

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Giacometti, F., Bardasi, L., Merialdi, G., Morbarigazzi, M., Federici, S., Piva, S., et al. (2016). Shelf life of donkey milk subjected to different treatment and storage conditions. JOURNAL OF DAIRY SCIENCE, 99(6), 4291-4299 [10.3168/jds.2015-10741].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/543107> since: 2020-02-23

*Published:*

DOI: <http://doi.org/10.3168/jds.2015-10741>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

1  
2  
3  
4  
5

6 This is the final peer-reviewed accepted manuscript of:

7 Federica Giacometti, Lia Bardasi, Giuseppe Merialdi, Michele Morbarigazzi, Simone Federici, Silvia  
8 Piva, and Andrea Serraino. Shelf life of donkey milk subjected to different treatment and storage  
9 conditions. Journal of Dairy Science. 99 (2016), pp. 4291-4299.

10 The final published version is available online at:

11 <http://dx.doi.org/10.3168/jds.2015-10741>

12

13 Rights / License:

14 The terms and conditions for the reuse of this version of the manuscript are specified in the  
15 publishing policy. For all terms of use and more information see the publisher's website.

16

*This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)*

***When citing, please refer to the published version.***

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

**Federica Giacometti,<sup>\*1</sup> Lia Bardasi,<sup>†</sup> Giuseppe Merialdi,<sup>†</sup> Michele Morbarigazzi,<sup>‡</sup> Simone Federici,<sup>‡</sup> Silvia Piva,<sup>\*</sup> Andrea Serraino<sup>\*</sup>**

<sup>\*</sup>University of Bologna - Department of Veterinary Medical Sciences - Via Tolara di Sopra 50-40064  
Ozzano Emilia - Bologna - Italy

<sup>†</sup>Experimental Institute for Zooprophyllaxis in Lombardy and Emilia Romagna - Via P. Fiorini, 5 -  
40127 Bologna – Italy

<sup>‡</sup>HPP Italia Srl, Via Carbognani, 6 – 43029 Traversetolo, Parma, Italy

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

<sup>1</sup>Corresponding author: federica.giacometti3@unibo.it

**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

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<sup>-1</sup>). 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 Zooprophyllaxis 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



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  $1.82 \pm 0.32$  SD log CFU/mL and  $0.89 \pm 1.54$  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 microorganisms 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 1<sup>st</sup> to the 3<sup>rd</sup> 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 10<sup>th</sup> 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 15<sup>th</sup>

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 3<sup>rd</sup> day of storage (table 5). No  
200 significant differences were observed between the pH values of raw milk versus HPP milk, till the 3<sup>rd</sup>  
201 day of storage (data not shown) and between pasteurized and pasteurized plus HPP milk till the 15<sup>th</sup>  
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<sup>-1</sup> in raw milk decreased to <100 – 103.0 mUL<sup>-1</sup> 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  $2 \times 10^2$  CFU/mL that reached  $1.3 \times 10^8$  and  $>3 \times 10^{10}$  at 3°C and 7°C respectively  
216 from the 3<sup>rd</sup> to the 28<sup>th</sup> 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 3<sup>rd</sup> and 21<sup>st</sup> 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  $1.2 \times 10^3$  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 ( $1.3 \times 10^2$  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 3<sup>rd</sup> 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 30<sup>th</sup> 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<sup>-1</sup>) 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<sup>-1</sup> 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<sup>-1</sup> 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

## 315 REFERENCES

- 316 Alberghini, L., P. Catellani, M.A. Norbiato, and V. Giaccone. 2012. Indagine preliminare sulle  
317 caratteristiche microbiologiche del latte d'asina. It. J. Food Safety 1(3):7-10.
- 318 Bartoszewicz, M., B.M. Hansen, and I. Swiecicka. 2008. The members of the *Bacillus cereus* group  
319 are commonly present contaminants of fresh and heat-treated milk. Food Microbiol. 25(4):588-596.
- 320 Beaufort, A., M. Cornu, H. Bergis, and A.L. Lardeux. 2008. Technical guidance document on shelf-  
321 life studies for *Listeria monocytogenes* in ready-to-eat foods. Maisons-Alfort, France: Agence  
322 Francaise de Sécurité Sanitaire des Aliments.
- 323 Carroccio, A., F. Cavataio, G. Montaldo, D.D'Amico, L. Alabrese, and G. Iacono. 2000. Intolerance  
324 to hydrolysed cow's milk proteins in infants: clinical characteristics and dietary treatment. Clin. Exp.  
325 Allergy. 30:1597–1603.

