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Running head: SHELF LIFE OF DONKEY MILK

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

21

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29

30 **Key words:** donkey milk, pasteurization, HPP, shelf life

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32

33 **ABSTRACT**

34 The aim of this study was to investigate the effect of different treatment conditions on the hygiene

35 microbiological indicators of donkey milk and their evolution during shelf life at 4 and 12°C from a

36 minimum of 3 to a maximum of 30 days simulating a farm-scale pasteurization and packing system.

37 Four treatment conditions were tested, respectively no treatment (raw milk), pasteurization (65°x30'),

38 high pressure processing (HPP), and pasteurization plus HPP.

39 The microbiological quality of the raw donkey milk investigated was not optimal: our results

40 highlight the importance of raw milk management with the need for animal hygiene management and

41 good dairy farming practices on donkey farms to improve handling procedures. The raw milk treated

42 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

69 50 – 100 L, and due to the long distances between donkey farms a logistic organization of both milk
70 collection and distribution is lacking.

71 Currently, donkey's milk for human consumption is sold as raw milk directly at farms or by vending
72 machines or heat-treated by pasteurization and, rarely, ultra-high temperature (UHT), or freeze-dried,
73 packed in cartons or PET or glass bottles and sold in shops, pharmacies or on-line. By Italian law,
74 raw milk has a shelf life of three days whereas the shelf life of pasteurized and UHT milk is usually
75 fixed by manufacturers at 4-6 days for pasteurized milk at storage conditions between 0 and 4°C and
76 6 months for UHT with the advice to refrigerate it at max 5°C after opening and consume it within 3
77 days. Nevertheless, these conditions raise some problems: i) the 3 days of donkey raw milk shelf life
78 limit the widescale marketing of this commodity and the development of donkey milk companies or
79 farms; ii) pasteurization extends the shelf life but not long enough to be a viable alternative for a wide
80 and efficient distribution given donkey farms logistic limitations; iii) UHT and freeze-drying
81 treatments guarantee commercially sterile products but entail irreversible changes in endogenous
82 milk compounds like whey protein and lipid components (Sorrentino et al., 2005), and could alter the
83 flavor. In addition, UHT treatment systems are very expensive for a single farm and need large
84 amounts of milk that donkey farms are not expected to produce. At the same time, the safety of
85 donkey milk is a potential concern for food-sensitive consumers or highly problematic patients.

86 For these reasons, it is useful to evaluate alternative approaches to donkey milk sanitation and shelf
87 life extension. High pressure processing (HPP) is a non-thermal food preservation technology with
88 minimal adverse effects on food quality (Cullen et al., 2012). It relies on the use of high pressures
89 (generally 100-600 MPa) to process liquid or solid foods to inactivate spoilage and pathogenic
90 microorganisms and extend the shelf life (Evelyn and Silva, 2015). HPP effects on foods were first
91 studied in the late 19th century, when processing cow milk at 670 MPa for 10 min resulted in five to
92 six logarithmic microbial reductions, extending shelf life up to 4 days after processing (Hite, 1899).
93 However, the complexity of foods and the wide variety of phenomena that occur under pressure make

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

96 Few literature data are available on the effects of heat treatments on the chemical and microbiological
97 parameters of donkey milk, and no study has hitherto addressed the effects of HPP on its microbial
98 contents. The aim of this study was to investigate the effect of different treatment conditions on the
99 hygiene microbiological indicators of donkey milk and their evolution during shelf life at different
100 temperatures from a minimum of 3 and a maximum of 30 days simulating a farm-scale pasteurization
101 and packing system.

