Shelf life of donkey milk subjected to different treatment and storage conditions

Federica Giacometti,^{*1} Lia Bardasi,[†] Giuseppe Meriardi,[†] Michele Morbarigazzi,[‡] Simone Federici,[‡] Silvia Piva,^{*} and Andrea Serraino^{*}

^{*}University of Bologna, Department of Veterinary Medical Sciences, Via Tolara di Sopra 50-40064 Ozzano Emilia, Bologna, Italy

[†]Experimental Institute for Zoonoses in Lombardy and Emilia Romagna, Via P. Fiorini 5, 40127 Bologna, Italy

[‡]HPP Italia Srl, Via Carognani 6, 43029 Traversetolo, Parma, Italy

ABSTRACT

The aim of this study was to investigate the effects of different treatment conditions on microbiological indicators of donkey milk hygiene and their evolution during shelf life at 4 and 12°C from 3 to 30 d, simulating a farm-scale pasteurization and packing system. Four treatment conditions were tested: no treatment (raw milk), pasteurization (65°C × 30 min), high-pressure processing (HPP), and pasteurization plus HPP. The microbiological quality of the raw donkey milk investigated was not optimal; our results highlight the importance of raw milk management with the need for animal hygiene management and good dairy farming practices on donkey farms to improve handling procedures. The raw milk treated with HPP alone showed visible alterations with flocks, making the milk unfit for sale. The microbiological risk posed by consumption of raw donkey milk was significantly reduced by heat treatment but farm-scale packing systems cannot guarantee an extended shelf life. In contrast, the pasteurization plus HPP treatment was the most effective method to maintain microbiological milk quality. Microflora growth had little effect on pH in donkey milk: pH values were significantly different only between raw milk and pasteurized and pasteurized plus HPP milk stored at 12°C for 3 d. Alkaline phosphatase activity and furosine could be used as indicators of proper pasteurization and thermal processing in donkey milk. Moreover, the presence and growth of *Bacillus cereus* in the case of thermal abuse hamper the wide-scale marketing of donkey milk due to the potential consequences for sensitive consumers and therefore further tests with time/temperature/high-pressure protocols associated with *B. cereus* are needed. Finally, our study shows that an HPP treatment of pasteurized milk after packing extends the shelf life of donkey milk and assures its microbial criteria up to 30 d if properly stored at

4°C until opening; therefore, combined heat treatment and storage strategies are recommended to enhance the shelf life of donkey milk.

Key words: donkey milk, pasteurization, high-pressure processing (HPP), shelf life

INTRODUCTION

Even though nonruminant milk accounts for less than 0.1% of global milk production (Claeys et al., 2014), donkey milk is receiving increasing interest in Europe as an alternative to breast milk and infant formula for babies allergic to cow milk (Iacono et al., 1992; Monti et al., 2007, 2012; Mansueto et al., 2013), in case of multiple food intolerance (Carroccio et al., 2000), or when breastfeeding is not possible (Sarno et al., 2012). In addition, donkey milk is appreciated by people eager to try new foods and purchase locally grown produce (Scatassa et al., 2011).

To meet this demand, donkey farming is undergoing a revival in Italy, with new donkey dairies opening in several regions. With few exceptions, farms are small (<10 to 150 donkeys and from 5 to 30 milking jennies), family-run, and usually located in mountainous or hilly areas. Jennies are milked once a day using milking machines adapted from goat or cow milking equipment (Cavallarin et al., 2015) and usually produce about 1.5 L of milk a day. Daily milk production on the farm does not usually exceed 50 to 100 L and, due to the long distances between donkey farms, logistical organization of milk collection and distribution is lacking.

Currently, donkey milk for human consumption is sold as raw milk directly at farms or from vending machines, or it is pasteurized (or, rarely, UHT-treated or freeze-dried), packed in cartons or polyethylene terephthalate (PET) or glass bottles, and sold in shops, pharmacies, or online. By Italian law, raw milk has a shelf life of 3 d, whereas the shelf life of pasteurized and UHT milk is usually fixed by manufacturers at 4 to 6 d for pasteurized milk under storage conditions between 0 and 4°C and at 6 mo for UHT, with the recommenda-

Received December 10, 2015.

Accepted February 9, 2016.

¹Corresponding author: federica.giacometti3@unibo.it

tion to refrigerate it at a maximum temperature of 5°C after opening and to consume it within 3 d. Nevertheless, these conditions raise some problems: (1) the 3-d shelf life of raw donkey milk limits the wide-scale marketing of this commodity and the development of donkey milk companies or farms; (2) pasteurization extends the shelf life but not sufficiently to be a viable alternative for wide and efficient distribution given the logistic limitations of donkey farms; (3) UHT and freeze-drying treatments guarantee commercially sterile products but entail irreversible changes in endogenous milk compounds such as whey protein and lipid components (Sorrentino et al., 2006), and could alter flavor. In addition, UHT treatment systems are very expensive for a single farm and require large amounts of milk, which donkey farms are not expected to produce. At the same time, the safety of donkey milk is a potential concern for food-sensitive consumers or individuals with allergies or other problems.

