Research Article

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Addition of polyphenolic extracts of *Myrtus communis* and *Arbutus unedo* fruits to whey: valorization of a common dairy waste product as a functional food

Cassandra Detti,[a] Luana Beatriz Dos Santos Nascimento,[a] Antonella Gori,[a,b] Giulia Vanti,[c] Giuseppe Amato,[d] Filomena Nazzaro,[d] Francesco Ferrini,[a,b] Mauro Centritto,[b] Anna Rita Bilia[c†] and Cecilia Brunetti[b†*]

Abstract

BACKGROUND: Whey, a nutrient-rich byproduct of the dairy sector, possesses high potential for creating novel nutraceutical products. The present study investigates a potential new functional food by incorporating polyphenolic extracts from *Myrtus communis* and *Arbutus unedo* fruits into whey in both liquid (LA) and powder (PA) addition forms. Chemical, microbiological, physical stability and antioxidant activity were monitored for 60 days (from T_0 to T_{60}).

RESULTS: Both LA and PA of fruit extracts remained chemically stable for the whole period, except for *A. unedo* PA, which showed a decline in polyphenols after T_{45}. Enriched whey samples showed higher antioxidant activity than pure whey. Microbiological analysis revealed the presence of lactic acid bacteria, indicating potential prebiotic effects. However, the high tannin concentration of *A. unedo* extracts partially modified the casein micelle structure.

CONCLUSION: Whey enriched with Mediterranean fruit extracts shows great potential as a functional food, combining the benefits of plant antioxidants, probiotic bacteria and good stability.

Supporting information may be found in the online version of this article.

Keywords: *Arbutus unedo*; *Myrtus communis*; polyphenols; whey valorization; stability analysis; antioxidant activity

INTRODUCTION

Myrtus communis L. (myrtle, Myrtaceae) and *Arbutus unedo* L. (strawberry tree, Ericaceae) are two typical evergreen species of the Mediterranean maquis. Myrtle is an aromatic perennial shrub producing dark blue berries with a ripening period from October to February.[1] Strawberry tree is an evergreen tree producing fruits ripening in Autumn with a color ranging from red to deep crimson.[2]

Traditionally used in folk medicine, the fruits of these species are abundant in polyphenols, including phenolic acids, flavonoids, tannins and anthocyanins.[3,4] Notably, flavonols and anthocyanins have shown potent antioxidant properties against oxidative stress-related diseases and are important for preventing and treating chronic inflammatory conditions.[5–8] Consequently, the food industry has shown an increasing interest in *M. communis* and *A. unedo* fruit extracts for their health-promoting properties.[9,10] In particular, the incorporation of Mediterranean plant fruit extracts into dairy products, such as yogurt, ice cream and cheese, is of interest to create novel functional foods.[11]

* *Correspondence to: C Brunetti, National Research Council of Italy (CNR), Institute for Sustainable Plant Protection, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy. E-mail: cecilia.brunetti@ipsp.cnr.it*

† *These authors contributed equally to this work.*

a *Department of Agriculture, Food, Environment and Forestry, University of Florence, Florence, Italy*

b *National Research Council of Italy (CNR), Institute for Sustainable Plant Protection, Florence, Italy*

c *Department of Chemistry, Ugo Schiff, University of Florence, Florence, Italy*

d *National Research Council of Italy (CNR), Institute of Food Sciences, Avellino, Italy*

Whey, the by-product of cheese manufacturing, is rich in proteins (e.g. β-lactoglobulin, α-lactalbumin, immunoglobulins, small quantities of lactoferrin and transferrin), lactose, fats, vitamins and minerals. Whey also possesses various functional properties (e.g. emulsifying stabilizer, foaming agent, aroma binder and mineral carrier) and offers various health benefits, such as promoting beneficial intestinal microflora and enhancing the immune system.[12] Despite being rich in several nutritional compounds, whey lacks nutraceutical compounds such as polyphenols (anthocyanins, tannins and flavonols). Therefore, the incorporation of polyphenolic fruit extracts into whey represents an opportunity to create innovative and health-enhancing functional foods, thereby elevating their commercial value.[13] Furthermore, the antioxidant and antimicrobial activities conferred by polyphenols could also contribute to increase the shelf-life of the new functional product.[14] However, adding fruit extracts to whey requires careful consideration because the interaction between polyphenols and proteins may affect the structure and the properties of the product.[15] Furthermore, polyphenolic extracts may negatively affect important sensorial and physical characteristics of whey-based product, the form in which polyphenols are added to whey needs to be carefully evaluated.[16] In this regard, it is essential to select the most appropriate extraction methodology to achieve the highest yield in polyphenols, prioritizing green extraction techniques such as ultrasound-assisted extraction (UAE) and decoction.[17] These methods align with current industry trends and are desirable from both economic and environmental perspective.[18] It is also important to evaluate the best way to incorporate the rich polyphenol extract into the food matrix to enhance its quality and to prevent its deterioration.[19]

As a result of limited research on fictionalization of dairy by-products with fruit extracts, the addition of polyphenolic extracts from *M. communis* and *A. unedo* fruits to whey was investigated together with the evaluation of the quality of this innovative functional food.[20,21] Specifically, this study focused on three key aspects: (i) selecting the best green extraction process (UAE or decoction) to maximize the yield of polyphenols in the extracts to be included in whey; (ii) evaluating the most appropriate form to incorporate fruit extracts into whey (liquid or powder forms); and (iii) testing the chemical, physical, and microbiological stability of the final products (whey with the addition of the fruit extracts).

MATERIALS AND METHODS

Plant material

Arbutus unedo and *M. communis* berries were collected from plants cultivated in the farm 'Olive grove partner Srl', located in Southern Tuscany (43°00′17.0″N; 11°13′20.1″E, Italy) at the end of November 2019. No soil tillage, fertilization or pesticide treatments were performed in the field. The shrub species were cultivated at planting distances of 6 × 3 m for *A. unedo* and 4 × 2 m for *M. communis*. The fruits were harvested at full maturity (well-red pigmented berries for *A. unedo* and fully dark violet for *M. communis*), taking 10 fruits (randomly distributed in the canopy) of 12 plants. After harvesting, the fruits were immediately frozen in liquid nitrogen and kept at −80 °C until analyses.

