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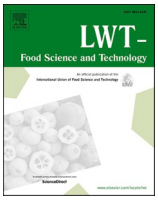

Effect of continuous flow HTST treatments on donkey milk nutritional quality

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ABSTRACT

Nutritional quality of raw donkey milk (DM) may be impaired during sanitization with the current batch holder pasteurization systems (62.5 °C for 30 min). In this paper, we present the preliminary results concerning the effects of high temperature for short time (HTST) protocols using an innovative in continuous low flow rate pasteurization plant (60 dm^3/h) on B-vitamins group, antioxidant capacity, lysozyme and β-lactoglobulin in DM. Lysozyme, β-lactoglobulin and antioxidant power decreased after the thermal treatments, with characteristics depending on the extent of the heat treatment. The lysozyme content was substantially reduced between 20 and 60%, while the degradation of β-lactoglobulin was lower (2–22%). No vitamin B1 and B12 were found in raw milk, whereas were detected vitamin B2 (0.17 μmol/L), nicotinic acid (13.28 μmol/L), B6 (2.06 μmol/L) and B9 (0.75 μmol/L). The heat treatments carried out with the innovative plant ensured vitamin retention, as no significant differences were found against the raw milk ($p > 0.05$). The preliminary results from this study represent a guidance to the establishment of DM pasteurization standards parameters with the perspective to improve DM nutritional quality.

1. Introduction

Non-ruminates milk production for human consumption, with special reference to mare and donkey milk (DM), represent 0.1% of global milk production (Claeys et al., 2014). DM production is becoming increasingly important because it has high nutritional value and represents an opportunity for children affected by Cow's Milk Protein Allergy (CMPA) that is defined as "a hypersensitivity reaction against cow's milk proteins by specific immunological mechanisms" (Iacono et al., 1992). CMPA is the most frequent form of allergy in early childhood in industrialized countries, with an incidence up to 3.5% under 5 years, it is mainly due to the presence of caseins (mainly αs1-, β-casein and K-casein) and β-lactoglobulin (β-lg), although there is some contrasting evidence with respect to β-lg (Souroullas, Aspri, & Papademas, 2018; Amati et al., 2010). The production of DM milk greatly differs from other species, both in terms of yield (1.54–1.73 kg/day) and nutritional composition (Trinchese et al., 2015; Pal, Kumar, Mohanty, & Bhardwaj, 2016, pp. 31–32), allowing it to be used for various industrial purposes (i.e. food, cosmetic, pharma) (Bhardwaj et al., 2020). DM has lower fat and protein content compared to cow's milk, while has higher lactose content that makes it very sweet and palatable. In addition, lactose is a rapid energy source and represents the ideal substrate for the growth of various species of lactobacilli (Brumini et al., 2013; Guo et al., 2007; Shin, Hayasawa, & Lönnerdal, 2001). DM could represent a considerable functional food for the nutrition of both children and adults as it is a source of vitamins, minerals, proteins, essential amino acids and polyphenols which exert significant biological activities promoting health well-being (Ozturkoglu-Budak et al., 2016; Perna, Intaglietta, Simonetti, & Gambacorta, 2015). Compared to cow's milk, DM has a different protein pattern characterized by dephosphorylated forms of αs-1 and β-casein, which probably determine its lower allergenicity (Zhang, Zhao, Jiang, Dong, & Ren, 2008). Elimination of phosphorylation sites may

