Raw donkey milk versus raw cow's milk. A preliminary study to compare the growth of Listeria monocytogenes *and* Staphylococcus aureus

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Summary
Gram-positive foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* can grow in a wide variety of foods, including raw milk. The aim of the study was to compare the growth of *L. monocytogenes* and *S. aureus* inoculated in donkey and cow samples of raw milk during a storage time of 11 days at 8 °C. Moreover, the study aimed to evaluate the influence of lactic acid bacteria (LAB) content on the growth of the two microbiological populations considered. LAB content was lower in raw donkey milk than in raw cow's milk during the entire analyses; on the other hand, pH levels were higher in the donkey milk rather than in the cow's milk, although both values showed a decrease at the day 11. *S. aureus* showed no significant differences in the two types of milk. From day 0 to 11, *L. monocytogenes* increased from 3.68 ± 0.02 log CFU/mL to 6.31 ± 0.07 log CFU/mL and from 3.64 ± 0.04 log CFU/mL to 4.59 ± 1.04 log CFU/mL, in donkey milk and in cow's milk, respectively. Our results showed that donkey milk is a more favourable matrix to support the growth of *L. monocytogenes* than cow's milk.

Introduction

Milk is a nutritious food product for humans, and it is obtained from a variety of animal sources, such as cows, goats, sheep, donkeys and buffaloes (Mehmeti *et al.* 2017). Since milk contains many important nutrients and provides a suitable physical environment, it represents an ideal growth medium for both non-pathogenic and pathogenic bacteria (Quigley *et al.* 2013, White 2001).

Donkey milk (DM) is considered the best substitute for human milk in infant nutrition when breast-feeding is not available (Monti *et al.* 2007). So, due to its tolerability (i.e. digestibility, palatability, low allergenicity) and bioactivity (i.e. lysozyme activity), DM could be used as a dietary supplement (Souroullas *et al.* 2018). Nevertheless, DM is still a "niche product" which often can only be retailed in farms for direct consumption, while a smaller part is destined for the cosmetics and food industries (Brumini *et al.* 2016, Soto Del Rio *et al.* 2017).

Pasteurized DM is usually sold directly from the farms; however, considering its nutritional properties, it can be sold raw, with three days of shelf-life (Giacometti *et al.* 2016). Some authors (Pilla *et al.* 2010) highlighted that foodborne pathogens are generally absent in raw DM and somatic cells and total bacterial count (TBC) are often low, suggesting it could be a safe food, provided that the mammary gland is healthy and the animals are milked in good hygienic conditions. Previous studies (Carminati *et al.* 2014, Quigley *et al.* 2013) showed that DM has different microbial flora, mainly composed of lactic acid bacteria (LAB). These are characterized by bacteriocins production active against some Gram-negative bacteria (Mottola *et al.* 2018, Murua *et al.* 2013), although the presence of undesirable pathogens responsible of food-borne diseases have

been described (Cavallarin *et al.* 2015, EFSA Biohaz Panel 2015).

Lactoferrin, lysozyme, immunoglobulins and lactoperoxidase carry out an antimicrobial activity in milk (Baldi *et al.* 2005, Yamauchi *et al.* 2006) and their content is different among species, breeds and individuals because of genetic or breeding variants (Brumini *et al.* 2016). The low microbial count of DM (Aspri *et al.* 2017) is related to the excellent natural anatomical position of the udder and its small size (Doreau and Martin-Rosset 2011), as well as the presence of natural antimicrobial components. This antimicrobial activity of DM is mainly attributed to lysozyme and, to a lesser extent, to lactoferrin (Uniacke-Lowe *et al.* 2010).