Fruit extraction procedures

To identify the most efficient extraction processes, we performed extractions on fresh, oven dried, and lyophilized fruits of *A. unedo* and *M. communis*. The dried fruits were prepared by desiccating 50 g of fresh fruits at 50 °C in a thermostatic oven for 3 days (until constant weight). Similarly, the freeze-dried fruits were prepared by lyophilizing 50 g of fresh fruits for 3 days using Lyovac GT freeze drier (Leybold-Heraeus, Cologne, Germany). Before the extraction, samples were ground in liquid nitrogen using a sterile mortar and pestle. Two types of extractions were made: an ultrasound-assisted extraction (UAE), using EtOH 75% as the solvent (50% w/v) for 10 min, and a decoction extraction (50% w/v), boiling the grinded sample in Milli-Q Water (Millipore, Burlington, MA, USA) for 10 min. For UAE, an ultrasonic bath (Bioclass, Pistoia, Italy) with a constant frequency of 39 kHz and an input power of 100 W was used, whereas the decoction was made on a heating plate (Velp Scientifica, Usmate Velate, Italy). All extractions were performed in triplicate. Subsequently, the extracts were filtered using a laboratory paper filter, and 1 mL of each extract was reduced to dryness under vacuum and then used for chromatographic analyses.

Extracts addition to whey

The whey milk was thermally treated at 90 °C for 30 min during the cheese production without adding any starter. The acid whey milk sample was then transported from the farm (Fattoria Pianporcino, Pienza, Italy) at 4 °C and stored in the fridge at the same temperature until the moment of the analysis. Before the addition of the extracts, the pH of the whey samples, measured using a pH meter (Mettler toledo, Milan, Italy), was adjusted to 4.40 with citric acid monohydrate 0.01 M (Sigma-Aldrich, Milan, Italy). Based on the qualitative and quantitative results of the polyphenolic content (refer to the section on 'Chemical characterization and quantification of polyphenols in *Myrtus communis* and *Arbutus unedo* fruit extracts'), decoctions of fresh fruits of *A. unedo* and *M. communis* were selected as the extracts to be added to whey. Particularly, the chosen extracts were added to 20 mL of whey in two distinct forms: liquid addition (LA), where 1 mL of the extract was directly mixed with the 20 mL of whey, and powder addition (PA), where 1 mL of the extract was freeze-dried and then combined with the 20 mL of whey. Freeze-dried powder was obtained by lyophilizing the extracts for 1 day using Lyovac GT freeze drier (Leybold-Heraeus). After incorporating the extracts into whey, the samples were submitted to microfiltration (0.2 µm) at 20 °C (room temperature) as preservation treatment. The samples (hereafter referred to as enriched whey) were first analyzed (sampling point T_0) and then stored in the fridge at 4 °C for 60 days to perform subsequent analyses every 15 days (sampling points T_{15}, T_{30}, T_{45} and T_{60}). At the sampling points T_0, T_{15}, T_{30}, T_{45} and T_{60}, samples were collected for pH measurements, high-performance liquid chromatography with diode-array detection (HPLC-DAD) analyses, antioxidant activity assays and microbiological analyses. The physical stability analyses were performed only at the sampling points T_0, T_{15}, T_{30} and T_{45}. Pure whey samples were used as controls. The entire experimental process, including the extraction, addition to whey and the analyses, is illustrated in the Supporting information (Fig. S1).

Characterization and quantification of polyphenols

The analyses of polyphenols in the extracts, in the enriched whey samples, and in pure whey (used as a control) were performed using a PerkinElmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (PerkinElmer, Bradford, CT, USA). To perform the analyses, aliquots (5 µL) of the samples were injected in a stationary phase consisted of a Zorbax® C-18 column (Agilant, Santa Clara, CA,

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USA), whereas the mobile phases consisted of (A) acidified water (0.1% HCOOH) and (B) acetonitrile (0.1% HCOOH). The separation was conducted using the gradient: 1 min (3% B), 1–55 min (3–40% B), 55–60 min (40% B), 60–61 min (40–3% B), with 62 min of total analysis time, at a flow rate of 0.6 mL min^{-1}. All analyses were conducted in triplicate, and chromatograms were obtained at 280 nm for gallic acid and catechin derivatives, 350 for flavonols and 520 nm for anthocyanins. The identification and quantification of polyphenols in the extracts (UAE and decoction) were carried out by comparing the UV-visible spectral characteristics of the peaks and the retention times of authentic standards (gallic acid, rutin, myricetin, catechin, myrtillin, kuromanin, petunidin and oenin, purity > 95%) (Sigma-Aldrich) injected under the same conditions. In addition, the compounds were identified by comparing the obtained information with available data reported in the literature. For the quantification of the polyphenols in the extracts, five-point calibration curves of the authentic standards were used. The analyses were conducted in triplicate and the results expressed in mg g^{-1} fresh weight (FW) (for fruit extracts).

Because of the complexity of the matrix of both pure whey and whey enriched with LA and PA, the matrix effect and recovery of the polyphenols was evaluated. Pure whey was first analyzed to identify possible interference compounds that could have co-eluted with the polyphenols. For the quantification of the polyphenols, five-point calibration curves of the authentic standards were prepared using whey matrix. Furthermore, to calculate the recovery of the polyphenols, rosmarinic acid and cyanin were used as internal standards (at 0.1 mg mL^{-1}) because these compounds are not present in *A. unedo* and *M. communis* fruit extracts. For preparation of the whey samples, samples were reduced to dryness using a rotavapor coupled to a vacuum controller (Buchi P12 with V-855; Buchi Italia, Cornaredo, Italy), and the residue was re-suspended with 1.0 mL of the solution MeOH: Milli-Q Water (1:1 v/v, pH 2.5, adjusted with HCOOH) and centrifuged twice before injection in HPLC-DAD (conditions as described above). The analyses were conducted in triplicate and the content of polyphenols in the enriched whey samples was determined in mg mL^{-1}.