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reduce IgE response to caseins (Souroullas et al., 2018). The hypoalergenicity of DM could be linked not only to the low concentration of αs2-and k-casein, but due to a lower amino acid presence of the main allergenic linear epitopes of αs1-and β-casein, and the low ratio of casein to the whey protein fraction (Vincenzetti et al., 2012). As reported by Salimei (2010, p. 42), donkey whey proteins are represented by β-lg (29.9%), α-lactalbumin (22.6%), lysozyme (21%), immunoglobulins (11.5%), serum albumin (6.2%) and lactoferrin (4.5%), owning some important biological activities. Many scientific evidences have highlighted the anti-inflammatory, immunomodulatory, anti-hypertensive and antibacterial activity of DM (Souroullas et al., 2018; Amati et al., 2010; Trinchese et al., 2015; Pal et al., 2016, pp. 31–32; Bhardwaj et al., 2020). The main biological activity is linked to those proteins owning antibacterial and anti-inflammatory activity, such as lactoferrin, lactoperoxidase and lysozyme. Lactoferrin is an iron-binding glycopeptide that has local anti-inflammatory action (Polidori & Vincenzetti, 2013). The lactoperoxidase system exerts its bactericidal action through oxidative mechanisms, however its inhibitory action occurs in the presence of thiocyanate and oxygen peroxide (H_2O_2). Lysozyme is an endogenous protein that mainly has a spectrum of action against gram +, as it breaks the glycosidic bonds between N-acetyl-muramic acid and N-acetyl-glucosamine in the peptidoglycan. However, several authors have shown an *in situ* inhibitory effect of DM against some gram-*bacteria* (*Salmonella cholaresuis*, *Shigella dysenteria)* (Zhang et al., 2008) and greater microbiological stability at 37 and 4 °C than other milks (Giribaldi et al., 2017; Xiao-Ying, Zhao, Jiang, Dong & Ren, 2008). DM vitamins pool is a source of both essential water and fat-soluble vitamins that cannot be synthesized by the human body, and its consumption could supply the vitamins daily intake recommended by international dietary recommendations (RDIs) of the EC 1169/2011. DM vitamin profile has been characterized by several authors, who have highlighted the abundance of vitamin C (0.28–0.50 mmol/L) and vitamin E (3.4 μmol/L). Both vitamin C and E are antioxidants, they protect cell membranes, DNA and proteins from oxidizing agents, such as reactive oxygen species (ROS). Water-soluble B-vitamin pool have recently been identified in Italian Amiata and Indian small grey breed milk (Nayak et al., 2020; Vincenzetti et al., 2020). Both products were characterized by a high vitamin B3 (1.27–1.87 μmol/L), moderate vitamin B1 (0.66 μmol/L), B2 (0.16 μmol/L), B6 (5.38 μmol/L), B9 (0.83 μmol/L) contents and absence of vitamin B12 (cyanocobalamin). In the European countries, marketability of DM often is limited by the scaled-down dimension of donkey livestock and by the yields, which do not allow its production at industrial level. For this reason, it is often commercialized raw or processed using thermal treatments carried on in discontinuous plant at low temperature (62.5 °C) for long periods (≈30 min) (LTLT), also called "holder pasteurization" (Di Renzo, Altieri, & Genovese, 2013). In addition, are not yet available sale criteria (i.e. pasteurization protocols) for processed DM, as happens with raw DM or heat-treated cow's milk (EC 2074/2005). However, being a high perishable product, DM should be processed into a more stable product, in liquid or powder form, through several processing which preserve the stability such as high temperatures (72–85 °C) for short periods (>15 s) (HTST) (Matera, Altieri, Genovese, & Di Renzo, 2019), eventually followed by a spry drying operation if the milk powder is required (Altieri, Di Renzo & Genovese, 2007, 2011; Altieri, Genovese, Admane, & Di Renzo, 2016). The industrial production of fresh-pasteurized cow's milk takes place using continuous plant relying on plate heat exchangers with tubular stop, with a working capacity over 600 dm^3/h (Di Renzo et al., 2013) which represent the minimum to economically processes in 8 h the daily livestock production. While, in the case of DM production, a working capacity of 60 dm^3/h could be enough to process in 1 h the daily production of livestock. The effectiveness of the heat exchanger during the heating and the cooling of the product is essential to preserve the heat sensitive compounds with biological activity (e.g. proteins and vitamins) and the sensory characteristics of the treated product. The non-instantaneous heating and subsequent cooling phases in holder pasteurization (LTLT) cause substantial and irreversible changes of the nutritional and organoleptic characteristics of the product. Modifications of the activity or amount of nutritional factors after heating treatments have been detected by several authors in milk from different animal sources (Table 1). The development of highly efficacy sanitation plant with low processing capacity is essential to produce high quality DM. In addition, the ease management represents a fundamental feature of the plant. According to the authors knowledge, there are only batch systems that perform treatments of 65 °C for 30 min or 72 °C for 2 min, while there are few HTST system designed to process very low amounts of food liquids. Cavallarin et al. (2015) developed a continuous flow pasteurizer for small amounts of liquid food, which was tested on DM (72 °C, 15 s) and was evaluated the sanitization efficacy and protein pattern change, comparing it with a holder pasteurization process (Giacometti et al., 2016). Whilst, there are no investigations in the literature on the effect of HTST treatments on B-vitamins group and phenolic component of DM. The paper presents the preliminary results on the effect of continuous flow HTST treatments on DM carried out through a low-flow rate continuous pasteurizer (60 dm^3/h) for food liquids (LFCP), developed and built by the research team of the Laboratory of Machines and Plants for the Food Industry of University of Basilicata (MAC-Lab, https://machimplab.wordpress.com/). The effect of nine HTST treatments on lysozyme, β-lg, B-vitamins pool (B1, B2, B6, B9, B12, Nicotinic acid), phenolic content and antioxidant capacity was investigated.

2. Materials and methods

2.1. Sample collection

Raw milk was kindly supplied from a donkey farm placed in southern Italy (Laterza). The research was carried out on bulk milk of 120 Martina Franca multiparous jennies that were fed in open polyphite-based. The milk was collected in wintertime (February 2019). As soon as the milking operation, it was refrigerated at 4 °C and delivered to the MAC-Lab in ten tanks (Vol. 5 L). At the arrival, the milk was pooled in an industrial tank refrigerator and kept mixed up the processing.

2.2. Plant description

Raw DM underwent HTST treatments using the LFCP designed by MAC lab's. The plant is made up of stainless steel: the pasteurization group is equipped with a plate heat exchanger AISI 316, while the structure and pipe unions are made up AISI 304. The milk is pasteurized through the tubular stop section where it stays the required time adjusted by managing the upstream milk flow, and the stop pipe length. The stop-section is interchangeable, due to the experimental purpose of the plant. The pasteurization residence time can be adjusted by the use of three different lengths (0.74, 0.93, 1.85 m) of the tubular stop-sections, having the same diameter (9.5×10^{-3} m) and were tested three residence times (3, 6, 12 s). Briefly, the flow-rate adjustable pump feeds the raw milk in the thermal recovery section of the plate exchanger. In the heat exchanger inlet milk is upstream preheated using the pasteurized outlet milk to get a heat recovery. Then the preheated milk enters into the heating section of the plate exchanger where it is upstream heated to the pasteurization temperature using hot water. For each residence time were tested three temperatures (75, 85, 92 °C) and nine experimental trials were accomplished. Three probes placed upstream and downstream the plant detects the following temperature:

- T1 untreated product inlet the thermal recovery section
- T2 product pasteurization temperature
- T3 treated product outlet the thermal recovery section

The parameters of the processes, including the plant and milk characteristics (e.g. the number of plates, flow, milk density), were used to

Table 1
Influence of heating treatments on nutritional compounds (B-group vitamins, antioxidant power, β-lg) in milk from different animal source from she-ass.

