LWT - Food Science and Technology 173 (2023) 114404

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Processing of raw donkey milk by pasteurisation and UV-C to produce freeze-dried milk powders: The effect on protein quality, digestibility and bioactive properties

Tullia Tedeschi [a], Maria Aspri [b], Cecilia Loffi [a], Luca Dellafiora [a], Gianni Galaverna [a], Photis Papademas [b,*]

[a] Department of Food and Drug, University of Parma, Parma, Italy
[b] Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

ARTICLE INFO

Keywords:
Donkey milk
Bioactivity
Non-thermal processing
UV-C
Protein functionality

ABSTRACT

Non-thermal processing of milk has been considered over the past decade as an alternative or adjunct to thermal processes. UV-C radiation in combination with turbulent flow of opaque liquids seems to be a promising non-thermal method for the reduction of bacterial populations in milk. Apart from confirming the efficacy of UV-C in destroying pathogens and spoilage bacteria, there is a need for assessment of the quality characteristics of the end-product and especially in added-value dairy products where bioactivity of constituents should be preserved during processing. Under this context, freeze-dried donkey milk powder processing by UV-C was studied and the effect on protein quality, digestibility and bioactive properties were assessed. Results show that UV-C treatment retains the protein's quality characteristics highly comparable to the not-treated milk (i.e. raw) rather than the pasteurized milk where some deterioration (i.e. lower bioactivities) was detected.

1. Introduction

Donkey milk is steadily gaining attention and due to its similar chemical composition to human milk, there has been a growing interest for its commercialization. It is considered suitable for infants with cow milk protein allergy, the immunocompromised, and for the elderly (Souroullas et al., 2018). Donkey milk's protein composition; i.e. low casein: whey ratio is responsible for its lower allergenicity, which make it a possible alternative or a supplement in those cases where infants are not breastfed and thus need to be fed with milk-based infant formulas. The latter are usually based on cow-milk and are the main cause of protein allergy in children younger than three years (Martini et al., 2015). The hypo-allergenicity of donkey milk and its low casein content can be ascribed to the formation of a softer curd during digestion; in the stomach, the acidic environment (pH = 2–3) of the stomach donkey milk forms a more easily digestible clot because of the lower protein content and, especially, for the lower casein content (Claeys et al., 2014; Fiocchi et al., 2022; Marletta et al., 2016).

In more detail, donkey milk has a relatively lower protein content (1.3%–1.8%) than bovine milk (3.1%–3.8%), whereas it is more similar to human milk (0.9%–1.7%). Despite inter-individual fluctuations, the casein-to whey ratio is also different; the latter is approximately 40/60 in human milk, 55/45 in donkey milk, and 80/20 in bovine milk (Aspri et al., 2017; Fantuz et al., 2016). Donkey milk has its caseins in a decreasing order as follows: β- (54.3% of total caseins) > as1- (35.6%) > as-2 (7.19%) > k-CN (2.79%) (Cosenza et al., 2019). The three most abundant whey proteins in donkey milk are α-lactalbumin (1.80 mg mL^{-1}, 22.7% of total whey proteins), β-lactoglobulin (3.75 mg mL^{-1}, 29.9%) and lysozyme (1.00 mg mL^{-1}, 21.0%). Other whey proteins were reported to be present in donkey milk, like immunoglobulins (Igs) (11.5% of total whey proteins), blood serum albumin (6.2%), and lactoferrin (4.5%) (Salimei et al., 2004). Regarding the amino acid composition of donkey milk, there is not much information in the literature. Donkey milk contains higher values of serine, glutamate, arginine and valine than bovine milk, and higher levels of most essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, valine), while less cysteine (Aspri et al., 2017; Fantuz et al., 2016). Fat content of donkey milk is approximately 0.5%–0.7%, where its fatty acid profile is similar to human milk, with a high content of essential fatty acids and a lower one of saturated fatty acids (SFAs). Similarly, to human milk, donkey milk fat also contains also high

* Corresponding author.
E-mail address: photis.papademas@cut.ac.cy (P. Papademas).

https://doi.org/10.1016/j.lwt.2022.114404
Received 9 June 2022; Received in revised form 22 December 2022; Accepted 27 December 2022
Available online 28 December 2022

List of abbreviations

SFAs	Saturated Fatty Acids
PUFAs	Polyunsaturated Fatty Acids
UV	Ultraviolet light
PTFE	Polytetrafluoroethylene Polymer
MW	Molecular Weight
SSF	Simulated Salivary Fluid
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
DH	Degree of Hydrolysis
SDS	Sodium Dodecyl Sulfatesulphate
NAC	N-acetylcysteine
ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2, 2 diphenyl-1-picryl hydrazyl
HHL	Hyppuryl–Histidil–Leucine (HHL
HA	Hippuric Acid
SD	Standard Deviation
XICs	Extract Ion Chromatograms (XICs)
EFSA	European Food Safety Authority
OPA	o-phthaldialdehyde

levels of polyunsaturated fatty acids (PUFAs), with a high amount of linoleic acid, a lowω-6 to ω-3 ratio and a high unsaturated/SFA ratio (Salimei & Fantuz, 2013). The lactose content of donkey milk is approximately 6.5%, thus higher than bovine milk (4.1%–4.4%), which makes donkey milk less suitable for lactose intolerant people. Moreover, the high lactose amount of donkey milk is also responsible for its very acceptable (sweet) taste.

In vitro studies showed that donkey milk β-lactoglobulin is more digestible than the bovine milk one, being more easily degraded (70%) by human gastric and duodenal juice (Tidona et al., 2014). α-lactalbumin, which is the main protein in human milk, is the second more abundant whey protein in donkey milk, followed by lysozyme; the latter contributes to its antimicrobial activity and relatively long shelf life, by reducing bacterial growth. Moreover, lysozyme has an important role in stimulating intestinal immune response of gut microbiota in people with a low immunity, like children and the elderly (Brand-Williams, Cuvelier, & Berset, 1995).

Despite being effective against food pathogens and spoilage bacteria, thermal treatments could lead to the disruption of important bioactive compounds, modifications of proteins and to impair the technological and organoleptic properties of milk (e.g., prolong the enzymatic milk protein coagulation and the formation of undesirable flavor compounds) and loss of nutrients (i.e. vitamins) (Papademas et al., 2021). Similarly, Hazeleger and Beumer (2016) argued that equine milk heating negatively affects the beneficial to health effects, therefore it needs to be consumed raw. Martini, Altomonte, Licitra, and Salari (2018) proved that the antioxidant activity after HTST treatment is reduced, probably due to the reduction of peptides and lysozyme, while illustrated that in dry-powdered donkey milk, thermal treatment at 70 °C initiated denaturation of lysozyme. Ozturkoglu-Budak (2018), conducted a study about thermal treatment of donkey milk and when milk was heated to 85 °C/2 min, lactoferrin was not detectable (by RP-HPLC) and the amount of lysozyme and β-lactoglobulin was reduced significantly.

UV-C treatment has been described to offer several advantages including faster microbial inactivation, less flavor and nutritional loss, and lower energy use (Koutchma et al., 2019). The information on changes occurring to the components and properties of donkey milk, after UV-C processing, is rather limited. In a previous study by Papademas et al. (2021) the use of turbulent flow UV-C was described as an alternative non-thermal method for milk sanitation when compared to low temperature long time (62.5 °C/30 min) pasteurization.

In the present study UV-C treated (max temperature 35 °C) and pasteurized (62.5 °C/30 min) donkey milk samples were produced. These samples were freeze-dried to produce milk powders. The possible effect of processing (heat vs non-heat treatment) on milk protein quality, digestibility and bioactive properties was studied. Furthermore, the milk's protein quality and bioactive properties were assessed after applying the in vitro gastrointestinal digestion protocol to the freeze-dried (UV–C and pasteurized) samples.

2. Material and methods

2.1. Materials

Unless otherwise specified, all reagents and standards (i.e. peptides, whey proteins) were from Sigma Aldrich (St. Louis, MO, USA). All solvents used for UPLC-MS, LC-HRMS/MS and LTQ-Orbitrap analysis were HPLC grade (HiPerSolv CHROMANORM®) and purchased from VWR International, Ltd. (Poole, United Kingdom) and from Scharlab S.L. (Scharlab, Barcelona, Spain). The fluorophore employed to derivatize amino acids (AccQ*Fluor Reagent kit) was from Waters (Milford, MA, USA), and the amino acids standard mix (Amino Acid Standard H) from Thermo Fischer Scientific (Waltham, MA, USA). Moreover, 5 μm and 0.45 μm filters were from Millipore Co. (Burlington, MA, USA).