Antioxidant activity assays of the enriched whey samples

To verify the antioxidant activity during the 60 day-storage period, the enriched whey samples were prepared as follows: 40 mg of the samples (enriched whey with LA, enriched whey with PA and whey without extracts) were dried in a concentrator plus/Vacofuge (Eppendorf, Hamburg, Germany) for 5 h and then dissolved in 2.5 mL of water acidified with formic acid (pH 2.5), reaching a final concentration of 16 mg mL^{-1} of a stock solution. Then the antioxidant activity was measured at the sampling points of T_0, T_{30} and T_{60} with two different methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing-antioxidant assay (FRAP).

DPPH radical scavenging activity assay

The DPPH assay was performed according to the method described by Kandi and Charles.[22] Briefly, diluted samples (0.250 μL) were added to 0.250 μL of DPPH solution (0.1 mM in methanol; Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany) and the mixture was incubated at room temperature for 45 min in the dark. Then, the absorbance was spectrophotometrically measured at 518 nm, using a PerkinElmer UV-visible spectrophotometer (Lambda 25; Perkin Elmer). The absorbance of the blank (0.250 μL of methanol and 0.250 μL of samples) and negative controls (0.250 μL of methanol and 0.250 μL of DPPH solution) was also measured. The analyses were conducted in triplicate, and the percentage of antioxidant activity was calculated as: $AA\ \% = 100 - \{[(ABS_{sample} - ABS_{blank}) \times 100]/ABS_{negative\ control}\}$. EC_{50} (effective concentration at 50%) values were calculated using Excel (Microsoft Corp., Redmond, WA, USA).

FRAP assay

The FRAP assay is based on the reduction at low pH of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe (III)-TPTZ] to the ferrous complex, developing an intense blue color, followed by spectrophotometric analysis of the sample with maximum absorption at 594 nm. The FRAP assay was performed using a FRAP assay kit (Sigma-Aldrich). Briefly, aliquots of the samples (10 μL) were added to a solution containing buffer (152 μL), $FeCl_3$ (19 μL of) and FRAP probe (19 μL). Blanks were prepared using the same mixed solutions without the FRAP probe. After the reaction time (60 min at 37 °C) absorbance of the mixture was measured using a SpectraMax® Microplate reader (Molecular Devices, San Jose, CA, USA) at 594 nm. Known solutions of FeII ($FeSO_4X7H_2O$) in the range of 0–20 nmol were used for calibration.

Antibacterial properties and microbiological stability of the enriched whey samples

For this 2 mL of both LA and PA enriched whey and pure whey (control) samples were collected at T_0, T_{15}, T_{30}, T_{45} and T_{60} to conduct the antibacterial activity and microbiological stability analysis.

Antibacterial activity

The antibacterial activities of the samples were investigated through the inhibition halo test on sterile Luria Bertani agar plate against the pathogenic Gram-positive, *Staphylococcus aureus* subsp. *aureus* (ATCC 25923) and *Listeria monocytogenes* (ATCC 7644), and the Gram-negative, *Escherichia coli* (DSM 8579). The strains were incubated at 37 °C for 18 h in Luria Bertani broth (Sigma-Aldrich). The microbial suspensions [1×10^7 colony forming units (CFU) mL^{-1}] were uniformly spread onto the Luria solid media plates (dish diameter = 90 mm) and two aliquots (10 and 20 μL per spot) of samples were used for the test. After 30 min under sterile conditions at room temperature, the plates were incubated at 37 °C for 24 h. The diameter of the clear zone shown on the plates was accurately measured, and the antibacterial activity was expressed in millimeters. Dimethyl sulfoxide (10 μL) was used as a negative control. Tetracycline (7 μg per spot), previously dissolved in a sterile physiological solution, served as a positive control. Samples were tested in triplicate. The results are expressed as the mean ± SD.

Microbiological analysis

To evaluate the potential presence of lactic acid bacteria in samples, two different aliquots (10 and 20 μL) of each sample were inoculated in a 96-wells microplate and De Man, Rogosa and Sharpe (MRS) broth (Sigma-Aldrich) previously sterilized was added to each well to reach a final volume of 250 μL. Concurrently, 10 μL of four standard lactic acid bacteria strains (*Lactobacillus. acidophilus*, *Lactobacillus gasseri*, *Lacticaseibacillus paracasei* and *Lacticaseibacillus rhamnosus*) (Creative Enzymes, Upton, NY, USA) were inoculated under the same conditions. The microplates were then incubated at 37 °C for 24 h. Next, 100 μL of each well was spread onto sterile MRS solid media in a flow laminar hood under sterile conditions. After 30 min, plates were closed and

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incubated at 37 °C for 24 h. The test was performed in triplicate. The results are expressed as CFU/mL (mean ± SD).

Dynamic light scattering and scanning transmission electron microscopy analysis

Samples of both LA and PA enriched whey and pure whey at T_0, T_{30} and T_{45} were analyzed by the dynamic light scattering (DLS) technique using the Zetasizer Pro Red of Malvern Panalytical (Alfatest Srl, Milan, Italy) equipped with a He—Ne gas laser with a maximum output power of 10 mW, a beam wavelength of 632.8 nm and an avalanche photodiode detector. The measurements were performed at 25 °C, with a backscatter detection angle of 173°. The analyses allowed determination of the average hydrodynamic diameter (size, nm) and polydispersity index (PdI, dimensionless parameter). Each measurement was performed in triplicate.

The samples were also analyzed using a scanning electron microscope Gaia 3 (Tescan s.r.o, Brno, Czech Republic), focused ion beam scanning electron microscope operating in high-vacuum mode with an electron beam voltage of 20 kV, and bright-field transmission electron microscope. Gaia 3 was equipped with an EDS-X-ray microanalysis system (EDAX, AMETEK, Pleasanton, CA, USA) and TeamEDS Basic Software Suite (TEAM; EDAX) and was delivered with a scanning transmission electron microscopy (STEM) detector, which provides a complementary method for image acquisition of transmitted electrons. The detector consisted of several semiconductor sensors for bright- and dark-field imaging. The transmitted electron signal was collected by placing the detection system below the specimen.

Statistical analysis

All data were analyzed using Systat, version 12.5 (Systat Software, Inc. San Jose, CA, USA). Two-way analysis of bvariance followed by Tukey's post-hoc test was used. For the comparison among the extraction methods, the two factors were the type of fruit (three types: dried, lyophilized or fresh) and the type of extraction (two extractions: decoction or UAE). For the analysis of enriched whey samples, the two factors were the sampling time (i.e. number of sampling points depending on the analysis) and incorporation type (two types: LA or PA). For all tests, differences were considered statistically significant at $P \leq 0.05$. The results are presented as the mean ± SD ($n = 3$).