Source	Heat treatment			Nutritional factors variation (%) after heat treatment									References
	Heating	Residence time (s)	T (°C)	Vit. B1	Vit. B2	Vit. B3	Vit. B6	Vit B9	Antioxidant power			β-lg	
									TPC	FRAP	DPPH		
Cow	Continuous-flow microwaves	23.56	91	−1.0	–	–	+1.0	–	–	–	–	–	Sierra et al. (2001)
		30.59	111	NS			+4.0						
		38.87	119	NS			+11.0						
	Tubular heat exchange	23.56	91	NS	–	–	NS	–	–	–	–	–	
		30.59	110	NS			+5.0						
		38.87	120	−1.0			+10						
Goat	Tubular heat exchange	15	72	–	–	–	–	–	–	–	−42.5	–	Prasantha and Wimalasiri (2019)
		25	72								−31.0		
		15	75								−36.7		Lee, Barbano and Drake (2017)
		25	75								−22.4		
		15	81								−42.5		
		25	81								−45.4		
Cow	Tubular heat exchange	4	80	–	–	–	–	–	–	–	–	−33.3	Sakkas, Moutafi, Moschopoulou, and Moatsou (2014)
			90									−72.2	
			100									−90.0	
			110									−95.0	
			120									−96.8	
			130									−98.6	
			140									−99.9	
Cow	Static/Household	300	100	−8.1	−9.5	−9.68	−8.0	−14.3	–	–	–	–	Asadullah (2010)
		600		−16.2	−16.5	−17.7	−16.0	−32.4					
		900		−27.0	−26.9	−29.0	−24.0	−36.1					
Cow	Static/Holder	15	75	NS	NS	–	–	–	−24.3	–	–	–	Bendicho, Espachs, Arantegui, and Martin (2002)
		1800	63	NS	NS				−51.3				
Goat	Static/Holder	1800	63	–	–	–	–	–	−27.9	−22.3	−22.4	–	Chávez-Servín et al. (2018)

compute the heating/cooling times in the heating recovery sections through NTU methods (Singh & Heldman, 2014, p. 340), and overall thermal loading for each treatment (Supplementary Table 1).

2.3. Chemical analyses

2.3.1. Quantitative analyses of lysozyme and β-lg

Lysozyme and β-lg levels in DM were accomplished by reversed-phase high performance liquid chromatography (RP-HPLC) as reported by Ozturkoglu-Budak (2016). Ten millilitres of milk were heated up 45 °C in a water bath and kept stirred for 10 min. Afterwards, caseins were removed by adding HCl 1 mol/L up to pH 4.6 and centrifuging at 17,500 g per 15 min (Amiconmicrocentrifuge MC-13; Amicon, Beverly, MA, USA). The collected supernatant was filtered with Whatman n. 42 filter and stored at −18 °C. Prior to the injection into HPLC column, the samples were filtered through 0.20 μm cellulose acetate filter (Ministar, NML). Twenty μL of the sample at a flow rate of 1 mL/min was injected into HPLC system through a C18 ViVa column (5 μm, 4.6 mm, 150 mm; Restek, USA). The HPLC apparatus was equipped with Varian ProStar Pump model 210, Rheodyne injector with a 20-μL loop, UV–VIS detector Varian ProStarmodel 325, and Galaxie chromatography software (Varian Inc., Walnut Creek, CA). Gradient elution was achieved with two mobile phases. Solvent A consisted of trifluoracetic acid (TFA) 1 mL/L in deionised water and solvent B consisted of 1 mL/L TFA, 950 mL/L acetonitrile in deionised water. Elution was performed using the solutions A and B as follows: 100% solvent A for 5 min followed by a linear gradient to 50% B (v/v) over 15 min, increasing to 60% B (v/v) over 5 min and running at 60% B (v/v) for 10 min. The eluted proteins from RP-HPLC were monitored at 280 nm. Quantitative determination was achieved according to the external standard methods, using standard solutions of lysozyme from chicken egg and bovine β-lg (Sigma-Aldrich, St Loius, MO, USA), in a range of 0.1 ÷ 2 and 0.5 ÷ 3 mg/mL, respectively.

2.3.2. Quantitative analyses of donkey milk B-vitamins pool

B-vitamins pool quantification was accomplished using a RP-HPLC method adapted by Albalà-Hurtado, Veciana-Nogue's, Izquierdo-Pulido, and Marine'-Font (1997), either for sample preparation steps and chromatographic conditions. Briefly, 10 g of DM were thoroughly mixed for 10 min with 1 g of solid TCA using a magnetic stirrer plate, then the solution was centrifuged at 1250× g for 10 min to separate the two phases. The supernatant was collected and placed in 10 mL volumetric flask. Afterwards, 3 mL of TCA 40 mg/L (w/v) was added to the obtained solid residue, mixed and centrifuged as before. The supernatant was collected and combined to the 10 mL volumetric flask, filled with TCA 40 mg/L (w/v). Prior to analysis, acid extracts were filtered through 0.45 μm membrane filter (Millipore, Bedford, MA, USA). The separation of compounds was accomplished using an HPLC Agilent 1260 Infinity LC System (Agilent Technologies. Data were acquired by the OpenLab Software (Agilent technologies). The column was a C18 HiQSil HS, 5 μm, 4.6 mm i.d, 250 mm (Kya Tech Corporation, Tokyo, Japan). The mobile phase was: methanol (85:15), containing trimethylamine 5 g/L (w/v), glacial acetic acid 24 g/L (w/v), and octane sulfonic acid 5 mmol/L (pH 3.6). The analyses were carried out at 25 °C, with a flow rate of 0.9 mL/min. An injection volume of 100 μL was used. The eluent was detected at 254 and 270 nm. Standard solutions of vitamin B1 (0.1–18.7 μmol/L), vitamin B2 (0.5–15 μmol/L), nicotinic acid (0.75–20.0 μmol/L), vitamin B6 (0.75–20.0 μmol/L) and folic acid (0.3–7.5 μmol/L) were prepared in ultra-pure water to prepare the standard curve. The area of each standard peak was measured using the valley-to-valley integration mode, and the quantification of the vitamins was achieved relating the peaks to the standard curves.

