Extended use of gamma irradiation in wild mushrooms conservation: validation of 2 kGy dose to preserve their chemical characteristics

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ABSTRACT

Irradiation is recognized by international organizations as a conservation technology, and its application to wild mushrooms has been tested in some species. Our research group evaluated the effectiveness of gamma irradiation to conserve different samples of highly appreciated species, particularly, *Lactarius deliciosus*, *Macrolepiota procera*, *Boletus edulis* and *Hydnum repandum*. From those results and considering also international recommendations on this subject, the 2 kGy dose was chosen for further studies. Therefore, the application of gamma irradiation at 2 kGy dose was extended to *Boletus pinophilus* Pilát & Dermek and *Clitocybe subconnexa* Murrill to validate the proposed technology. Considering the obtained results, some of the analysed chemical parameters (specially sugars and fatty acids), as well as the antioxidant activity, showed significant changes after irradiation treatment, particularly in *B. pinophillus*, probably due to its higher water content. Nevertheless, the obtained differences did not seem to be sufficient to change the organoleptic characteristics of these mushrooms. Furthermore, the antioxidant activity was generally higher in irradiated samples. In conclusion, the detected chemical changes might be considered as acceptable, when considering the high advantages of gamma irradiation at decontamination and/or disinfestation level.

Keywords: Gamma irradiation; Wild mushrooms; Chemical composition; Antioxidant activity.

1. Introduction

Wild edible mushrooms, especially abundant in woods and forests, are natural resources of growing significance and market search. In recent years, mushrooms' picking ceased to be a family activity, of reduced size, to become a booming business, with annual trading indicators of thousands of tons of mushrooms in particular countries like Portugal (Koune, 2001; Marques, 2005).

The seasonal consumption of fresh mushrooms (mostly wild species that are highly consumed in fresh, being only available in specific periods) is mainly related to its high perishable nature. After harvest, the signs of deterioration include dehydration, loss of texture, enzymatic browning and bacterial lesions (Aguirre, Frias, Barry-Ryan & Grogan, 2008; Kulshreshtha, Singh & Deepti-Vipul, 2009). Mushroom' conservation and distribution are critical points, demanding appropriate methods of preservation once the level of losses during marketing achieves 40% (Lacroix & Ouattara, 2000).

Applying irradiation might be an alternative to minimize these losses, being recognized by international organizations as a valid conservation technology (WHO, 1991; Nagar, Hajare, Saroj & Bandekar, 2012), with recognized benefits such as extending the shelf life of many foods, stopping the maturation process, decontaminating and lowering the presence of bacteria and fungi (Minnaar, Taylor & McGill, 1995).

Our research group evaluated the effectiveness of gamma irradiation in the maintenance of wild mushrooms' quality, using fresh and/or processed samples, with focus on *Lactarius deliciosus* (Fernandes et al., 2013a), *Macrolepiota procera* (Fernandes et al., 2014a), *Boletus edulis* and *Hydnum repandum* (Fernandes et al., 2013b). Gamma irradiated (up to 2 kGy) samples of *B. edulis* (fresh), maintain the amounts of fat, carbohydrates, ash, fructose, glucose, mannitol, trehalose, citric and

fumaric saturated fatty acids as those observed in non-irradiated samples. Likewise, the antioxidant activity was preserved after irradiation (Fernandes et al., 2013b). The same dose had a similar effect on *H. repandum* fresh samples, as verified by the preservation of carbohydrates, mannitol, trehalose, ash, δ-tocopherol, saturated fatty acids, oxalic, malic, citric and fumaric acids, as well as its antioxidant activity (Fernandes et al., 2013b).

In different countries (Argentina, China, Croatia, Hungary, Israel, Korea, Poland, United Kingdom, Mexico) the recommended dose for extending the shelf life of fresh mushrooms is 1-3 kGy (Akram & Kwon, 2010). Moreover, ICGFI (1999) reported that irradiation of mushrooms at 2 to 3 kGy inhibits cap opening, stem elongation and has been shown to have minimal effect on flavour, aroma and colour (ICGFI, 1999, Sommer et al., 2009, 2010). The texture of mushroom is often the first of many quality attributes judged by the consumer and is, therefore, extremely important in overall product acceptance. However, many studies indicate that mushrooms' firmness is similar among irradiated and non-irradiated samples (Jiang et al., 2010; Fernandes et al., 2012).