RESULTS

Chemical characterization and quantification of polyphenols in *M. communis* and *A. unedo* fruit extracts

The identification by HPLC-DAD revealed the presence of 29 polyphenols in fresh, dried and lyophilized fruit extract of *M. communis* obtained using the UAE or decoction method. The identified compounds were mainly galloyl derivatives, lavonoids, and anthocyanins and are reported in detail reported in the Supporting information (Fig. S2).

Extracts obtained from fresh and lyophilized fruits showed a similar phenolic profile, regardless of the extraction method applied (UAE or decoction). Conversely, extracts of dried fruits, obtained with both UAE and decoction, had a lower variety of phenolics, with no anthocyanins and fewer galloyl derivatives (see Supporting information, Table S1). Moreover, all polyphenols were more abundant in extracts of fresh and lyophilized fruits compared to the dried ones (Table 1). In particular, the highest content of tannins and flavonoids was obtained using the decoction of fresh and lyophilized fruits compared to the extracts obtained with dried fruits ($P \leq 0.001$). In addition, UAE was the best methodology to obtain the highest amounts of total anthocyanins ($P \leq 0.001$). Because of the high polyphenol content in the *M. communis* fresh fruit decoction, this extract was chosen to be added to the whey samples.

In all *A. unedo* fruit extracts, three classes of compounds were identified, namely tannins, flavonoids, and anthocyanins. Twenty-seven peaks were detected and their identification is reported in detail in the Supporting information (Fig. S3).

All extracts obtained by UAE and decoction showed comparable phenolic profiles. However, extracts obtained from dried fruits showed notable differences compared to the other two types (fresh and lyophilized), primarily because of the absence of anthocyanins (see Supporting information, Table S2). As observed for myrtle, the decoction of fresh fruits was the most efficient method for obtaining the highest amounts of tannins, flavonoids, and polyphenols ($P \leq 0.001$) (Table 2). Consequently, extracts obtained by decoction were added into the whey samples for the subsequent phase of the experiment.

Analysis of enriched whey samples with *M. communis* and *A. unedo* extracts: evaluation of polyphenolic stability, antioxidant activity and pH

Chemical stability of polyphenols

The addition of the extracts of myrtle and strawberry tree fruits to whey enriched this by-product with polyphenols, otherwise not present in pure whey (data not shown). However, whey exerted a matrix effect on all classes of phenolics present in *M. communis* and *A. unedo* fruit extracts, resulting in a statistically significant decrease in the detection of total phenolics, flavonols, anthocyanins, and gallic and ellagic acid derivatives content across all samples (see Supporting information, Table S3). We observed a recovery rate of 15% for anthocyanins in both PA and LA of both species, whereas the recovery of gallic acid derivatives was 50% for *A. unedo* and 10% for *M. communis* (see Supporting information, Table S3). In whey enriched with *M. communis* and *A. unedo* LA extracts, the analysis of the chemical stability during the 60-day storage period revealed no significant differences in the amount of any class of polyphenols ($P > 0.05$) (Fig. 1A–C; see aso Supporting information, Figs S4–S6). Whey enriched with *M. communis* PA extracts resulted also chemically stable during the 60-day storage period ($P > 0.05$) (Fig. 1B), whereas whey enriched with *A. unedo* PA extracts showed a decreased polyphenol content from T_{45} to T_{60} ($P > 0.01$) (Fig. 1D).

pH stability

The addition of fruit extracts in whey did not cause any initial change in pH, as these values were similar to those of the pure whey (control) (see Supporting information, Fig. S7). However, as the storage period progressed, whey enriched with either liquid (LA) or powder (PA) extracts from both species showed a decreasing trend in pH, whereas the pH of pure whey was stable until T_{45}. In whey enriched with both *M. communis* LA and PA extracts, the initial pH values were similar to those of control at T_0. After 15 days of storage, the pH in PA enriched whey samples started to decrease until T_{60}, whereas, in LA enriched whey samples, it began to significantly decrease after 30 days of storage. Whey enriched with *M. communis* PA extract showed the lowest pH values by the end of the storage period (T_{60}) (see Supporting information, Fig. S7A). Similarly, in the case of *A. unedo*, pH of

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Table 1. Comparison among the polyphenol content (mg g^{-1} FW) in different extracts of *Myrtus communis* fresh, dried or lyophilized fruits obtained using ultrasound-assisted extraction (UAE) or decoction

		Total content (mg g^{-1} FW)			
Type of fruit	Extraction methods	Galloyl derivatives	Anthocyanins	Flavonols	Polyphenols
Fresh	Decoction	0.67 ± 0.01 a	1.25 ± 0.14 c	1.48 ± 0.15 a	3.40 ± 0.20 ab
	UAE	0.27 ± 0.05 c	2.12 ± 0.36 b	0.61 ± 0.04 c	3.00 ± 0.07 b
Dried	Decoction	0.24 ± 0.01 b	ND	0.20 ± 0.05 d	0.45 ± 0.03 c
	UAE	0.03 ± 0.01 d	ND	0.05 ± 0.01 e	0.08 ± 0.03 d
Lyophilized	Decoction	0.67 ± 0.15 a	0.88 ± 0.03 d	1.46 ± 0.23 a	3.01 ± 0.53 b
	UAE	0.27 ± 0.08 c	3.28 ± 0.70 a	0.83 ± 0.03 b	4.38 ± 0.79 a

Note: The values are presented as the mean ± SD ($n = 3$). Different lowercase letters show the significant differences for the same class of compounds ($P \leq 0.001$). ND, not detected.

