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Arbutus unedo leaf extracts as potential dairy preservatives: case study on quark cheese†

Nabila Derbassi,[a,b] Mariana C. Pedrosa, [a] Sandrina Heleno, [a] Filipa Fernandes,[a] Maria Inês Dias, [a] Ricardo C. Calhelha,[a] Paula Rodrigues,[a] Marcio Carocho, *[a] Isabel C. F. R. Ferreira [a] and Lillian Barros *[a]

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The plant kingdom is an endless source of molecules that can be applied in almost all realms of society. The food industry has profited from the use of plants and their derived materials for many decades. Recently, the food industry has been looking into plants to find different ways of either preserving, coloring or sweetening foods. In this work, leaf extracts of *Arbutus unedo* L. obtained by dynamic maceration and ultrasound assisted extraction with prior optimization of their extraction conditions through the response-surface methodology, were incorporated in quark cheese as natural preservatives and analyzed over 8 days of shelf-life. Both extracts showed antioxidant activity with no toxicity towards primary cell lines at the maximum tested concentration, as well as antibacterial activity, especially against Gram-positive strains. After their incorporation in quark cheese, no significant changes were observed in the nutritional profile and physical traits of the quark cheeses, while the microbial load was highly reduced in the cheese, especially using the extracts obtained from dynamic maceration. Thus, leaf extracts of *A. unedo* can be promising candidates for use in the food industry as natural preservatives.

1. Introduction

In recent years, the increasing knowledge and awareness of consumers towards their diet and its relation to many diseases has led consumers to reduce the consumption of unhealthy foods, some of which with high amounts of food additives.[1–4] Today, knowledgeable consumers tend to prefer natural food additives, or nature-based additives such as rosemary extract (E 392), ascorbic acid (E 300), tocopherols (E 306-E 309), anthocyanins (E 163), chlorophylls (E 140), curcumin (E 100), steviol glycosides (E 960), glycyrrhizin (E 958), and erythritol (E 968), among others, all approved by the European Food Safety Authority EFSA (EFSA) for use in the European Union.[3,5,6] Consequently, scientists have been focused on finding natural alternatives to synthetic additives, mostly from plants. Thus, several plant extracts with high concentrations of phenolic compounds, as well as sesquiterpenes and terpenoids, have been reported for their antioxidant and antimicrobial activities.[7]

Phenolic compounds (or polyphenols) are one of the most widely distributed secondary metabolites throughout the plant kingdom, depending on the plant species.[8] These molecules work as natural protectors from herbivores, various microbial infections (parasites and predators) and the harmful effects of ultraviolet radiation.[9] They are common constituents of plant foods (fruits, vegetables, cereals, among others) and beverages (tea, coffee, beer, among others) being partially responsible for the global organoleptic properties of plant foods.[10] Polyphenols also present a strong antioxidant activity, which acts in collaboration with vitamins and enzymes as a defense against oxidative stress caused by excess reactive oxygen species (ROS), thus preventing biomolecules (proteins, nucleic acids, lipids, and sugars) from oxidative damage.[11,12] Currently, more than 8000 structures of phenolic compounds are known and can be categorized into several classes. Carocho and Ferreira (2013),[14] classified them according to their basic carbon skeleton, among which flavonoids constitute the largest group of phenolic compounds, classified into flavonols (such as kaempferol, quercetin, isorhamnetin, and myricetin), flavones (apigenin, luteolin, wogonin, and baicalein), flavanones (naringenin and hesperetin), flavanols or flavan-3-ols (catechins), isoflavones (as phytoestrogens), and anthocyanidins (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin).[13]

Arbutus unedo L. (known as a strawberry tree) belongs to the Ericaceae family and Vaccinioideae subfamily, and is a small

[a]*Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal. E-mail: mcarocho@pb.pt, lillian@ipb.pt*

[b]*Higher Institute of Biotechnology of Monastir, University of Monastir, Monastir, Tunisia*

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Mediterranean plant found mostly in Southern Europe, North-Eastern Africa, Ireland, Palestine, and the Canary Islands.[15]

Different parts of *A. unedo* tree (roots, bark, fruits, flowers, and leaves) have been studied for their chemical composition. Fruits and leaves of *A. unedo* L. have been widely studied for their diverse chemical composition namely phenolic acids, flavonoids, tannins, and anthocyanins, which have been applied in traditional medicine for centuries. A study of *A. unedo* from the Natural Park of Montesinho (Bragança, Northeastern Portugal) showed that the leaf extracts contain flavonols (kaempferol, glucosides of myricetin, quercetin), flavanols (procyanidin dimers, respective gallate esters, and catechins), several gallotannins and ellagitannins derivatives and the amount of these compounds was higher compared to those of the fruit extracts.[16] In addition, the leaves are effective against inflammatory and cardiovascular diseases, and in the treatment of diabetes, and are also used for their purgative and astringent potentials.[15,17] Oliveira *et al.* (2009)[18] showed that strawberry tree leaves may also be valuable in the pharmaceutical industry and food applications.

Due to the advancement of food preservation technologies, fresh cheese production has been growing around the world, this type of cheese is consumed almost by all cultures and countries, making it one of the most attractive products within the dairy industry.[19] Quark (as known in Germany) or Tvorog (in Eastern European countries) cheese, is classified under acid-rennet coagulated fresh cheeses. It is basically a milk protein paste and a soft fermented cheese, with a white to yellowish color, slightly acidic flavor, and medium sour taste, which is produced from pasteurized skimmed or full fat milk at pH 4.6 to 4.8. Its shelf-life is defined between 2 and 4 weeks (prior to opening) at <8 °C because of the elevated moisture content (82%) and is reduced if no preservatives are used.[20–22] Furthermore, its shelf-life after opening expires after few days, after which the flavor, texture and color change. Due to the high propensity of this cheese to spoilage and pathogenic microorganism growth, which reduces the shelf life, some synthetic preservatives have been used in Quark cheese production, namely cheese ripening salts, calcium carbonate or sodium hydrogen carbonate, sorbic acid and sorbates (E 200–203), although most of the cheese sold in the EU does not have any additives. Thus, finding suitable natural preservatives for such an important commodity would benefit both consumers and industry with the extension of the shelf-life through a natural additive.

Therefore, this work aimed at studying the hydroethanolic extracts of *A. unedo* in terms of their antioxidant and antimicrobial activity, for further use as natural preservatives for quark cheese.

2. Material and methods

2.1. Plant material

The leaves of *Arbutus unedo* L. (strawberry tree) were purchased from "Deifil-Green Technology, Lda", aromatic, medicinal and seasoning plant retailers. The leaves were dried for 15 days protected from light, after which they were milled down to a size of 20 mesh (~0.8 mm) in a shredder (Moulinex A320, Mayenne, France).