For these reasons, it is useful to evaluate alternative approaches to donkey milk sanitation and shelf-life extension. High-pressure processing (HPP) is a nonthermal food preservation technology with minimal adverse effects on food quality (Cullen et al., 2012). It relies on the use of high pressure (generally 100–600 MPa) to process liquid or solid foods to inactivate spoilage and pathogenic microorganisms and extend the shelf life (Evelyn and Silva, 2015). The effects of HPP on foods were first studied in the late 19th century, when processing cow milk at 670 MPa for 10 min resulted in 5- to 6-log microbial reductions, extending shelf life up to 4 d after processing (Hite, 1899). However, the complexity of foods and the wide variety of phenomena that occur under pressure make it difficult to predict effects of HPP on foods (Palou et al., 2007). For these reasons, HPP conditions must be evaluated in each specific food.

Little data exists in the literature on the effects of heat treatments on the chemical and microbiological parameters of donkey milk, and no study has hitherto addressed the effects of HPP on its microbial contents. The aim of this study was to investigate the effect of different treatment conditions on the microbiological indicators of donkey milk and their evolution during shelf life at different temperatures from a minimum of 3 d and a maximum of 30 d, simulating a farm-scale pasteurization and packing system.

MATERIALS AND METHODS

Milk Sample Preparation

Four treatment conditions were tested: no treatment (raw milk), pasteurization, HPP, and pasteurization

plus HPP. Three batches of raw donkey milk were collected from local farms for 3 consecutive weeks in June 2015. After postmilking refrigeration, each batch (30 L) was transported to the cheese factory of the Department of Veterinary Medical Sciences, Bologna, and then it was divided into 2 portions: (i) 20 L of raw donkey milk was pasteurized (65°C for 30 min) using a commercial farm-scale pasteurization system (Caseus, Plastitalia group, Milan, Italy) and packed into 26 PET spout pouches (250 mL each): 10 were used for the pasteurization test and 16 were transported to a local industry for HPP treatment for the pasteurization plus HPP test; ii) 10 L of raw donkey milk were packed into 16 PET spout pouches (250 mL each): 6 for the raw milk test and 10 for HPP treatment. The HPP treatment was performed by Avure Technologies (Quintus Food Press QFP350L-600; Middletown, OH): milk packs were initially treated at a constant pressure of 600 MPa and at temperatures in the range of 4 to 6°C for 180 s; under working conditions, the temperature increased by approximately 10°C due to pressure buildup (approximately 100 MPa/min). After HPP treatment, the milk was visually inspected for any changes that could affect the marketing of donkey milk. Due to the appearance of clotting in the HPP-treated milk, the pressure was reduced from 600 to 400 MPa for 180 s for pasteurized milk and the HPP treatment was further reduced to 400 MPa for 100 s for raw milk destined for the HPP-only treatment.

For each treatment condition, all samples were divided and stored at 4 and 12°C to simulate optimal storage conditions and domestic storage, respectively (Beaufort et al., 2008): raw milk samples were stored for 3 d (according to Italian legislation); pasteurized and HPP samples for 15 d; and pasteurization plus HPP samples for 30 d.

Samples were analyzed from each PET spout pouch at d 0 (before treatment) and for each storage condition at d 1 and 3 for raw milk; at d 1, 3, 7, 10, 15 for pasteurized and HPP milk; and additionally at d 21, 25, and 30 for the pasteurization plus HPP samples.

Microbiological and Chemical Analyses

The following microbiological analysis were performed in each sample of type of milk as described above: total mesophilic colony count (TMC; UNI EN ISO 4833-2:2013/Cor.1:2014; ISO, 2013a); enumeration of *Enterobacteriaceae* (ISO, 2004), *Pseudomonas* spp. (ISO, 2009), presumptive *Bacillus cereus* (ISO, 2005a), and only for raw and HPP samples, enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species; ISO, 1999). The pH value of each sample was measured by an automatic temperature

compensation device (HI 223, Hanna Instruments, Milan, Italy).

According to Italian legislation (Ministerial Decree 16-05-1996), alkaline phosphatase activity (ALP; ISO, 2013b) and furosine were determined by HPLC technique in raw donkey milk samples at d 0 and 1 (after each type of treatment). All analyses were performed in the laboratories of the Experimental Institutes for Zooprophyllaxis in Lombardy and Emilia Romagna, accredited according to International Organization for Standardization (ISO) method 17025 (ISO, 2005b) by Accredia, the Italian accreditation body, except for furosine determination which was performed at Chelab S.r.l. (Resana, Treviso, Italy).

Statistical Analysis

The results were analyzed statistically for the comparison, within each day of storage, of all microbiological and pH data between the different treatments as follows: (1) raw versus HPP milk, raw versus pasteurized milk, and raw versus pasteurized plus HPP milk for the first 3 d; and (2) HPP versus pasteurized milk, pasteurized versus pasteurized plus HPP milk, and HPP versus pasteurized plus HPP milk for the first 15 d of storage. Comparison was performed for both storage conditions. The data were analyzed by repeated-measures 2-way ANOVA and Bonferroni post-tests; we used PRISM 5.0 software (GraphPad Software, La Jolla, CA) and statistical significance was set at $P < 0.05$.

RESULTS

The raw milk treated directly with HPP (both at 600 MPa for 180 s and at 400 MPa for 180s and 100 s)

showed visible alterations with flocks, making the milk unfit for sale. For this reason, the microbiological data and their statistical analysis are not shown in detail. The HPP treatment at 600 MPa for 180 s caused the same alterations when applied to pasteurized milk, but to a lesser extent. Pasteurized milk treated at 400 MPa for 180 s showed no alterations.