Table 2. Comparison among the polyphenol content (mg g^{-1} FW) in different extracts of *Arbutus unedo* fresh, dried or lyophilized fruits obtained using ultrasound-assisted extraction (UAE) or decoction

		Total content (mg g^{-1} FW)			
Types of fruits	Extraction methods	Galloyl and ellagic acid derivatives	Anthocyanins	Flavonols	Polyphenols
Fresh	Decoction	1.12 ± 0.11 a	0.04 ± 0.01 b	0.04 ± 0.001 a	1.20 ± 0.14 a
	UAE	0.17 ± 0.01 d	0.01 ± 0.01 d	ND	0.18 ± 0.0 1e
Dried	Decoction	0.71 ± 0.08 b	ND	0.03 ± 0.002 b	0.74 ± 0.08 d
	UAE	0.20 ± 0.02 c	ND	ND	0.20 ± 0.02 e
Lyophilized	Decoction	0.95 ± 0.07 a	0.02 ± 0.01 c	0.02 ± 0.004 c	1.00 ± 0.06 b
	UAE	0.68 ± 0.12 b	0.14 ± 0.01 a	0.03 ± 0.0004 b	0.85 ± 0.11 c

Note: The values are presented as the mean ± SD ($n = 3$). Different lowercase letters show the significant differences for the same class of compounds ($P \leq 0.001$). ND, not detected.

whey enriched with liquid or powder extracts slightly decreased from T_0 to T_{60}. Moreover, pH values in whey added with *A. unedo* PA extracts were consistently lower than those of whey enriched with *A. unedo* LA extracts at all sampling times (see Supporting information, Fig. S7B).

Antioxidant activity

The results of antioxidant activity of whey enriched with *M. communis* extracts evaluated using both methods (DPPH and FRAP) showed no significant effect of the type of inclusion (LA or PA) nor of the storage time. Similarly, LA of *A. unedo* extracts did not show a significant difference during storage. Furthermore, these extracts displayed a higher antioxidant activity compared to those enriched with PA which declines from T_{30} to T_{60} in both assays, particularly in the DPPH. Pure whey did not show any antioxidant activity (ND) (Table 3).

Antibacterial activity and microbiological evaluation

Antibacterial activity

None of the whey samples enriched with fruit extracts of myrtle or strawberry trees had antibacterial activity against *E. coli* or *L. monocytogenes* at the two concentrations used in the test. However, whey samples enriched with PA of *A. unedo* extracts demonstrated inhibition of the growth of *S. aureus*, giving halos of inhibition of 4.25 mm and 11 mm, using 10 µL per spot and 20 µL per spot of sample, respectively. This result is particularly interesting, considering that tetracycline, used as a positive control, produced an inhibition halo of 5.7 mm (see Supporting information, Table S4).

Microbiological analysis

Throughout the storage period, all tested samples showed the presence of lactic acid bacteria (LAB).

The comparison between control and whey enriched with liquid and powder extracts of both species is reported in Fig. 2 expressed in CFU mL^{-1}.

Whey enriched with LA of *M. communis* extract showed an increasing trend throughout the storage time, starting at a level similar to the control at T_0, increasing and reaching the highest number of LAB at T_{60}. In the whey enriched with PA of *M. communis* extract, no differences were found at T_0 compared to control, but a lower number of LAB was observed at all the other sampling points (Fig. 2A).

Whey samples enriched with *A. unedo* extracts showed a different trend compared to pure whey during the storage. There was a significant increase in the number of LAB from T_0 to T_{15} followed by a reduction from T_{15} until the end of the experiment (1422 CFU mL^{-1}). Whey samples enriched with PA of *A. unedo* showed an increase in LAB from T_0 to T_{30}, followed by a decrease from T_{30} to T_{60} (Fig. 2B).

To assess the suitability of the growth medium, four lactic acid bacteria (*L. acidophilus*, *L. gasseri*, *L. paracasei* and *L. rhamnosus*) were used as a control. These strains, tested under identical conditions, confirmed that the medium employed in the experiments was suitable (see Supporting information, Table S5).