2.3.3. Antioxidant power of donkey milk

DM was centrifuged at 5000×g at 4 °C for 20 min, and the supernatant was separately filtered through a 0.45 μm membrane filter and used to measure the antioxidant activity. Radical scavenging activity of water-soluble extracts of DM was evaluated by 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and ferric-reducing antioxidant power (FRAP) assays, as adapted by Perna et al. (2015). Results were expressed as milligrams of Trolox equivalents (TE) per gram of DM.

2.3.4. Determination of total phenolic (TPC) and flavonoid contents (TFC) and free thiol groups (SH)

Quantification of total phenolic compounds was carried out with the Folin–Ciocalteu method as reported by Perna, Simonetti, and Gambacorta (2019). Gallic acid (0–200 mg/L) was used as reference standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per mL of sample. TFC was determined as described by Chang, Zuo, Chow, and Ho (2006). Rutin (5–100 μg/mL) was used as reference standard and the results expressed as μg of rutin equivalent (RE) per mL of sample. The free thiol groups (SH) of DM were determined according to Ellman's method (1959), with some modifications, as reported by Perna et al. (2015). A molar extinction coefficient of 14.150 $M^{-1}cm^{-1}$ was used to calculate moles of thiol groups (SH).

2.4. Statistical analysis

Data were analysed using Matlab™ software (Matlab R2016a, The MathWorks Inc., Natick, MA, USA). Two-way analysis of variance (ANOVA) was carried out to find out whether there were significant differences among the treatments. The effect of time, temperature and their interaction on each parameter was investigated. Tukey's post hoc test was used to determine whether there was any significant difference ($\alpha = 0.05$) between treatments. Finally, Principal Component (PCA) and Simple Correspondence analyses (SCA) were performed to explain how the parameters and treatments correlate with each other.

3. Results and discussions

3.1. Heat load of the treatments

The standardized heat load (Q) of each treatment is graphically represented in Fig. 1. The thermal load refers to the underlying area obtained by plotting the temperature (°C) and time (s) values computed for the main groups of the plate exchanger (i.e. recovery, heating, cooling). The heat load on the milk treated for 6 and 12 s is almost similar, whatever the temperatures used. One-way ANOVA suggest as when the milk was heated up to 75 or 85 °C for 6 or 12 s the heat load is not significantly different, whilst at 92 °C it was different. When the milk was treated for 3 s the heat load was greatly reduced, up to 30–40%. The reasons why the treatments at 6 and 12s have high similarity need further investigations, preliminary results suggest this may due the different flows used in order to accomplish the desired holding-pasteurization time using the stop-sections. In this paper we are interest in evaluate the effect of the extent of the heating treatments on nutritional quality of DM, in order to set up the process parameters able either to preserve the compounds of nutritional interest and to sanitize milk against pathogenic and spoilage. To date, compared to cow's milk, the main effort in establish pasteurization parameters of milk from other animal sources lies into the presence at very different level of alkaline phosphatase enzyme (Clawin-Radecker et al., 2021), which is the marker of the efficiency of sanitation treatment for cow's milk (EC 2074/2005). Therefore, the scouting of further marker to evaluate the effectiveness of the pasteurization and the quality of heated DM is of great interest.

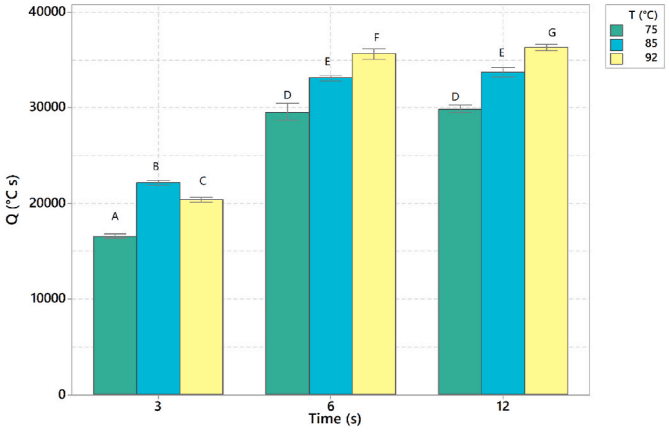

Fig. 1. Standardized heat load (Q) computed for each treatment. Bars that do not shear the same letter are significantly different (Tukey's LSD, $p < 0.01$).

3.2. Lysozyme and β-lg content in heat-treated donkey milk samples

In Table 2 is reported the lysozyme and β-lg content in the DM samples. The lysozyme content in raw milk was 1.17 g/L, this is in agreement with what reported by other authors (1–4 g/L) (14,16,21). Heat treatments significantly reduced the lysozyme content ($p = 0.000$), with characteristics depending on both the temperature and the duration of treatments, between 19.6 and 60.6%. However, the reduction occurring in treated samples followed a heat load-depending trend. Statistically, treatments at 75 °C for 3 and 6 were equivalent to that at 85 °C for 3 s ($p < 0.05$), the lysozyme content among the samples treated under these conditions did not differ significantly, and there was a reduction between 19 and 24% compared to the raw milk. These results are in line with other authors what reported by (Chandan, Shahani, & Holly, 1964; Giribaldi et al., 2017) who highlighted the decrease of lysozyme decreasing up to 30% after HTST treatments, due to the partial inactivation of the enzyme during pasteurization. The other tested treatments reduced the lysozyme content, ranging between 35 and 60%. The Pearson correlation statistical analysis found, as expected, a negative correlation between lysozyme and the intensity of the treatment (−0.849; $p = 0.000$). Several reports confirmed that thermal instability of lysozyme (irreversibility and inactivation of the enzyme) is due to both physical and chemical degradation such as asparagine deamination (Tomizawa, Yamada, Tanigawa, & Imoto, 1995), disulphide scrambling (Tomizawa, Yamada, & Imoto, 1994) and aggregation (Guo, Harn, Robbins, Dougherty, & Middaugh, 2006). The β-lg content in raw milk was 3.43 g/L, in agreement with what reported by Ozturkoglu-Budak (2016). The heat treatment decreased the β-lg content between 2 and 25%, with characteristics dependent on the time ($p = 0.000$) rather than temperature ($p = 0.074$) of treatment. A similar decrease of β-lg content was reported by Ozturkoglu-Budak (2016). Our results are in agreement with those of Lee et al. (2017), who reported approximately 20% serum protein denaturation after HTST treatment of milk. After the milk was treated for 3 s at 75, 85 and 92 °C the reduction in β-lg content was 2.7, 6 and 9%, respectively. This result was consistent with Griffiths (2010) that reported that HTST pasteurization only denatures 5–15% of β-lg. The treatments at 85 and 92 °C for 12 s, i.e. the most energetic ones, contributed significantly ($p < 0.01$) to the denaturation of β-lg. The greatest reduction was found when the milk was treated at 85 °C for 12 s, leading to a drastic and significant reduction in β-lg (2.6 g/L) compared to raw milk. In all other treatments the final β-lg content was not