The initial contamination of the 3 batches of raw milk at d 0 showed a variability in TMC, *Pseudomonas* spp., and *Enterobacteriaceae* counts (see Tables 1, 2, and 3); 2 of the 3 analyzed batches of raw donkey milk did not meet criteria established by Italian law for raw milk sold by vending machines or directly on farms that require a TMC $\leq 100,000$ cfu/mL. Storage of raw milk for 3 d at 4°C and at 12°C resulted in increased TMC, *Pseudomonas* spp., and *Enterobacteriaceae* counts. Presumptive *B. cereus* was always <10 cfu/mL and coagulase-positive staphylococci were detected at 0.77 to 1.00 log cfu/mL with no increase during storage.

Both pasteurization and pasteurization plus HPP resulted in significant 3- to 5-log reductions of contaminant microflora with respect to raw milk but did not guarantee the absence of contaminants, which were found to grow after 3 d of storage at 12°C and after 7 d when stored at 4°C; in fact, when milk was stored at 4°C, TMC and *Pseudomonas* spp. counts in pasteurized milk increased during the different sampling days in different batches but were acceptable up to 7 d of storage (see Tables 1 and 2). We sporadically detected a low count of presumptive *B. cereus*. In contrast, all microbiological parameters increased up to 6 to 11 log cfu/mL in milk stored at 12°C, which was associated with a bluish coloration in batch 3.

The pasteurized plus HPP milk stored at 4°C showed very moderate contamination for all microbiological

Table 1. Evolution of total mesophilic count (log cfu/mL or cfu/mL if <10) during storage of donkey milk stored at 4°C and 12°C; data represent mean $\pm$ SD of 3 batches for each investigated milk (raw, pasteurized, and pasteurized plus high-pressure processed, HPP)

Storage time	Storage at 4°C			Storage at 12°C		
	Raw	Pasteurized	Pasteurized plus HPP	Raw	Pasteurized	Pasteurized plus HPP
Day 0 (raw)	5.23 $\pm$ 0.55 ^a	5.23 $\pm$ 0.55 ^a	5.23 $\pm$ 0.55 ^a	5.23 $\pm$ 0.55 ^a	5.23 $\pm$ 0.55 ^a	5.23 $\pm$ 0.55 ^a
Day 1	6.05 $\pm$ 1.03 ^a	1.85 $\pm$ 1.60 ^b	0.43 $\pm$ 0.75 ^b	6.53 $\pm$ 1.31 ^a	0.65 $\pm$ 1.12 ^b	$<10^b$
Day 3	6.31 $\pm$ 0.87 ^a	$<10^b$	0.49 $\pm$ 0.85 ^b	8.33 $\pm$ 0.65 ^{a,1}	4.12 $\pm$ 0.82 ^b	2.14 $\pm$ 1.15 ^b
Day 7	NP ²	1.61 $\pm$ 2.79 ^a	0.55 $\pm$ 0.95 ^a	NP	7.35 $\pm$ 0.88 ^a	6.36 $\pm$ 0.93 ^a
Day 10	NP	4.43 $\pm$ 3.98 ^a	0.98 $\pm$ 0.87 ^b	NP	8.42 $\pm$ 2.15 ^a	5.26 $\pm$ 2.64 ^b
Day 15	NP	6.90 $\pm$ 3.61 ^a	1.12 $\pm$ 0.99 ^b	NP	10.13 $\pm$ 1.23 ^a	7.69 $\pm$ 0.41 ^a
Day 21	NP	NP	0.93 $\pm$ 1.62	NP	NP	7.94 $\pm$ 0.63
Day 25	NP	NP	1.82 $\pm$ 0.32	NP	NP	8.69 $\pm$ 1.15
Day 30	NP	NP	0.93 $\pm$ 1.62	NP	NP	8.69 $\pm$ 0.68

^{a,b}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

¹Samples in which milk presented anomalous blue coloration.

²Analysis not performed.

Table 2. Evolution of *Pseudomonas* spp. count (log cfu/mL) during storage of donkey milk stored at 4°C and 12°C; data represent mean $\pm$ SD of 3 batches for each investigated milk (raw, pasteurized, and pasteurized plus high-pressure processed, HPP)

Storage time	Storage at 4°C			Storage at 12°C		
	Raw	Pasteurized	Pasteurized plus HPP	Raw	Pasteurized	Pasteurized plus HPP
Day 0 (raw)	4.56 $\pm$ 1.31 ^a	4.56 $\pm$ 1.31 ^a	4.56 $\pm$ 1.31 ^a	4.56 $\pm$ 1.31 ^a	4.56 $\pm$ 1.31 ^a	4.56 $\pm$ 1.31 ^a
Day 1	4.79 $\pm$ 1.69 ^a	<10 ^b	<10 ^b	6.05 $\pm$ 1.65 ^a	<10 ^b	<10 ^b
Day 3	6.99 $\pm$ 1.13 ^a	<10 ^b	<10 ^b	7.95 $\pm$ 0.34 ^{a,1}	1.63 $\pm$ 2.83 ^b	<10 ^b
Day 7	NP ²	1.61 $\pm$ 2.79 ^a	0.89 $\pm$ 1.54 ^a	NP	3.84 $\pm$ 3.77 ^a	0.73 $\pm$ 1.27 ^a
Day 10	NP	4.40 $\pm$ 3.94 ^a	<10 ^a	NP	3.61 $\pm$ 6.26 ^a	2.70 $\pm$ 2.45 ^a
Day 15	NP	5.32 $\pm$ 4.62 ^a	<10 ^b	NP	6.57 $\pm$ 5.84 ^a	1.90 $\pm$ 3.29 ^a
Day 21	NP	NP	<10	NP	NP	1.91 $\pm$ 3.31
Day 25	NP	NP	<10	NP	NP	<10
Day 30	NP	NP	<10	NP	NP	2.32 $\pm$ 4.02

^{a,b}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

¹Samples in which milk presented anomalous blue coloration.