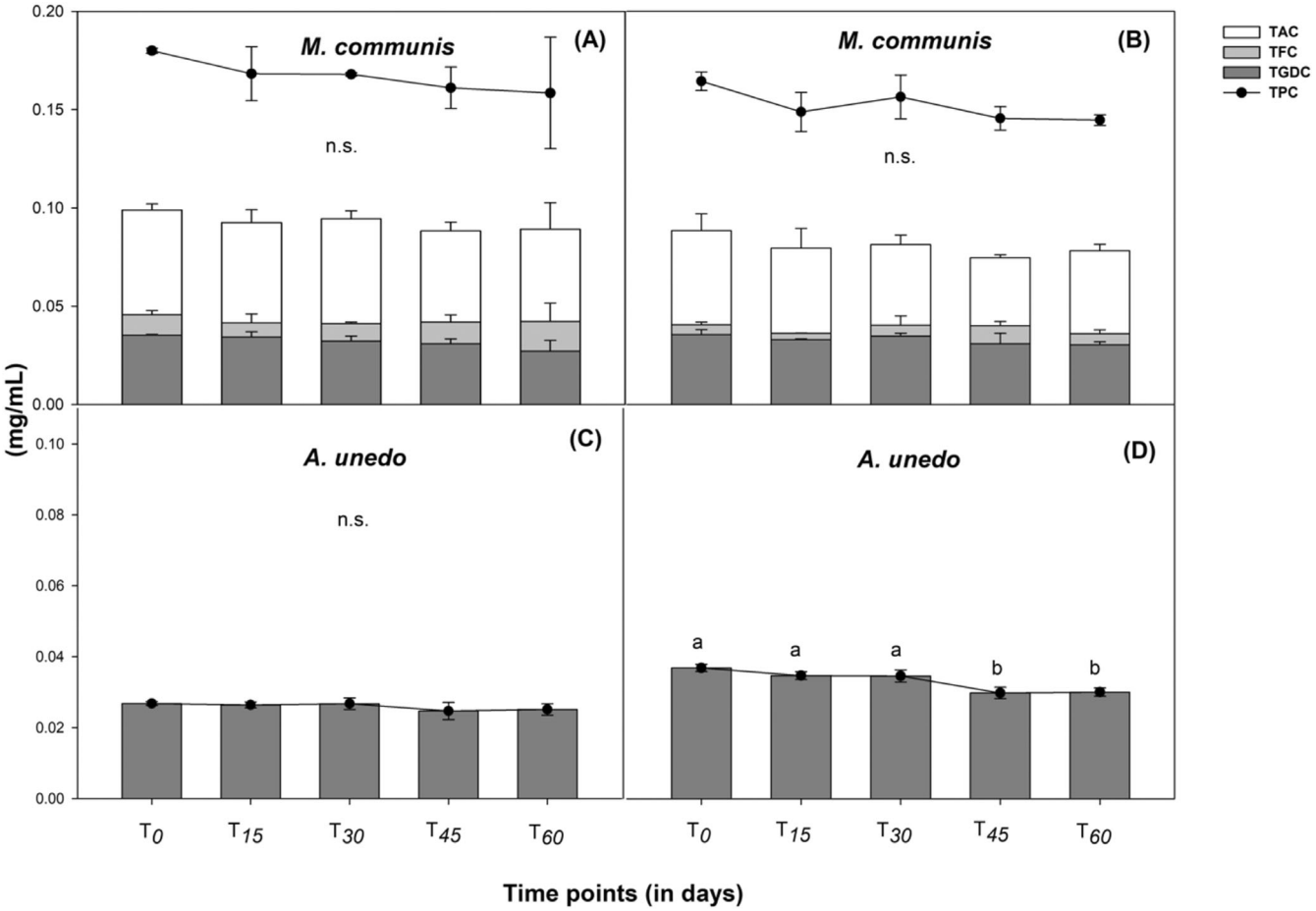

Figure 1. Stability analysis of phenolic content in enriched whey samples. Total polyphenol (TPC, line), flavonols (TFC, light gray bars), anthocyanin (TAC, white bars) and galloyl derivatives (TGDC, dark gray bars) contents (mg mL^{-1}) after addition of liquid (LA) (A, C) or powder (PA) (B, D) extracts of *Myrtus communis* (A, B) and *Arbutus unedo* (C, D). Values are presented as the mean ± SD ($n = 3$). Different letters represent significant differences ($P \leq 0.001$) among the time points; n.s., no significant differences.

Physical stability

DLS of pure whey (data not reported) revealed a very complex system with numerous clusters and a high polydispersity index (more than 0.5). By contrast, whey samples (T_0) added with the extracts of *M. communis* and *A. unedo* fruits exhibited two distinct clusters of structures with sizes of 200–300 nm and 2000–2500 nm as reported in the Supporting information (Table S6). During storage, the samples maintained two groups of structures with similar size distributions and a polydispersity index of approximately 0.3. However, the polydispersity index of the whey samples indicated a more homogeneous distribution after the addition of the extracts, as reflected by the two well-separated peaks related to the cluster structures clearly identified (see Supporting information, Fig. S8).

In addition, the samples analyzed by STEM evidenced the morphology and architecture of the array structures of the whey dispersed phase after the addition of the extracts. Pictures of pure whey (Fig. 3A–C) show large white spherical structures (yellow arrow) and small white spheres (green arrow) with sizes ranging between 0.025 and 5 µm associated with fat globules. Moreover, Fig. 3(A–C) also shows smaller black architectures with sizes between 50 and 200 nm, associated with casein micelles (red arrow), except for some more giant micelles around 500 nm (light blue arrow), and interactions between a large fat globule and small casein micelles adhering to the globule surface (yellow arrow in Fig. 3A). Instead, from the STEM result, the addition of the liquid and powder extracts of the two species to whey affects casein micelles to variable degrees. Both the LA (Fig. 3D) and PA (Fig. 3E) of *M. communis* extract added to whey slightly affect the architecture of casein micelles, as some casein chains dispersed in the resulting preparation (pink arrows) as well as intact casein micelles (blue arrows) are visible (Fig. 3D,E). Clear fat globules are still intact after the addition of the extracts. In both LA (Fig. 3F) and PA (Fig. 3G) of *A. unedo* extract to whey, the STEM result of the final product was different from pure whey, in particular some casein chains dispersed in the sample (pink arrow in Fig. 3F) and a few intact casein micelles (blue arrow in Fig. 3G) are detectable.

DISCUSSION

The present study demonstrates that decoction constitutes the most effective extraction technique for supplementing whey with fresh fruit extracts of *M. communis* and *A. unedo*, allowing the maintainance of a total polyphenol content similar to that achieved with UAE, but without the need for sophisticated technical apparatus. Decoction was used to prepare extracts both in powder form (PA), after lyophilization, and in liquid form (LA) for

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Table 3. Antioxidant activity (in terms of EC_{50} and in nmol of Fe μL^{-1}) determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and by ferric reducing-antioxidant assay (FRAP) assay of enriched whey samples with liquid (LA) or powder (PA) extracts of *Myrtus communis* and *Arbutus unedo* fruits

DPPH assay

EC_{50} values (mg mL^{-1})

	Myrtus communis (LA)	*Myrtus communis* (PA)	Statistical analysis	*Arbutus unedo* (LA)	*Arbutus unedo* (PA)	Statistical analysis	Control (whey)
T_0	0.77 ± 0.02	0.74 ± 0.04	NS	0.75 ± 0.02 a	0.62 ± 0.02 c	$P < 0.01$	ND
T_{30}	0.76 ± 0.01	0.75 ± 0.03	NS	0.73 ± 0.02 a	0.62 ± 0.02 c	$P < 0.01$	ND
T_{60}	0.73 ± 0.04	0.73 ± 0.03	NS	0.73 ± 0.01 a	0.67 ± 0.05 b	$P < 0.027$	ND

FRAP assay

nmol μL^{-1}

	Myrtus communis (LA)	*Myrtus communis* (PA)	Statistical analysis	*Arbutus unedo* (LA)	*Arbutus unedo* (PA)	Statistical analysis	Control (whey)
T_0	13.4 ± 0.06	13.5 ± 0.42	NS	12.6 ± 0.21a	12.5 ± 0.19 a	$P < 0.247$	ND
T_{30}	13.7 ± 0.03	13.6 ± 0.08	NS	12.6 ± 0.07 a	12.2 ± 0.23 b	$P < 0.003$	ND
T_{60}	13.7 ± 0.02	13.6 ± 0.10	NS	12.5 ± 0.14 a	12.3 ± 0.07 b	$P < 0.034$	ND

Note: The values are presented as mean ± SD ($n = 3$). Letters represent significant differences among time-points (T_0, T_{30}, or T_{60}) within the same sample. ND, not detected; NS, not significant.