According to Lacroix & Ouattara (2000), a dose of 2 kGy appears to be necessary for satisfactory prolongation of the shelf life of mushrooms at 10 ºC.

Therefore, the aim of the present work was validating the use of 2 kGy of gamma irradiation as a technology able to maintain chemical parameters of wild mushrooms, extending previous studies to unreported edible species: *Boletus pinophilus* Pilát & Dermek and *Clitocybe subconnexa* Murrill.

2. Materials and methods

2.1. Standards and Reagents

For irradiation: To estimate the dose and dose rate of irradiation it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Co., UK). To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

For chemical analyses: Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Lab-Scan (Lisbon, Portugal). 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, organic acid, tocopherol and sugar standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA).

For antioxidant potential analysis: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and gallic acid were from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were of analytical grade and obtained from common sources. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples irradiation

B. pinophilus wild samples were obtained in Trás-os-Montes, in the Northeast of Portugal, in November 2012 and *C. subconnexa* in November 2013. Subsequently, the samples were divided in two groups with five mushrooms per group for *B. pinophilus*

and seven mushrooms in each group, for *C. subconnexa*: control (non-irradiated, 0.0 kGy) and irradiated (2 kGy). The estimated dose rate for the irradiation position was obtained with Fricke dosimeter, and the irradiation of the samples was performed in a Co-60 experimental chamber with four sources, total activity 198 TBq (5.33 kCi) in November 2012 (Precisa 22, Graviner Manufacturing Company Ltd, U.K.), following the procedure previously described by the authors (Fernandes et al., 2013a). The estimated doses, dose rates and dose uniformity ratios (Dmax/Dmin) were: 2.09±0.16 kGy, 1.56 kGy/h, 1.18 for *B. pinicola*; and 1.95±0.22 kGy, 1.95 kGy/h, 1.33 and for *C. subconnexa*.

All the samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenized samples for subsequent analysis.

2.3. Chemical parameters

2.3.1. Nutritional value

Moisture, protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 1995). Moisture content was evaluated by lyophilization (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), crude protein content (N×4.38) of the samples was estimated by the macro-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 ^{o}C using a Chamber furnace Lenton Thermal Designs Ltd, model ECF 12/22. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × ($g_{protein}$) + 3.75 × ($g_{carbohydrate}$) + 9 × (g_{fat}).

2.3.2. Free sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) according to the extraction procedure described by Heleno et al., (2011), using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). Data were analyzed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.3. Fatty acids

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID), according to the extraction and derivatization procedures described previously (Heleno et al., 2011). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) at 260 ºC and a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50%phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50

ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the Clarity DataApex 4.0 Software and expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols

Tocopherols were determined as previously described, using tocol as IS (Heleno, Barros, Sousa, Martins & Ferreira, 2010). The analysis was carried out in the HPLC system described above connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method, and tocopherols content was further expressed in µg per 100 g of dry weight (dw).

2.3.5. Organic acids

Organic acids were determined following a procedure previously optimized and described by the authors (Barros, Pereira & Ferreira, 2013). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Cooperation). Detection was carried out in a PDA, using 215 and 245 nm as preferred wavelengths. The organic acids 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 mg per 100 g of dry weight (dw).

2.3.6. Phenolic compounds

Phenolic compounds were determined in the UFLC system mentioned above, as previously described by the authors (Barros, Dueñas, Ferreira, Baptista, & Santos-Buelga, 2009). DAD detection was carried out using 280 nm and 370 nm as preferred wavelengths. The phenolic compounds were characterized according to their UV spectra and retention times, and comparison with authentic standards. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in µg per g dw.

2.4. Antioxidant parameters

2.4.1. Extraction procedure

Lyophilized powdered mushrooms samples (1 g) were stirred with methanol (30 mL) at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland), re-dissolved in methanol at 20 mg/mL (stock solution) for both mushrooms; and stored at 4 ºC for further use. Successive dilutions were made from the stock solution and submitted to *in vitro* assays already described by the authors (Heleno, Barros, Sousa, Martins & Ferreira, 2010) to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages

(DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

2.4.2. DPPH radical scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Winooski, VT, USA). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\% RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.

2.4.3. Reducing power

Two different procedures were used to evaluate the reducing power:

A) The first methodology was performed using the microplate reader described above. The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). For each concentration, the mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

B) The second methodology followed the Folin-Ciocalteu assay. The extract solution (1 mL) was mixed with Folin-Ciocalteu reagent (5 mL, previously diluted with water 1:10,

v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 ºC for color development. Absorbance was then measured at 765 nm. Gallic acid was used to obtain the standard curve (0.0094-0.15 mg/mL), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.4.4. Inhibition of β-carotene bleaching

β-carotene (2 mg) was dissolved in chloroform (10 mL) and 2 mL of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance) × 100.