²Analysis not performed.

parameters considered up to 30 d of storage; the higher values detected during 30 d of storage resulted 1.82 ± 0.32 (SD) log cfu/mL and 0.89 ± 1.54 log cfu/mL for TMC and *Pseudomonas* spp. Count, respectively (Tables 1 and 2); the *Enterobacteriaceae* and *B. cereus* counts were <10 cfu/mL in all samples during storage (Tables 3 and 4). In contrast, an increase was observed in TMC at d 3 when milk was stored at 12°C. The *Enterobacteriaceae* and *Pseudomonas* spp. counts were generally below the limit of detection, with only some exceptions, and we observed an increase in presumptive *B. cereus*, in particular in batches 2 and 3 from d 3 of storage.

The pH values at d 0 were always above neutrality (range from 7.26 to 7.37). During storage at 4°C, the pH remained substantially unchanged for all milk samples, except in batch 3 of raw milk, in which we observed a decrease of pH associated with milk coagulation on d

3 of storage. The pH of milk stored at 12°C decreased after different times depending on the sample and the batch: we observed a pH decrease associated with milk coagulation after 1, 7, 8, and 15 d of storage at 12°C for raw, HPP, pasteurized, and pasteurized plus HPP milk respectively (see Table 5).

The statistical evaluation of the effects of pasteurization and pasteurization plus HPP treatments on the count of the investigated microorganisms through the shelf-life showed (1) a significant difference of pasteurized and pasteurized plus HPP milk versus raw milk for TMC, *Pseudomonas* spp., and *Enterobacteriaceae* counts from d 1 to 3 of storage at 4°C (see Tables 1 and 2); (2) a significant difference in TMC of pasteurized versus pasteurized plus HPP milk stored at 4 and 12°C from d 10 of storage (see Table 1 and Figure 1); (3) a significant difference in *Pseudomonas* spp. count of pasteurized versus pasteurized plus HPP milk stored

Table 3. Enumeration of *Enterobacteriaceae* (log cfu/mL) during storage of donkey milk stored at 4°C and 12°C; data represent mean $\pm$ SD of 3 batches for each investigated milk (raw, pasteurized, and pasteurized plus high-pressure processed, HPP)

Storage time	Storage at 4°C			Storage at 12°C		
	Raw	Pasteurized	Pasteurized plus HPP	Raw	Pasteurized	Pasteurized plus HPP
Day 0 (raw)	2.20 $\pm$ 1.69 ^a	2.20 $\pm$ 1.69 ^a	2.20 $\pm$ 1.69 ^a	2.20 $\pm$ 1.69 ^a	2.20 $\pm$ 1.69 ^a	2.20 $\pm$ 1.69 ^a
Day 1	2.92 $\pm$ 1.80 ^a	<10 ^b	<10 ^b	3.73 $\pm$ 1.84 ^a	<10 ^b	<10 ^b
Day 3	3.53 $\pm$ 1.42 ^a	<10 ^b	<10 ^b	6.33 $\pm$ 0.80 ^{a,1}	0.69 $\pm$ 1.20 ^b	<10 ^b
Day 7	NP ²	<10 ^a	<10 ^a	NP	2.16 $\pm$ 3.74 ^a	<10 ^a
Day 10	NP	<10 ^a	<10 ^a	NP	3.37 $\pm$ 5.83 ^a	<10 ^a
Day 15	NP	<10 ^a	<10 ^a	NP	3.72 $\pm$ 6.42 ^a	<10 ^a
Day 21	NP	NP	<10	NP	NP	2.76 $\pm$ 4.79
Day 25	NP	NP	<10	NP	NP	2.96 $\pm$ 5.12
Day 30	NP	NP	<10	NP	NP	2.69 $\pm$ 4.66

^{a,b}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

¹Samples in which milk presented anomalous blue coloration.

²Analysis not performed.

Table 4. Enumeration of *Bacillus cereus* (log cfu/mL or cfu/mL if <10) during storage of donkey milk stored at 4°C and 12°C; data represent mean $\pm$ SD of 3 batches for each investigated milk (raw, pasteurized, and pasteurized plus high-pressure processed, HPP)

Storage time	Storage at 4°C			Storage at 12°C		
	Raw	Pasteurized	Pasteurized plus HPP	Raw	Pasteurized	Pasteurized plus HPP
Day 0 (raw)	<10 ^a	<10 ^a	<10 ^a	<10 ^a	<10 ^a	<10 ^a
Day 1	<10 ^a	<10 ^a	<10 ^a	<10 ^a	<10 ^a	<10 ^a
Day 3	<10 ^a	0.49 $\pm$ 0.69 ^a	<10 ^a	<10 ^{a,1}	2.90 $\pm$ 0.87 ^b	1.91 $\pm$ 1.41 ^b
Day 7	NP ²	0.33 $\pm$ 0.47 ^a	<10 ^a	NP	6.47 $\pm$ 0.61 ^a	4.49 $\pm$ 3.17 ^a
Day 10	NP	0.33 $\pm$ 0.47 ^a	<10 ^a	NP	6.69 $\pm$ 0.58 ^a	4.56 $\pm$ 3.24 ^a
Day 15	NP	0.33 $\pm$ 0.47 ^a	<10 ^a	NP	6.61 $\pm$ 0.82 ^a	4.95 $\pm$ 3.50 ^a
Day 21	NP	NP	<10	NP	NP	4.53 $\pm$ 3.21
Day 25	NP	NP	<10	NP	NP	4.26 $\pm$ 3.04
Day 30	NP	NP	<10	NP	NP	3.48 $\pm$ 3.48

^{a,b}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

¹Samples in which milk presented anomalous blue coloration.