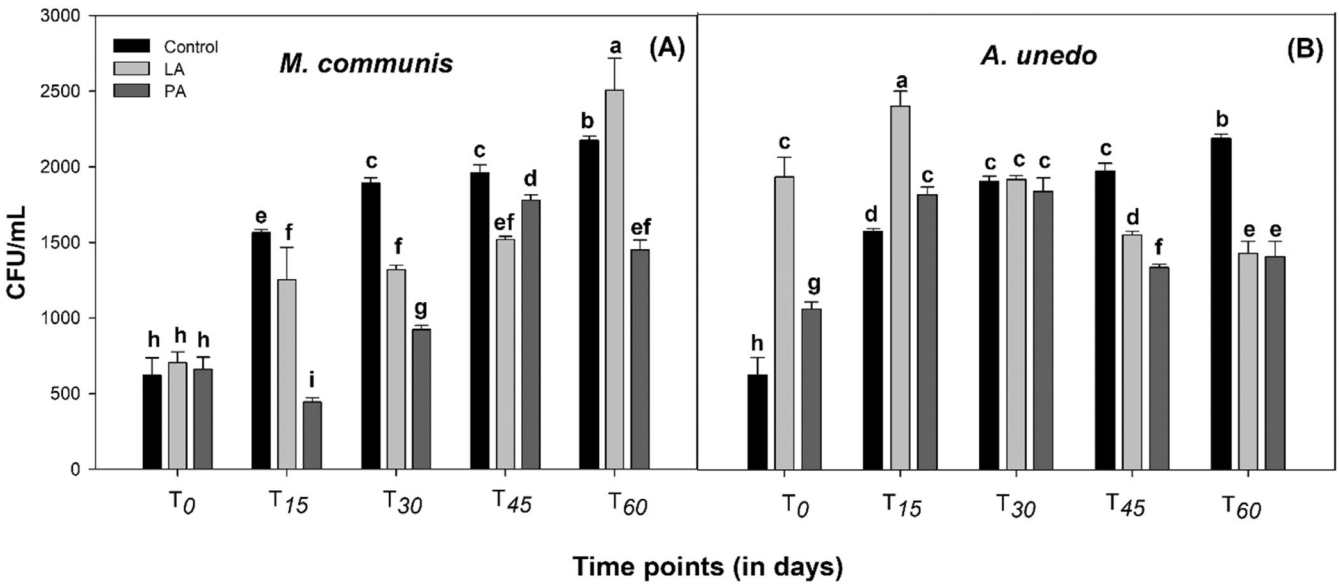

Figure 2. Monitoring of lactic acid bacteria present in pure whey (black bar) and in whey enriched with the liquid (LA, dark gray bar) or powder (PA, light gray bar) extracts of *Myrtus communis* (A) and *Arbutus unedo* (B), during the entire analysis period (60 days of storage). Values are presented as the mean ± SD ($n = 3$). Letters represent significant differences ($P \leq 0.001$) among time points within the same sample.

the development of the biofortified whey. The majority of polyphenols present in the two extract forms showed a remarkable chemical stability for 60 days, and this may have also contributed to the microbiological stability of the final products.[23] The slight decrease in polyphenol content in whey enriched with *A. unedo* PA extracts could be attributed to the formation of milk-polyphenol interactions that might have hampered the detection of polyphenols.

Whey contains approximately 50% of the nutrients of milk, mainly lactose (approximately 70%), proteins (approximately 14%, including caseins), vitamins, minerals and small amounts of fat.[24] The complex matrix of whey may explain the interactions between the hydroxyl groups of polyphenols with whey protein, leading to a lower detection of polyphenols in whey enriched with PA and LA extracts compared to the pure extracts. This matrix effect has already been showed in milk added with extracts of blueberry and black tea because it resulted in a reduction in the apparent content of the phenolic compounds in the final beverage compared to the total phenolic content of the added blueberry and black tea extracts.[25,26] However, further studies have