2.2. Optimization of the extraction conditions for *Arbutus unedo* L.

For each of the two extraction techniques – ultrasound-assisted extraction (UAE) and dynamic maceration (DM), – extraction optimization was implemented. For DM, the Box–Behnken design (BBD) was used, and for the UAE the central composite design (CCD) was chosen. Three independent variables were varied [X_1 (t, min), X_2 (T, °C or P, W) and X_3 (S, %)], where "t" represents time, "T" represents temperature, "P" represents power, measured in "W" watts and "S" represents the solvent percentage, ethanol being the solvent. The BBD included 17 runs (independent combinations), with 5 repetitions of the center point to evaluate the precision of the method. The CCD included 20 independent combinations and 4 repetitions of the center point. The upper and lower limits of each individual variable were selected based on reports from the literature, according to extractions related to the products of the strawberry tree.[23–27] The analyzed responses for each extraction technique were the solid residue after extractions ($R1$) expressed in mg, and the most abundant phenolic compounds, expressed in mg g^{-1} extract, namely catechin ($R2$), isorhamnetin ($R3$), quercetin ($R4$), luteolin ($R5$) and the total amount of phenolic compounds ($R6$) also expressed in mg g^{-1} extract.

2.2.1. Ultrasound assisted extraction. The extraction of bioactive compounds assisted by UAE of *A. unedo* was performed using an ultrasonic device (Optic Ivymen System CY-500 Comecta S.A, Scharlab, Madrid, Spain) with a titanium probe, operating under the conditions provided by the experimental design.

2.2.2. Dynamic maceration extraction. The dynamic maceration (DM) extraction was performed using a thermostatic water bath under continuous electromagnetic stirring for different extraction times and solvent percentages as defined by the experimental design, in amber airtight containers at specific temperatures.

The obtained extracts from both the UAE and DM were filtered through a Whatman paper filter no 4. Afterwards, 1.5 mL of each extract was filtered through a 0.2 μm nylon filter (Whatman) and collected in vials for chromatographic analysis. Then, 5 mL of each extract was placed in weighed crucibles and left to dry in an oven at 100 °C for 4 days. After the fourth day the crucibles were weighed again, and the amount of dry residue was used as a response ($R1$) for the optimization protocol, while the most abundant individual polyphenols were identified as other responses.

2.2.3. Identification and quantification of phenolic compounds. Chromatographic analysis was carried out in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system. High-performance liquid chromatography (HPLC) was coupled with a diode array detector, and an electrospray

ionization mass detector (LC-DAD-ESI/MSn) allowing the detection of compounds at wavelengths of 280 nm, 330 nm and 370 nm. Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column, working at 35 °C. MS detection was performed in the negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Nitrogen served as the sheath gas (50 psi); the system operated with a spray voltage of 5 kV, a capillary voltage of −20 V, and a source temperature of 325 °C. The identification of compounds was performed through the comparison of their UV-VIS, mass spectra and retention times to available standard compounds or using the literature data. For quantitative analysis, a 7-level calibration curve of standard phenolic compounds was plotted based on the UV signal: catequin (y = 84.950x − 23.200, R^2 = 0.999, (LD) limit of detection = 0.17 μg mL^{-1}; LQ (limit of quantification) = 0.68 μg mL^{-1}) and quercetin-3-*O*-glucoside (y = 34.843x − 160.173, R^2 = 0.9998, LD = 0.21 μg mL^{-1}; LQ = 0.71 μg mL^{-1}). For quantitative analysis, a calibration curve of standard phenolic compounds was plotted based on the UV signal. The results were expressed in mg g^{-1} extract.

2.3. Bioactivity evaluation of extracts

2.3.1. Antioxidant activity. The antioxidant activity of the extracts was tested in two different types of assays, namely the thiobarbituric acid reactive substance (TBARS) assay which uses porcine brain homogenates and a cellular antioxidant assay (CAA) which uses a tumor cell line.

2.3.1.1. TBARS assay. The TBARS assay was carried out following a procedure described by Gómez-Mejía *et al.* (2021).[28] Porcine brains were obtained from a local slaughterhouse and dissolved in Tris-HCl buffer (20 mM, pH = 7.4). The solution was then centrifuged at 3500*g* for 10 minutes. Each dilution of the sample solutions (200 μL) was pipetted into Eppendorf tubes, adding them with 100 μL of $FeSO_4$ (10 mmol L^{-1}), 100 μL of ascorbic acid (0.1 mmol L^{-1}), and 100 μL of the supernatant of brain tissue homogenate. Two blanks were prepared, one with Tris-HCl buffer and the other with deionized water. The Eppendorf's were incubated at 37.5 °C for 1 h., after which trichloroacetic acid (500 μL, 28% w/v) was added to stop the reaction, and at the same time 380 μL of thiobarbituric acid (TBA, 2% w/v) was added, followed by the subsequent incubation of tubes at 80 °C for 20 min. Then, to eliminate the precipitated protein, the tubes were centrifuged at 3000 rpm for 5 min, and the supernatant samples were measured at 532 nm. The equation, inhibition ratio (%) = $[(A - B)/A] \times 100$; (A – absorbance of the control and B – absorbance of the sample solution) was used to calculate the percentage of inhibition, which was then transformed into EC_{50} values (concentration of extracts responsible for 50% of lipid peroxidation inhibition) in mg mL^{-1} extract solution.

2.3.1.2. Cellular antioxidant assay. The cellular antioxidant assay was also used to analyze the extract activity using tumour cell lines. For this assay, a murine macrophage cell line (RAW264.7) was obtained from the ECAAC – European Collection of Authenticated Cell Cultures and grown in DMEM medium containing 10% heat inactivated FBS, glutamine (2 mM), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37° C in a humidified air incubator containing 5% CO_2. For determination of intracellular ROS, the cells were incubated with antioxidant compounds and 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), to promote the formation of free radicals. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was used as a fluorescent marker.[29] Oxidation is inhibited by the action of antioxidant compounds that will reduce the antioxidant potential of ROS. The decrease in fluorescence emission compared to control cells indicated the antioxidant activity of the tested molecules.[29,30] Cellular antioxidant measurements were performed following the method of Wolfe and Liu (2007)[31] with slight modifications.

2.3.2. Toxicity. The toxicity was determined using the sulforhodamine B (SRB) assay for the evaluation of the effects of the extracts in cell lines to a precise subtoxic concentration, following the work by Corrêa *et al.* (2015).[32] In brief, a non-tumor cell line obtained from the porcine liver (PLP2) was maintained as adherent cell cultures in RPMI-1640 containing heat inactivated FBS (10%), penicillin (100 U mL^{-1}), glutamine (2 mM), and streptomycin (100 μg mL^{-1}), incubated at 37 °C with 5% CO_2 and humidified air. The PLP2 cells were placed in 96-well microplates to which varying concentrations (dilutions) of the extracts were added. Then, the plate was incubated at 37 °C with 5% CO_2 for 48 h, and directly after incubation, 10% of previously refrigerated trichloroacetic acid (100 μl) was added to allow the fixation of the adherent cells, followed by another incubation at 4 °C for 60 minutes. The last step consisted of the solubilization of the adhered cell line with 10 mM Tris (200 μl), and measurement at 540 nm using the microplate reader (ELX800). The results were expressed in GI_{50} values (sample concentration inhibiting cell growth by 50%).