Salimei and colleagues (Salimei *et al.* 2004) showed that the average concentration of lysozyme in DM is three times higher than in human milk, while this component is absent in the milk of cows, ewes and goats (Vincenzetti *et al.* 2007). Lysozyme in DM ranges from 0.67 to 3.74 g/L and maintains the same high percentage over the total protein during 150 days of lactation (Guo *et al.* 2007, Vincenzetti *et al.* 2011, Šarić *et al.* 2012, Šarić *et al.* 2014). The interaction between lactoferrin and the lipopolysaccharidic layer (LPS) causes disruption of the outer membrane. Moreover, this situation promotes the susceptibility of Gram-negative bacteria to the lysozyme by increasing the membrane permeability (Benkerroum 2008, Ellison and Giehl 1991, Farnaud and Evans 2003). Because of this mechanism, Gram-negative bacteria are less sensible to lysozyme than Gram-positive due to their outer layer, which does not allow the entry of lysozyme molecules into the target places in peptidoglycan structure (Floris *et al.* 2003).

The abundance of lactose seems to favour the growth and survival of adapted probiotic lactobacilli, although there is a high content of lysozyme (Chiavari *et al.* 2005, Coppola *et al.* 2002). Studies conducted by Zhang and colleagues (Zhang *et al.* 2008) on the ability of the LAB microflora to grow in DM, showed that enterococci could be the major portion of growing bacteria. Enterococci, in fact, are more resistant to lysozyme than lactobacilli and, among lactobacilli, sensitivity to lysozyme is species-specific or strain specific (Neviani *et al.* 1991). Therefore, the high content of lysozyme in DM is responsible for the presence of only coccus-shaped species (Carminati *et al.* 2014).

According to Regulation (EC) No. 853/2004[1], raw milk is produced by the secretion of the mammary gland of farmed animals, it has not been heated to more than 40 °C or undergone any treatment with an equivalent effect. The direct sale of raw milk from farms to consumers is allowed in several European countries, provided that the operation complies with the hygienic criteria in Regulation (EC) No. 853/2004 and the General Food Law [Regulation (EC) No. 178/2002[2]]. On the basis of Regulation (EC) No. 853/2004, DM is included under the section "other milk producing species," where the TBC is less than 1.500.000 CFU/mL at 30 °C. In addition, Regulation (EC) No. 2073/2005[3] includes the microbiological "food safety criteria" for *Listeria monocytogenes* in ready-to-eat (RTE) foods and the "process hygiene criteria" for coagulase-positive staphylococci (CPS). Annex I of Regulation (EC) No. 2073/2005 sets out the microbiological criteria for foodstuffs, including the criteria for *L. monocytogenes* in RTE foods (criteria 1.1 to 1.3); in particular, in RTE foods able to support the growth of *L. monocytogenes*, when food business operator (FBO) is not able to demonstrate that the product will not exceed the limit of 100 CFU/g throughout the shelf-life, the criteria is the absence of the pathogen. Annex II of this regulation specifies that FBOs shall conduct, as necessary, studies to evaluate the growth of *L. monocytogenes* that may be present in the product during the shelf-life under reasonably foreseeable storage conditions. Considering that consumers not always respect the advice to boil the raw milk before consuming it (Claeys *et al.* 2013), in the present study we considered the raw milk as a RTE product. Thus, the aim of the study was to compare the growth of *L. monocytogenes* and *S. aureus* inoculated in donkey and cow samples of raw milk during a storage time of 11 days at 8 °C. Moreover, we aimed to evaluate the influence of LAB content on the growth of the two microbiological populations considered.

Materials and methods

Milk contamination and sampling

The study was carried out during years 2017 and 2018. Two different batches of DM and raw cow's milk were supplied from local farms, collected into sterilized 1-litre laboratory bottles and transported in coolers to IZSLER's laboratories (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy) immediately after

[1] E1 European Commission (EC) 2004. Commission Regulation of 29 April 2004 laying down specific hygiene rules on the hygiene of foodstuffs. *Off J.* **L139**, 05/08/2004, 55.

[2] European Commission (EC) 2002. Commission Regulation of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Off J.* **L31**, 30/09/2002, 1-24.