326 Cavallarin, L., M. Giribaldi, M. Soto-Del Rio, E. Valle, G. Barbarino, M.S. Gennero, and T. Civera.  
 327 2015. A survey on the milk chemical and microbiological quality in dairy donkey farms located in  
 328 NorthWestern Italy. *Food Control* 50:230-235.

329 Claeys, W.L., S. Cardoen, G. Daube, J. De Block, K. Dewettinck, K. Dierick, L. De Zutter, A.  
 330 Huyghebaert, H. Imberechts, P. Thiange, Y. Vandenplas, and L. Herman. 2013. Raw or heated cow  
 331 milk consumption: review of risks and benefits. *Food Control* 31(1):251-262.

332 Claeys, W.L., C. Verraes, S. Cardoen, J. De Block, A. Huyghebaert, K. Raes, K. Dewettinck, and L.  
 333 Herman. 2014. Consumption of raw or heated milk from different species: an evaluation of the  
 334 nutritional and potential health benefits. *Food Control* 42:188-201.

335 **European Commission (2007).** Commission Regulation (EC) No 1441/2007 of 5 December 2007  
 336 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

337 Conte, F., and A. Passantino. 2009. Guidelines for physical, chemical and hygienic quality and safety  
 338 control of donkey's milk. *Milchwissenschaft* 64(1):85-88.

339 Conte, F., T. Rapisarda, G. Belvedere, and S. Carpino. 2010. Shelf-life del latte d'asina: batteriologia  
 340 e componente volatile. *It. J. Food Safety* 7:25-29.

341 Cullen, P.J., B.K.Tiwari, and V.P. Valdramidis. 2012. Status and trends of novel thermal and non-  
 342 thermal technologies for fluid foods. In: Valdramidis, P.J.C.K.T.P. (Ed.), *Novel thermal and non-  
 343 thermal technologies for fluid foods*. Academic Press, San Diego, pp. 1–6.

344 **European Commission (2006).** Commission Regulation (EC) No 1664/2006 of 6 November 2006  
 345 amending regulation (EC) No 2074/2005 as regards implementing measures for certain products of  
 346 animal origin intended for human consumption and repealing certain implementing measures.  
 347 *Official Journal*, L320, 13–45.

348 European Commission (2007). Commission Regulation (EC) No 1441/2007 of 5 December 2007  
 349 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official Journal*,  
 350 L322/12, 12-29.

351 Evelyn and F.V.M. Silva. 2015. High pressure processing of milk: modeling the inactivation of  
352 psychrotrophic *Bacillus cereus* spores at 38–70 °C. J Food Eng. 165:141-145.

353 Hite, B.H. 1899. The effect of pressure in the preservation of milk. Bull. West Virginia Univ. Agric.  
354 Exper. Stn. 58:15-35.

355 Iacono, G., A. Carroccio, F. Cavataio, G. Montalto, M.Soresi, and V. Balsamo. 1992. Use of ass' milk  
356 in multiple food allergy. J. Pediatr. Gastroenterol. Nutr. 14(2):177e181.

357 ISO (International Organization for Standardization). 1999. Microbiology of food and animal feeding  
358 stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus*



359 aureus and other species) - Part 2: Technique using rabbit plasma fibrinogen agar medium. ISO 6888-  
 360 2:1999/Amd. 1:2003. ISO, Geneva, Switzerland.

361 ISO (International Organization for Standardization). 2004. Microbiology of food and animal feeding  
 362 stuffs - Horizontal methods for the detection and enumeration of *Enterobacteriaceae* - Part 2: Colony-  
 363 count method. ISO 21528-2:2004. ISO, Geneva, Switzerland.