102

103 **MATERIALS AND METHODS**

104 *Milk Sample Preparation*

105 Four treatment conditions were tested: no treatment (raw milk), pasteurization, HPP, and
106 pasteurization plus HPP. Three batches of raw donkey milk were collected from local farms for three
107 consecutive weeks in June 2015: after post-milking refrigeration, each batch (30 L) was transported
108 to the cheese factory of the Department of Veterinary Medical Sciences, Bologna, and then, it was
109 divided into 2 portions: i) 20 L of raw donkey milk was pasteurized (65°C for 30') using a commercial
110 farm scale pasteurization system (Caseus, Plastitalia group, Italy) and packed into 26 PET spout
111 pouches (250 mL each): 10 were used for the pasteurization test and 16 were transported to a local
112 industry for HPP treatment for the pasteurization plus HPP test; ii) 10 L of raw donkey milk were
113 packed into 16 PET spout pouches (250 mL each): 6 for the raw milk test and 10 were subjected to
114 HPP treatment. The HPP treatment was performed by Avure Technologies (Quintus Food Press
115 QFP350L-600): milk packs were initially treated at a constant pressure of 600 MPa and at
116 temperatures in the range of 4-6°C for 180''; under working conditions, the temperature increased by
117 approximately 10°C due to pressure buildup (approximately 100 MPa min⁻¹). After HPP treatment,
118 the milk was visually inspected for any changes that could affect donkey milk marketing. Due to
119 appearance of clotting in the HPP-treated milk, the pressure was reduced from 600 Mpa to 400 Mpa

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

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

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

129

130 ***Microbiological and Chemical Analyses***

131 The following microbiological analysis were performed in each sample of type of milk as described
132 above: total mesophilic colony count (TMC) (UNI EN ISO 4833-2:2013/Cor.1:2014); enumeration
133 of *Enterobacteriaceae* (ISO 21528-2:2004), *Pseudomonas* spp. (ISO/TS 11059:2009 (IDF/RM 225:
134 2009), presumptive *Bacillus cereus* (UNI EN ISO 7932:2005), and only for raw and HPP samples,
135 enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) (ISO
136 6888-2:1999/Amd.1:2003). The pH value of each sample was measured by an automatic temperature
137 compensation device (Hanna Instruments HI 223, Milan, Italy).

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

145

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 $TMC \leq 100.000$ 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

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

176 The pasteurized plus HPP milk stored at 4°C showed a very moderate contamination for all the
177 microbiological parameters considered up to 30 days of storage; the higher values detected during 30
178 days of storage resulted 1.82 ± 0.32 SD log CFU/mL and 0.89 ± 1.54 log CFU/mL SD for TMC and
179 *Pseudomonas* spp. count respectively (Table 1 and 2); the *Enterobacteriaceae* and *B. cereus* counts
180 resulted <10 CFU/mL in all the samples during storage (Table 3 and 4). On the contrary an increase
181 was observed in TMC at day 3 when the milk was stored at 12°C. The *Enterobacteriaceae* and
182 *Pseudomonas* spp. counts were generally below the limit of detection, with only some exceptions,
183 and we observed an increase in presumptive *B. cereus*, in particular in batches II and III from the
184 third day of storage.

185 The pH values at day 0 were always above neutrality (min 7.26-max 7.37). During storage at 4°C the
186 pH remained substantially unchanged for all milk samples, except batch III of raw milk in which we
187 observed a decrease of pH associated with milk coagulation on the third day of storage. The pH of
188 milk stored at 12°C decreased after different times depending on the sample and the batch: we
189 observed a pH decrease associated with milk coagulation after 1, 7, 8 and 15 days of storage at 12°C
190 for raw, HPP, pasteurized and pasteurized plus HPP milk respectively (see Table 5).

191 The statistical evaluation of the effects of pasteurization and pasteurization plus HPP treatments on
192 the count of the investigated microorganisms through the shelf-life showed: i) significant difference
193 of pasteurized and pasteurized plus HPP milk versus raw milk for TMC, *Pseudomonas* spp. and
194 *Enterobacteriaceae* counts from the 1st to the 3rd days of storage at 4°C (see tables 1 and 2); ii) a
195 significant difference of the TMC of pasteurized versus pasteurized plus HPP milk stored both at 4
196 and 12°C since the 10th day of storage (see table 1 and figure 1); iii) a significant difference of
197 *Pseudomonas* spp. count of pasteurized versus pasteurized plus HPP milk stored at 4°C at the 15th

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

203 Table 6 reports the results of ALP and furosine: ALP concentration from an initial value of 2533.4 –
204 4500.0 mUL⁻¹ in raw milk decreased to <100 – 103.0 mUL⁻¹ in the two types of heat-treated milk
205 (pasteurized and pasteurized plus HPP). HPP treatment performed without pasteurization did not
206 significantly affect the ALP concentration. Similarly, furosine concentration increased from 5.27 to
207 18.9-19.3 in the two types of heat-treated milk (pasteurized and pasteurized plus HPP).