2.3.3. Antibacterial activity. For the antimicrobial activity, the used bacterial strains were isolated from patients hospitalized at the Hospital of Trás-os-Montes and Alto Douro (Vila Real, Portugal) and in various departments of the Northeastern Local health unit (Bragança, Portugal). Five Gram-negative bacteria were clinically isolated, *Escherichia coli* (from urine), *Klebsiella pneumoniae* (from urine), *Proteus mirabilis* (from wound exudate), *Morganella morganii* (from urine) and *Pseudomonas aeruginosa* (from expectoration) and three Gram-positive bacterial strains, namely: *Listeria Monocytogenes* (from the cerebrospinal fluid), *Enterococcus faecalis* (from urine), and methicillin-resistant *Staphylococcus aureus* (MRSA) (from expectoration) were tested. The assay was carried out following the procedure previously described[33] through the microplate microdilution method to obtain the minimum inhibitory concentration (MIC) which is known as the lowest concentration that inhibits visible bacterial growth, and minimum bactericidal concentrations (MBC) that present the lowest concentration that effectively kills bacteria.

The preparation of the stock solution with 20 mg mL^{-1} as the final concentration consisted in dissolving the extracts initially in 5% (v/v) dimethyl sulfoxide and 95% of Mueller–Hinton Broth/Tryptic Soy Broth (MHB/TSB). Afterwards, 190 μL of this solution was added to a well in a 96-well microplate, followed by the addition of 90 μL of medium MHB or TSB in the remaining wells. The final step was the addition of 10 μL of the inoculum (standardized at 1.5×10^8 Colony Forming Unit (CFU) ml^{-1}) in all wells. Ampicillin and vancomycin were used as positive controls for *E.s faecalis* and MRSA. Ampicillin and Imipenem were selected for *L. monocytogenes* and the five Gram-negative bacteria. The microplates were incubated at 37 °C for 24 h. The detection of MIC of samples was performed after adding 40 μL of 0.2 mg ml^{-1} *p*-iodonitrotetrazolium chloride and subsequent incubation at 37 °C for 30 minutes following the MIC detection. The wells that did not acquire color were used to obtain the MBC, 10 μL of which was plated on solid medium, blood agar (7% sheep blood) and incubated at 37 °C for 24 h.

2.4. Quark cheese preservation

2.4.1. Incorporation of *A. unedo* extracts (DM and UAE) as natural preservatives in Quark cheese. After analyzing the bioactive properties of *A. unedo* extracts obtained through DM and UAE, these extracts were incorporated into quark cheese to understand their preserving effects on this dairy product and compare their activity with an artificial dairy food preservative (potassium sorbate). All three preservatives (*A. unedo* from UAE, *A. unedo* from DM, and potassium sorbate) were added to a lot of cheese (0.1 g/100 g cheese). A fourth lot of cheese was prepared, and no additive was added to it, serving as the negative control. The four lots were further divided into other three groups, namely, samples to analyze immediately after preparation (*T*0), after three days (*T*3) and after 8 days (*T*8) of storage. The samples were kept in air-tight containers until analysis and kept at a constant temperature of 4 °C.

2.4.2. Chemical analysis. Following the standard AOAC procedures, the nutritional profile (moisture, proteins, fat, and ash) was determined and expressed in g/100 g of fresh weight and energy was calculated based on the following formula, approved by the European Parliament and Council in 2011: Energy (kcal per 100 g) = 4 × [protein (g) + carbohydrates (g)] + 9 × fat (g).

The quantification of proteins was based on the amount of nitrogen (N) in the sample, in which the conversion factor 6.38 (N × 6.25) was used in the macro-Kjeldahl method for protein analysis (Kjeldahl Pro-Nitro-S, JP Selecta, Abrera, Barcelona, Spain). The moisture analysis was performed using a moisture analyser (Adam Equipment company, model PBM 163, Oxford, USA), while the crude fat content was based on the extraction of fat by petroleum ether in a Soxhlet apparatus. Finally, the ash content was obtained by incineration at 550 °C for 5 h.

Organic acids were determined after extraction with 25 mL of metaphosphoric acid, using an ultra-fast liquid chromatograph (UFLC – Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled to a photodiode array diode detector (PDA). Individual compounds were identified and quantified by comparison with commercial standards, retention times, and their respective standard calibration curves.[34]

Soluble sugars were determined using defatted samples by HPLC coupled to a refraction index (RI) detector, quantified considering the internal standard (melezitose) and identified by comparison with standards.[35]

To determine the fatty acids, the previously described procedure[35] was applied and gas chromatography (DANI 1000, Contone, Switzerland) coupled with a flame ionization detection (GC-FID)/capillary column was used. The identification and quantification of fatty acids were accomplished through the comparison of the relative retention times of fatty acid methyl ester peaks with commercial standards. The results were treated and presented as a relative percentage for each fatty acid using CSW 1.7 software (DataApex 1.7, Prague, Republic Czech).

2.4.3. Physical analysis. The external color was analyzed for the cheese samples in distinct points with three repetitions, using a portable CR400 colorimeter from Konica Minolta (Chiyoda, Tokyo, Japan). The international Commission's Illumination (CIE), D_{65} illuminant with an 8 mm aperture and 10° degrees of observation were used, which characterize the midday light in Europe. According to the colour space of the CIE L^* a^* b^* of 1976, L^* represented lightness (L = 0 black, L = 100 white), a^* represented greenness–redness ($-a$ = 0 green, $+a$ = red), and b^* represented blueness–yellowness ($-b$ = blue; $+b$ = yellow).

To perform the texture profile analysis, a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA.XT Plus Texture Analyzer with a 30 kg load cell was used, along with a *P*/45 45 mm aluminium cylinder probe, a pre-test speed of 10 mm s^{-1}, a test and post-test speed of 5 mm s^{-1}, as well as a trigger force of 3 g, in order to comprehend the effects of the extract on the texture profile of the cheese. A standard texture profile analysis (TPA) was carried out, which intends to imitate human chewing by compressing the sample twice, managing to extract enough parameters for analysis, namely firmness, consistency, cohesiveness and work of cohesion.

The water activity "aw" determination was based on the dew point methodology with an absolute error of 0.003, at 20 °C for 5 min on the surface of each sample, using the activity measuring instrument AQUALAB 4TE (MeterGroup, München, Germany).

The pH of the cheese samples was measured at three different points with a portable food pH meter 99161 (Hanna Instruments, Woonsocket, Rhode Island, USA).

2.4.4. Microbial load. The cheese samples were prepared according to the procedures of the International Organization for Standardization (ISO) 6887-1:2003.[36]

In the microbial load analysis during the shelf life of 8 days, the following microorganisms were analyzed: total aerobic mesophiles, Enterobacteriaceae, molds, yeasts, phychrotrophic lactic acid bacteria and *Staphylococcus aureus*.

The microorganism's analyses were performed immediately after cheese preparation (T0), after 3 days (T3) and after 8 days

(T8) of storage at 4–5 °C. Briefly, 10 g of the cheese sample were mixed in 90 mL of peptone water in Stomacher bags and further homogenized in the Stomacher equipment (Star Blender, VWR, Radnor, USA) for 1 min at 300 units, followed by serial dilutions ranging from 10^{-1} to 10^{-12} (ISO6887-1:2003).