[3] European Commission (EC) 2005. Commission Regulation of 15 November 2005 on microbiological criteria for foodstuffs. *Off J.* **L338**, 07/12/2005, 1-26.

milking. For each pathogen considered, a mixture consisting of three different strains was formed: one registered reference strain and two field strains previously isolated from cow's milk and cheese; in particular, ATCC® 19115TM (reference strain), LM 273250 and LM 332764 for *L. monocytogenes*, and *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC® 25923™ (reference strain), CPS 54057 and CPS 283463 for *S. aureus*. The strains, stored in a freezer at - 80 °C, were individually revitalized in BHI (brain heart infusion) liquid culture medium and incubated at 37 °C for at least 15-18 hours in aerobic conditions.

Then, each strain was re-suspended in BHI at a lower temperature in order to adapt the microorganism to the storage conditions of 8 °C as suggested by Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE products (EUCRL 2017). All strains were separately diluted in physiological solution and then each pathogen was separately mixed in equal volume to obtain a multi-strain cocktail of *L. monocytogenes* and a multi-strain cocktail of *S. aureus*.

DM and cow's milk were divided in 3 groups and inoculated with 1% v/v of physiological solution to obtain control samples or 1% v/v of each multi-strain cocktail to obtain the contaminated samples. Samples were incubated at 8 °C for 11 days. The sampling was carried out on single replicates (9 mL each) for each sampling time at 0, 3, 5, 7 and 11 days during the milk storage and the analyses were performed.

Analysis and test methods

The presence/absence of natural contaminations of milk were evaluated on control samples (not contaminated samples) at time 0 by ISO 11290-1:2017 to detect the *L. monocytogenes* presence and by ISO 6888-1:1999/Amd. 1:2003 to enumerate the CPS concentration in milk.

During the milk storage, on control samples, the enumeration of TBC, Enterobacteriaceae (ENT), LAB and CPS (*S. aureus* and other species) was performed by ISO 4833:2003, ISO 21528:2017, ISO 15214:1998 and ISO 6888-1:1999/Amd. 1:2003, respectively. The pH was determined using an instrument with automatic temperature compensation (Hanna Instruments HI 223).

On contaminate samples, the enumeration of *L. monocytogenes* was performed using the ISO 11290-2:2017 while the enumeration of CPS *S. aureus* was carried out using the ISO 6888-1:1999/Amd. 1:2003.

Statistical analysis

Microbiological results were expressed as log CFU/mL. For each analysed parameter and for each type of studied milk, the individual means and standard deviations were determined on the basis of the average of the single replicate of two milk batches. Three different increasing rates were evaluated starting from the observation of different tendency in LAB, *L. monocytogenes* and *S. aureus* between the two different types of milk from the day 0 to the day 11, divided by the level found at time 0.

Results

The results were expressed as mean value and standard deviation (SD) of the two samples of DM and the two samples of cow's milk used in the present study during the pre-established time intervals (0, 3, 5, 7 and 11 days). LAB concentration was lower in raw DM than in raw cow's milk during the entire experiment; on the other hand, pH levels

Table I. *Values of pH and enumeration of lactic acid bacteria (LAB), total bacterial count (TBC), enterobacteriaceae (ENT) and coagulase-positive staphylococci (CPS) (expressed in log CFU/mL) in raw DM and raw cow's milk during the storage at 8 °C for 11 days.* The results are expressed as mean ± standard deviation (SD).