Table 2
Lysozyme and β-lg content in raw milk (RM) and treated samples. For each parameters, values in the same columnwith different capital or small letters are significantly different, respectively, at $\alpha = 0.01$ and $\alpha = 0.05$ (Tukey's LSD).

T (°C)	Time (s)	Lysozyme (g/L)	β-lg (g/L)
RM	RM	$1.17 \pm 0.01^{a,A}$	$3.43 \pm 0.30^{a,A}$
75	3	$0.94 \pm 0.04^{b,B}$	$3.33 \pm 0.29^{ab,AB}$
	6	$0.89 \pm 0.02^{b,B}$	$3.04 \pm 0.10^{abc,AB}$
	12	$0.76 \pm 0.02^{c,C}$	$2.97 \pm 0.11^{abc,AB}$
85	3	$0.90 \pm 0.02^{b,C}$	$3.22 \pm 0.27^{abc,AB}$
	6	$0.76 \pm 0.04^{c,BC}$	$2.87 \pm 0.11^{abc,AB}$
	12	$0.67 \pm 0.04^{c,C}$	$2.60 \pm 0.16^{c,B}$
92	3	$0.70 \pm 0.00^{c,C}$	$3.11 \pm 0.19^{abc,AB}$
	6	$0.54 \pm 0.02^{d,D}$	$2.78 \pm 0.02^{bc,AB}$
	12	$0.46 \pm 0.06^{d,D}$	$2.66 \pm 0.09^{c,B}$

significantly reduced compared to the control. Pereira, Costa, Rodrigues, and Villa (2020) reported that HTST at 90 °C for 1 s affected the protein secondary structure and increased the content of reactive aggregates. It is known that the thiol/disulphide groups due to the unfolding of whey proteins forms aggregates among denatured whey proteins through covalent bonds (Tolkach & Kulozik, 2020). Whey proteins, to which β-lg belongs, are heat sensitive compounds. Sensitivity is in the order α-lactalbumin (α-la) <β-lactoglobulin (β-lg) < bovine serum albumin (BSA) < immunoglobulins (Igs). Considering the denaturation kinetics in the heating phase, cow milk α-la and β-lg could be used as markers of heat treatment (Tolkach & Kulozik, 2020). Our study could confirm this insight on DM, since we recorded a significant correlation between the extent of the heat treatment and the residual content of β-lg (Pearson's coefficient = −0.77). Our finding also adds further knowledge regarding the study on the milk proteins behaviour during heating, as we found that the lysozyme content in DM, surprisingly, is highly and significantly correlated (Table 4) with the heat load of the treatment (Pearson's coefficient = −0.84), more than β-lg. As lysozyme may have a significant role in preserving the intestinal ecosystem from potentially harmful microbes (Polidori & Vincenzetti, 2013), the possibility to manage the lysozyme content in DM through proper heat treatment designing could bring interesting prospective to increase the nutritional quality.

3.3. B-vitamin content in heat treated donkey milk samples

The content of B-vitamins pool in raw milk and heat-treated samples is shown in Table 3. Row milk was characterized by the presence of vitamin B2 (0.17 μmol/L), Nicotinic acid (13.28 μmol/L), B6 (μmol/L), B9 (μmol/L). These concentrations are similar to those found by other Authors (Lee et al., 2017; Griffiths, 2010), except for the vitamins B1 and B12, which were not found both in raw and treated milk. In this experiment, the tested HTST treatments had no effect on the degradation of B-vitamins group ($p > 0.05$). Surprisingly, we recorded a retention effect or low increasing compared to the control, although only in some conditions it was significant. The B-vitamins pool are heat sensitive, a dramatic decrease was recorded with UHT household treatments (Asadullah, Tarar, Ali, Jamil, & Begum, 2010; Macdonald et al., 2010). On other hand, our data are in agreement with the study by Sierra and Vidal-Valverde (2001), who observed retention of vitamin B1 and B6 in cow's milk at 90 °C with HTST in continuous treatments, and vitamin B6 increasing up to 4–6% when the heating was more severe (110 °C). However, don't appear in bibliography studies on the effect of HTST in continuous treatments on quantitative profile of B-vitamins in DM, therefore the results are poorly comparable.

Table 3
B-Vitamins pool content in raw milk (RM) and treated sample expressed as μmol/L. For each parameters, values in the same columnwith different capital or small letters are significantly different, respectively, at α = 0.01 and α = 0.05 (Tukey's LSD).