2.4.5. TBARS (thiobarbituric acid reactive substances) assay

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the samples solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µL), followed by thiobarbituric acid

(TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.5. Statistical analysis

All extractions were performed in triplicate and each replicate was analyzed three times. Data were expressed as mean±standard deviation. The normal distribution of the residuals and the homogeneity of variance was tested by means of the Shapiro-Wilk and the Levene's tests, respectively. Results were classified using a *t*-test for equality of means. All statistical tests were performed at a 5% significance level using the SPSS software, version 20.0 (SPSS Inc).

3. Results and discussion

3.1. Effects on chemical parameters

As far as we know, there are no reports describing the nutritional and chemical composition or the antioxidant activity of *C. subconnexa*. Hence, comparisons with previous works could not be made.

The proximate composition and energetic value of *B. pinophilus* and *C. subconnexa* (**Table 1**) were quite distinct, which is an advantageous feature, considering the main purpose of validating gamma irradiation at 2 kGy as a suitable technology to increase wild mushrooms shelf life, preserving the wholesomeness of their chemical characteristics. Either way, water was the predominant component (≈93% in *B.*

pinophilus; ≈70% in *C. subconnexa*). In foods with high water percentages, irradiation might generate three primary free radicals (hydroxyl, hydrogen atoms and hydrated electrons). Therefore, several types of chemical compounds and quality attributes should be evaluated in irradiated foods, in order to understand the potential damages induced by irradiation treatment. Herein, a special attention was dedicated to those parameters more prone to suffer significant changes when treated with gamma irradiation. Other chemical substances, which typically occur in the same level as that observed in different heat treatments were not considered at this stage (Institute of Food Science and Technology, 2015).

Regarding the composition in dry basis, *B. pinophilus* stood out for its content in proteins (≈55 g/100 g dw), indicating its adequacy to be included in vegetarian diets, carbohydrates (≈31 g/100 g dw), ash (≈8 g/100 g dw) and fat (≈5 g/100 g dw) contents. The detected values are generally in agreement with previous works (Manzi, Marconi, Aguzzi, & Pizzoferrato, 2004; Heleno et al., 2011; Fernandes et al., 2014b). In *C. subconnexa*, carbohydrates were the major component (≈90 g/100 g dw), followed by ash (≈6 g/100 g dw), proteins (≈3 g/100 g dw) and fat (≈1 g/100 g dw) contents. In terms of gamma irradiation effects, dry matter, ash and carbohydrates revealed significant changes in both mushrooms. The effect on the protein content of *B. pinophillus* was also significant, as verified in other *Boletus* species submitted to gamma irradiation (Fernandes et al., 2013b). These changes might be associated to scissions in the C-N bonds in the polypeptide chain, splitting of the dissulfide bonds, or physical changes like unfolding or aggregation (Molins, 2001).

Mannitol was the sugar detected in highest amount (**Table 2**) in *C. subconnexa*, while trehalose was the main sugar in *B. pinophillus*, in agreement with previous reports studying the *Boletus* genus (Heleno et al., 2011; Fernandes et al., 2013b, 2014b). All

sugars showed a significant decrease in irradiated samples for both mushrooms, except for mannitol in *C. subconnexa*. The verified degradation may be explained by a reduction in the optical rotation of sugars, which is a common occurrence when sugars are irradiated (Molins, 2001).

Regarding fatty acids profiles (**Table 3**), the studied mushrooms were surprisingly similar, with C18:1 (≈45% in *B. pinophilus*; ≈50% in *C. subconnexa*), C18:2 (≈42% in *B. pinophilus*; ≈36% in *C. subconnexa*) and C16:0 (≈7% in *B. pinophilus*; ≈8% in *C. subconnexa*) as major compounds. Due to the magnitude of their relative percentages, these results were ultimately reflected in the amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The fatty acids profile of *B. pinophilus* showed percentages typically reported for *Boletus* genus (Hanuš, Shkrob & Dembitsky, 2008; Heleno et al., 2011). *B. pinophilus*, most likely because of its higher fat content, proved to be less sensitive to irradiation, since C18:3, C20:0 and C20:1 did not show significant changes. On the other hand, all fatty acids (except C16:1) quantified in *C. subconnexa* gave significant changes among unirradiated and irradiated samples. In most cases, these differences were characterized as slight decreases, which may result from oxidative or radiolytic processes. In fact, the observed changes were more likely produced by autoxidation, since the presence of oxygen accelerates the formation of free radicals and causes the breakdown of hydrogen peroxide and the destruction of antioxidants that would normally scavenge the free radicals formed (Nawar, 1977). The same reasoning might be applied to the α-tocopherol (in *C. subconnexa*) and δ-tocopherol (in *B. pinophilus*) contents, which showed the same decreasing effect (**Table 3**).