Aerobic mesophiles (ISO4833-1:2013): 1 mL of each dilution of the sample was placed in a Petri dish, and 15 mL of Plate Count Agar (PCA, Liofilchem, Italy) were added, using the pour plate technique, in duplicate. After homogenization and solidification, the plates were incubated at 30 °C in an inverted position for 72 h. Counting was performed on plates containing between 15 and 300 colonies (LOQ = 1 $\log_{10}$(Colony Forming Units) CFU g^{-1}).

Enterobacteriaceae (ISO 4832:2006): 1 mL of each dilution was mixed with 20 mL of violet red bile glucose agar (VRBG, Liofilchem, Italy) using the pour plate technique. Subsequently, the plates were incubated at 30 °C for 48 h in an inverted position. Counting was performed on plates containing between 15 and 150 colonies (LQ = 1 $\log_{10}$ CFU g^{-1}).

Psychrotrophic lactic acid bacteria (ISO 4832:2006): 1 mL of each dilution was mixed with 20 mL of Man, Rogosa and Sharpe (MRS, Liofilchem, Italy) agar using the pour plate technique. After solidification of the medium, 5 mL of medium was added to create anaerobiosis. The plates were then incubated at 22 °C for 5 days in an inverse position and counted (LOQ = 1 $\log_{10}$ CFU g^{-1}).

Yeasts and molds (ISO 21527-2:2008): corresponding to the spread plate technique and in duplicate (LOQ = 1.7 $\log_{10}$ CFU g^{-1}), 0.2 mL of each suspension was distributed in Petri dishes containing 20 mL of Dichloran Rose Bengal Chloranfenicol (DRBC, Liofilchem, Italy) agar. The plates were incubated at 25 °C for 72 h for yeasts and 120 h for molds in an upright position, and then counted.

Staphylococcus aureus (ISO 688-2:1999): corresponding to the spread plate technique and in duplicate (LOQ = 1.7 $\log_{10}$ CFU g^{-1}), 0.2 mL of each suspension was distributed in Petri dishes containing 20 mL of Baird Parker (BP) agar. The plates were incubated at 37 °C for 24 h in an inverse position, and then counted.

2.4.5. Statistical analysis. Throughout the whole document, all data were expressed as mean ± standard deviation. The optimization protocol was performed using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA), and relied on the Response Surface Methodology using Central Composite Design and Box–Behnken models for the design of experiments followed by an optimization of the solid residue response.

Furthermore, after incorporating the preservatives in the cheese, to better understand the effects of these different preservatives (PT) and their storage time (ST), a two-way ANOVA with type III sums of squares using the SPSS Software, version 25, was used for analysis. This multivariate general linear model treats the two factors, PT and ST as independent, thus allowing the effect of each one to be analyzed independently, providing more insight into their contribution towards the changes. If a significant interaction (<0.05) was recorded among the two factors (PT × ST), these were evaluated simultaneously, and if possible, some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a Tukey's multiple comparison test when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using Levene's test. All analyses were carried out using a significance level of 0.05.

3. Results and discussion

3.1. Extraction optimization

3.1.1. Dynamic maceration and ultrasound assisted extraction. Upon analyzing the different runs, 4 major phenolic compounds were identified by HPLC-DAD-ESI/MS through their retention time, UV-VIS spectra, and deprotonated molecules. The first peak was identified as (+)-catechin, having a λ_{max} at 279 nm and a deprotonated ion $[M - H]^-$ at *m*/*z* 289, according to its retention time, mass, and UV-vis characteristics in comparison with the commercial standard (ESI†). The second compound was tentatively identified as isorhamnetin-*O*-deoxyhexoside, according to the deprotonated ion $[M - H]^-$ at *m*/*z* 461 and MS^2 fragment released at *m*/*z* 315 ($[M - H - 146]^-$, loss of the deoxyhexosyl moiety). Similarly, the third ($[M - H]^-$ at *m*/*z* 447) and fourth compounds ($[M - H]^-$ at *m*/*z* 431) were tentatively assigned quercetin-*O*-deoxyhexoside and luteolin-*O*-deoxyhexoside, respectively. The identification of the phenolic compounds is added in the ESI.†

The dynamic maceration (DM) for *A. unedo* used the Box–Behnken model, which only considers points within the given range of values for the three factors (*F*). Thus, for the DM, the range was set at 10 to 60 minutes (*F*1), 30 to 80 °C (*F*2) and 0 to 100% ethanol (*F*3). The analysed responses included the solid dry residue of the extraction solution after complete evaporation of the solvent (*R*1), the quantity of catechin (C) (*R*2), isorhamnetin-*O*-deoxyhexoside (IOD) (*R*3), quercetin-*O*-deoxyhexoside (QOD) (*R*4), luteolin-*O*-deoxyhexoside (LOD) (*R*5) and the total amount of phenolic compounds detected in each extraction (*R*6), from *R*1 to *R*6 obtained by HPLC-DAD-ESI/MS analysis. The experimental design included three levels and 17 independent combinations (runs) and 5 replicates of the central position. The runs were executed randomly to avoid errors arising from the variability of the observed responses (ESI†). The response chosen for the optimization protocol was *R*1, the dry residue of *A. unedo* due to a positive correlation between a higher quantity of dry residue translating into a higher number of extracted compounds, indicating increased bioactive properties.

The optimal conditions were set at 60 minutes, 73 °C and 66% ethanol, using the maximize function of the Design Expert Software. Fig. 1 shows three plots, the plots of two varying factors representing the optimal conditions, where the red zones represent higher yields (Fig. 1a). Overall, considering