²Analysis not performed.

at 4°C on d 15 of storage (see Table 2 and Figure 2); and (4) a significant differences in pH values of raw milk versus pasteurized and pasteurized plus HPP milk stored at 12°C on d 3 of storage (Table 5). No significant differences were observed between the pH values of raw milk versus HPP milk until d 3 of storage (data not shown) or between pasteurized and pasteurized plus HPP milk until d 15 of storage.

Table 6 reports the results of ALP and furosine: the concentration of ALP from an initial value of 2,533.4 to 4,500.0 mU/L in raw milk decreased to <100 to 103.0 mU/L in the 2 types of heat-treated milk (pasteurized and pasteurized plus HPP). The HPP treatment without pasteurization did not significantly affect the ALP concentration. Similarly, furosine concentration increased from 5.27 to 18.9 and 19.3 in the 2 types of heat-treated milk (pasteurized and pasteurized plus HPP, respectively).

DISCUSSION

The microbiological quality of the investigated raw donkey milk was not optimal, resulting in 2 of the 3 batches analyzed not being compliant with requirements of the applicable regional regulation; the initial viable count was higher than in most literature studies that report low bacterial counts (<4 log cfu/mL for bulk tank donkey milk; Pilla et al., 2010; Alberghini et al., 2012; Salimei and Fantuz, 2012; Sarno et al., 2012) but in line with the study of Cavallarin et al. (2015), which reported counts 1 order of magnitude higher (mean 5.38 log cfu/mL). Conte et al. (2010) found an initial total mesophilic flora of 2×10^2 cfu/mL, which reached 1.3×10^8 and $>3 \times 10^{10}$ at 3°C and 7°C, respectively, from d 3 to 28 of storage. The *Enterobacteriaceae* count in our study was in line with literature reports of mean raw milk values in the range

Table 5. Evolution of pH values during storage of donkey milk stored at 4°C and 12°C; data represent mean $\pm$ SD of 3 batches for each investigated milk (raw, pasteurized, and pasteurized plus high-pressure processed, HPP)

Storage time	Storage at 4°C			Storage at 12°C		
	Raw	Pasteurized	Pasteurized plus HPP	Raw	Pasteurized	Pasteurized plus HPP
Day 0 (raw)	7.30 $\pm$ 0.05 ^a	7.30 $\pm$ 0.05 ^a	7.30 $\pm$ 0.05 ^a	7.30 $\pm$ 0.05 ^a	7.30 $\pm$ 0.05 ^a	7.30 $\pm$ 0.05 ^a
Day 1	7.31 $\pm$ 0.16 ^a	7.54 $\pm$ 0.19 ^a	7.55 $\pm$ 0.21 ^a	7.32 $\pm$ 0.13 ^{a,1}	7.51 $\pm$ 0.23 ^a	7.51 $\pm$ 0.23 ^a
Day 3	7.26 $\pm$ 0.12 ^a	7.49 $\pm$ 0.14 ^a	7.49 $\pm$ 0.15 ^a	6.89 $\pm$ 0.20 ^a	7.51 $\pm$ 0.16 ^b	7.50 $\pm$ 0.12 ^b
Day 7	NP ²	7.50 $\pm$ 0.20 ^a	7.49 $\pm$ 0.10 ^a	NP	6.98 $\pm$ 0.15 ^a	7.24 $\pm$ 0.11 ^a
Day 10	NP	7.37 $\pm$ 0.07 ^a	7.48 $\pm$ 0.10 ^a	NP	6.83 $\pm$ 0.24 ^{a,1}	7.07 $\pm$ 0.31 ^a
Day 15	NP	7.19 $\pm$ 0.23 ^a	7.42 $\pm$ 0.12 ^a	NP	6.54 $\pm$ 0.06 ^{a,3}	6.69 $\pm$ 0.52 ^{a,1}
Day 21	NP	NP	7.43 $\pm$ 0.13	NP	NP	6.53 $\pm$ 0.51 ¹
Day 25	NP	NP	7.44 $\pm$ 0.18	NP	NP	6.40 $\pm$ 0.74 ¹
Day 30	NP	NP	7.44 $\pm$ 0.15	NP	NP	6.03 $\pm$ 1.11 ¹

^{a,b}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

¹Samples in which milk appeared completely coagulated in 1 or 2 batches.

²Analysis not performed.

³Samples in which milk appeared completely coagulated in all the batches.

Figure 1. Evolution of total mesophilic count (TMC; log cfu/mL) during storage of donkey milk stored at 4°C (A) and 12°C (B). Data represent mean and standard deviation of 3 batches for pasteurized and pasteurized plus high-pressure processed (HPP) milk; *statistical difference between the 2 treatments ($P < 0.05$).