2.2. Samples

Donkey milk (2 batches, 7 L per batch) were supplied by the "Golden Donkeys Farm", Larnaca, Cyprus. All donkeys were fed the same diet consisting of hay, barley, corn, and a concentrate of minerals, vitamins, and salt, according to the European Directive 98/58/EC. Donkeys were healthy, and were not administered with antibiotics before sampling. Milking was carried out in the stable, and donkeys were milked manually from the same milker. During milking, the udder was cleaned using sterile wet wipes and the nipples using 70% ethanol and dried with sterile gauze. Samples were subjected firstly to UV-C and pasteurization treatments followed by freeze-drying. In this study three milk samples were used: 1-Raw donkey milk powder, 2-Pasteurized donkey milk powder, 3-UV-C treated donkey milk powder. They were subjected to an in vitro gastrointestinal digestion protocol, producing the following other samples: 4- Digesta-raw donkey milk, 5- Digesta- Pasteurized donkey milk, 6- Digesta- UV-C treated donkey milk.

2.3. Milk processing

2.3.1. UV-C

Donkey milk (2 batches, 7 L per batch) was treated with UV-light in a

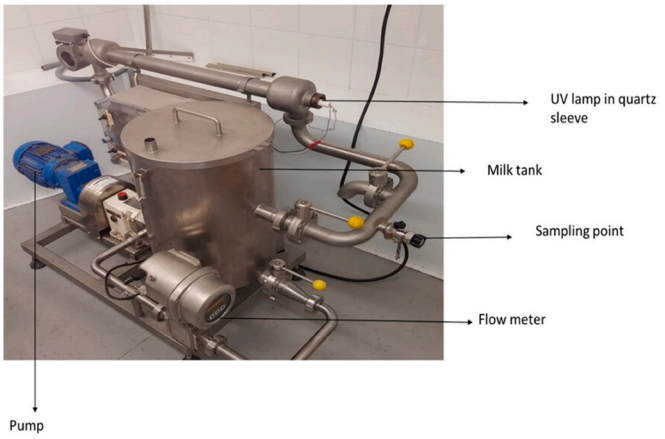

Fig. 1. SurePure SP1 UV-C system.

pilot-scale, low-power UV-C system (SP-1, Sure Pure) (Fig. 1). The system contained a UV lamp enclosed in an optical quartz sleeve which separates the milk from the UV-C light. The "SurePure Turbulator™" UV-C device creates turbulent flows and is designed to inactivate turbid fluids such as milk in a continuous flow. The SP-1 unit was operated at a flow rate of 4000 L h^{-1} (1.11 L^{-1}) and the UV dose was 1000 JL^{-1}. The UV-C dose was chosen based on a previous study carried out by Papademas et al. (2021).

2.3.2. Pasteurization

Pasteurization of donkey milk (7 L) has been carried out at Golden Donkeys Farm at 62.5 °C for 30 min with continuous stirring using an Evopasto 30 pasteurizer (Telme, Codogno, Italy), which is the gold standard treatment of a (milder) heat processing of donor human milk (Peila et al., 2017).

2.3.3. Freeze-drying of UV-C, pasteurized and raw donkey milk samples

Raw, UV-C and pasteurized donkey milk were poured into stainless steel rectangular trays and frozen to −80 °C, overnight before being transferred to the freeze -dryer. The next day, the samples were freeze-drying using a freeze-dryer Sublimator 3 × 4 × 5 (Zirbus Technology, Bad Grunt, Germany). The samples were transferred to the freeze-dryer operating at a maximum temperature of 35 °C in a condensation chamber under vacuum at a maximum pressure of 0.3 mbar. The freeze-drying was completed in 72 h. The obtained powders were kept under vacuum in sealed polyethylene bags for further analysis.

2.4. Total nitrogen content

To determine the total nitrogen content of raw, pasteurized, and UV-C treated donkey milk powder (samples 1–3) the standard Kjeldahl protocol, EC 152/2009 was applied., using a DKL Heating Digester and UDK 139 Semi-Automatic Distillation Unit (VELP SCIENTIFICA, Usmate Velate, MB, Italy). The analysis was performed in duplicate. A conversion factor of 6.38 was employed to calculate the protein content (g/100 g) from Kjeldahl nitrogen content.

2.5. Extraction of soluble whey proteins fraction

0.9 g of each milk powder (samples 1, 2, 3) was reconstituted in 10 mL of Milli-Q water. The reconstituted milk was skimmed by centrifugation Eppendorf 5810 R (Eppendorf, Hamburg DE) (920×*g*, 15 min, 4 °C) and the upper fat layer was removed manually: this procedure was repeated twice. Caseins were precipitated from skimmed milk by addition of 10% (v/v) acetic acid to adjust the pH to 4.6 and by centrifugation (920×*g*, 15 min, 4 °C). The recovered supernatant was centrifugated again to remove remaining caseins. Filtration on 0.45 μm polytetrafluoroethylene polymer (PTFE) filter was performed on the obtained supernatant, yielding the desired donkey milk whey proteins solution. Samples were prepared as duplicates.

2.6. SDS-PAGE analysis

Whey proteins samples obtained as described in section 2.5 were analysed with SDS-PAGE according to an experimental procedure already reported in the literature (Gasparini et al., 2020).

2.7. UPLC-MS analysis

Whey proteins extract solutions obtained as described in section 2.5 were analysed by UPLC-MS. Prior to analysis, samples were centrifugated (3220 g, 10 min, 4 °C) and diluted 1:3 with Milli-Q water.LC-MS analysis was performed following the method set up by Buhler et al. (2019) with slight modifications. An ACQUITY UPLC® separation mode equipped with an Acquity UPLC© Protein BEH C4 column (300 Å, 1.7 μm, 2.1 mm × 150 mm) and coupled with an ACQUITY SQ ESI-MS system (Waters, Milford, MA, USA).was employed for UPLC-MS analysis. Gradient elution was set as follows employing eluent A (H_2O + 0.2% CH_3CN + 0.1% HCOOH) and eluent B (CH_3CN + 0.1% HCOOH)): 0–2 min 85% A, 2–15 min: 85% A to 67% A, 15–20 min 67% A, 20–23 min: 67% A to 0% A, 23–25 min 0% A, 25–27 min: 0% A to 85% A, and 27–32 min 85% A. The flow rate was 0.20 mL min^{-1}, injection volume 5 μL, column temperature 35 °C and sampler temperature 18 °C. The samples were analysed in the Full Scan mode with the following conditions: ionization type: positive ions, scan range: 100–2000 m/z, capillary voltage: 3.2 kV, cone voltage: 30 V, source temperature: 150 °C, desolvation temperature: 300 °C, cone gas flow: 100 Lh^{-1}, desolvation gas flow: 650 Lh^{-1}. All the samples were analysed in triplicate.

To quantify whey proteins, α-lactalbumin and β-lactoglobulin standards from bovine milk were employed to prepare a calibration curve, as reported by Gasparini et al. (2020). The prepared standard solutions were run according to a chromatographic method which has been already reported in the literature (Buhler et al., 2019). MassLynxTM V4.0 software was employed to process data from UPLC-MS (Waters Corporation, Milford MA, USA).

Donkey α-lactalbumin, β-lactoglobulin (isoforms I and II) and lysozyme in their native and modified forms were extracted from the MS spectra by means of Extract Ion Cromatogram Technique (XIC), employing characteristic ions of each protein. Identification of the lactosylated forms was obtained by considering an increase of native protein's molecular weight (MW) of 324 Da (lactose MW). After protein identification, the corresponding areas underlying each chromatographic peak from the XICs were integrated with QuanLynx software to quantify whey proteins (Waters Corporation, Milford MA, USA).

The degree of lactosylation of each protein was determined by the ratio of the area of the lactosylated form to the total amount of the protein as reported by Gasparini et al. (2020). The total amount of each protein was calculated by the sum of the areas of the same protein in the native and lactosylated forms. For β-lactoglobulin, the sum of the areas of isoforms I and II was considered in the total protein amount.

2.8. Total amino acids determination

Samples were treated and analysed according to a protocol already reported in literature (Buhler et al., 2019) with slight modifications. Briefly, the samples were hydrolyzed with HCl, then the amino acid residues were mixed with 20 μl of 2.5 mM Norleucine in 0.1M HCl.

