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ABSTRACT

The aim of this study was to investigate the effect of different treatment conditions on the hygiene microbiological indicators of donkey milk and their evolution during shelf life at 4 and 12°C from a minimum of 3 to a maximum of 30 days simulating a farm-scale pasteurization and packing system. Four treatment conditions were tested, respectively no treatment (raw milk), pasteurization (65°x30'), 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 directly with HPP showed visible alterations with flocks making the milk unfit for sale. The

43 microbiological risk posed by raw donkey milk consumption is significant reduced by heat treatment
44 but farm-scale packing system cannot guarantee an extended shelf life whereas the pasteurization
45 plus HPP treatment resulted the most effective method to maintain the microbiological milk quality.
46 Microflora growth seems to have few influence on pH in donkey milk: pH values were significant
47 different only between raw milk versus both pasteurized and pasteurized plus HPP milk stored at
48 12°C at day 3. Alkaline phosphatase activity and furosine could be used as indicators of proper
49 pasteurization and for thermal processing in donkey milk. Moreover, the presence and growth of *B.*
50 *cereus* in the case of thermal abuse hamper the widescale marketing of donkey milk due to the
51 potential consequences for sensitive consumers and therefore further tests with time/temperature/high
52 pressure protocols associated with *B. cereus* are needed. Finally, our study shows that a HPP
53 treatment of pasteurized milk after packing extends the shelf life of the produce and assures its
54 microbial criteria up to 30 days if properly stored at 4°C until opening, therefore combined heat
55 treatment and storage strategies are suggested to enhance the shelf life of donkey milk.
56
57 **INTRODUCTION**
58 Even if non-ruminant milk accounts for less than 0.1% of global milk production (Claeys et al., 2014),
59 donkey's milk is receiving increasing interest in Europe as an alternative to breast milk and infant
60 formula for babies allergic to cow's milk (Iacono et al., 1992; Mansueto et al., 2013; Monti et al.,
61 2007, 2012) or in case of multiple food intolerance (Carroccio et al., 2000) or when breastfeeding is
62 not possible (Sarno et al., 2012). In addition, donkey's milk is appreciated by people eager to try new
63 foods and purchase locally grown produce (Scatassa et al., 2011).
64 To meet this demand, donkey farming is undergoing a revival in Italy with new donkey dairies
65 opening in several Regions. With few exceptions, farms are small (<10 to 150 donkeys and from 5 to
66 30 milking jennies), family-run and usually located in mountain or hilly areas. Jennies are milked
67 once a day using milking machines adapted from goat or cow milking equipment (Cavallarin et al.,
68 2015) and usually produce about 1.5 L of milk a day. Daily milk production does not usually exceed

50 – 100 L, and due to the long distances between donkey farms a logistic organization of both milk collection and distribution is lacking.

Currently, donkey's milk for human consumption is sold as raw milk directly at farms or by vending machines or heat-treated by pasteurization and, rarely, ultra-high temperature (UHT), or freeze-dried, packed in cartons or PET or glass bottles and sold in shops, pharmacies or on-line. By Italian law, raw milk has a shelf life of three days whereas the shelf life of pasteurized and UHT milk is usually fixed by manufacturers at 4-6 days for pasteurized milk at storage conditions between 0 and 4°C and 6 months for UHT with the advice to refrigerate it at max 5°C after opening and consume it within 3 days. Nevertheless, these conditions raise some problems: i) the 3 days of donkey raw milk shelf life limit the widescale marketing of this commodity and the development of donkey milk companies or farms; ii) pasteurization extends the shelf life but not long enough to be a viable alternative for a wide and efficient distribution given donkey farms logistic limitations; iii) UHT and freeze-drying treatments guarantee commercially sterile products but entail irreversible changes in endogenous milk compounds like whey protein and lipid components (Sorrentino et al., 2005), and could alter the flavor. In addition, UHT treatment systems are very expensive for a single farm and need large amounts of milk that 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 highly problematic patients.

For these reasons, it is useful to evaluate alternative approaches to donkey milk sanitation and shelf life extension. High pressure processing (HPP) is a non-thermal food preservation technology with minimal adverse effects on food quality (Cullen et al., 2012). It relies on the use of high pressures (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). HPP effects on foods were first studied in the late 19th century, when processing cow milk at 670 MPa for 10 min resulted in five to six logarithmic microbial reductions, extending shelf life up to 4 days after processing (Hite, 1899). However, the complexity of foods and the wide variety of phenomena that occur under pressure make

it difficult to predict HPP effects on foods (Palou et al., 2007). For these reasons, HPP conditions must be evaluated in each specific food.