Figure 2. Evolution of *Pseudomonas* spp. (log cfu/mL) during storage of donkey milk stored at 4°C. Data represent mean and standard deviation of 3 batches for pasteurized and pasteurized plus high-pressure processed (HPP) milk; *statistical difference between the 2 treatments ($P < 0.05$).

of 0 and 0.32 log cfu/mL, and peaks after 8 d at 3°C or 3-log increases after 8 d at 8°C (Sarno et al., 2012). After the pasteurization and pasteurization plus HPP treatments, the *Enterobacteriaceae* count, a hygiene criterion indicative of heat-treatment efficiency and prevention of recontamination, was always below the legal limit (European Commission, 2007; Regulation CE 1441/2007) for pasteurized milk stored at 4 and 12°C until the end of the shelf-life periods investigated. The only exception was the second batch, which gave unsatisfactory results at 12°C from d 3 and 21 of storage in pasteurized and pasteurized plus HPP milk, respectively.

In agreement with Cavallarin et al. (2015), high *Pseudomonas* spp. counts seem to be frequent in raw donkey milk, suggesting possible contamination due to the use of water not provided by a municipal supply system, poor cleaning of milking machines and other dairy equipment (bulk tank), or biofilm formation. This finding highlights the need to improve hygiene practices during milking and milk storage at donkey dairy farms.

A nonnegligible variability between the batches was noted both for raw donkey milk and for milk after the different treatments for all microorganisms considered in the study: the variability we observed among the batches could be due to differences in the native microbial population of raw milk used and to the fact that, although we used autoclaved equipment, the milk was packed in unsterilized commercial containers, as used

on most donkey milk farms. This suggests that the milk should be treated after packing to reduce postprocessing contamination.

Of particular interest was *B. cereus* found in donkey milk after heat treatments: contamination of milk by this microorganism is significant not only because of its spoilage capability but especially for its potential to cause human diseases. In fact, pasteurization may induce germination of *B. cereus* spores, which subsequently grow and produce toxins during the preservation of pasteurized milk (Claeys et al., 2013). Contamination of cow milk by *B. cereus* group species has been found, with 40 to 50 and 40 to 170 cfu/L spores in UHT and pasteurized milk, respectively (Bartoszewicz et al., 2008). Scatassa et al. (2011) reported the first isolation of *B. cereus* in bulk jennet milk samples with a maximum concentration of 1.2×10^3 cfu/mL and in individual milk samples at levels of 10, 20, and 60 cfu/mL, whereas Cavallarin et al. (2015) found similar *B. cereus* counts (1.3×10^2 cfu/mL). Inactivation of microbial spores by high pressure alone is not feasible (Evelyn and Silva, 2015) and, therefore, the combination of HPP with a mild thermal process is required. Few studies exist in the literature on HPP efficacy in inactivating *B. cereus* spores in milk and they were performed exclusively in artificially contaminated cow milk. Milk treated at 500 MPa at 60°C for 30 min resulted in 6-log spore inactivation (Van Opstal et al., 2004) or from 2 to 4.8 log units when milk was treated twice at 500 MPa at 40°C for 5 min in the presence of nisin (Black et al., 2008); in reconstituted milk treated at 600 MPa, a 5.3-log spore inactivation was registered after 40 min at 70°C, whereas only a 1.8-log reduction was observed at 38°C (Evelyn and Silva, 2015; a 3- to 6-log reduction was observed when reconstituted skim milk was treated at 500 MPa at 72°C (Scurrah et al., 2006). In any case, complete spore inactivation was not achieved by using the aforementioned treatments, and large variations were observed between different *B. cereus* strains (Black et al., 2008). Although the potential HPP ability was highlighted, these treatments are not applicable in raw donkey milk due to appearance of visible flocks in milk after HPP treatment.

We did not detect *B. cereus* in raw milk samples in the current study, but after pasteurization or pasteurization plus HPP, we sporadically isolated the bacterium in pasteurized milk stored at 4°C and continuously detected it in all milk batches stored at 12°C from d 3 of storage, with values ranging between 1.91 ± 1.41 (SD) and 6.69 ± 0.58 log cfu/mL. This high level of contamination also represents a potential risk to food-sensitive consumers. In fact, 1 of the 2 syndromes caused by *B. cereus*—diarrheal illness—results from the ingestion of spores or vegetative cells and production of enterotoxins in the small intestine: infective doses range from 10^4 to 10^9 cells per gram of food (Logan, 2012). Based on this evidence, improper storage after milk treatment will influence the capacity of spores to germinate and the capacity of vegetative cells to multiply; it is thus a key issue for safety reasons and a critical point requiring strict regulation.

In the comparison of the effects of pasteurization and pasteurization plus HPP treatments on the count of the investigated microorganisms throughout the shelf life, the results show that both treatments were effective methods to increase microbiological quality compared with raw milk, and that the pasteurization plus HPP treatment, together with proper storage, can be an effective method to preserve the microbial quality of the milk and to maintain the process hygiene criteria in compliance with EC Regulation 1441/2007 until d 30 of storage.

The pH values recorded in this study were in line with the literature data (Conte et al., 2010; Alberghini et al., 2012; Sarno et al., 2012; Cavallarin et al., 2015), even if Conte and Passantino (2009) reported lower values. Unlike cow milk, in donkey milk, microflora growth seems to have less influence on pH.