208 DISCUSSION

209 The microbiological quality of the investigated raw donkey milk was not optimal resulting in two of
210 the three batches analyzed not compliant with requirements of the applicable regulation: the initial
211 viable count was higher than in most literature studies that report low bacterial counts (under 4 log
212 CFU/mL for bulk tank donkey's milk) (Pilla et al., 2010; Salimei and Fantuz, 2012; Sarno et al.,
213 2012; Alberghini et al., 2012), but in line with the study of Cavallarin et al. (2015) which reported
214 one order of magnitude higher (mean 5.38 log CFU/mL). Conte et al. (2010) found an initial total
215 mesophilic flora of 2x10² CFU/mL that reached 1.3x10⁸ and >3x10¹⁰ at 3°C and 7°C respectively
216 from the 3rd to the 28th day of storage. The *Enterobacteriaceae* count in our study was in line with
217 literature reports of mean raw milk values in the range of 0 and 0.32 log CFU/mL, and peaks after 8
218 days at 3°C or 3-log increases after 8 days at 8°C (Sarno et al., 2012). After the pasteurization and
219 pasteurization plus HPP treatments, the *Enterobacteriaceae* count, a hygiene criterion indicative of
220 heat treatment efficiency and prevention of recontamination, was always below the legal limit
221 (Regulation CE 1441/2007) for pasteurized milk stored at both 4 and 12°C until the end of the shelf
222 life periods investigated. The only exception was the second batch, that gave unsatisfactory results at
223 12°C from the 3rd and 21st days of storage in pasteurized and pasteurized plus HPP milk respectively.

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

229 A not negligible variability between the batches has to be noted both for raw donkey milk and for
230 milk after the different treatments for all the microorganisms considered in the study: the variability
231 we observed among the batches could be due both to differences in the native microbial population
232 of raw milk used and to the fact that, although we used autoclaved equipment, the milk was packed
233 in unsterilized commercial containers as used in most donkey milk farms. This suggests the milk
234 should be treated after packing to reduce post-processing contamination.

235 Of particular interest is *B. cereus* found in donkey milk after heat treatments: contamination of milk
236 by this microorganism is significant not only because of its spoilage capability but especially for its
237 potential to cause human diseases. In fact, pasteurization may induce the germination of *B. cereus*
238 spores, which subsequently grow and produce toxins during the preservation of pasteurized milk
239 (Clayes et al., 2013). Contamination of cow's milk by *B. cereus* group has been found, with 40-50
240 and 40-170 CFU/L spores in UHT and pasteurized milk respectively (Bartoszewicz et al., 2008).
241 Scatassa et al. (2011) reported the first isolation of *B. cereus* in bulk jennet milk samples with a
242 maximum concentration of 1.2×10^3 CFU/mL and in individual milk samples at levels of 10, 20 and
243 60 CFU/mL, while Cavallarin et al. (2015) found similar *B. cereus* counts (1.3×10^2 CFU/mL).

244 Few data were in literature on efficiency of HPP treatment in inactivating *B. cereus* spores in milk,
245 and most of the tests were performed on artificially contaminated cow milk. Generally, a high rate of
246 inactivation could be obtained in a single step with high pressure >1000 Mpa (used only for studies
247 and not be reasonable used for food applications) or at temperatures of 80-110°C or with high pressure
248 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
249 germination of spores followed by heat treatment at 60°C for 10 min to kill the germinated spores.