Few literature data are available 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 hygiene microbiological indicators of donkey milk and their evolution during shelf life at different temperatures from a minimum of 3 and a maximum of 30 days 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 three consecutive weeks in June 2015: after post-milking 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') using a commercial farm scale pasteurization system (Caseus, Plastitalia group, 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 were subjected to HPP treatment. The HPP treatment was performed by Avure Technologies (Quintus Food Press QFP350L-600**)**: milk packs were initially treated at a constant pressure of 600 MPa and at temperatures in the range of 4-6°C for 180''; under working conditions, the temperature increased by approximately 10°C due to pressure buildup (approximately 100 MPa min^{-1}). After HPP treatment, the milk was visually inspected for any changes that could affect donkey milk marketing. Due to appearance of clotting in the HPP-treated milk, the pressure was reduced from 600 Mpa to 400 Mpa

for 180'' for pasteurized milk and for raw milk the HPP treatment was further reduced at 400 Mpa for 100''.

For each treatment condition, all the 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 days (according to Italian legislation), pasteurized and HPP samples for 15 days and pasteurization plus HPP samples for 30 days.

Samples were analyzed from each PET spout pouch at day 0 (before treatment) and for each storage condition at days 1 and 3 for raw milk, at days 1, 3, 7, 10, 15 for pasteurized and HPP milk and additionally at days 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); enumeration of *Enterobacteriaceae* (ISO 21528-2:2004), *Pseudomonas* spp. (ISO/TS 11059:2009 (IDF/RM 225: 2009), presumptive *Bacillus cereus* (UNI EN ISO 7932:2005), and only for raw and HPP samples, enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) (ISO 6888-2:1999/Amd.1:2003). The pH value of each sample was measured by an automatic temperature compensation device (Hanna Instruments HI 223, Milan, Italy).

According to Italian legislation (Ministerial Decree 16-05-1996), alkaline phosphatase activity (ALP) (ISO 11816-1:2006) and furosine were determined by HPLC technique in raw donkey milk samples at day 0 and at day 1 (after each type of treatment). All analyses were performed in the laboratories of the Experimental Institutes for Zooprophylaxis in Lombardy and Emilia Romagna, accredited according to International Organization for Standardization (ISO) method 17025:2005 by ACCREDIA, the Italian accreditation body, except for furosine determination which was performed at Chelab S.r.l. (Resana, Treviso, Italy).

146 **Statistical analysis**

147 The results were analyzed statistically for the comparison, within each day of storage, of the
148 microbiological and pH data between the different treatments : i) raw versus HPP milk, raw versus
149 pasteurized milk and raw versus pasteurized plus HPP milk for the first 3 days; ii) HPP versus
150 pasteurized milk, pasteurized versus pasteurized plus HPP milk and HPP versus pasteurized plus HPP
151 milk for the first 15 days of storage. Comparison was performed for both the two different storage
152 conditions. The data were analyzed by repeated measures two-way ANOVA and Bonferroni post-
153 tests; we used u PRISM 5.0 software and statistical significance was set at $p < 0.05$.

154

155 **RESULTS**

156 The raw milk treated directly with HPP (both at 600 MPa for 180'' and at 400 MPa for 180" and
157 100'') showed visible alterations with flocks making the milk unfit for sale. For this reason, the
158 microbiological data and their statistical analysis are not shown in detail. HPP treatment at 600 MPa
159 for 180'' caused the same alterations when applied to pasteurized milk, but to a lesser extent.
160 Pasteurized milk treated at 400 MPa for 180'' showed no alterations.

161 The initial contamination of the three batches of raw milk at day 0 showed a variability in the TMC,
162 *Pseudomonas* spp. and *Enterobacteriaceae* counts (see tables 1-3); two of the three analyzed batches
163 of raw donkey milk didn't meet criteria fixed by Italian law for raw milk sold by vending machines
164 or directly at farms that require a TMC≤ 100.000 CFU/mL. Storage of raw milk for 3 days at 4°C and
165 at 12°C resulted in an increased TMC, *Pseudomonas* spp. and *Enterobacteriaceae* counts.
166 Presumptive *B. cereus* was always <10 CFU/mL and coagulase-positive staphylococci were detected
167 at 0.77–1.00 log/ CFU/mL with no increase during storage.