T (°C)	Time (s)	Vitamin B2	Nicotinic acid	Vitamin B6	Vitamin B9
RM	RM	0.17 ± 0.01^{a,A}	13.28 ± 0.44^{a,A}	2.06 ± 0.60^{a,A}	0.75 ± 0.06^{a,A}
75	3	0.11 ± 0.10^{a,A}	14.88 ± 2.23^{ab,A}	4.69 ± 0.70^{a,A}	0.80 ± 0.02^{ab,A}
	6	0.16 ± 0.04^{a,A}	14.12 ± 0.76^{ab,A}	3.20 ± 0.18^{a,A}	0.79 ± 0.02^{ab,A}
	12	0.15 ± 0.06^{a,A}	15.02 ± 1.77^{ab,A}	3.44 ± 0.85^{a,A}	0.81 ± 0.05^{ab,A}
85	3	0.12 ± 0.03^{a,A}	14.06 ± 0.24^{ab,A}	2.12 ± 0.87^{a,A}	0.80 ± 0.06^{ab,A}
	6	0.18 ± 0.03^{a,A}	16.61 ± 0.50^{ab,A}	4.16 ± 0.43^{a,A}	0.76 ± 0.04^{a,A}
	12	0.14 ± 0.12^{a,A}	15.96 ± 1.61^{ab,A}	2.40 ± 2.27^{a,A}	0.87 ± 0.05^{ab,A}
92	3	0.14 ± 0.02^{a,A}	15.33 ± 2.01^{ab,A}	2.35 ± 0.14^{a,A}	0.81 ± 0.04^{ab,A}
	6	0.18 ± 0.02^{a,A}	18.25 ± 3.94^{b,A}	4.46 ± 0.32^{a,A}	0.99 ± 0.17^{b,A}
	12	0.16 ± 0.10^{a,A}	15.75 ± 1.46^{ab,A}	3.82 ± 1.81^{a,A}	0.79 ± 0.02^{ab,A}

Table 4
Pearson correlation among parameters and heat loading (Q).

Parameter	Person coefficient	p value
Lysozyme	−0.849	0.000
β-lg	−0.770	0.000
Vitamin B2	−0.084	0.650
Nicotinic Ac.	0.479	0.007
Vitamin B6	0.337	0.069
Vitamin B9	0.338	0.068
ABTS	−0.925	0.000
FRAP	−0.915	0.000
TFC	−0.828	0.000
TPC	−0.920	0.000
SH	−0.931	0.000

3.4. Antioxidant activity of donkey milk

DM total antioxidant power, as expected, is highly correlated with intensity of treatments (Table 4), although each assay returned a different response in terms of percentage reduction. The lack of a widely accepted standardized methods for evaluation of antioxidant properties of foods and the complex reactivity of bioactive compounds are the reasons why were employed three different antioxidant capacity assays (FRAP, ABTS, SH). The antioxidant power was reduced from 20 to 31% with ABTS, from 8 to 15% with FRAP and from 11 to 37% with SH assays (Fig. 2). This variability is due to the principle of the methodology. The ABTS assay is applicable to both hydrophilic and lipophilic antioxidant systems. FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. The thiols assay measures the number of thiol groups (SH-), such as glutathione and protein thiol groups, which play an essential role as antioxidants. This assay reflects the ability to detoxify lipid and other peroxides in biological samples (Chang et al., 2006). It is known that DM, and dairy products in general, have an antioxidant power mainly correlated with the production technology, in particular fermentation brings greater antioxidant power (Perna et al., 2015) due to the presence of many bioactive peptides of low molecular weight (≤5000 kDa) released from milk proteins through the proteolytic activity of lactic acid bacteria and yeasts. Piovesana et al. (2015) observed that bioactive peptides and amino acids of DM derive mainly from the enzymatic degradation of αS1- and β-casein and, with less extent, from β-lg. As known, DM has lower protein percentage compared to cow milk, but its casein/whey protein ratio is clearly shifted to whey proteins (Salimei et al., 2004). Bučević-Popović, Delaš, Međugorac, Pavela-Vrančić & Kulišić-Bilušić (2014) reported that whey from donkey milk showed exceptionally high DPPH radical scavenging activity due to wealth in-OH groups in their compounds. Anyway, short heat treatments may be responsible for the decrease of antioxidant properties of milk (Şanlidere Aloğlu, 2013). Also, in this study, significant inverse relationship between disulphide reactivity and the intensity of thermal treatment of different thermal-treated milk was observed (- 0.931). These results are in agreement with Carbonaro, Cappelloni, Sabbadini, and Carnovale (1997), as positive and significant ($p < 0.01$) correlation (Table 5) was found between β-lg content and antioxidant power detected by FRAP, ABTS, SH assays (0.6<*Person's coefficient*<0.8). Polyphenols are strong antioxidants mainly due to their low redox potential and capacity to donate electrons or hydrogen atoms and contribute to the overall antioxidant power of the foodstuffs. Polyphenols cluster natural compounds with antioxidant, modulatory and anti-inflammatory activity, whose intake at certain levels can have

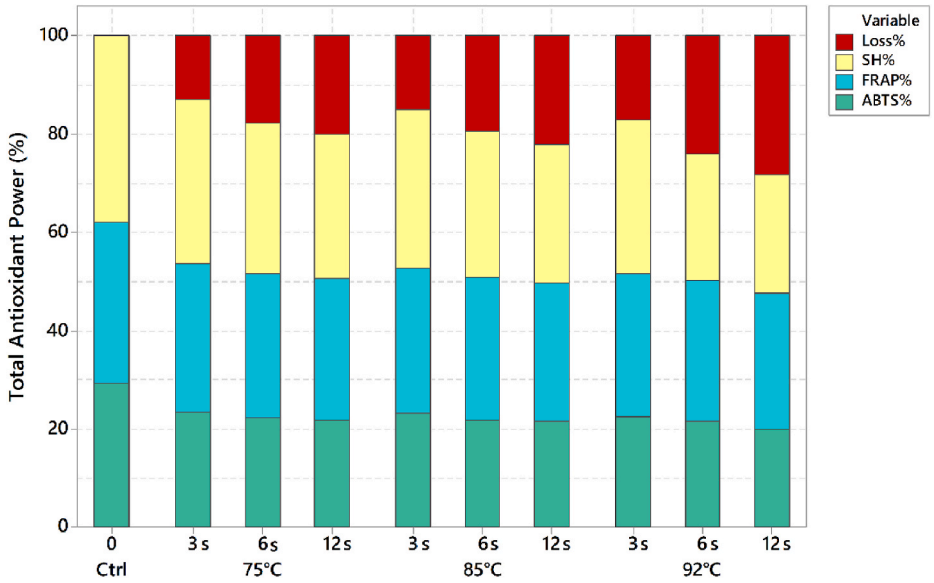

Fig. 2. Distribution of % antioxidant activity determined by each assay and of its loss after the heat treatments.