For methionine and cysteine, the acid hydrolysis was preceded by performic acid oxidation. Briefly, 50 mg of sample were added with freshly prepared performic acid (9 vol of 95% Formic acid mixed with hydrogen peroxide) and kept at 0 °C for 16 h. A calibration standard solution was also prepared mixing a standard mixture 2.5 mM (Thermo Scientific, Waltham, MA, USA) with a mixture of amino acids 2.5 mM (Nor-Leucine, Cysteic acid, Methionine sulfone - Sigma Aldrich, St. Louis, MO, USA) in ratio 1:1. Samples were then derivatised, according to the manufacturer instructions, using AccQ-Fluor reagent kit (Waters, Milford, MA, USA) and analysed using a UPLC ACQUITY system coupled with an ACQUITY SQ ESI-MS system (Waters, Milford, MA, USA). The analysis was performed with an ACQUITY UPLC Protein BEH C18 (300 Å, 1.7 μm, 2.1 mm 170 × 150 mm) column (Waters, Milford, MA, USA) with an ACQUITY UPLC Peptide CSH™ C18 VanGuard™ (130 Å, 1.7171 μm, 2.1 mm × 5 mm) pre-column (Waters, Milford, MA, USA).

2.9. In vitro simulated gastrointestinal digestion of donkey milk samples

Freeze-dried donkey milk samples were subjected to in vitro gastrointestinal digestion according to the updated protocol recently described by Brodkorb et al. (2019). This model was developed by the COST action FA1005 INFOGEST and is based on human gastrointestinal physiologically relevant conditions. Prior to digestion, the activity of all enzymes and the concentration of bile extract were evaluated. Additionally, the donkey milk powder was reconstituted with water to milk

with 10% total solids. The entire digestion process was performed at 37 °C, and all solutions were preheated prior use to eliminate temperature fluctuations. Briefly, the protocol is based on the use of 3 simulating fluids, saliva at pH 7 (simulated salivary fluid, SSF), gastric juice at pH 3 (simulated gastric fluid, SGF) and intestinal (duodenal) juice at pH 7 (simulated intestinal fluid, SIF). For the oral phase digestion, donkey milk samples (5 g) were mixed with SSF and $CaCl_2$ (1.5 mM) in a ratio of 1:1 (v/v) and incubated at 37 °C for 2 min with continuous agitation. Then, the oral bolus was diluted 1:1 (v/v) with the pre-warmed SGF, which contained pepsin (2000 U mL^{-1} in the final digestion mixture) and 0.15 mM $CaCl_2$. pH was adjusted to 3.0 using HCl and incubated under shaking at 37 °C for 2 h. The sample from the gastric phase was mixed 1:1 (v/v) with SIF containing trypsin (100 U mL^{-1}), bile salts (10 mM in the final mixture) and $CaCl_2$ (0.6 mM). After adjusting to pH 7.0, the sample was incubated at 37 °C for 2 h with continuous shaking. The intestinal phase was stopped using 5 mM Pefabloc SC (4-(2- aminoethyl) benzenesulfonyl fluoride hydrochloride) and all samples were stored at −80 °C for further use. A control sample, which consisted of the gastrointestinal juices, enzymes, and water instead of donkey milk powder, was included in the experimental trials to evaluate the possible impact of the digestive enzymes on the subsequent analyses.

2.10. Sample preparation for peptide identification of in vitro digested samples

Digested milk (samples 4, 5, 6) were analysed to identify the peptides resulting from the in vitro gastrointestinal digestion. Samples were then centrifuged (11,000 g, 30 min, 4 °C) and the supernatants filtered on 0.45 μm PTFE filters and concentrated under nitrogen flux.

2.11. LC-HR MS/MS analysis of in vitro digested samples

In vitro digested samples were analysed according to a procedure already reported in the literature (Di Nunzio et al., 2022).using a Vion IMS QTof Mass Spectrometer (Waters, Milford, MA, USA) with the following parameters. Experiment type: peptide map (IMS), experiment type: MSe, source type: ESI, polarity: positive, analyser mode: sensitivity, mode: standard transmission, capillary: 3.00 kV, sample cone voltage: 40 V, source offset voltage: 80 V, source temperature: 120 °C, desolvation temperature: 450 °C, cone gas flow: 50 Lh^{-1}, desolvation gas flow: 800 Lh^{-1}. MSe mode: high definition MSe, acquisition time: 0–58.2 min, scan range: 100–2000 m/z, scan time: 0.4 s, low collision energy: 6V, high collision energy ramp: 20–45 V, automatic lock correction (leucine enkephaline).

Data from LC-HR MS/MS were processed with UNIFI software (Waters Corporation, Milford MA, USA), employing the following protein Uniprot protein codes: Q5XLE4 (Albumin · *Equus asinus*), P86273 (Beta-casein · *Equus asinus*), P86272 (Alpha-S1-casein · *Equus asinus*), P28546 (Alpha-lactalbumin · *Equus asinus*), P19647 (Beta-lactoglobulin-2 · *Equus asinus*), P13613(Beta-lactoglobulin-1 · *Equus asinus*), P11375 (Lysozyme C · *Equus asinus*), F0V6V5 (Kappa-casein · *Equus asinus africanus*), D6QX32 (Beta-lactoglobulin II variant C · *Equus asinus*), D6QX31 (Beta-lactoglobulin II variant B · *Equus asinus*), D2EC27 (D2EC27_EQUAS Beta-casein · *Equus asinus*), C1L3G3 (Alpha-S2-casein · *Equus asinus*), B7VGF9 (Alpha-S2-casein · *Equus asinus*). Allowed variable amino acid modifications were: deamidation (N, Q) pyroglutamic acid N-term (E, Q), oxidation (single or double, M or W), phosphorylation (S, T, Y). Nonspecific digestion reagent, minimum sequence length: 3.

2.12. Hydrolysis degree determination of in vitro digested donkey milk samples

Hydrolysis degree was calculated with the o-phthaldialdehyde (OPA) assay (Spellman et al., 2003). The degree of hydrolysis (DH) was calculated using the OPA method described by Spellman et al. (2003) [16] with some modifications. Briefly, the assay was performed adding 20 μL of the sample - suitably diluted - to 2.4 mL of OPA/NAC reagent, composed by: 5 mM o-phthaldialdehyde (OPA) (Sigma Aldrich, St. Louis, MO, USA), 5 mM N-acetylcysteine (NAC) (Merck Millipore, Burlington, MA, USA), 2% Sodium dodecyl sulfatesulphate (SDS) (Sigma Aldrich, St. Louis, MO, USA), 75 mM Borate buffer, in 1:9 Methanol: Milli-Q water, pH 9.5.

The measurement of absorbance was carried out at 340 nm using a JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklahoma City, OK, USA). For each sample, the measurement was taken three times and the mean was used for the calculation Also, the intrinsic absorbance of samples was measured adding 20 μL of the sample (diluted 1:20 with Milli-Q water) to 2.4 mL of Milli-Q water. To determine the DH a calibration curve was performed using L-isoleucine (2 $mgmL^{-1}$, 1 $mgmL^{-1}$, 0.5 $mgmL^{-1}$, 0.25 $mgmL^{-1}$, 0.125 $mgmL^{-1}$). The calibration curve was analysed in the same way as the samples. The DH% was calculated as the percentage of the ratio between free amino groups determined with the procedure and total amino groups of the sample.

2.13. Bioinformatic analysis

Peptides bioactivity was analysed with bioinformatics to identify peptide sequences previously chracaterized and stored in a reference database for bioactive peptides (i.e., BIOPEP-UWM; Minkiewicz et al., 2019). Specifically, peptide sequences resulting from LC-HRMS/MS analysis were iteratively searched into BIOPEP-UWM database, which included information of 4485 bioactive peptide (last database access 19th July 2022), employing a script developed "in-house" (available upon request).

2.14. Bioactivity assays

2.14.1. Antioxidant activity

The antioxidant activity of UV-C, pasteurized and raw donkey milk powders before and after in vitro gastrointestinal digestion was assayed using two different methods, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS) and 2, 2 diphenyl-1-picryl hydrazyl (DPPH) radical assays according to Aspri et al. (2018).

2.14.2. Antimicrobial activity

The antimicrobial activity of UV-C, pasteurized and raw donkey milk powders before and after in vitro gastrointestinal digestion was tested using well diffusion assay according to Aspri et al. (2018) against *Listeria monocytogenes* strains.