Matrix	Parameter	Sampling interval (days)				
		0	3	5	7	11
Raw donkey milk	pH	7.31 ± 0.05	7.50 ± 0.13	7.46	7.26 ± 0.08	6.95
	LAB	1.30 ± 0.30	1.65 ± 0.05	1.65 ± 0.05	1.54 ± 0.54	1.81 ± 0.81
	TBC	5.66 ± 0.47	6.18 ± 0.93	7.48 ± 0.20	7.90 ± 0.20	8.43 ± 0.07
	ENT	< 1	< 1	< 1	< 1	< 1
	S+	1.26 ± 0.37	1.20 ± 0.28	< 1	1.23 ± 0.33	1.75 ± 1.05
Raw cow's milk	pH	6.69 ± 0.02	6.68 ± 0.02	6.43 ± 0.11	6.02 ± 0.62	5.61 ± 0.85
	LAB	3.48 ± 0.39	4.33 ± 0.74	5.23 ± 1.37	5.78 ± 2.06	6.17 ± 2.16
	TBC	5.16 ± 1.31	8.08 ± 0.69	8.45 ± 0.42	8.82 ± 0.47	8.70 ± 1.44
	ENT	2.26 ± 1.78	4.59 ± 1.73	5.31 ± 1.97	5.97 ± 3.36	2.15 ± 0.98
	S+	1.75 ± 1.05	< 1	< 1	2.08 ± 1.52	1.83 ± 1.17

Table II. *Enumeration of* Staphylococcus aureus *and* Listeria monocytogenes *(expressed in log CFU/mL) inoculated in raw DM and row cow's milk during the storage at 8 °C for 11 days.* The results are expressed as mean ± standard deviation (SD).

Matrix	Parameter	Sampling interval (days)				
		0	3	5	7	11
Raw donkey milk	*Staphylococcus aureus*	3.36 ± 0.35	3.31 ± 0.32	3.24 ± 0.42	3.15 ± 0.42	2.95 ± 0.23
	Listeria monocytogenes	3.68 ± 0.02	3.65 ± 0.08	4.41 ± 0.42	5.72 ± 0.27	6.31 ± 0.07
Raw cow's milk	*Staphylococcus aureus*	3.53 ± 0.37	3.30 ± 0.27	3.37 ± 0.41	3.34 ± 0.47	3.45 ± 0.78
	Listeria monocytogenes	3.64 ± 0.04	3.98 ± 0.35	4.16 ± 0.50	4.54 ± 1.50	4.59 ± 1.04

were higher in the DM rather than in the cow's milk, although both values showed a decrease at the day 11 (Table I).

S. aureus had no significant differences in the two types of milk considered (Table II); specifically, in the raw cow's milk *S. aureus* showed almost the same value at time 0 (3.53 ± 0.37 log CFU/mL) and at time 11 (3.45 ± 0.78 log CFU/mL), conversely in the raw donkey milk *S. aureus* decreased from time 0 (3.36 ± 0.35 log CFU/mL) to time 11 (2.95 ± 0.23 log CFU/mL). On the other hand, *L. monocytogenes* increased from the value of 3.68 ± 0.02 log CFU/mL at time 0 (the day of the inoculation) to the value of 6.31 ± 0.07 log CFU/mL in the DM and from the value of 3.64 ± 0.04 log CFU/mL at time 0 to the value of 4.59 ± 1.04 log CFU/mL in the cow's milk (Table II).

L. monocytogenes revealed a great variation between the inoculation (day 0) and the day 11 in the DM and a low variation in the cow's milk (Table III).

Discussion

This preliminary study estimated the growth of *L. monocytogenes* and *S. aureus* experimentally added to DM and cow's milk during a storage time of 11 days at 8 °C, to evaluate which of the two matrices resulted more favourable to support the growth of the two bacteria considered.

The pH value of both raw DM and raw cow's milk at time 0 was similar to the values reported in literature by Salimei and colleagues (Salimei *et al.* 2004) and by Guo and colleagues (Guo *et al.* 2007). The average pH value (7.31 ± 0.05 at time 0 and 6.95 after the storage of 11 days) of DM was higher than that of cow milk (6.69 ± 0.02 at time 0 and 5.61 ± 0.85 after 11 days). This may be explained by the lower casein N and phosphate contents in DM than in cow milk (Salimei *et al.* 2004). Moreover, the slight change of the pH values in DM could be associated with the presence of natural concentration of antimicrobial compounds like lactoferrin and lysozyme, which act directly on bacteria (Chiavari *et al.* 2005), maintaining almost unvarying pH values (Coppola *et al.* 2002, Zhang *et al.* 2008). On the basis of Regulation (EC) No 853/2004, DM respected the limit of TBC concentration (1,500,000 CFU/mL at 30 °C) until the third day of storage, while in cow's milk the limit was exceeded earlier.