Table 5
Pearson correlation matrix of the investigated parameters.

		1	2	3	4	5	6	7	8	9	10	11
1	Lysozyme	–										
2	β-lg	0.77*	–									
3	Vitamin B2	0.20	0.08	–								
4	Nicotinic Ac	−0.49	−0.41**	−0.05	–							
5	VitaminB6	−0.30	−0.19	−0.37**	0.37**	–						
6	Vitamin B9	−0.36**	−0.32	−0.25	0.22	0.18	–					
7	ABTS	0.85*	0.68*	0.19	−0.38	−0.34	−0.24	–				
8	FRAP	0.88*	0.73*	0.30	−0.42**	−0.31	−0.22	0.96*	–			
9	TPC	0.86**	0.84**	0.07	−0.39**	−0.19	−0.27	0.83*	0.87*	–		
10	TFC	0.91*	0.71*	0.11	−0.44**	−0.14	−0.32	0.83*	0.88*	0.85**	–	
11	SH	0.93*	0.77*	0.17	−0.47*	−0.33	−0.39**	0.87**	0.89*	0.91*	0.88*	–

Significant for $p < 0.01$* or $p < 0.05$**.

beneficial effects on human health. Total phenols content (TPC) in raw DM was of 155.09 ± 4.58 μg/mL, expressed as Gallic Acid Equivalent (Fig. 3). Flavonoids (TFC), which represent a class of phenols, in raw milk were 31.06 ± 2.05 μg/mL, expressed as Rutin Equivalent. These values are ten times lower than those found from other authors in DM milk (Yirmibeşoğlu & TefonÖztü), who found, respectively, 1873 ± 344 μg/mg and 1560 ± 16 μg/mL of phenol and flavonoid, although in the latter case it was expressed as quercetin equivalent. However, this results may due the different livestock areas and breeds involved in the trails. After heating treatments TPC and TFC were reduced upon the intensity. TPC ranged within 127 and 149 μg/mL, while TFC within 19.7 and 28.3 μg/mL. Heating up 75 and 85 °C per 3 s guaranteed no significant reduction of both parameters. Differences within treatments were significant up to α = 0.001 (Fig. 3). However, TPC slows down up

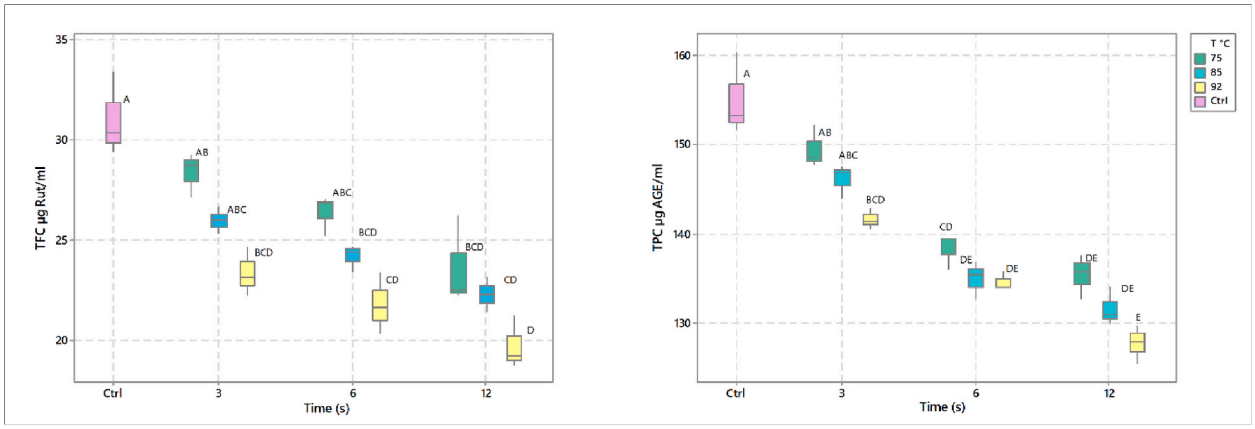

Fig. 3. Boxplot of TFC (left-side) and TPC (right-side) of the milk samples. Treatments (bars) that do not shear the same letter are significantly different (Tukey's LSD, p < 0.01).

to a maximum of 15% and flavonoid up to 36%, suggesting that other class of polyphenols may be enclose within DM total phenols. It is known that thermal processing reduced TPC by triggering complex physical and chemical reactions that affected phenolic composition, the release of phenolic compounds from their bonded forms, the degradation polyphenols and the breakdown, hydrolysis and transformations of phenols; all reactions are due to compounds' structure and the phenolic subclass to which they belong (Chen, Yu, & Rupasinghe, 2013; De et al., 2014).