2.15. ACE-inhibitory activity

ACE-inhibitory activity of donkey milk powders after in vitro gastrointestinal digestion was determined according to the method described by Li et al. (2005). Briefly, a solution of hyppuryl–histidil–leucine (HHL, 5 mM) was prepared in sodium phosphate buffer (NaPB, 0.1 M, pH 8.3) containing NaCl (0.3 M). For each analysis, a donkey milk sample (20 μl) with 50 μL of 5 mM HHL was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 10 ml of ACE solution (100 $mUml^{-1}$). Samples were incubated for 30 min at 37 °C, and then 100 μL of HCl (1 M) were added to stop the reaction. Sodium borate buffer was then added to the reaction mixture to a volume of 0.5 mL. The concentration of hippuric acid (HA) produced at the end of the reaction was determined spectrophotometrically (Infinite PRO 200, Tecan, Switzerland) at 492 nm. All measurements were performed in triplicate. ACE inhibitory activity of each sample was calculated as follows: $ACE\ inhibitory\ activity\ (\%) = \frac{(B-A)}{(B-C)} * 100$, where B is the absorbance of control (buffer added instead of test sample), C the absorbance of the reaction blank (HCl was added before ACE), and A the absorbance in the presence of sample.

2.16. Statistical analysis

Statistical analysis was performed employing SPSS software (IBM SPSS Statistics Data Editor, Armonk, NY, USA). Univariate Analysis of Variance (ANOVA) was performed employing post-hoc Tukey's test and Duncan's test. The differences among samples were considered significant with $P < 0.05$.

3. Results and discussion

3.1. Protein content characterization

The protein content (%) of raw, pasteurized, and UV-C treated donkey milk powders were calculated on dry matter and values reported were 15.29 ± 0.34, 15.34 ± 0.18, 15.67 ± 0.20 respectively. The values are expressed as mean ± standard deviation (S.D.) of two sample replicates. The protein content was not significantly different between the three samples. (ANOVA, post-hoc Tukey's test, $p < 0.05$). The protein profile of each sample was analysed through SDS-PAGE as shown in Fig. S1, Supplementary Information.

The three most abundant whey proteins in donkey milk, namely; β-lactoglobulin, α-lactalbumin, and lysozyme, are present in all samples. More specifically, the band of β-lactoglobulin appears at nearly 20 kDa, at 15 kDa the band of lysozyme and at around 14 kDa the one of α-lactalbumin. Furthermore, in all samples (except pasteurized sample, entry 2) it is possible to see three light bands at around 75 kDa; that in the middle (exactly at 75 kDa) can be ascribed to lactoferrin, that below to serum albumin, and the above one to high MW immunoglobulins. Finally, the light band immediately above 50 kDa may correspond to low MW immunoglobulins and the bands between 25 and 37 kDa to some remaining caseins. The protein pattern is consistent with those already published for donkey and mare milk (Guo et al., 2007; Miranda et al., 2004; Salimei et al., 2004), and does not show particular differences among samples subjected to different treatments.

3.2. Identification of whey proteins

Identification of donkey whey proteins in their native and lactosylated forms was performed with UPLC-MS analysis according to the analytical method reported in Section 2.8. Each compound was searched in the MS spectra employing its characteristic ions to obtain Extract Ion Chromatograms (XICs). MS ions associated to raw and pasteurized samples are reported in Table S1, Supplementary Information (S·I.).

As an example, the full-scan chromatogram of raw donkey milk sample is reported in Fig. 2A. In Fig. 2B, the XIC of native α-lactalbumin is shown, with its retention time at 16.73 min; in Fig. 2C and D, the XICs of the native form of β-lactoglobulin II (β-lac II) and β-lactoglobulin I (β-lac I), with a retention time of 17.04 and 17.48 min, respectively. The same retention times are also indicative of those present in the other samples.

β-lactoglobulin I MS spectra from raw donkey milk and pasteurized donkey milk samples are reported in Fig. 3A and B, respectively. Raw (A) and pasteurized (B) donkey milk samples shared the same ion pattern in the multicharged MS spectra. In Fig. 3A and B the ions corresponding to the lactosylated and dilactosylated forms of the protein are also present.

On the other hand, the UV-C treated sample (C) led to a different ion pattern in β-lactoglobulin I MS spectra. The different spectral pattern in the UV-C treated sample can be associated to modified forms of β-Lg I

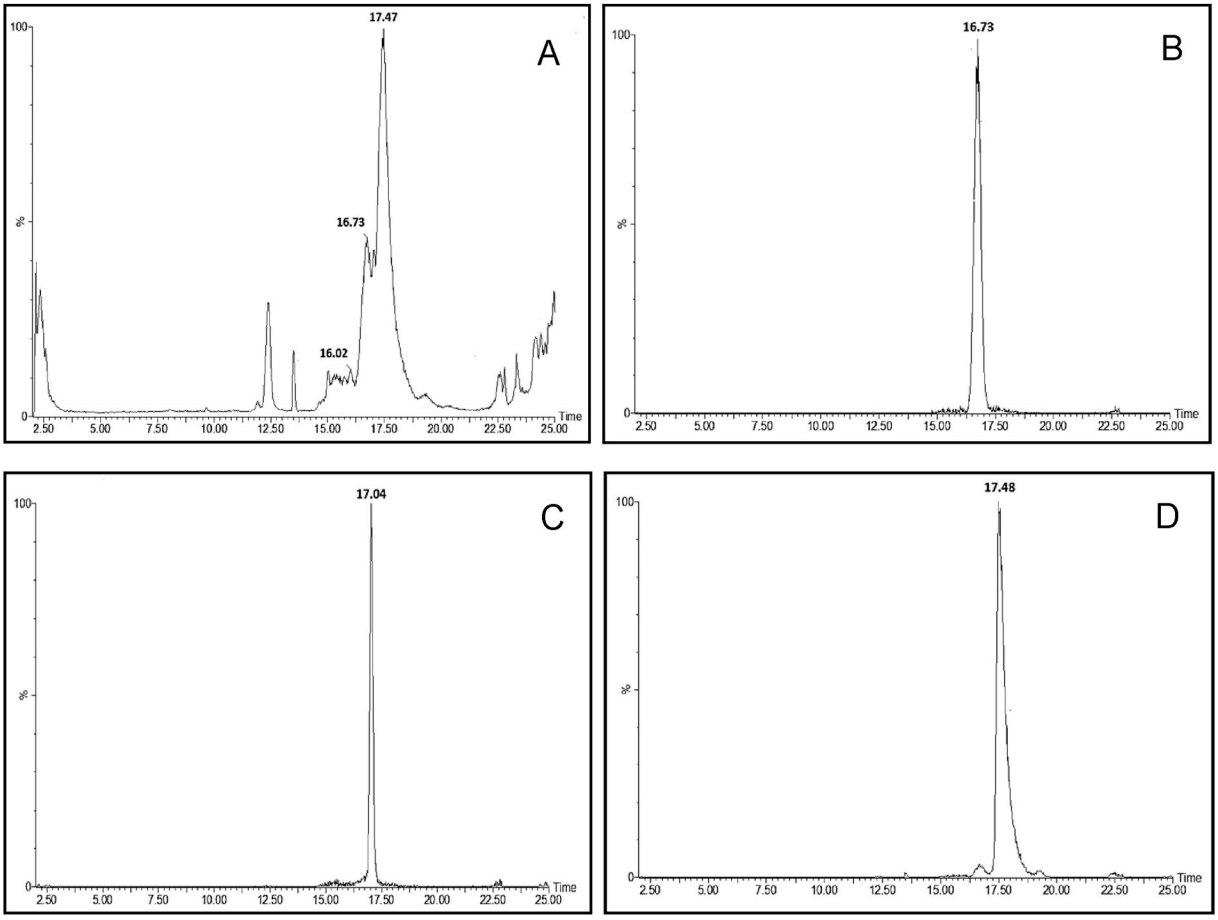

Fig. 2. UPLC-MS Full-scan chromatogram of whey proteins from raw donkey milk sample (A) and the respective extract-ion chromatograms (XICs) of native α-lactalbumin (B), native β-lactoglobulin II (C), and native β-lactoglobulin I (D). Relative intensity is on the y axis, time on the x axis.