S. aureus showed no changes in its concentration during the entire period of analysis, both in raw DM and in row cow's milk. On the other hand, *L. monocytogenes* showed a greater increase rate (0.717) in the DM than in the cow's milk (0.260). The increase rates regarding LAB highlighted an inverse trend to *L. monocytogenes*, showing a growth of 0.316 in DM and of 0.750 in cow's milk.

The inversely related growth between *Listeria* and LAB can be explained considering that LAB produced undetermined antimicrobials such as organic acids, hydrogen peroxide, antifungal peptides and bacteriocins that can inhibit the growth of *Listeria* spp. by competitive exclusion (Zhao *et al.* 2004, 2006). Studies conducted by Balla and colleagues (Balla *et al.* 2000) and by Gilmore and colleagues (Gilmore *et al.* 2014) reported that many enterocins from various enterococcal species isolated from many different environments are bactericidal to *L. monocytogenes*. These include enterocin Q (Cintas *et al.* 2000), enterocin A (Nilsen *et al.* 1998), enterocin P (Kang and Lee 2005), bacterocin 31 (Tomita *et al.* 1996), bacteriocin 51

Table III. *Mean values (M), standard deviation (SD) and standard error (SE) of the increase rates among two times (day 0 and day 11).* M from time 0 to time 11 expresses the difference between the parameter recorded in the day 11 and in the day 0, divided by the level found at time 0.

		Raw donkey milk	Raw cow's milk
		From day 0 to day 11	
LAB	M	0.316	0.750
	SD	0.446	0.425
	SE	0.316	0.300
Listeria monocytogenes	M	0.717	0.260
	SD	0.029	0.274
	SE	0.021	0.194
Staphylococcus aureus	M	-0.120	-0.028
	SD	0.023	0.117
	SE	0.017	0.083

(Yamashita *et al.* 2011) and several others. Similarly, Amézquita and Brashears (Amézquita and Brashears 2002) observed that some LAB could competitively inhibit *L. monocytogenes* in ready-to-eat meats at refrigeration temperature even though the competitive bacteria did not grow.

Moreover, although some literature sources reported strong antibacterial activity of DM, the majority of these reports are related to its activity toward Gram-negative bacteria members of the Enterobacteriaceae (Šarić *et al.* 2012, Tidona *et al.* 2011, Zhang *et al.* 2008). Indeed, in our study, the enumeration of ENT in donkey milk was fewer (1 log CFU/mL) than in cow's milk during the 11 days of analysis (Table I).

The results indicate that DM represents a more favourable matrix for support the growth of *L. monocytogenes* compared to cow's milk. Although the data obtained are relative to a limited number of samples, it is possible to state that probably the high concentration of lysozyme in the DM is not able to compensate for the poor concentration of LAB. Moreover, in a particularly delicate matrix like milk, the concentration of LAB is relevant, thanks to the Jameson effect, to bio-compete with any pathogens present in the raw material. In fact, the pH profile relative to cow's milk suggests that the LAB population present, even at a temperature of + 8 °C, may be able to cause an evident pH reduction, directly through the production of bacteriocins or indirectly through the fermentation activity carried out on the sugars. Therefore, the results obtained suggest a particular caution in the consumption of raw DM and confirm the need to give this product a very short shelf life, as correctly established by the Order of 10 December 2008 of the Ministry of Labour, Health and Social Policy, according to which the shelf life of raw milk indicated by the producer may not exceed three days from the date on which it is made available to the consumer. Regarding the data obtained from the contamination of milk with *S. aureus*, this pathogen does not seem to be particularly influenced by the different concentration of LAB and lysozyme. This result suggests the need for further studies to better assess which other enzymatic components can help to ensure a higher level of hygienic and safety in DM compared to raw cow's milk.

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