168 Both pasteurization and pasteurization plus HPP resulted in a significant 3-5 log reduction of
169 contaminant microflora with respect to raw milk but did not guarantee the absence of contaminants,
170 which were found to grow after 3 days of storage at 12°C and 7 days when stored at 4°C (Tables 2
171 and 3). When stored at 4°C, the TMC and *Pseudomonas* spp. counts of pasteurized milk increased

during the different sampling days in the different batches but were acceptable up to 7 days of storage (see Tables 2 and 3). We sporadically detected a low count of presumptive *B. cereus*. By contrast, all microbiological parameters increased up to 6-11 log CFU/mL in milk stored at 12°C, associated with a bluish coloration in batch III.

The pasteurized plus HPP milk stored at 4°C showed a very moderate contamination for all the microbiological parameters considered up to 30 days of storage; the higher values detected during 30 days of storage resulted 1.82±0.32 SD log CFU/mL and 0.89±1.54 log CFU/mL SD for TMC and *Pseudomonas* spp. count respectively (Table 1 and 2); the *Enterobacteriaceae* and *B. cereus* counts resulted <10 CFU/mL in all the samples during storage (Table 3 and 4). On the contrary an increase was observed in TMC at day 3 when the 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 II and III from the third day of storage.

The pH values at day 0 were always above neutrality (min 7.26-max 7.37). During storage at 4°C the pH remained substantially unchanged for all milk samples, except batch III of raw milk in which we observed a decrease of pH associated with milk coagulation on the third day 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 days 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 microrganisms through the shelf-life showed: i) significant difference of pasteurized and pasteurized plus HPP milk versus raw milk for TMC, *Pseudomonas* spp. and *Enterobacteriaceae* counts from the 1st to the 3rd days of storage at 4°C (see tables 1 and 2); ii) a significant difference of the TMC of pasteurized versus pasteurized plus HPP milk stored both at 4 and 12°C since the 10th day of storage (see table 1 and figure 1); iii) a significant difference of *Pseudomonas* spp. count of pasteurized versus pasteurized plus HPP milk stored at 4°C at the 15th

day of storage (see table 2); iv) significant differences in pH values of raw milk versus both pasteurized and pasteurized plus HPP milk stored at 12°C at the 3rd day of storage (table 5). No significant differences were observed between the pH values of raw milk versus HPP milk, till the 3rd day of storage (data not shown) and between pasteurized and pasteurized plus HPP milk till the 15th day of storage.

Table 6 reports the results of ALP and furosine: ALP concentration from an initial value of 2533.4 – 4500.0 mUL^{-1} in raw milk decreased to <100 – 103.0 mUL^{-1} in the two types of heat-treated milk (pasteurized and pasteurized plus HPP). HPP treatment performed without pasteurization did not significantly affect the ALP concentration. Similarly, furosine concentration increased from 5.27 to 18.9-19.3 in the two types of heat-treated milk (pasteurized and pasteurized plus HPP).

DISCUSSION

The microbiological quality of the investigated raw donkey milk was not optimal resulting in two of the three batches analyzed not compliant with requirements of the applicable regulation: the initial viable count was higher than in most literature studies that report low bacterial counts (under 4 log CFU/mL for bulk tank donkey's milk) (Pilla et al., 2010; Salimei and Fantuz, 2012; Sarno et al., 2012; Alberghini et al., 2012), but in line with the study of Cavallarin et al. (2015) which reported one order of magnitude higher (mean 5.38 log CFU/mL). Conte et al. (2010) found an initial total mesophilic flora of $2x10^2$ CFU/mL that reached $1.3x10^8$ and $>3x10^{10}$ at 3°C and 7°C respectively from the 3rd to the 28th day of storage. The *Enterobacteriaceae* count in our study was in line with literature reports of mean raw milk values in the range of 0 and 0.32 log CFU/mL, and peaks after 8 days at 3°C or 3-log increases after 8 days 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 (Regulation CE 1441/2007) for pasteurized milk stored at both 4 and 12°C until the end of the shelf life periods investigated. The only exception was the second batch, that gave unsatisfactory results at 12°C from the 3rd and 21st days 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 not negligible variability between the batches has to be noted both for raw donkey milk and for milk after the different treatments for all the microrganisms considered in the study: the variability we observed among the batches could be due both 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 in most donkey milk farms. This suggests the milk should be treated after packing to reduce post-processing contamination.