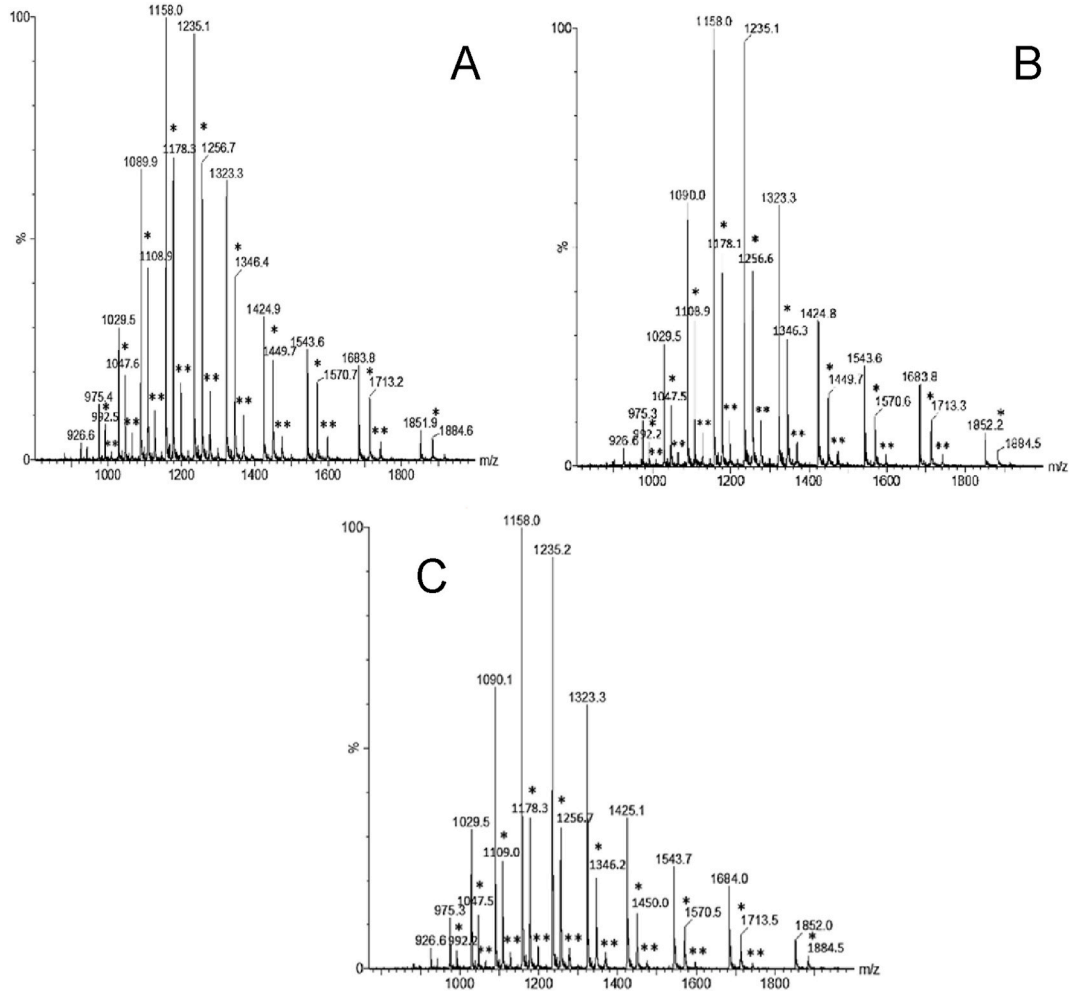

Fig. 3. β-Lg I MS spectrum from raw (A) pasteurized (B), UV-C treated (C) donkey milk samples. Single star and double star highlight the MS ions referred to the lactosylated and dilactosylated forms of the protein, respectively. Relative intensity is reported on the y axis, while m/z on the x axis.

and β-Lg II. Spectral deconvolution yielded a molecular weight corresponding to that of the native proteins plus 15 Da or 31 Da, that could be ascribed to single or doubly oxidized Met or Trp residues (Scheidegger et al., 2010). A similar trend was observed also in B-type lysozyme, which was found in its unmodified forms in raw and pasteurized samples, whereas only in its modified forms in the UV-C treated sample. For all the three whey proteins, lactosylated forms were also identified, as shown in Tables S1 and S2 (S·I.).

Whey proteins were quantified in all the samples employing an external calibration curve, as described previously (Gasparini et al., 2020). The values of the concentrations (mg mL^{-1}) of total, native and lactosylated forms of α-La and β-Lg (I and II isoforms) are listed in Table S3 (S·I.). Regarding β-Lg, the total amount of protein, comprising of native and lactosylated derivatives, was significantly lower in the pasteurized sample than the raw and the UV-C treated ones (ANOVA, post-hoc Tukey's test, $p < 0.05$). On the other hand, the total α-La content was significantly lower in the UV-C sample, when compared to raw and pasteurized. As a general trend, the total content of whey proteins seems to slightly decrease ranging from raw samples, to pasteurized and UV-C treated, as shown in Table S3.

The corresponding percentages of lactosylation were also calculated for each whey protein, as shown in Table S4. Regarding α-La, its native form was reported to be significantly different between raw, pasteurized and UV-C samples. The same trend was observed for the lactosylated forms. For β-Lg the raw sample was significantly different from pasteurized and UV-C, for both native and lactosylated forms, whereas no significant differences were present between pasteurized and UV-C samples, regarding lactosylation. (One-way ANOVA, post-hoc Tukey's test, $p < 0.05$). The corresponding percentages of native and lactosylated forms of α-La and β-Lg are reported in Fig. 4. In general, it seems that processing affects more the lactosylation degree of α-La than that of β-Lg.

The UV-C treated sample has the lowest amount of lactosylated derivatives and mostly in their monolactosylated forms, whereas dilactosylated derivatives are totally absent in α-La and in very low amount in β-Lg. Hence, UV-C irradiation does not negatively affect the whole protein quality, but, instead, leads to a lower lactosylation degree if compared to other technological treatments. These results agree with previous studies regarding cow's milk, in which the lactosylation degree acts as a control marker of the quality of the process. It has been widely reported that milk processing which involved heat (e.g., pasteurization) affects the protein integrity by promoting the glycation reaction (Buhler et al., 2019).

3.3. Total amino acids determination

In order to evaluate the nutritional value of the different samples, the total amino acid content was determined. Concentration of the amino acids as mean ± standard deviation (S.D.) for two replicates, as reported in Table S5. Regarding the distribution of each amino acid, i.e. total amino acid content and sulphured amino acids (cysteic acid and methionine sulfone), no significant differences were observed among

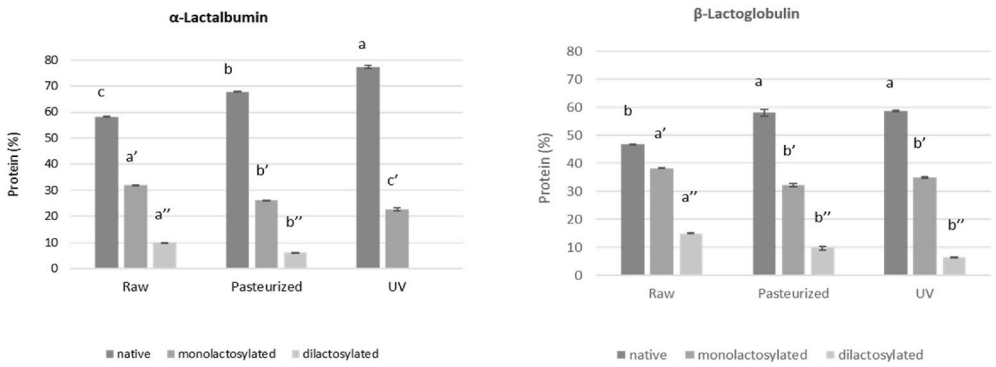

Fig. 4. Percentages of α-lactalbumin and β-lactoglobulin (as sum of the isoforms I and II) in their native and lactosylated forms in raw, pasteurized and UV samples. Different letters a, b, c indicate different significances, whereas three different statistics performed over the three different forms of α-La and β-Lg (a,b,c = native forms; a',b',c' = monolactosylated; a'',b'',c'' = dilactosylated) (One-way ANOVA, post-hoc Tukey's test, $p < 0.05$).

samples subjected to the three different technological treatments (One-way ANOVA, post-hoc Tukey's test, $p < 0.05$). The same can be stated for the ratio between methionine and methionine sulfone, for which no significant difference were reported.

Even if LC-MS analysis of UV-C treated samples showed the presence of some oxidized methionine residues, this modification seems not to affect the nutritional quality of the final product, as the total amino acid content is totally comparable to that of raw and pasteurized samples (see Fig. 5). Methionine was in fact determined also as the sulfone derivative, and the ratio between the two species has been found comparable for all the samples, thus indicating that there is no loss that affects the nutritional value of products. Very similar results are reported by European Food Safety Authority (EFSA, 2016), in the scientific report on UV-treated milk as a novel food, where potential protein oxidation was evaluated; the amino acids used as markers for oxidation (i.e. Methionine) showed no significant differences between UV-C treated and pasteurized milks. Moreover, EFSA (2016) assessed that oxidation products in UV-C treated milk does not affect milk quality and is not of safety concern.