The organic acids profile (**Table 4**) showed only oxalic acid and fumaric acid in both mushrooms, which revealed also similar amounts of each compound. Among the

assayed parameters, the organic acids seemed to be the most resistant to gamma-irradiation, since only fumaric acid showed a significant increase in irradiated samples of *C. subconnexa*.

Regarding phenolic acids composition (**Table 5**), *p*-hydroxybenzoic acid was the only compound detected in *B. pinophilus*, while *C. subconnexa* showed also gallic acid and protocatechuic acid, despite the lower quantified amounts in this species. Cinnamic acid was only detected in *B. pinophilus*, as it was previously reported for *B. reticulatus* (Heleno et al., 2011). Protocatechuic acid (in *C. subconnexa*) and cinnamic acid (in *B. pinophilus*) showed a significant decrease in irradiated samples, while *p*-hydroxybenzoic acid (in *C. subconnexa*) increased significantly with irradiation.

3.2. Effects on antioxidant parameters

In order to know the effect of gamma irradiation in the antioxidant activity, five chemical and biochemical assays were tested (**Table 6**). *B. pinophilus* extracts showed to be more active as radical scavengers and reducing agents, while *C. subconnexa* showed higher activity as a lipid peroxidation inhibitor. Except for the results obtained from reducing power (as assessed through Folin-Ciocalteau assay) and β-carotene bleaching assays in *B. pinophilus* extracts, the antioxidant activity showed a significant increase in irradiated samples (except for TBARS formation inhibition in *C. subconnexa* extracts). Since phenolic acids and tocopherols decreased with irradiation, these results indicate that other antioxidant molecules (*e.g.*, ascorbic acid, flavonoids or carotenoids) might be present in higher amounts in mushroom samples submitted to irradiation.

4. Conclusions

Overall, some of the analysed chemical parameters showed significant changes after irradiation treatment. Nevertheless, the magnitude of the obtained differences did not seem to be sufficient to affect the chemical profiles of the assayed mushrooms. The higher effects observed in *B. pinophilus* might be related to its higher water content, which make it more prone to radiolysis and the consequent formation of oxidizing hydroxyl radicals and reducing aqueous electrons and hydrogen atoms that can cause several reactions such as addition to carboxylic acids, ketones, aldehydes, thiols, aromatic and olefinic compounds or abstracting hydrogen atoms from C-H bonds (Molins, 2001). The effect observed in the antioxidant activity of both mushrooms was quite interesting, since it showed an increased bioactivity in most cases, reflecting an additional advantage of irradiating mushrooms with the suggested dose. In conclusion, the detected chemical changes might be considered as allowable, in view of the high advantages offered by gamma irradiation at decontamination and/or disinfestation level.

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Table 1. Nutritional parameters, lactose (g/100 g fw) and energy (kcal/100 g) in the cottage cheese samples along shelf life. Results are presented as estimated mean±standard deviation.

		Water	Fat	Protein	Ash	Carbohydrates	Lactose	Energy
				Foeniculum vulgare				
ST	0 days	70±1	14±1	12.0±0.3	1.3±0.1	2.5±0.4	1.9±0.1	186±7
	7 days	66±1	17±1	12.4±0.2	1.1±0.1	3.6±0.4	1.1±0.4	215±3
	14 days	54±2	24±1	18.0±0.5	1.0±0.1	3.7±0.5	0.2±0.1	301±9
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001
FT	None	63±7	18±4	15±3	1.1±0.1	3.2±0.5	1.3±0.4	238±49
	Microspheres	64±6	18±4	14±2	1.2±0.1	2.9±0.5	1.0±0.5	229±46
	Extract	63±8	18±4	14±3	1.1±0.1	3.6±0.5	0.9±0.5	235±55
	p-value (n=27)	0.443	0.532	0.546	<0.001	<0.001	0.906	0.459
ST×FT	*p*-value (n=81)	<0.001	<0.001	<0.001	0.013	<0.001	<0.001	<0.001
				Matricaria recutita				
ST	0 days	69±1	15±1	12±1	1.2±0.1	3±1	1.8±0.1	193±4
	7 days	66±1	17±1	12±1	1.1±0.1	4±1	1.6±0.1	215±6
	14 days	53±2	24±1	18±1	1.1±0.1	4±1	0.2±0.1	304±13
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
FT	None	63±7	19±4	15±3	1.1±0.1	3±1	1.3±0.5	238±49
	Microspheres	64±6	18±4	14±3	1.2±0.1	3±1	1.2±0.5	228±44
	Extract	62±8	19±4	14±3	1.1±0.1	5±1	1.2±0.5	245±54