3.5. Multivariate analyses

Principal component analysis (PCA) and simple correspondence analysis (SCA) are two multivariate statistical approaches used to compute how investigated parameters correlate along the experiment each other and with the samples. PCA allow to identify a smaller number of uncorrelated variables, that are linear combinations of the observed variables. The goal of PCA is to explain the maximum amount of variance with the fewest number of principal components. Figs. 4 and 5 shows as PCA and SCA allow to explain, respectively, the 83.2 and 86.6% the variance among data, therefore SCA would be more accurate to describe the variability among the dataset, albeit slightly. It's worth noting as the samples spread out on the graph from the right to the left side of the PC1 and Component 1 on both PCA and SCA with characteristics dependent to the thermal loading. Samples produced by lowest treatments cluster closely together and are placed on the right side. Basically, those sample are characterized by higher content of lysozyme, β-lg, phenols and higher antioxidant activity (ABTS, FRAP, SH). On other hand, when the heating treatment intensity rises the sample are characterized by lower content of those compounds, and a slight increasing or retention of B-vitamins pool. The main output of these analyses relies on the possibility to identify the samples whose chemical characteristics match closer with the control sample (raw milk). Therefore, according to both PCA and SCA results, the samples 75_3, 75_6, 85_3, 92_3 have very close features with the raw milk (Ctrl).

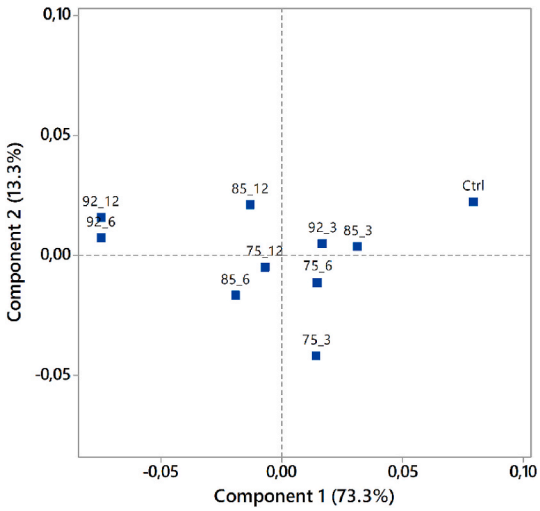

Fig. 5. Simple correspondence analysis plot. Each symbol identifies a treatment, indicating the temperature (75, 85, 92 °C) and duration (3, 6, 12 s). Ctrl is the raw milk.

4. Conclusions

DM is notable source of compounds with high nutritional value, especially B-vitamins pool, antioxidants and lysozyme. These compounds play a very important role in childhood and can also contribute to health well-being in majority, but being heat sensitive their functionality may impaired by sanitation treatment needed to marketability. In addition, the lack of European polices establishing the pasteurization parameters for donkey's milk, as for cow's milk, and the lack of facilities that allow its sanitization and marketing while preserving its nutritional properties, is a hindrance to the industrial manufacturing and marketing of this product. This study provides preliminary information on the nutritional profile of heat-treated donkey milk under different conditions, allowing to select and define process conditions intended to

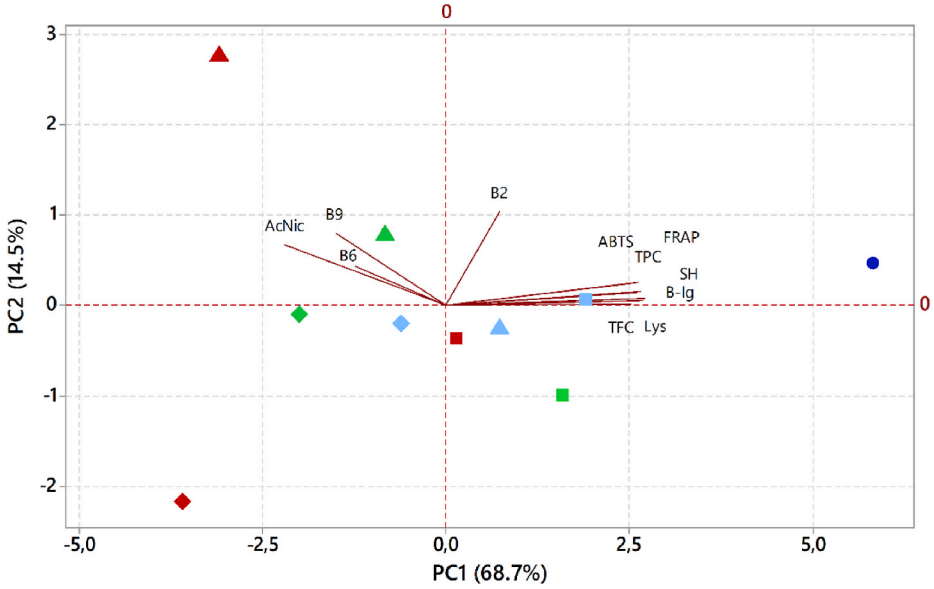

Fig. 4. Principal component analysis Bi-Plot (achieved by overlapping score plot and loading plot of data). Each symbol identifies a treatment, indicating the temperature (● 75, ● 85, ● 92 °C) and duration (□ 3, △ 6, ◇ 12 s). ● is the raw milk (Ctrl).

optimize the DM sanitation without impair the nutritional quality. The noteworthy result concerns the quantitative profile of B-group vitamins (B2, Nicotinic Acid, B6, B9) in treated samples, since some HTST treatments tested guaranteed the retention of these compounds. However, further investigation considering to evaluate the matrix effect on the retention influence of vitamin after HTST treatment of DM could be.

CRediT authorship contribution statement

Attilio Matera: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Giuseppe Altieri:** Conceptualization, Methodology, Software, Validation, Writing – review & editing, Supervision. **Francesco Genovese:** Methodology. **Paolo Polidori:** Conceptualization, Formal analysis, Resources. **Silvia Vincenzetti:** Formal analysis, Investigation. **Annamaria Perna:** Resources, Formal analysis. **Amalia Simonetti:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Mahdi Rashvand Avei:** Software. **Augusto Calbi:** Resources. **Giovanni Carlo Di Renzo:** Conceptualization, Methodology, Resources, Supervision.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112444.

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