3.4. Digestibility study

Raw, pasteurized and UV-C freeze-dried donkey milk samples underwent a simulated gastrointestinal digestion according to the updated protocol recently described by Brodkorb et al. (2019) to study the effect of technological treatment on digestion. The digestibility was evaluated through the calculation of the hydrolysis degree (DH%) by the spectrophotometric OPA method. Results are expressed as mean ± standard deviation over two replicates (digesta of raw donkey milk 37.02 ± 1.64; digesta UV-C treated donkey milk 37.19 ± 0.88, digesta of pasteurized donkey milk 44.31 ± 3.14). No significant differences were reported between raw, and UV-C treated digesta, whereas the pasteurized sample showed a significantly higher hydrolysis degree than the other two treatments (One-way ANOVA, Duncan's test, $p < 0.05$). This confirms that UV-C treatment does not have a significant influence on hydrolysis degree, and thus on protein digestibility, i.e. raw and UV-C samples are comparable after digestion. In vitro digested samples were analysed by LC-HRMS/MS analysis with Vion IMS QTof Mass Spectrometer (Waters, Milford, MA, USA) and processed by UNIFI (Waters, Milford, MA, USA) software. In Table S6 each peptide was classified according to the sample. Peptides were derived from both caseins (β-CN, αs1-CN, αs2-CN, κ-CN) and whey proteins (β-Lg I, β-Lg II, α-La, serum albumin and lysozyme). Regarding the whey protein fraction most peptides originated from β-Lg I and II, a few peptides from α-La and only one from lysozyme. This is in accordance with the fact that all caseins, the whey protein fraction as well as other high molecular weight proteins such as lactoferrin, serum albumin and γ-immunoglobulins, were completely hydrolyzed during the simulated gastrointestinal digestion protocol, whereas lower degradation profile was observed for α-lactalbumin and lysozyme (Inglingstad et al., 2010; F. Tidona et al., 2014; Aspri et al., 2018). It is possible to observe that the lowest number of peptides was found in raw milk samples (16 peptides), followed by UV-C samples (30 peptides), whereas the highest one was found in pasteurized samples (36 peptides). Interestingly, some sequences were found only in one type of sample and not in the other ones. This behaviour seems to indicate that the processing could affect the digestibility of the milk proteins.

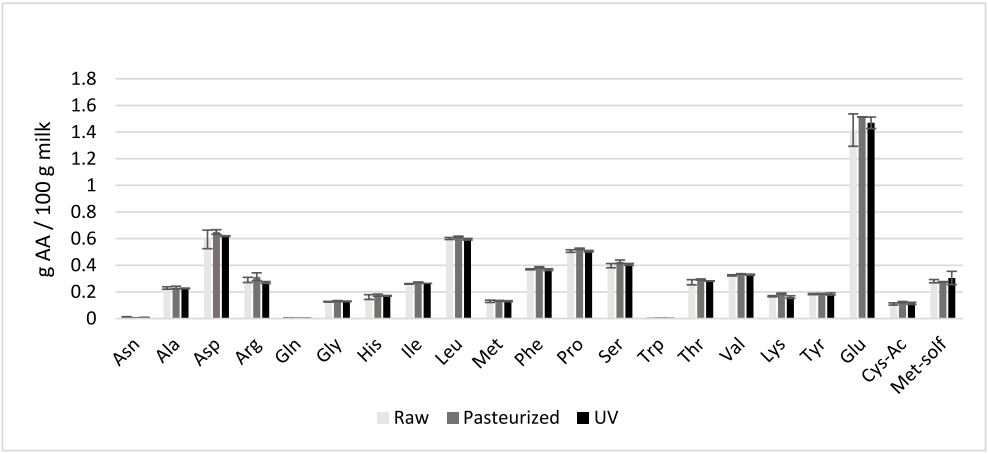

Fig. 5. Total amino acids (g AA/100 g milk) in donkey milk samples subjected to different technological treatments. (One-way ANOVA, post-hoc Tukey's test, $p < 0.05$, no statistical differences were observed).

A total of 27 non-redundant peptide sequences were identified from in vitro digested donkey milk samples, analysed as described above and further analysed in order to find any structure-activity relation with potential bioactivities. After an automatic search thorough an "in-house" bioinformatic workflow, none of those sequences matched the reference database of bioactive peptides (i.e., BIOPEP-UWM; Minkiewicz et al., 2019). On this basis, it was assumed that their biological activity must be assessed and characterized.

This evidence suggested that the bioactivity of the exact sequences identified in donkey milk samples has not been assessed yet. However, as a second step of analysis, the presence of bioactive peptides recorded in the BIOPEP-UWM database within the donkey's sequences was studied to describe the fraction of bioactive peptides encrypted in the list of peptides identified in donkey milk samples. The following bioactive peptides were found encrypted in 3 sequences from donkey milk samples (i.e. SEEAP, WQVLP and VGPPLPS).

As shown in Table 1, three peptides identified in donkey milk samples were encrypted in seven already characterized ACE inhibitory peptides. At present, the only peptide present in the MBPDP database (mbpdb.nws.oregonstate.edu) and established to have an ACE-inhibitory activity is the VAPFPQPVVP sequence (Bidasolo et al., 2012).

It is noteworthy that, for ACE-inhibitory activity, the presence of a proline (P), lysine (K), or phenylalanine (F) residue at the C-terminus can favour peptide-ACE enzyme binding (Aspri et al., 2018; Bidasolo et al., 2012). Indeed, VGP and GPP sequences reported in Table 1 revealed a quite potent inhibitory activity with the IC50 in the μM range: these fragments share the same structural motif as other potent ACE-inhibitory peptides, like IPP and VPP (Nakamura et al., 1995). These sequences were isolated from bovine β-casein f (74–76), IPP and f (84–86), VPP and also in bovine κ-casein for IPP peptide, as f (108–110).

On this basis, SEEAP, WQVLP and VGPPLPS may act as a source of highly potent bioactive peptides with an antihypertensive potential. In particular WQVLP and VGPPLPS have been found in all the digested samples coming from the three type of donkey milk (raw, pasteurized and UV treated), as it is shown in Table S6. However, their relevance as a source of antihypertensive peptides should be then assessed with in vitro tests to better understand the possible biological role of donkey milk samples under investigation.

3.5. Bioactivity assays

3.5.1. Antioxidant activity

The antioxidant activity of all donkey milk powder samples before and after in vitro gastrointestinal digestion was assessed with two different methods; ABTS and DPPH radical assays (see Fig. 6). All samples before digestion demonstrated antioxidant activity in both assays. Raw and UV-C donkey milk powder showed similar antioxidant activity while the lowest antioxidant activity was observed in pasteurized donkey milk powder. An enhanced antioxidant activity was assessed in all the samples after in vitro gastrointestinal digestion and followed the same pattern with undigested samples; this result agrees with previous studies, showing that the antioxidant activity of casein and whey proteins may be enhanced upon enzymatic hydrolysis. (Aspri et al., 2018; Hernández-Ledesma et al., 2005). This demonstrates that antioxidant compounds in donkey milk digesta are either resistant to the reaction with digestive enzymes and/or these enzymes are likely to generate novel antioxidant compounds, yielding an enhanced bioactivity upon digestion.

Table 1
Bioactive peptides encrypted in SEEAP, WQVLP and VGPPLPS sequences.

Sequence	Activity*	IC50(μM)*
EAP	ACE inhibitor	407.4
VLP	ACE inhibitor	320.0
VGP	ACE inhibitor	26.3
PLP	ACE inhibitor	430.0
GPP	ACE inhibitor	23.1
PPL	ACE inhibitor	427.2
PPLP	ACE inhibitor	not reported

•As per BIOPEP_UWM database.

3.5.2. Antimicrobial activity

Antimicrobial activity of raw, UV-C and pasteurized donkey milk powder samples before and after in vitro *gastrointestinal* digestion was assessed against a wide range of *L. monocytogenes strains* using agar diffusion assay (see Fig. 7). No antimicrobial activity was determined before digestion in all of the samples. Upon digestion, raw donkey milk showed the highest antimicrobial activity, followed by UV-C and pasteurized donkey milk. Similar results were obtained for pathogens like *Salmonella typhimurium*, *Bacillus cereus* and *Staphylococcus aureus* (data not shown). This may be due to the fact that the content of some bioactive proteins (i.e. Immunoglobulins, Lysozyme) were partially decreased due to pasteurization of the milk (data not shown), resulting in lower antimicrobial activity. These results agree with what Tidona et al. (2011) report regarding the effect of digestion with human digestive enzymes on donkey milk antimicrobial activity Additionally, Blackshaw et al. (2021) and Silvestre et al. (2008) reported the negative effect of thermal treatments on the bactericidal capability of human milk. On the other hand, Abu Bakar et al., 2021 demonstrated that the bioactivity of the most human milk proteins can be negatively affected by heat treatment.