Alkaline phosphatase is an indigenous milk enzyme present in the raw milk of all mammals at levels varying among species (Marchand et al., 2009), but no data on ALP in donkey milk are available in the literature. Our results show that ALP activity in donkey milk is similar to that reported in equine milk (Marchand et al., 2009), and that ALP values were comparable in raw milk and HPP-treated milk (from 1,939.9 to 4,500 mU/L) and

Table 6. Determination of alkaline phosphatase activity (data represent mean $\pm$ SD of 3 batches) and of furosine (1 batch) for each typology of donkey milk considered in the study

Milk	Alkaline phosphatase activity (mU/L)	Furosine (mg/100 g of protein)
Raw	36,046.8 $\pm$ 1,008.78	5.27 $\pm$ 0.57
High-pressure processed (HPP)	3,343.63 $\pm$ 1,267.42	4.83 $\pm$ 0.52
Pasteurized	106.06 $\pm$ 10.47	19.3 $\pm$ 2.1
Pasteurized plus HPP	100.33 $\pm$ 2.30	18.9 $\pm$ 2.1

were comparable in pasteurized and pasteurized plus HPP-treated milk (from <100 to 118.1), showing that ALP can be used as an indicator of proper pasteurization in donkey milk. Furosine values describe the extent of lactose isomerization and early Maillard reaction and increase linearly with increased heating temperature and heating time. We found a lower furosine content in raw donkey milk than that reported by Salimei and Fantuz (2012), who adapted the data of Sorrentino et al. (2006) (5.27 vs. 15.43 mg/100 g of protein, respectively), but similar values were found after pasteurization and thermal treatment at 63°C for 30 min (19.3 vs. 18.53 mg/100 g of protein, respectively). We found similar furosine values in raw and HPP-treated milk and in pasteurized and pasteurized plus HPP-treated milk, indicating that furosine could be used, like ALP, as an indicator for thermal processing.

The growing interest in donkey milk as an alternative food for infants with food allergies should be supported by appropriate studies showing its suitability for human consumption and in terms of milk safety. Limited data are available in the literature on donkey milk hygiene and safety, and no studies have hitherto investigated the frequency of pathogens occurring in raw donkey milk, hindering an accurate risk definition. Our results show that the total bacterial count of 2 of the 3 batches of raw donkey milk sold by vending machines or directly at farms did not meet criteria established by Italian law in terms of safety or hygiene quality. These data highlight the importance of raw milk management, with the need for animal hygiene management and good dairy farming practices on donkey farms to improve handling procedures and the control of low temperature at the farm and during milk transport.

The microbiological risk posed by raw donkey milk consumption is reduced by heat treatment. However, the presence and growth of *B. cereus* after moderate thermal abuse impede the wide-scale marketing of donkey milk due to the potential consequences for sensitive consumers. Therefore, combined heat treatment and storage strategies are recommended to control bacterial spores or reduce the viability of *B. cereus*.

CONCLUSIONS

Our study showed that a farm-scale packing system for pasteurized milk cannot guarantee extended shelf life and that the shelf life of donkey milk varies. The HPP treatment of pasteurized milk performed after packing extended the shelf life of donkey milk and assured its microbial safety up to 30 d if properly stored at 4°C. As food business operators bear the primary responsibility for food safety and the shelf life of produce should be based on scientific evidence, our results

could be used to define the shelf life of donkey milk and conduct further tests with time-temperature-high pressure protocols associated with *B. cereus*.

REFERENCES

- Alberghini, L., P. Catellani, M. A. Norbiato, and V. Giaccone. 2012. Indagine preliminare sulle caratteristiche microbiologiche del latte d'asina. Ital. J. Food Safety 1:7–10.
- Bartoszewicz, M., B. M. Hansen, and I. Swiecicka. 2008. The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. Food Microbiol. 25:588–596.
- Beaufort, A., M. Cornu, H. Bergis, and A. L. Lardeux. 2008. Technical guidance document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods. Agence Francaise de Securité Sanitaire des Aliments, Maisons-Alfort, France.
- Black, E. P., M. Linton, R. D. McCall, W. Curran, G. F. Fitzgerald, A. L. Kelly, and M. F. Patterson. 2008. The combined effects of high pressure and nisin on germination and inactivation of *Bacillus* spores in milk. J. Appl. Microbiol. 105:78–87.
- Carroccio, A., F. Cavataio, G. Montaldo, D. D'Amico, L. Alabrese, and G. Iacono. 2000. Intolerance to hydrolysed cow's milk proteins in infants: clinical characteristics and dietary treatment. Clin. Exp. Allergy 30:1597–1603.
- Cavallarin, L., M. Giribaldi, M. Soto-Del Rio, E. Valle, G. Barbarino, M. S. Gennero, and T. Civera. 2015. A survey on the milk chemical and microbiological quality in dairy donkey farms located in northwestern Italy. Food Contr. 50:230–235.
- Claeys, W. L., S. Cardoen, G. Daube, J. De Block, K. Dewettinck, K. Dierick, L. De Zutter, A. Huyghebaert, H. Imberechts, P. Thiange, Y. Vandenplas, and L. Herman. 2013. Raw or heated cow milk consumption: R of risks and benefits. Food Contr. 31:251–262.
- Claeys, W. L., C. Verraes, S. Cardoen, J. De Block, A. Huyghebaert, K. Raes, K. Dewettinck, and L. Herman. 2014. Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. Food Contr. 42:188–201.
- Conte, F., and A. Passantino. 2009. Guidelines for physical, chemical and hygienic quality and safety control of donkey's milk. Milchwissenschaft 64:85–88.
- Conte, F., T. Rapisarda, G. Belvedere, and S. Carpino. 2010. Shelf-life del latte d'asina: Batteriologia e componente volatile. Ital. J. Food Safety 7:25–29.
- Cullen, P. J., B. K. Tiwari, and V. P. Valdramidis. 2012. Status and trends of novel thermal and non-thermal technologies for fluid foods. Pages 1–6 in Novel Thermal and Non-thermal Technologies for Fluid Foods. P. J. Cullen, B. K. Tiwari, and V. Valdramidis, ed. Academic Press, San Diego, CA.
- European Commission. 2007. Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. Official Journal L322:12–29.
- Evelyn, and F. V. M. Silva. 2015. High pressure processing of milk: Modeling the inactivation of psychrotrophic *Bacillus cereus* spores at 38–70°C. J. Food Eng. 165:141–148.
- Hite, B. H. 1899. The effect of pressure in the preservation of milk. Bull. West Virginia Univ. Agric. Exper. Stn. 58:15–35.
- Iacono, G., A. Carroccio, F. Cavataio, G. Montalto, M. Soresi, and V. Balsamo. 1992. Use of ass' milk in multiple food allergy. J. Pediatr. Gastroenterol. Nutr. 14:177e181.
- ISO (International Organization for Standardization). 1999. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)—Part 2: Technique using rabbit plasma fibrinogen agar medium. ISO 6888-2:1999/Amd. 1:2003. ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization). 2004. Microbiology of food and animal feeding stuffs—Horizontal methods for the detection and enumeration of *Enterobacteriaceae*—Part 2: Colony-count method. ISO 21528-2:2004. ISO, Geneva, Switzerland.