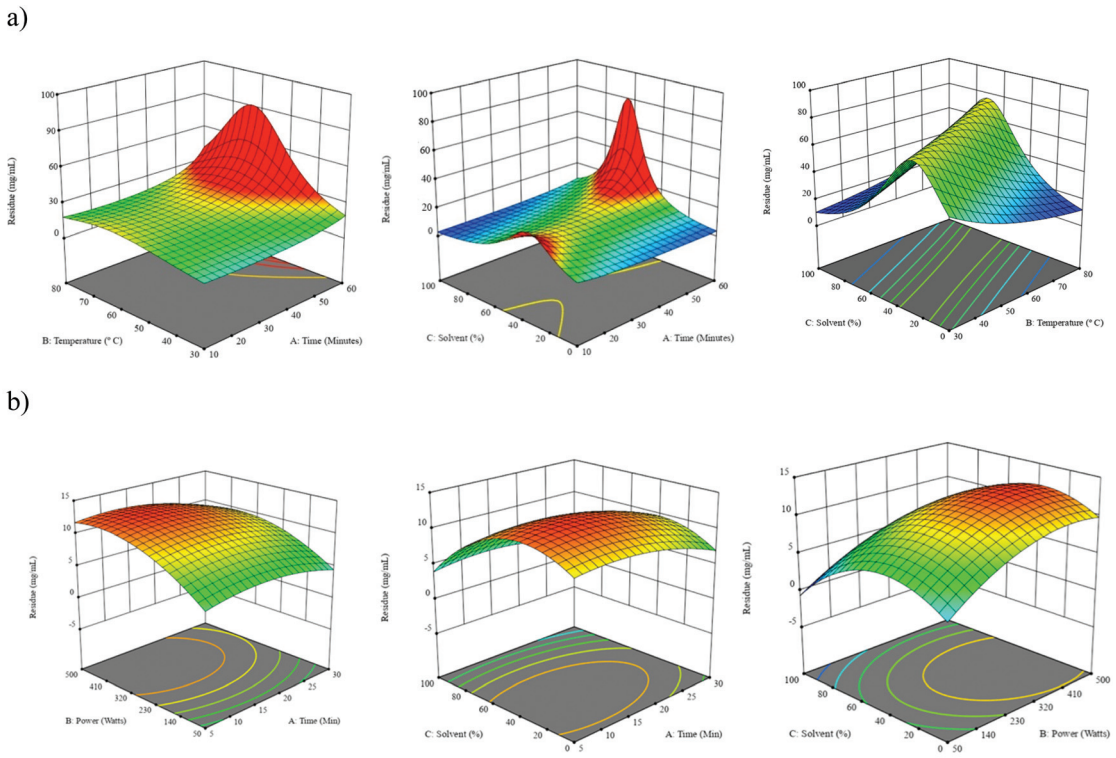

Fig. 1 3D plots of the optimal points for *R*1 (dry residue) for dynamic maceration (a) and ultrasound assisted extraction (b) using the response surface methodology.

temperature *vs.* time, the highest yield was found between temperatures near 60 °C (left graph), but higher extraction times, of around 60 minutes. Still, in terms of solvent *vs.* time, a considerable yield was obtained at about 10 minutes, but in terms of solvent, the addition of 60% ethanol yielded the highest (middle graph). Finally, in the right graph, (solvent *vs.* temperature), the highest amounts of residue were promoted by 20 to 60% ethanol, with temperature having a lower influence.

The second experimental design was performed for UAE using the central composite design, which allows for axial points to be placed outside the desired range. This model was chosen due to higher uncertainty pertaining to the extraction of *A. unedo* by ultrasound. Time and solvent were also used as varying factors, while temperature (used in DM optimization) was swapped for ultrasonic power, measured in watts, which ranged from 50 to 500 weeks. The table with different runs can be found in the ESI,† while the optimal conditions were approximately 13 minutes, 36% ethanol and 402 watts, shown in Fig. 1b. Regarding the right graph for the ultrasound assisted extraction (power *vs.* time), the highest yield was found for higher power, but lower time, which can also be seen in the middle graph, where the highest yields are found in the shortest extraction time and between 20 to 40% ethanol. Finally, the right graph shows that yields are higher at a higher wattage, and once again between 20 to 40% ethanol.

3.1.2. Bioactive properties of *A. unedo* under optimal extraction conditions. After the assessment of the optimal points for maximization of dry residue of *A. unedo*, both by DM and UAE, the extracts resulting from the application of these optimal conditions were subjected to the measurement of the bioactive properties. Regarding the TBARS assay, the DM showed a stronger potential by presenting lower EC_{50} values when compared with the UAE (140 ± 1 and 230 ± 11 μg mL^{-1}, respectively). On the other hand, for CAA, UAE showed higher activity, with an oxidation–reduction of 37 ± 2% while DM only achieved a reduction of 24 ± 1%. While the difference between the two assays could seem odd, it is quite normal due to the different antioxidant mechanisms that each method detects. Regarding the cytotoxic effect in normal cells, both samples revealed no toxicity against a primary cell line PLP2 at the maximum tested concentration of 400 μg mL^{-1}, further ruling the use of *A. unedo* leaves as safe to be applied in foodstuff. While extracts of *A. unedo* fruits have already been used to functionalize cheese,[37] this is, to the authors' best knowledge, the first report on the antioxidant potential of the leaves as functionalizing and preserving agents.

With regards to the antibacterial potential (Table 1), both extracts presented antibacterial activity against most of the tested strains, showing higher activity against Gram-positive bacteria, probably due to higher affinity between the polyphenols of the extract and the higher amount of peptidoglycan in

Table 1 Antibacterial activity of DM and UAE extracts

	DM		UAE		Ampicillin (20 mg mL^{-1})		Imipenem (1 mg mL^{-1})		Vancomycin (1 mg mL^{-1})	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Escherichia coli	**2.5**	>20	**10**	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	**10**	>20	>20	>20	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	**10**	>20	**20**	>20	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	**10**	>20	>20	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>20	>20	>20	>20	>20	>20	0.5	1	n.t.	n.t.
Gram-positive bacteria										
Enterococcus faecalis	**2.5**	>20	**10**	>20	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	**10**	>20	**10**	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	**2.5**	>20	**2.5**	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

n.t.: not tested; MRSA: methicillin resistant *S. aureus*.

the cell walls of these microorganisms. This increased activity against Gram-positive bacteria is in agreement with other reports in the literature,[38–40] The DM extract was more efficient against *E. faecalis*, MRSA and *E. coli* with MIC values of 2.5 mg mL^{-1}, showing no activity against *P. aeruginosa*. On the other hand, the UAE extract presented stronger potential against MRSA and was ineffective against *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*. Dib *et al.* (2013)[41] also identified higher antimicrobial activity against *E. coli* and *S. aureus* in *A. unedo* root aqueous extracts, as well as poor activity against *P. aeruginosa*, which was also found by Mrabti *et al.* (2021).[42] The antimicrobial activity may be due to the presence of flavonoids, which are produced to act against microbial infection, as well as tannins, which are another class of molecules that act against fungi and bacteria.[41] The phenolic compounds may affect the constituents of the cell membrane of microorganisms, such as enzymatic bonds, adhesins and polypeptides. The action of the phenolics can be explained by the formation of protein complexes that can inactivate and cause loss of the protein function, leading to membrane disruption.[43] The structural difference in the membrane between Gram-positive and Gram-negative bacteria might be the reason for the difference in inhibition. Gram-positive bacteria have a peptidoglycan outer layer with high permeability. Gram-negative bacteria, on the other hand, have an outer layer with lipopolysaccharide, which acts as an impermeable barrier for lipophilic compounds.[40,44] These results are important due to the activity exhibited against MRSA, which is a resistant bacterium and considered a major issue worldwide, given its resistance to antibiotics.

Although it was possible to obtain promising MIC values, it was not possible to determine the MBC, meaning that the extracts were able to act only as bacteriostatic agents, with no capacity to kill the tested bacteria.

3.2. Incorporation into quark cheese

After confirming the antioxidant and the antibacterial activities guaranteeing the absence of toxicity, each extract was incorporated into lots of quark cheese with subsequent analysis over a period of 8 consecutive days. Then, a two-way ANOVA was used to interpret the results; the results are shown in the following tables.