3.5.3. ACE inhibitory activity

Hypertension nowadays represents one of the major causes of cardiovascular diseases. A conscious lifestyle and a healthy diet including the consumption of milks containing bioactive compounds could be helpful in regulating blood pressure and avoiding hypertension. Angiotensin Converting Enzyme (ACE) is an enzyme that plays an important role in the regulation of blood pressure; an effective strategy to control and prevent high blood pressure is the inhibition of this enzyme. In this frame, the ACE inhibitory activity was measured only in samples after in vitro gastrointestinal digestion since ACE-inhibitory peptides have to reach the bloodstream in an active form and resist intestinal peptidases' degradation to exert their antihypertensive activity. Our study has shown that pasteurized donkey milk powder had the lowest ACE-inhibitory activity (36 ± 2.05%) while the highest one was determined in raw milk powder (66 ± 1.70%). UV-C treated donkey milk powder gave a result of 56 ± 3.84%, relatively close to the raw donkey milk powder sample. Captopril, a well-known antihypertensive medication, was used as a control and its %ACE inhibitory activity was determined at 110 ± 4.27%. These results agree with the in-silico analysis performed in section 3.4, which highlights the presence of several ACE-inhibitory peptides.

4. Conclusion

This study illustrated to what extent could processing of donkey milk (pasteurization and UV-C) affect the protein quality, digestibility and bioactivities, when compared to a raw milk (i.e. control-no treatment). More specifically, UV-C (as a no-heat treatment) results were closer to the raw milk indicating that pasteurization (i.e. heat treatment) could lead to a higher degree of protein lactosylation. The digestibility of protein as expressed by the degree of hydrolysis showed a similar pattern i.e. similar values for raw and UV-C treated milk and higher values for pasteurized milk. Heat treatment of milk seems to decrease the functional (bioactive) character of the samples i.e. antibacterial, antioxidant and ACE inhibitory activity. As somewhat expected the general trend of the results obtained show that the UV-C treatment proves to be a minimal process comparable to raw milk (i.e. no treatment) whereas pasteurization negatively affects, to a certain degree, milk bioactivities and protein quality.

However, further studies are required to characterize the stability

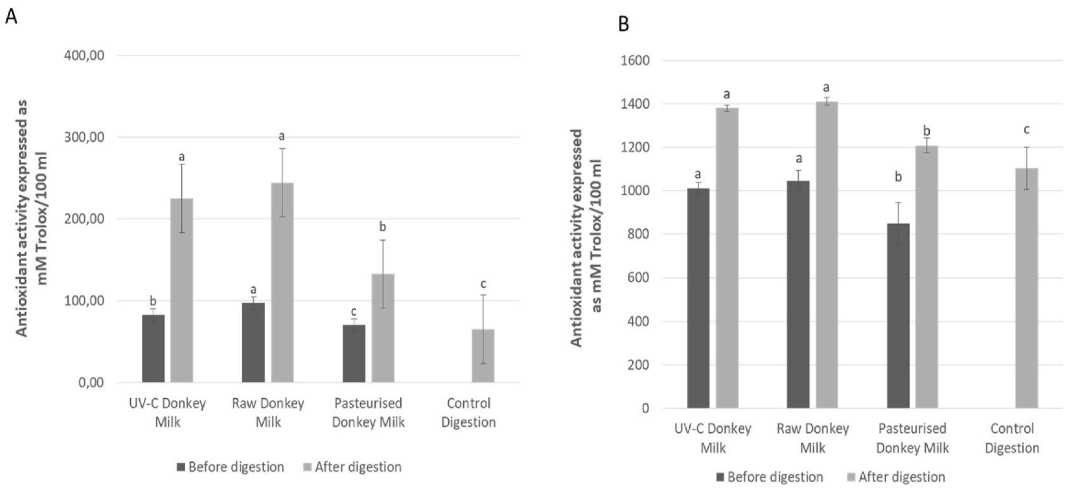

Fig. 6. Antioxidant activity of UV-C, pasteurized and raw donkey milk freeze-dried powders before and after in vitro gastrointestinal *digestion* using A) DPPH and B) ABTS assays. One-way ANOVA, post-hoc Tukey's test, $p < 0.05$.

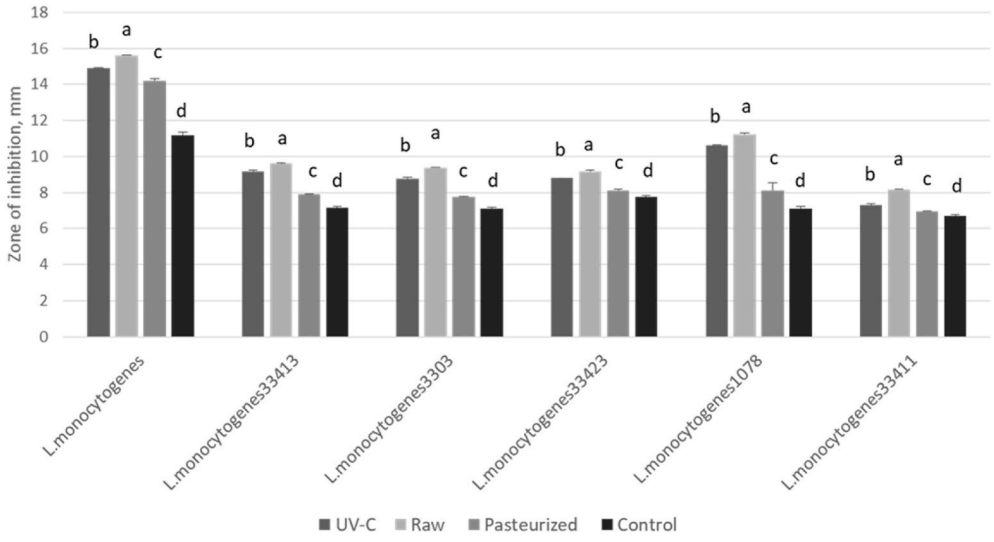

Fig. 7. Antimicrobial activity of UV-C, pasteurized and raw donkey milk freeze-dried powders against *Listeria monocytogenes* strains using the well diffusion assay. (One-way ANOVA, post-hoc Tukey's test, $p < 0.05$).

(chemical, microbiological) and the sensory profile during shelf-life of the UV-C treated, freeze-dried milk powders. This is essential in order to satisfy the quality criteria of infant food and to be considered as a supplement to existing milk formulae.

Funding

The project DELIVER (Enterprises/0916/0083) was funded by the Research and Innovation Foundation, Cyprus.

CRediT authorship contribution statement

Tullia Tedeschi: Investigation, Data curation, Supervision, Writing – review & editing, Writing – original draft. **Maria Aspri:** Investigation, Writing – original draft. **Cecilia Loffi:** Investigation, Writing – original draft. **Luca Dellafiora:** Investigation. **Gianni Galaverna:** Supervision, Writing – review & editing, Writing – original draft. **Photis Papademas:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors wish to acknowledge the contribution of Golden Donkeys Farm, Larnaca, Cyprus in providing the milk samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114404.

References

Abu Bakar, S. Y., Salim, M., Clulow, A. J., Nicholas, K. R., & Boyd, B. J. (2021). Human milk composition and the effects of pasteurisation on the activity of its components. *Trends in Food Science & Technology, 111*, 166–174. https://doi.org/10.1016/j.tifs.2021.02.055

Aspri, M., Economou, N., & Papademas, P. (2017). Donkey milk: An overview on functionality, technology, and future prospects. *Food Reviews International, 33*(3), 316–333. https://doi.org/10.1080/87559129.2016.1175014

Aspri, M., Leni, G., Galaverna, G., & Papademas, P. (2018). Bioactive properties of fermented donkey milk, before and after in vitro simulated gastrointestinal digestion. *Food Chemistry, 268*, 476–484. https://doi.org/10.1016/j.foodchem.2018.06.119

Bidasolo, I. B., Ramos, M., & Gomez-Ruiz, J. A. (2012). In vitro simulated gastrointestinal digestion of donkeys' milk. Peptide characterization by high performance liquid chromatography-tandem mass spectrometry. *International Dairy Journal, 24*(2), 146–152. https://doi.org/10.1016/j.idairyj.2011.04.014

Blackshaw, K., Wu, J., Valtchev, P., Lau, E., Banati, R. B., Dehghani, F., & Schindeler, A. (2021). The effects of thermal pasteurisation, freeze-drying, and gamma-irradiation on the antibacterial properties of donor human milk. *Foods, 10*(9), 2077. https://doi.org/10.3390/foods10092077

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT, 28*, 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carriere, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Buhler, S., Solari, F., Gasparini, A., Montanari, R., Sforza, S., & Tedeschi, T. (2019). UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey. *LWT, 105*, 127–134. https://doi.org/10.1016/j.lwt.2019.01.051

Claeys, W. L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., Dewettinck, L., & Herman, L. (2014). Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Control, 42*, 188–201. https://doi.org/10.1016/j.foodcont.2014.01.045

Cosenza, G., Mauriello, R., Garro, G., Auzino, B., Iannaccone, M., Costanzo, A., … Pauciullo, A. (2019). Casein composition and differential translational efficiency of casein transcripts in donkey's milk. *Journal of Dairy Research, 86*(2), 201–207. https://doi.org/10.1017/S0022029919000256. Cambridge University Press.

