9). In each row and within each sample (Control cottage cheese, Cottage cheese with fennel powder and Cottage cheese with fennel decoction), different letters mean significant statistical differences along shelf life ($p<0.05$).


mAU
MP
1750
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1250
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Time (min)

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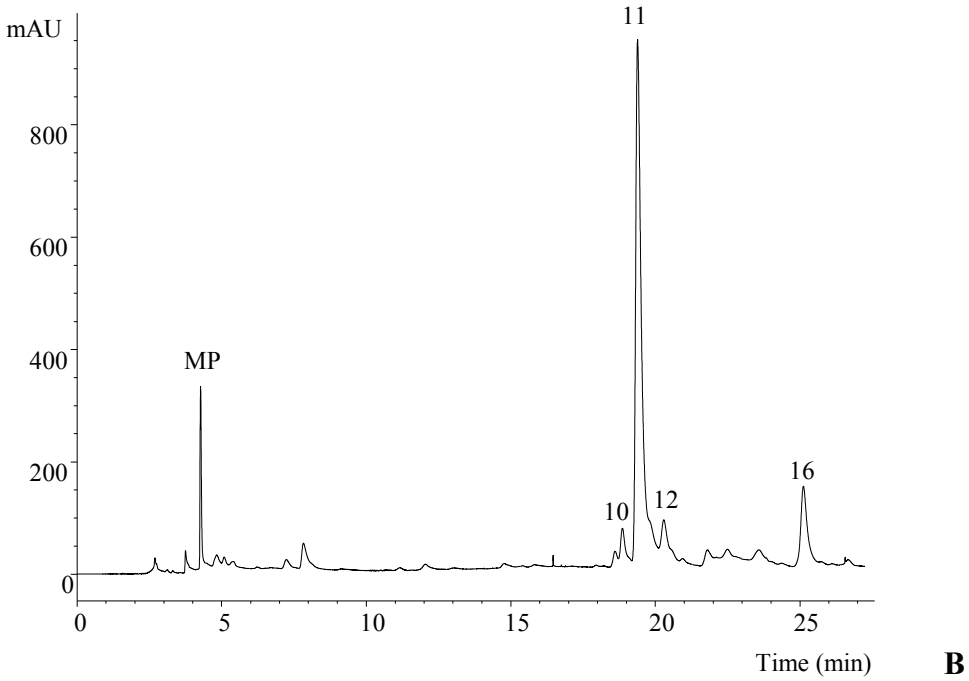
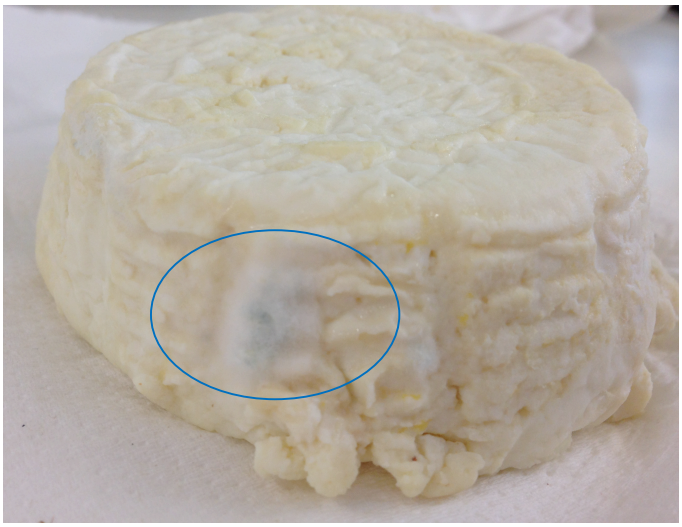


Figure 1. Profile of phenolic compounds in fennel deccotion, recorded at 280 nm (**A**) and 370 nm (**B**).


Control cottage cheese
Cottage cheese with fennel powder
Cottage cheese with f decoction
0 days
7 days
14 days

(A)



(B)

Figure 2. (A) Appearance of cottage cheese samples along shelf life. **(B)** Control sample of cottage cheese after 14 days of storage, showing signs of degradation.