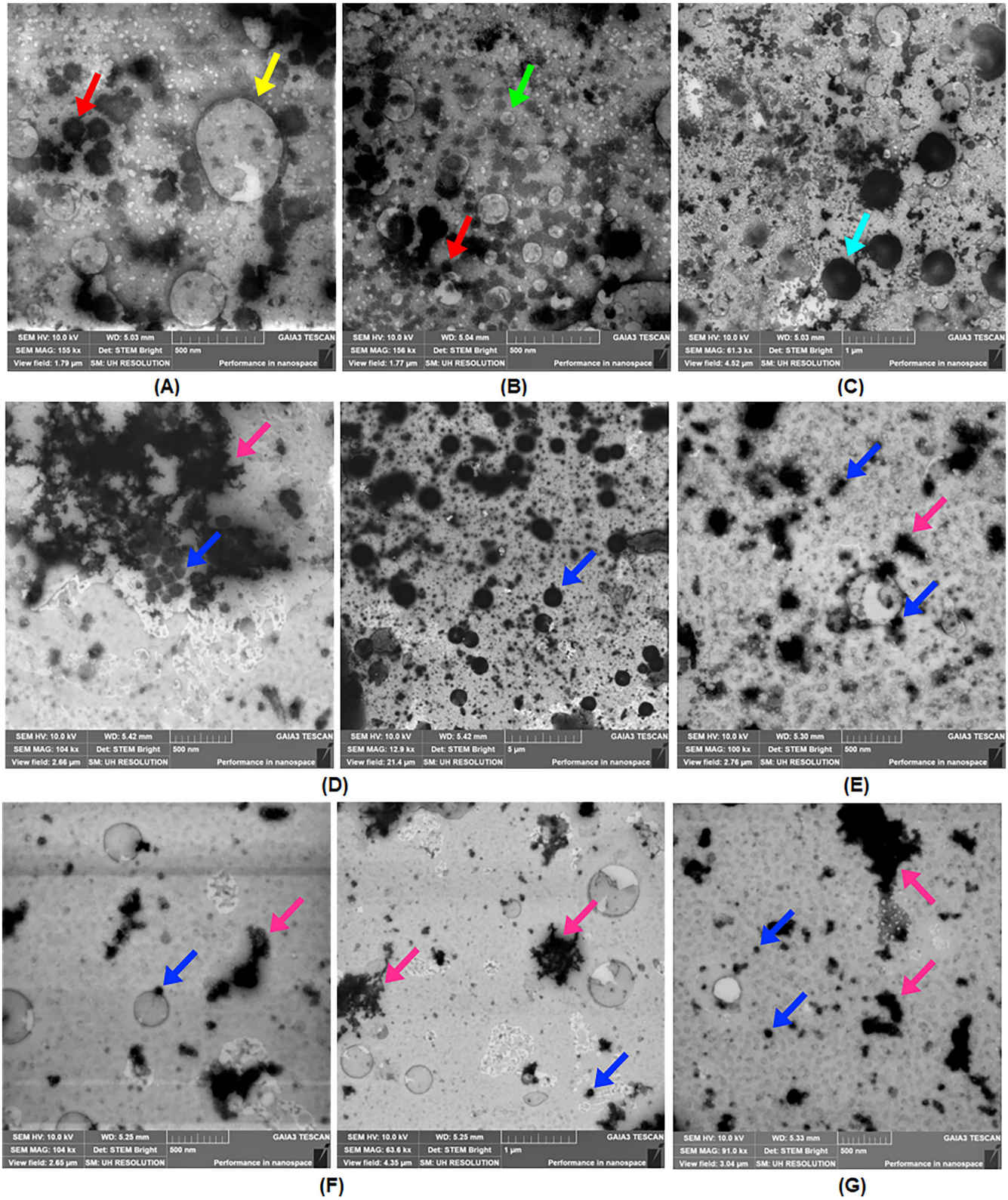

Figure 3. Size, morphology and distribution of aggregated structures and complex clusters of the whey dispersed phase (scale bars = 500 nm) (A,B) and 1 μm (C), and structure of whey sample with LA (D) and PA (E) of *Myrtus communis* (scale bars = 500 nm and 5 μm) and whey sample with LA (F) and (PA) (G) of *Arbutus unedo* (scale bars = 500 nm and 1 μm). Pink arrows indicate casein chains dispersed in the preparation; blue arrows indicate intact casein micelles.

shown that the protein–polyphenol interaction is reversible during digestion, leading to the release of phenolics and ensuring the final bioavailability of these compounds.[26,27]

Regarding the chemical stability of enriched whey, pH is a crucial parameter as its variations can affect both the technological and sensory properties of whey. In the present study, the gradual

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decrease in pH during storage period may be attributed to the metabolic activity of LAB, which can inhibit the development of pathogenic bacteria.[28] These results are consistent with previous studies, where a decrease in pH during storage was observed in milk products after the addition of polyphenolic extracts, even at cool temperatures.[15]

The antioxidant activity of whey added with the extracts was stable throughout the storage time, following the same trend of the polyphenolic content. Whey enriched with *A. unedo* extracts showed an antioxidant activity comparable to that added with *M. communis* extracts, although strawbery tree fruits have a lower polyphenol content compared to myrtle fruits. We speculate that this result may have been caused by other antioxidant compounds, such as terpenoids (betulinic acid and lupeol) and vitamins C (ascorbic acid) and E (α-tocopherol, γ-tocopherol), generally present in *A. unedo* extracts,[10] that were not evaluated in our study.

Polyphenols have been recently recognized as potential prebiotics with positive effects on the human microbiota because these compounds may positively affect the LAB metabolism.[29] The presence of lactic acid bacteria in all the tested samples indicates that the addition of polyphenol fruit extracts from *A. unedo* and *M. communis* to whey may positively affect the growth of lactic acid microflora, thus enhancing its prebiotic properties. Our results confirm the positive effect exerted by myrtle and strawberry fruit extracts on lactobacilli in dairy products.[27,30] In addition, M'Hir *et al.*[31] also obtained an increase in LAB after the addition of the myrtle juice in whey permeate and kefir and this was explained by the prebiotic effect of myrtle polyphenols. The present study also demonstrates the potential antimicrobial activity of whey enriched with PA of *A. unedo* fruit extracts, which also inhibited the growth of *S. aureus* compared to the other extracts.

The DLS analysis, as used to explore the dimensions and architectures of aggregated micellar systems and clusters containing complex arrays, showed a more homogeneous distribution after adding the extracts with the polydispersity index around 0.3, a rather very high value for such a complex matrix.[32] Despite the more homogeneous size distribution measured by DLS, adding PA and LA extracts to whey affected casein micelles to different extents as evidenced by STEM analyses. As previously reported, caseins exist in pure whey as micellar dispersions because the amphoteric nature of casein molecules allows electrostatic interactions and the formation of possible complex arrays with a relatively uniform distribution of non-polar, polar and charged groups.[33] The morphology and architecture of the array structure of the tested samples clearly show that mainly PA and LA of *A. unedo* extracts affected casein micelles, probably because of the high amount of tannins that can partially modify casein micelles as a result of their high affinity towards the proline groups of this class of proteins.[34] The higher total tannin content and different qualitative profiles of tannins (catechin derivatives, gallic and ellagic acid, and arbutin) in the extracts of *A. unedo* with respect to *M. communis* may be considered responsible of insoluble complexes with milk proteins including β-casein.[35]

CONCLUSIONS

The liquid and powder addition of decoction of *M. communis* and *A. unedo* to whey confers extra healthy properties to this by-product. The addition of *M. communis* extracts to whey, in both forms, resulted in a product with a high polyphenolic content that remained chemically and microbiologically stable for at least 60 days. Regarding *A. unedo* extracts, the utilization of the liquid addition was the best method for the functionalization of whey because the product was chemically stable during the experimental period. The presence of high amounts of polyphenols in the fruit extracts resulted in enriched whey with a high antioxidant activity. Furthermore, our study demonstrated the presence of lactic acid bacteria in all samples, supporting the potential application of this new functional food in the nutraceutical sector as prebiotics. In conclusion, we propose the development of a novel functional product obtained by the addition of the decoction of two Mediterranean plants in a dairy waste product with numerous potentially beneficial effects on human health. The addition of these extracts, either in liquid or powder form, to whey can be applied by the food industry to create new whey-based products characterized by antioxidant properties, prebiotic activity and good stability over time.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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