Of particular interest is *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 the germination of *B. cereus* spores, which subsequently grow and produce toxins during the preservation of pasteurized milk (Clayes et al., 2013). Contamination of cow's milk by *B. cereus* group has been found, with 40-50 and 40-170 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 $1.2x10^3$ CFU/mL and in individual milk samples at levels of 10, 20 and 60 CFU/mL, while Cavallarin et al. (2015) found similar *B. cereus* counts ($1.3x10^2$ CFU/mL).

Few data were in literature on efficiency of HPP treatment in inactivating *B. cereus* spores in milk, and most of the tests were performed on artificially contaminated cow milk. Generally, a high rate of inactivation could be obtained in a single step with high pressure >1000 Mpa (used only for studies and not be reasonable used for food applications) or at temperatures of 80-110°C or with high pressure at 600 Mpa at 60°C for 30 min or with a two-step treatment at 200 Mpa at 45°C for 30 min for germination of spores followed by heat treatment at 60°C for 10 min to kill the germinated spores.

All these treatments cannot be used in donkey milk treatment due to the appearence of flocks appearence we noted and already noted also by Reviewer 1 in a previous study and, for the latter hypothesis, because the pasteurization after the HPP treament is not feasible in case of food industry. To be noted that industrial HPP processing relies on elevated pressure (about 400-600 Mpa) treatments at refrigerated or room temperature (between 4 and 25°C).

Our study never detected *B. cereus* in raw milk samples, 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 the 3rd storage day with values ranging between 1.91±1.41SD and 6.69±0.58 SD log CFU/mL. This high level of contamination also represents a potential risk to food-sensitive consumers. In fact, one of the two syndromes caused by *B. cereus*, namely 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 et al., 2011). Based on this evidence, improper storage after milk treatment will influence the capacity of spores to germinate and of vegetative cells to multiply and 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 microrganisms through the shelf-life, the results show that both these treatments resulted effective methods to increase the microbiological quality, when compared to raw milk, and that the pasteurized plus HPP treatment, together with a 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 till the 30th day of storage.

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

Alkaline phosphatase is an indigenous milk enzyme present in the raw milk of all mammals at levels varying among species and from one species to another (Marchand et al., 2009), but no data on ALP

in donkey milk are available in the literature. Our results show that: i) ALP activity in donkey milk is similar to that reported in equine milk (Marchand et al., 2009); ii) ALP values in raw milk and HPP-treated milk (min 1939.9 max 4500 mU L^{-1}) and also in pasteurized and pasteurized plus HPP-treated milk (from <100 to 118.1) were comparable, 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 rise linearly with increased heating temperature and heating time. We found a lower furosine content in raw donkey milk than that reported by Salimei et al. (2012), who adapted the data of Sorrentino et al. (2006) (5.27 versus 15.43 mg 100 g^{-1} protein respectively), but similar values were found after pasteurization and thermal treatment at 63°C for 30'(19.3 versus 18.53 mg 100 g^{-1} protein respectively). We found similar furosine values for raw and HPP-treated milk and for pasteurized and pasteurized plus HPP-treated milk, indicating that furosine could be used, as ALP, as an indicator for thermal processing.

CONCLUSION

The growing interest in donkey milk as an alternative food for highly problematic patients like infants with food allergy should be supported by appropriate studies showing its suitability for human consumption, also in terms of milk safety. Only 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, hampering a correct risk definition. Our results show that the total bacterial count of two of the three batches of raw donkey milk sold by vending machines or directly at farms does not meet criteria fixed by Italian law in terms of safety for hygiene quality and does not guarantee hygienic quality standards for consumers. 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 farms 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 hamper the widescale marketing of donkey milk due to the potential consequences for sensitive consumers. Therefore combined heat treatment and storage strategies are needed to control bacterial spores or reduce the viability of *B. cereus*.

Our study shows that a farm-scale packing system for pasteurized milk cannot guarantee an extended shelf life and that the shelf life of donkey milk varies. HPP treatment of pasteurized milk performed after packing extends the shelf life of the produce and assures its microbial criteria up to 30 days if properly stored at 4°C, resulting a valid tool to assure the compliance of microbiological criteria until opening by the consumer, and, therefore, a valid choice for the donkey milk enterprises.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 further tests with time/temperature/high pressure protocols associated with *B. cereus*.

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