Table 2. Colour parameters and free radicals scavenging activity in the cottage cheese samples along shelf life. Results are presented as estimated marginal mean±standard error.

		DPPH scavenging activity	*L**	*b**	*a**
			Foeniculum vulgare		
ST	0 days	152±70	91±3 a	10±1	-2.3±0.2 b
	7 days	152±69	90±4 ab	11±1	-2.2±0.2 b
	14 days	144±44	89±2 b	16±2	-0.9±0.3 a
	p-value (n=27)	0.885	0.001	<0.001	<0.001
FT	None	200*	92±2 a	12±3	-2.0±0.4
	Microspheres	178±31	91±3 a	13±3	-1.8±0.5
	Extract	69±20	88±3 b	12±2	-1.7±0.5
	p-value (n=27)	<0.001	0.003	0.054	0.256
ST×FT	*p*-value (n=81)	<0.001	0.052	0.005	0.263
			Matricaria recutita		
ST	0 days	145±80	92±2 a	11±1	-2.3±0.2
	7 days	151±70	91±2 a	11±1	-2.3±0.4
	14 days	142±60	89±2 b	17±2	-0.8±0.2
	p-value (n=27)	0.856	0.009	<0.001	<0.001
FT	None	200*	92±2 a	12±3	-2.0±0.5
	Microspheres	188±19	90±2 b	13±3	-1.6±0.5
	Extract	50±12	90±2 b	14±4	-1.8±0.5
	p-value (n=27)	<0.001	<0.001	0.460	0.292

ST×FT *p*-value (n=81)	<0.001	0.076	<0.001	0.034

*Corresponds to the maximum assayed concentration. Different letters mean significant statistical differences.

Table 3. Fatty acids profile (%) of the cottage cheese samples along shelf life. Results are presented as estimated mean±standard deviation.

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	SFA	MUFA	PUFA
Foeniculum vulgare													
ST	0 days	4.0±0.1	3.5±0.1	3.3±0.1	8.1±0.2	4.7±0.1	10.7±0.2	23.7±0.3	12±2	22±2	72±2	24±2	3.8±0.1
	7 days	3.7±0.3	3.8±0.1	3.6±0.1	8.8±0.2	5.0±0.1	11.1±0.3	24.1±0.5	12±2	20±1	74±1	22±1	3.6±0.2
	14 days	2.4±0.5	3.2±0.4	3.4±0.2	8.5±0.3	5.0±0.2	11.1±0.2	24.1±0.3	13±2	21±2	73±2	23±2	3.9±0.3
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.053	0.002	<0.001	<0.001	<0.001
FT	None	3.5±0.4	3.5±0.1	3.3±0.1	8.3±0.3	4.9±0.2	10.9±0.3	24.0±0.4	14±1	19±1	75±1	21±1	3.8±0.1
	Microspheres	3.7±0.5	3.7±0.2	3.5±0.2	8.6±0.4	4.9±0.1	11.1±0.2	24.3±0.2	10±1	22±1	72±2	25±2	3.8±0.1
	Extract	3.0±0.5	3.3±0.4	3.4±0.2	8.5±0.3	4.9±0.2	10.8±0.2	23.7±0.4	13±1	21±1	73±1	23±1	3.8±0.4
	p-value (n=27)	0.015	<0.001	<0.001	0.005	0.328	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	0.443
ST×FT	*p*-value (n=81)	<0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Matricaria recutita													
ST	0 days	3.9±0.1	3.5±0.1	3.3±0.1	8.2±0.2	4.7±0.1	10.7±0.2	23.7±0.3	12±2	22±1	73±1	24±1	3.7±0.1
	7 days	3.8±0.3	3.7±0.1	3.5±0.2	8.8±0.4	5.1±0.2	11.0±0.2	23.8±0.5	11±2	21±2	73±2	23±2	3.6±0.2
	14 days	3.0±0.2	3.4±0.1	3.3±0.1	8.3±0.2	4.9±0.1	11.0±0.2	24.2±0.4	13±2	21±2	73±2	23±2	3.7±0.1
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	0.234	0.155	0.201	0.004
FT	None	3.5±0.4	3.5±0.1	3.3±0.1	8.3±0.3	4.9±0.2	10.9±0.3	24.0±0.4	14±1	19±1	75±1	21±1	3.8±0.1
	Microspheres	3.6±0.3	3.5±0.1	3.3±0.1	8.4±0.3	4.8±0.1	10.9±0.2	23.9±0.5	11±1	23±1	72±1	25±1	3.6±0.1
	Extract	3.6±0.5	3.5±0.2	3.5±0.2	8.6±0.4	5.0±0.2	10.9±0.3	23.8±0.3	12±2	21±1	73±1	24±1	3.6±0.2
	p-value (n=27)	0.951	0.452	0.001	0.006	0.062	0.553	0.196	<0.001	<0.001	<0.001	<0.001	<0.001
ST×FT	*p*-value (n=81)	<0.001	<0.001	0.001	0.022	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005