Di Nunzio, M., Loffi, C., Chiarello, E., Dellafiora, L., Picone, G., Antonelli, G., Di Gregorio, C., Capozzi, F., Tedeschi, T., Galaverna, G., & Bordoni, A. (2022). Impact of a shorter brine soaking time on nutrient bioaccessibility and peptide formation in 30-months-ripened parmigiano reggiano cheese. *Molecules, 27*, 664. https://doi.org/10.3390/molecules27030664

EFSA. (2016). Safety of UV-treated milk as a novel food pursuant to Regulation (EC) No 258/97. *EFSA Journal, 14*(1), 4370. http://doi:10.2903/j.efsa.2016.4370.

Fantuz, F., Salimei, E., & Papademas, P. (2016). Macro-and micronutrients in non-cow milk and products and their impact on human health. In *Non-bovine milk and milk products* (pp. 209–261). Elsevier Inc. https://doi.org/10.1016/B978-0-12-803361-6.00009-0.

Fiocchi, A., Bognanni, A., Brożek, J., Ebisawa, M., Schünemann, H., Ansotegui, I. J., Arasi, S., Assa'ad, A. H., Bahna, S. L., Canani, R. B., & Bozzola, M. (2022). World allergy organization (WAO) diagnosis and rationale for action against cow's milk allergy (DRACMA) guidelines update – I – plan and definitions. *World Allergy Organization Journal, 15*(1), 57–161. https://doi.org/10.1016/j.waojou.2021.100609

Gasparini, A., Buhler, S., Faccini, A., Sforza, S., & Tedeschi, T. (2020). Thermally-induced lactosylation of whey proteins: Identification and synthesis of lactosylated β-lactoglobulin epitope. *Molecules, 25*(6). https://doi.org/10.3390/molecules25061294

Guo, H. Y., Pang, K., Zhang, X. Y., Zhao, L., Chen, S. W., Dong, M. L., & Ren, F. Z. (2007). Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science, 90*(4), 1635–1643. https://doi.org/10.3168/jds.2006-600

Hazeleger, W. C., & Beumer, R. R. (2016). Microbial quality of raw horse milk. *International Dairy Journal, 63*, 59–61. https://doi.org/10.1016/j.idairyj.2016.07.012

Hernández-Ledesma, B., Miralles, B., Amigo, L., Ramos, M., & Recio, I. (2005). Identification of antioxidant and ACE-inhibitory peptides in fermented milk. *Journal of the Science of Food and Agriculture, 85*(6), 1041–1048. https://doi.org/10.1002/jsfa.2063

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke, E. O., & Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science & Technology, 90*(5), 549–563. https://doi.org/10.1051/dst/2010018

Koutchma, T., Bissonnette, S., & Popovic, V. (2019). An update on research, development and implementation of UV and pulsed light technologies for nonthermal preservation of milk and dairy products. *Ref. Module Food Sci.*, 1–21.

Li, G.-H., Liu, H., Shi, Y.-H., & Le, G.-W. (2005). Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. *Journal of Pharmaceutical and Biomedical Analysis., 37*(2), 219–224. https://doi.org/10.1016/j.jpba.2004.11.004

Marletta, D., Tidona, F., & Bordonaro, S. (2016). Donkey milk proteins: Digestibility and nutritional significance. In *Milk proteins - from structure to biological properties and health aspects* (pp. 199–209). https://doi.org/10.5772/62597. IntechOpen.

Martini, M., Altomonte, I., Licitra, R., & Salari, F. (2018). Nutritional and nutraceutical quality of donkey milk. *Journal of Equine Veterinary Science, 65*, 33–37. https://doi.org/10.1016/j.jevs.2017.10.020

Martini, M., Altomonte, I., Manica, E., & Salari, F. (2015). Changes in donkey milk lipids in relation to season and lactation. *Journal of Food Composition and Analysis, 41*, 30–34. https://doi.org/10.1016/j.jfca.2014.12.019

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences, 20*(23), 5978. https://doi.org/10.3390/ijms20235978

Miranda, G., Mahé, M. F., Leroux, C., & Martin, P. (2004). Proteomic tools characterize the protein fraction of Equidae milk. *Proteomics, 4*(8), 2496–2509. https://doi.org/10.1002/pmic.200300765

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*(4), 777–783.

Ozturkoglu-Budak, S. (2018). Effect of different treatments on the stability of lysozyme, lactoferrin and β-lactoglobulin in donkey's milk. *International Journal of Dairy Technology, 71*(1), 36–45. https://doi.org/10.1111/1471-0307.12380

Papademas, P., Mousikos, P., & Aspri, M. (2021). Optimization of UV-C processing of donkey milk: An alternative to pasteurization? *Animals, 11*(1), 1–10. https://doi.org/10.3390/ani11010042

Peila, C., Emmerik, N. E., Giribaldi, M., Stahl, B., Ruitenberg, J. E., Van Elburg, R. M., Guido, M. E., Bertino, E., Coscia, A., & Cavallarin, L. (2017). Human milk processing: A systematic review of innovative techniques to ensure the safety and quality of donor milk. *Journal of Pediatric Gastroenterology and Nutrition, 64*(3), 353–361. https://doi.org/10.1097/MPG.0000000000001435

Regulation (EC) No 152/2009. (2009). *Laying down the methods of sampling and analysis for the official control of feed. European Parliament*. Council of the European Union. http://data.europa.eu/eli/reg/2009/152/2020-11-16.

Salimei, E., & Fantuz, F. (2013). Horse and donkey milk. In Y. W. Park, & G. F. W. Haenlein (Eds.), *Milk and Dairy Product in Human Nutrition: Production, Composition and Health* (pp. 594–613). Oxford, UK: John Wiley & Sons.

Salimei, E., Fantuz, F., Coppola, R., Chiofalo, B., Polidori, P., & Varisco, G. (2004). Composition and characteristics of ass's milk. *Animal Research, 53*(1), 67–78. https://doi.org/10.1051/animres

Scheidegger, D., Pecora, R. P., Radici, P. M., & Kivatinitz, S. C. (2010). Protein oxidative changes in whole and skim milk after ultraviolet or fluorescent light exposure. *Journal of Dairy Science, 93*(11), 5101–5109. https://doi.org/10.3168/jds.2010-3513

Silvestre, D., Ruiz, P., Martínez-Costa, C., Plaza, A., & López, M. C. (2008). Effect of pasteurization on the bactericidal capacity of human milk. *Journal of Human Lactation, 24*(4), 371–376. https://doi.org/10.1177/0890334408319158

Souroullas, K., Aspri, M., & Papademas, P. (2018). Donkey milk as a supplement in infant formula: Benefits and technological challenges. *Food Research International, 109*, 416–425. https://doi.org/10.1016/j.foodres.2018.04.051

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal, 13*(6), 447–453. https://doi.org/10.1016/S0958-6946(03)00053-0

Tidona, F., Criscione, A., Devold, T. G., Bordonaro, S., Marletta, D., & Vegarud, G. E. (2014). Protein composition and micelle size of donkey milk with different protein patterns: Effects on digestibility. *International Dairy Journal, 35*(1), 57–62. https://doi.org/10.1016/j.idairyj.2013.10.018

Tidona, F., Sekse, C., Criscione, A., Jacobsen, M., Bordonaro, S., Marletta, D., & Vegarud, G. E. (2011). Antimicrobial effect of donkeys' milk digested in vitro with human gastrointestinal enzymes. *International Dairy Journal, 21*(3), 158–165. https://doi.org/10.1016/j.idairyj.2010.10.008