364 ISO (International Organization for Standardization). 2005. Microbiology of food and animal feeding  
 365 stuffs. Horizontal method for the enumeration of presumptive *Bacillus cereus* and colony-count  
 366 technique at 30 °C. ISO 7932:2005. ISO, Geneva, Switzerland.

367 ISO (International Organization for Standardization). 2005. General requirements for the competence  
 368 of testing and calibration laboratories. ISO/IEC 17025:2005. ISO, Geneva, Switzerland.

369 ISO (International Organization for Standardization). 2009. Milk and milk products - Method for the  
 370 enumeration of *Pseudomonas* spp. ISO/TS 11059:2009. ISO, Geneva, Switzerland.

371 ISO (International Organization for Standardization). 2009. Milk and milk products - Determination  
 372 of hen's egg white lysozyme by HPLC. ISO/TS 27105:2009 IDF/RM 216:2009. ISO, Geneva,  
 373 Switzerland.

374 ISO (International Organization for Standardization). 2013. Milk and milk products - Determination  
 375 of alkaline phosphatase activity - Part 1: Fluorimetric method for milk and milk-based drinks. ISO  
 376 11816-1:2013. ISO, Geneva, Switzerland.

377 Logan, N.A. 2012. *Bacillus* and relatives in foodborne illness. J. Appl. Microbiol. 112:417-429.

378 Mansueto, P., G. Iacono, G. Taormina, A. Seidita, A. D'Alcamo, F. Adragna, G. Randazzo, M. Carta,

379 G. Rini, and A. Carroccio. 2013. Ass's milk in allergy to cow's milk protein: a review. *Acta Medica*  
380 *Mediterranea* 29(2):153-160.

381 Marchand, S., M. Merchiers, W. Messens, K. Coudijzer, and J. De Block. 2009. Thermal inactivation  
382 kinetics of alkaline phosphatase in equine milk. *Int. Dairy J.* 19:763-767.

383 Monti, G., E. Bertino, M.C. Muratore, A. Coscia, F. Cresi, L. Silvestro, C. Fabris, D. Fortunato,  
384 M.G. Giuffrida, and A. Conti. 2007. Efficacy of donkey's milk in treating highly problematic cow's  
385 milk allergic children: an in vivo and in vitro study. *Pediatr. Allergy Immunol.* 18(3):258-264.

386 Monti, G., S. Viola, C. Baro, F. Cresi, P.A. Tovo, G. Moro, M.P. Ferrero, A. Conti, and E. Bertino.  
387 2012. Tolerability of donkey's milk in 92 highly-problematic cow's milk allergic children. *J. Biol.*  
388 *Regul. Homeost. Agents* 26(3 Suppl):75-82.

389 Palou, E., A.Lopez-Malo, G.V. Barbosa-Cánovas, and B.G. Swanson. 2007.. High-pressure treatment  
390 in food preservation. In: *Handbook of food preservation*, Second ed. Rahman, M.S. (Ed.), Eds., CRC  
391 Press, Boca Raton.

392 Pilla, R., V. Dapra, A. Zecconi, and R. Piccinini. 2010. Hygienic and health characteristics of donkey  
393 milk during a follow-up study. *J. Dairy Res.* 77(4):392-397.

394 Salimei, E., and F. Fantuz. 2012. Equid milk for human consumption. *Int. Dairy J.* 24(2):146-152.

395 Sarno, E., A.M.L. Santoro, R. Di Palo, and N. Costanzo. 2012. Microbiological quality of raw donkey  
396 milk from Campania region. *Ital. J. Anim. Sci.* 11(3):266-269.

397 Scatassa, M.L., A. Carrozzo, B. Ducato, C. Giosué, V. Miraglia, L. Arcuri, and I. Mancuso. 2011.  
398 *Bacillus cereus* isolation in jennet milk. *It. J. Food Safety* 1:243-246.

399 Sorrentino, E., E. Salimei, M. Succi, D. Gammariello, T. Di Criscio, G. Panfili, and R. Coppola. 2006.  
400 Heat treatment of ass's milk, a hypoallergenic food for infancy. In C. Severini, T. DePilli, & R.  
401 Giuliani (Eds.), *Technological innovation and enhancement of marginal products*. pp. 569-574.  
402 Foggia, Italy: Claudio Grezi Editore.

403

404