Table 4. Organic acids (mg/100 g dw) of *Boletus pinophilus* and *Clitocybe subconnexa*. The results are presented as mean±SD.

		Oxalic acid	Fumaric acid	Organic acids
B. pinophilus	0 kGy	4.0±0.2	0.35±0.03	4.3±0.2
	2 kGy	4.1±0.2	0.37±0.04	4.4±0.2
Levene's test	*p*-value	0.098	0.206	0.146
t-test	*p*-value	0.408	0.192	0.284
C. subconnexa	0 kGy	3.2±0.2	0.27±0.03	3.5±0.3
	2 kGy	3.4±0.2	0.31±0.04	3.7±0.2
Levene's test	*p*-value	0.604	0.472	0.038
t-test	*p*-value	0.141	0.023	0.053

Table 5. Phenolic acids and related compounds (μg/100 g dw) of *Boletus pinophilus* and *Clitocybe subconnexa*. The results are presented as mean±SD.

		Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	Phenolic acids	Cinnamic acid
B. pinophilus	0 kGy	nd	nd	17.8±0.4	17.8±0.4	5.0±0.2
	2 kGy	nd	nd	17.9±0.3	17.9±0.3	4.7±0.2
Levene's test	*p*-value	-	-	0.618	0.618	0.415
t-test	*p*-value	-	-	0.434	0.434	<0.001
C. subconnexa	0 kGy	1.4±0.1	4.3±0.2	0.5±0.1	6.3±0.2	nd
	2 kGy	1.5±0.1	3.9±0.1	1.0±0.1	6.4±0.2	nd
Levene's test	*p*-value	0.061	0.159	0.015	0.954	-
t-test	*p*-value	0.139	<0.001	<0.001	0.198	-

Table 6. *In vitro* antioxidant properties obtained for the extracts of *Boletus pinophilus* and *Clitocybe subconnexa*. The results are presented as mean±SD. Values are presented as EC_{50} values (mg/mL) for all assays except Folin-Ciocalteau, expressed as mg GAE/g extract.

			Reducing power		Lipid peroxidation inhibition	
		DPPH scavenging activity	Ferricyanide/Prussian blue assay	Folin-Ciocalteu assay	β-Carotene bleaching inhibition	TBARS formation inhibition
B. pinophilus	0 kGy	1.78±0.01	0.69±0.01	43±1	0.8±0.1	2.2±0.1
	2 kGy	1.71±0.03	0.62±0.01	44±1	0.9±0.2	1.1±0.1
Levene's test	*p*-value	0.022	<0.001	0.330	0.039	0.194
t-test	*p*-value	<0.001	<0.001	0.061	0.144	<0.001
C. subconnexa	0 kGy	2.9±0.1	1.05±0.02	32±1	0.61±0.02	0.7±0.1
	2 kGy	2.2±0.2	0.92±0.01	34±1	0.58±0.03	1.3±0.1
Levene's test	*p*-value	0.856	0.020	0.586	0.271	0.003
t-test	*p*-value	<0.001	<0.001	0.002	0.005	<0.001