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

255 Our study never detected *B. cereus* in raw milk samples, but after pasteurization or pasteurization
256 plus HPP, we sporadically isolated the bacterium in pasteurized milk stored at 4°C and continuously
257 detected it in all milk batches stored at 12°C, from the 3rd storage day with values ranging between
258 $1.91 \pm 1.41 \text{SD}$ and $6.69 \pm 0.58 \text{SD log}$ CFU/mL. This high level of contamination also represents a
259 potential risk to food-sensitive consumers. In fact, one of the two syndromes caused by *B. cereus*,
260 namely diarrheal illness, results from the ingestion of spores or vegetative cells and production of
261 enterotoxins in the small intestine: infective doses range from 10^4 to 10^9 cells per gram of food (Logan
262 et al., 2011). Based on this evidence, improper storage after milk treatment will influence the capacity
263 of spores to germinate and of vegetative cells to multiply and is thus a key issue for safety reasons
264 and a critical point requiring strict regulation.

265 In the comparison of the effects of pasteurization and pasteurization plus HPP treatments on the count
266 of the investigated microorganisms through the shelf-life, the results show that both these treatments
267 resulted effective methods to increase the microbiological quality, when compared to raw milk, and
268 that the pasteurized plus HPP treatment, together with a proper storage, can be an effective method
269 to preserve the microbial quality of the milk and to maintain the process hygiene
270 criteria in compliance with EC Regulation till the 30th day of storage.

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

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

276 in donkey milk are available in the literature. Our results show that: i) ALP activity in donkey milk
277 is similar to that reported in equine milk (Marchand et al., 2009); ii) ALP values in raw milk and
278 HPP-treated milk (min 1939.9 max 4500 mU L⁻¹) and also in pasteurized and pasteurized plus HPP-
279 treated milk (from <100 to 118.1) were comparable, showing that ALP can be used as an indicator of
280 proper pasteurization in donkey milk. Furosine values describe the extent of lactose isomerization
281 and early Maillard reaction and rise linearly with increased heating temperature and heating time. We
282 found a lower furosine content in raw donkey milk than that reported by Salimei et al. (2012), who
283 adapted the data of Sorrentino et al. (2006) (5.27 versus 15.43 mg 100 g⁻¹ protein respectively), but
284 similar values were found after pasteurization and thermal treatment at 63°C for 30'(19.3 versus 18.53
285 mg 100 g⁻¹ protein respectively). We found similar furosine values for raw and HPP-treated milk and
286 for pasteurized and pasteurized plus HPP-treated milk, indicating that furosine could be used, as ALP,
287 as an indicator for thermal processing.

288

289

CONCLUSION

290 The growing interest in donkey milk as an alternative food for highly problematic patients like infants
291 with food allergy should be supported by appropriate studies showing its suitability for human
292 consumption, also in terms of milk safety. Only limited data are available in the literature on donkey
293 milk hygiene and safety, and no studies have hitherto investigated the frequency of pathogens
294 occurring in raw donkey milk, hampering a correct risk definition. Our results show that the total
295 bacterial count of two of the three batches of raw donkey milk sold by vending machines or directly
296 at farms does not meet criteria fixed by Italian law in terms of safety for hygiene quality and does not
297 guarantee hygienic quality standards for consumers. These data highlight the importance of raw milk
298 management with the need for animal hygiene management and good dairy farming practices on
299 donkey farms to improve handling procedures and the control of low temperature at the farms and
300 during milk transport.

301 The microbiological risk posed by raw donkey milk consumption is reduced by heat treatment.
302 However, the presence and growth of *B. cereus* after moderate thermal abuse hamper the widescale
303 marketing of donkey milk due to the potential consequences for sensitive consumers. Therefore
304 combined heat treatment and storage strategies are needed to control bacterial spores or reduce the
305 viability of *B. cereus*.

306 Our study shows that a farm-scale packing system for pasteurized milk cannot guarantee an extended
307 shelf life and that the shelf life of donkey milk varies. HPP treatment of pasteurized milk performed
308 after packing extends the shelf life of the produce and assures its microbial criteria up to 30 days if
309 properly stored at 4°C, resulting a valid tool to assure the compliance of microbiological criteria until
310 opening by the consumer, and, therefore, a valid choice for the donkey milk enterprises. As food
311 business operators bear the primary responsibility for food safety and the shelf life of produce should
312 be based on scientific evidence, our results could be used to define the shelf life of donkey milk and
313 further tests with time/temperature/high pressure protocols associated with *B. cereus*.

314

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