3.2.1. Proximate composition. Table 1 is divided into two sections, the upper represents the storage time (ST), but included on each day all different types of the preservatives,

Table 2 Nutritional profile of the quark cheeses incorporated with the different preservatives, represented in g/100 g of fresh weight

		Moisture	Proteins	Crude fat	Carbohydrates	Ash	kcal	kJ
Storage time (ST)	0 Days	78.0 ± 0.7	10.0 ± 0.6	4.3 ± 0.3	7.0 ± 0.2^{b}	0.8 ± 0.2^{a}	107 ± 4^{b}	448 ± 16^{b}
	3 Days	78.1±.05	11.3 ± 0.9	3 ± 1	6.6 ± 0.5^{a}	0.8 ± 0.1^{a}	100 ± 7^{a}	420 ± 10^{a}
	8 Days	78.4 ± 0.5	11.1 ± 0.2	4.2 ± 0.2	5.7 ± 0.6^{a}	1.0 ± 0.2^{b}	105 ± 3^{a}	439 ± 5^{a}
p-value (n = 3)	Tukey's test	0.361	0.011	0.109	0.001	<0.001	0.027	0.027
Preservative type (PT)	Control	78.0 ± 0.5	9.9 ± 0.6	4 ± 1	7 ± 2^{b}	0.9 ± 0.1^{a}	104 ± 7	437 ± 30
	Maceration	78.4 ± 0.8	10.2 ± 0.7	4.0 ± 0.8	6 ± 1^{a,b}	0.89 ± 0.07^{a,b}	102 ± 5	429 ± 22
	Ultrasound	78.2 ± 0.6	11.1 ± 0.2	4.1 ± 0.2	5.5 ± 0.3^{a}	1.0 ± 0.3^{b}	103 ± 3	433 ± 13
	Potassium sorbate	78.1 ± 0.4	11.2 ± 0.4	4 ± 1	6 ± 1^{a,b}	0.7 ± 0.2^{a}	103 ± 6	431 ± 25
p-value (n = 12)	Tukey's test	0.401	<0.001	0.740	0.008	0.001	0.870	0.870
ST × PT (n = 36)	*p*-value	0.895	0.007	0.357	0.135	0.076	0.470	0.470

In each row, different letters represent statistically significant differences with a significance level of 0.05. The SD were calculated from results obtained under different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

and, at the bottom, for each type of preservative extract, the three analyses over the time are also included. When each of the factors can be analysed independently (p-value ST × PT > 0.05), the classification is made using *post-hoc* tests (Tukey's test for homoscedastic samples and Tahmane T2 for non-homoscedastic ones); however, when p-value ST × PT < 0.05, then, no classification can be performed.

The nutritional profile, along with the energy values of the quark cheeses incorporated with different preservatives are presented in Table 2. After moisture, proteins are the most prevalent nutrient, followed by carbohydrates and crude fat, making these cheeses quite desirable for modern diets due to the high protein content, low fat, and carbohydrates. Regarding the effect of each factor (time and preservative), moisture, proteins and carbohydrates, significant interactions were studied and thus, although no significant changes were detected over time, for all the incorporated preservatives, their influence was limited. Despite an increase and a subsequent decrease in the protein contents, as well as the opposite for the fat content, statistical results showed no significant difference between them, *i.e.*, they can be considered to have similar values.

For carbohydrates, the influence of each factor was determined, showing that over time there was a decrease in carbohydrates, with a significant difference from T0 to T3, which could be explained by the consumption of lactose by lactic acid bacteria present in the cheese.

Furthermore, for the ash content, a slight increase over time with a significant statistical difference from T3 to T8 was observed, while also showing a significant statistical difference between the preservatives, although very slight variations were observed. Finally, in terms of energy values, energy values showed a reduction with time, with significant differences being observed from T0 to T3. Overall, different preservatives did not influence the nutritional quality of the cheeses, which is expected from food additives. Furthermore, longer storage periods should be further researched to understand the influence over a longer period. Still, due to the high moisture, quark cheese has a very limited storage time, which could be improved by these natural preservatives. As the present work is the first study with the incorporation of strawberry leaf extract, specific data for comparative purposes on the nutritional profile do not exist. Nevertheless, in relation to the incorporation of the fruit extract, research indicates maintenance of the nutritional profile of the food with slight variations, for example, in flatbreads and waffles, for shelf-life analysis of up to 7 days.[45]

3.2.2. Individual molecules

3.2.2.1. Fatty acids. Regarding the individual profiles of specific molecules found in the cheeses preserved with the different additives over 8 days, the fatty acids arise as one of the most important molecules. This relevance is due to the changes sought in unsaturated fatty acids over time that tend to lose their instaurations due to oxidation mechanisms. Thus, in Table 3, the profile in relative percentage of the fatty acids is shown. Only the fatty acids over 1% of relative percentage are shown in the table, where the most abundant fatty acid is C16:0 (palmitic acid), followed by C18:1 (oleic acid).

Table 3 Fatty acid profile of the quark cheeses, presented in relative percentage

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	SFA	MUFA
Storage time (ST)	0 Days	8.5 ± 0.9	4.1 ± 0.8	2.5 ± 0.3	6 ± 1	4.4 ± 0.5	11 ± 1	33 ± 1	10.8 ± 0.9	15 ± 1	82 ± 2	17 ± 2
	3 Days	8 ± 2	4.0 ± 0.4	2.4 ± 0.4	5 ± 1	4.5 ± 0.3	11 ± 1	33 ± 1	11.2 ± 0.4	16 ± 2	82 ± 3	18 ± 2
	8 Days	8 ± 2	4.1 ± 0.6	2.0 ± 0.2	5 ± 1	4.3 ± 0.5	11 ± 1	33 ± 1	11.4 ± 0.5	15 ± 2	81 ± 3	18 ± 2
p-value (n = 3)	Tukey's test	0.014	0.473	<0.001	<0.001	0.415	0.829	<0.001	0.001	0.136	<0.001	0.005
Preservative type (PT)	Control	9.6 ± 0.2	3.5 ± 0.5	2.3 ± 0.6	6.3 ± 0.7	4.4 ± 0.3	10.8 ± 0.1[a]	33.6 ± 0.4	11.6 ± 0.3	14.6 ± 0.3	83 ± 1	16.4 ± 0.5
	Maceration	6 ± 1	3.9 ± 0.5	2.2 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	12.3 ± 0.3[b]	32 ± 1	10.4 ± 0.8	18.6 ± 0.8	78 ± 1	21.2 ± 0.8
	Ultrasound	8.9 ± 0.5	4.4 ± 0.4	2.6 ± 0.4	6.7 ± 0.4	4.0 ± 0.2	10.4 ± 0.4[a]	33.2 ± 0.7	11.2 ± 0.5	14.3 ± 0.4	84.4 ± 0.8	16.5 ± 0.2
	Potassium sorbate	8.1 ± 0.7	4.6 ± 0.1	2.1 ± 0.2	4.1 ± 0.4	4.4 ± 0.6	12.4 ± 0.2[b]	34.5 ± 0.4	11.4 ± 0.2	14.8 ± 0.1	82.7 ± 0.6	16.8 ± 0.2
p-value (n = 12)	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST × PT (n = 36)	p-value	<0.001	0.004	<0.001	<0.001	0.140	0.446	<0.001	0.016	0.001	<0.001	<0.001

In each row, different letters represent statistically significant differences with a significance level of 0.05. The SD were calculated from the results obtained under different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Overall, nine fatty acids showed values over 1% with the saturated fatty acids (SFAs) showing about 78 to 83% and monounsaturated fatty acids (MUFA) representing about 16 to 21%. Regarding the effects of the preservatives and storage time, no significant changes were observed in the cheeses, except for C14:0 (myristic acid), in which different preservatives showed a higher influence than the storage time. The control cheese and the one preserved with the ultrasound extract showed the lowest change. Overall, no drastic changes were observed in the fatty acid profile, in accordance with what is expected for a preservative. Still, once again, eight days might not be enough time to allow the degradation of enough quantities of fatty acids to be observable.