- ISO (International Organization for Standardization). 2005a. Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of presumptive *Bacillus cereus* and colony-count technique at 30°C. ISO 7932:2005. ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization). 2005b. General requirements for the competence of testing and calibration laboratories. ISO/IEC 17025:2005. ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization). 2009. Milk and milk products—Method for the enumeration of *Pseudomonas* spp. ISO/TS 11059:2009. ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization). 2013a. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of microorganisms. Colony-count technique at 30°C by the surface plating technique. UNI EN ISO 4833-2:2013/Cor 1:2014. ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization). 2013b. Milk and milk products—Determination of alkaline phosphatase activity—Part 1: Fluorimetric method for milk and milk-based drinks. ISO 11816-1:2013. ISO, Geneva, Switzerland.
- Logan, N. A. 2012. *Bacillus* and relatives in foodborne illness. J. Appl. Microbiol. 112:417–429.
- Mansueto, P., G. Iacono, G. Taormina, A. Seidita, A. D'Alcamo, F. Adragna, G. Randazzo, M. Carta, G. Rini, and A. Carroccio. 2013. Ass's milk in allergy to cow's milk protein: A review. Acta Med. Mediter. 29:153–160.
- Marchand, S., M. Merchiers, W. Messens, K. Coudijzer, and J. De Block. 2009. Thermal inactivation kinetics of alkaline phosphatase in equine milk. Int. Dairy J. 19:763–767.
- Monti, G., E. Bertino, M. C. Muratore, A. Coscia, F. Cresi, L. Silvestro, C. Fabris, D. Fortunato, M. G. Giuffrida, and A. Conti. 2007. Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: An in vivo and in vitro study. Pediatr. Allergy Immunol. 18:258–264.
- Monti, G., S. Viola, C. Baro, F. Cresi, P. A. Tovo, G. Moro, M. P. Ferrero, A. Conti, and E. Bertino. 2012. Tolerability of donkey's milk in 92 highly-problematic cow's milk allergic children. J. Biol. Regul. Homeost. Agents 26(Suppl.):75–82.
- Palou, E., A. Lopez-Malo, G. V. Barbosa-Cánovas, and B. G. Swanson. 2007. High-pressure treatment in food preservation. Pages 815–853 in Handbook of Food Preservation. 2nd ed. M. S. Rahman, ed. CRC Press, Boca Raton, FL.
- Pilla, R., V. Dapra, A. Zeconi, and R. Piccinini. 2010. Hygienic and health characteristics of donkey milk during a follow-up study. J. Dairy Res. 77:392–397.
- Salimei, E., and F. Fantuz. 2012. Equid milk for human consumption. Int. Dairy J. 24:146–152.
- Sarno, E., A. M. L. Santoro, R. Di Palo, and N. Costanzo. 2012. Microbiological quality of raw donkey milk from Campania region. Ital. J. Anim. Sci. 11:266–269.
- Scatassa, M. L., A. Carrozzo, B. Ducato, C. Giosué, V. Miraglia, L. Arcuri, and I. Mancuso. 2011. *Bacillus cereus* isolation in jennet milk. Ital. J. Food Safety 1:243–246.
- Scurrah, K. J., R. E. Robertson, H. M. Craven, L. E. Pearce, and E. A. Szabo. 2006. Inactivation of *Bacillus* spores in reconstituted skim milk by combined high pressure and heat treatment. J. Appl. Microbiol. 101:172–180.
- Sorrentino, E., E. Salimei, M. Succi, D. Gammariello, T. Di Criscio, G. Panfili, and R. Coppola. 2006. Heat treatment of ass's milk, a hypoallergenic food for infancy. Pages 569–574 in Technological Innovation and Enhancement of Marginal Products. C. Severini, T. DePilli, and R. Giuliani, ed. Claudio Grezi Editore, Foggia, Italy.
- Van Opstal, I., C. F. Bagamboula, S. C. M. Vanmuysen, E. Y. Wuytack, and C. W. Michiels. 2004. Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. Int. J. Food Microbiol. 92:227–234.