3.2.2.2. Organic acids and soluble sugars. Table 4 shows the profile of organic acids and soluble sugars, both obtained through HPLC detection. Four organic acids were detected, namely oxalic, shikimic, lactic and citric acid, with the latter being the most abundant. Overall, the profile in organic acids did not show any change over the 8 days of storage, regardless of the preservative used, showing a significant interaction among the two factors. Inversely, the two detected soluble sugars, glucose and lactose did show significant changes. In the case of these sugars, the preservatives showed a significant effect on the profiles, while storage time showed minimal effects.

The cheeses incorporated with potassium sorbate and the maceration extracted *A. unedo* showed the lowest amount of glucose and lactose, followed by the control cheese, showing a significant difference. The control sample showed an average of 17 g/100 g lactose and 1.9 g/100 g glucose, statistically lower than the values obtained for the ultrasound extract incorporated cheese. These high values sought for the sugar may be due to higher extraction of these molecules during the ultrasound extraction, and thus contributed to a higher quantity of overall sugars in the cheese. Still, the maceration extract did not seem to alter the profile in any manner for either the organic acids or soluble sugars.

3.2.3. Microbial load. Fig. 2 shows the three graphs prepared for the microbial loads, including the aerobic mesophiles (AM), the psychrotrophic lactic acid bacteria (PLAB) and the yeasts (*Y*). *S. aureus* and molds were not detected in any of the cheeses.

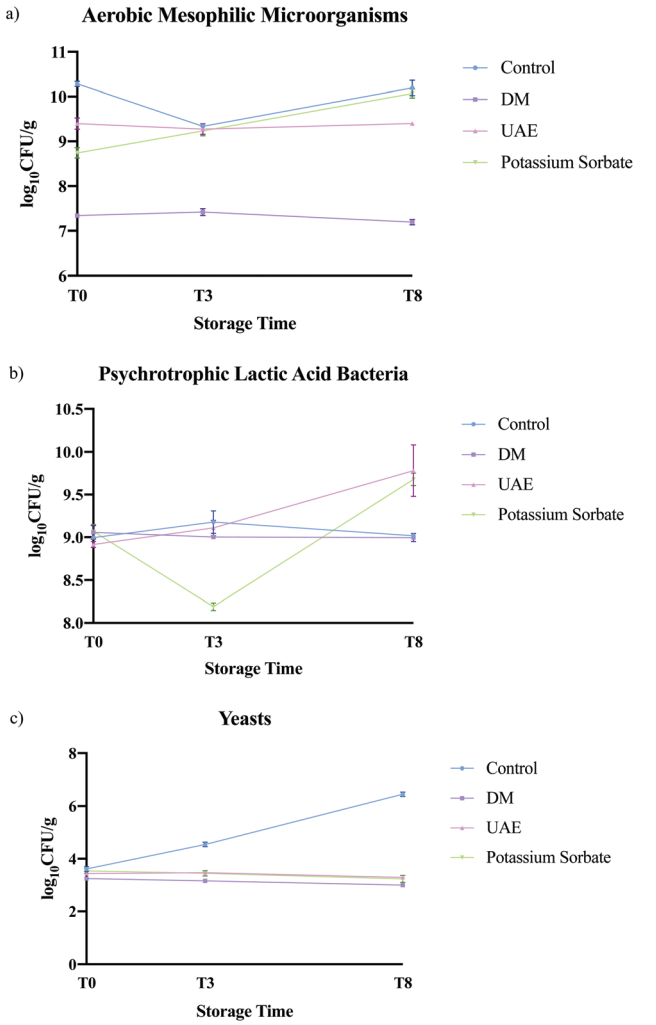

Fig. 2 Microbial load of the quark cheese over the 8 days: (a) aerobic mesophilic microorganisms; (b) psychrotrophic lactic acid bacteria; and (c) yeasts.

Table 4 Organic acids and soluble sugars profile of the cheeses, expressed in g/100 g dry weight

		Oxalic acid	Shikimic acid	Lactic acid	Citric acid	Glucose	Lactose
Storage time (ST)	0 days	0.05 ± 0.02	0.006 ± 0.004	48 ± 15	1.6 ± 0.6	2.0 ± 0.6	17 ± 4
	3 days	0.04 ± 0.03	0.004 ± 0.002	46 ± 7	1.5 ± 0.6	1.8 ± 0.4	16 ± 3
	8 days	0.02 ± 0.01	0.005 ± 0.001	52 ± 3	1.9 ± 0.7	2.0 ± 0.6	17 ± 4
p-value (n = 3)	Tukey's test	<0.001	<0.001	<0.001	<0.001	0.084	0.211
Preservative type (PT)	Control	0.05 ± 0.02	0.0047 ± 0.0001	56 ± 4	2.2 ± 0.1	1.9 ± 0.2^{b}	17 ± 2^{b}
	Maceration	0.05 ± 0.02	0.009 ± 0.003	54 ± 4	2.08 ± 0.09	$1.7 \pm 0.1^{a,b}$	$14.8 \pm 0.9^{a,b}$
	Ultrasound	0.02 ± 0.02	0.004 ± 0.001	47.8 ± 0.8	0.8 ± 0.2	2.7 ± 0.5^{c}	22 ± 3^{c}
	Potassium sorbate	0.026 ± 0.001	0.002 ± 0.001	37 ± 13	1.5 ± 0.5	1.5 ± 0.1^{a}	13.7 ± 0.9^{a}
p-value (n = 12)	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST × PT (n = 36)	*p*-value	<0.001	<0.001	<0.001	<0.001	0.208	0.181

In each row, different letters represent statistically significant differences with a significance level of 0.05. The SD were calculated from results obtained under different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Considering AM (Fig. 2a), at T0 of the cheese incorporated with macerated extract showed significantly lower counts, while the control sample showed the highest value, over 10 $\log_{10}$ CFU g^{-1}. Over time, the cheese with the maceration of *A. unedo* did not show statistically significant changes, maintaining the same load of these microorganisms.

Interestingly, the cheese with the UAE extract showed the same constant behavior over time, although with a significantly higher microbial load when compared to the maceration incorporated cheese. The cheeses preserved with potassium sorbate showed a statistically significant increase over time, while the control cheese significantly reduced the $\log_{10}$ CFU g^{-1} from T0 to T3 but increased from T3 to T8. Overall, due to maintaining a constant lower value of $\log_{10}$ CFU g^{-1} over the 8 days of storage, DM was the best extract in terms of preservation capacity, while potassium sorbate was less efficient against these microorganisms.

Considering the PLAB (Fig. 2b), no statistical differences were found at T0, where all cheeses showed a $\log_{10}$ CFU g^{-1} value of around 9. Then, from T0 to T3, the cheese preserved with potassium sorbate showed a significantly reduced $\log_{10}$ CFU g^{-1} value while the control cheese and the one incorporated with the maceration maintained the same value, with no statistically significant differences. Finally, at T8, the potassium sorbate preserved cheeses drastically increase their PLAB levels and showed no statistically significant differences from the control sample, which exhibits consistently increasing bacterial load over 8 days. Inversely, the natural extracts maintained their constant values from T3 to T8, showing no statistical differences between each other. Overall, the natural preservatives which did not significantly alter over time seem to better preserve the cheese against these bacteria, which, if left to grow freely will convert all lactose to lactic acid, thus changing the sensorial qualities of the cheese. Finally, for the Y (Fig. 2c), a very homogeneous behavior was found for all quark cheeses except for the control sample. This latter sample showed significantly increased Y over the 8 days of storage. Although a very slight decrease over time was detected for the incorporated cheeses, the one incorporated with the DM extract of *A. unedo* has a significant but slight reduction when compared to the other two from T0 to T3 and to T8 showing a slight yet better preservation against yeasts. Considering all the microorganisms, the quark cheese incorporated with the DM showed better preservation capacity when compared to the UAE incorporated cheese, and a much better effect when compared to the cheese preserved with potassium sorbate.

3.2.4. Physical properties. The physical properties of foods are paramount to understanding the effects that any preservative or ingredient has on food. Thus, the texture dimensions, external color, water activity and pH were analysed for all cheeses, as shown in Table 5.

Concerning the texture dimensions, firmness, consistency, cohesiveness and work of cohesion were analysed due to the cheese being a semi-solid food. Thus, the TPA test showed that while no changes were observed for cohesiveness and work of

Table 5 Texture, color profiles, water activity and pH of the cheese samples preserved with extracts over the storage time

		Firmness	Consistency	Cohesiveness	Work of Cohesion	L^*	a^*	b^*	aW	pH
Storage time (ST)	0 Days	365 ± 25	458 ± 38^{a}	−263 ± 20	−334 ± 23	92 ± 4	−2.3 ± 0.4	11.1 ± 0.7	0.986 ± 0.001	4.657 ± 0.006
	3 Days	420 ± 18	525 ± 49^{a,b}	−282 ± 20	−373 ± 44	80 ± 16	−2 ± 1	13 ± 3	0.981 ± 0.01	4.62 ± 0.03
	8 Days	383 ± 30	481 ± 63^{a,b}	−273 ± 23	−339 ± 44	92 ± 2	−2.4 ± 0.2	11.0 ± 0.6	0.986 ± 0.01	4.64 ± 0.02
p−value (n = 3)	Tukey's test	<0.001	0.014	0.017	0.061	0.004	0.142	0.064	0.041	<0.001
Preservative type (PT)	Control	370 ± 17	467 ± 50	−263 ± 16	−353 ± 47	87 ± 12	−2.5 ± 0.3^{a}	12 ± 2	0.990 ± 0.002^{b}	4.61 ± 0.02
	Maceration	379 ± 31	503 ± 64	−275 ± 26	−361 ± 46	86 ± 17	−2 ± 1^{b}	12 ± 4	0.989 ± 0.001^{b}	4.61 ± 0.01
	Ultrasound	389 ± 33	484 ± 60	−273 ± 26	−330 ± 36	90 ± 3	−2.1 ± 0.2^{a,b}	11.7 ± 0.3	0.970 ± 0.009^{a}	4.68 ± 0.01
	Potassium sorbate	388 ± 33	498 ± 57	−279 ± 24	−350 ± 34	88 ± 11	−2.5 ± 0.2^{a}	11.0 ± 0.7	0.987 ± 0.003^{b}	4.67 ± 0.05
p-value (n = 12)	Tukey's test	0.065	0.479	0.191	0.448	0.668	0.009	0.588	<0.001	<0.001
ST × PT (n = 36)	p-value	<0.001	0.522	<0.001	0.963	0.100	0.138	0.424	0.215	<0.001

In each row, different letters represent statistically significant differences with a significance level of 0.05. The SD were calculated from results obtained under different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

cohesion, the storage times had an influence on consistency, thus showing a significant increase over time. Specifically, for cohesiveness, it increased over time, which could be due to the migration of moisture from the intracellular to the intercellular space.[46]

For the color of the cheeses, an important parameter that can determine the success or failure of a food additive, lightness (L^*) showed a significant interaction among the two factors, while yellowness–blueness (b^*) did not show any changes. Redness–greenness (a^*) did show significant changes, especially for the different preservatives, in which the control cheese and the cheese preserved with potassium sorbate revealed lower values with a significant difference towards the two natural preservatives. While this is expected due to the almost transparent color of potassium sorbate and the dark color of the natural extracts, these values are only separated by 0.5 values in a total range of 200, thus being quite negligible. Water activity, an important parameter that shows the available water in the food, which could be related to an increase in the development of contaminants was also assessed. For this parameter, only the cheese preserved with the UAE showed a significantly lower value, with all other cheeses showing no statistical differences. Once again, the preservatives seemed to have a higher influence than the storage time on this parameter. Finally, pH was also evaluated as a determinant parameter due to its importance in the development of flavours and other sensory alterations in foods. Although a significant interaction was sought for both parameters, no statistical differences were found. *A. unedo* fruits had shown capacity to be used as food preservatives for loaf breads,[37] and while the leaves have already been studied[15,18] this is the first report, to the author's best knowledge, of the use of leaves as an additive in food. A limitation of this work is that it analyses only 8 days of storage time which could not have been enough to reveal the effects of the natural extracts. While quark cheese does not have any preservative in its composition and also because the recommended shelf life of this cheese is about 3 days after opening, only seven days were considered in this study, and thus, by adding these preservatives the shelf-life could be extended probably for an extra week.

4. Conclusions

Natural preservatives are one of the most demanded additives by the consumers and industry, especially food preservatives. In the present work two extracts of *A. unedo* were tested for their capacity to act as preservatives in quark cheese. The obtained extracts revealed strong antioxidant and antimicrobial properties and after incorporation in quark cheese were able to preserve it as efficiently or even better than the commercial preservative (potassium sorbate).

It can also be highlighted that among the two tested extracts, DM was the most promising one, presenting lower active concentrations without toxicity. Nevertheless, because none of them exhibits toxicity or change the profile of the foods (as expected from food additives), both are good candidates to be used in quark cheese.

Author contributions

Nabila Derbassi, Filipa Fernandes, Mariana Pedrosa, Maria Inês Dias, Ricardo C. Calhelha: investigation; Marcio Carocho, Sandrina Heleno: conceptualization; Marcio Carocho: formal analysis, writing – original draft. Sandrina Heleno, Paula Rodrigues: writing – review and editing. Lillian Barros: conceptualization, writing – review & editing. Isabel C. F. R. Ferreira: project administration.

Conflicts of interest

The authors declare that they have no competing financial interests.

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