TITLE: Calcium-dependent antibacterial activity of donkey's milk against *Salmonella*

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Calcium dependent antibacterial activity of donkey's milk against *Salmonella*

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Abstract

The aim of this study was to examine the antibacterial activity of raw donkey's milk toward *Salmonella* Enteritidis and *Salmonella* Typhimurium as well as to determine its dependence on calcium, lysozyme and lactoferrin content. Antibacterial assays were conducted in artificially contaminated donkey's milk incubated at 38 °C for eight hours. A strong influence of the calcium concentration on the antibacterial activity of donkey's milk was analysed in artificially contaminated samples with added $CaCl_2$ and EDTA. The strong calcium-dependant antibacterial activity of donkey's milk toward the tested *Salmonella* strains was observed, and the addition of $CaCl_2$ to donkey milk improved its antibacterial potential against both pathogens. *S.* Enteritidis appeared to be less sensitive to antimicrobial agents in donkey's milk. The calcium dependant antibacterial activity of donkey's milk could possibly be attributed to the calcium binding ability of its lysozyme. Lysozyme might be marked as the main antibacterial agent with the most probable nonenzymatic mode of action against tested *Salmonella* strains.

Keywords donkey's milk; antibacterial activity; *Salmonella*; calcium

Introduction

According to the literature, donkey's milk (DM) is traditionally consumed in Asia, Africa and Eastern Europe (Fernando and Starkey 2000), but it also finds its place on the developed countries market, taking into account its favourable effect on human health (Tidona et al. 2011). Functional properties and chemical composition of DM have been investigated in recent years, particularly in terms of its possible application for infant nutrition in the

cases of bovine milk protein allergy (Monti et al. 2007). High content of lactose, low fat and protein content, good balance of casein and whey proteins as well as high concentration of polyunsaturated fatty acids make this milk suitable for this application (Guo et al. 2007). There are reports attributed to potential in the prevention of atherosclerosis (Tafaro et al. 2007) and its anti-inflammatory activity (Jirillo et al. 2010). The DM has also assigned as anti-proliferative and anti-tumor agent (Mao et al. 2009). The knowledge about the functionality of DM constantly increases as well as the published data on antimicrobial activity of DM. Despite the fact that this aspect of its functionality could be deeply explored, there are different reports of DM antibacterial properties (Zhang et al. 2008; Tidona et al. 2011; Šarić et al. 2012; Cavallarin et al. 2015; Fratini et al. 2015). Lysozyme (LZ) is marked as the main antimicrobial agent in this milk (Tidona et al. 2011; Šarić et al. 2012). The secondary role in antibacterial activity of DM is ascribed to lactoferrin (LF) since it occurs at much lower concentration in DM in comparison to LZ (Tidona et al. 2011; Šarić et al. 2012). According to available literature data the antibacterial activity of LZ is primarily directed against Gram positive bacteria, while the Gram negative bacteria appear less sensitive to LZ owing to protection role of its outer membrane (Floris et al. 2003). However, the previous investigations of DM suggested its strong antibacterial activity against Gram negative bacteria, including some *Salmonella* species (Šarić et al. 2012). The LZ as the major antibacterial compound in mare's milk is reported as effective against Gram negative bacteria owing to its calcium binding ability (Bruhn et al. 2011). Since a high degree of similarity exists between DM and mare LZ (Bruhn et al. 2011), investigation of connections between intensity of antibacterial activity of DM and its calcium and LZ content can be a good way of clarifying the mode of DM antibacterial action toward *Salmonella* species.

In line with that, the aim of the present study was to investigate the antibacterial activity of DM against *Salmonella* Enteritidis and *Salmonella* Typhimurium as well as to access the dependence on the content of calcium, LZ and LF. In order to determine the impact of calcium content on DM antibacterial activity, $CaCl_2$ and EDTA were added to selected DM samples. $CaCl_2$ is used as the donor of calcium ions, whereas EDTA is used as calcium ions binding compound.

The second order polynomial (SOP) and Artificial Neural Network (ANN) models which are utilised in this paper give a reasonable fit to experimental data and successfully predict observed parameters. (Annadurai et al. 2007).

Materials and methods

Collection of samples

Individual raw milk samples were collected from 18 clinically healthy donkeys (Domestic Balkan donkey breed) in Special Nature Reserve „Zasavica“, located in the northwest of Serbia. At sampling donkeys were in different periods of lactation (65–220 days *post-partum*). After the morning hand-milking, samples were immediately chilled to 4 °C and transported to the laboratory where the samples kept frozen at – 20 °C.

Protein profile determination

Samples preparation was performed using a modified method of Tidona et al. (2011). Dilution of milk samples was done in the buffer (0.125 M Tris-HCl, 4% SDS, 2% glycerol, 2% β-mercaptoethanol, pH 6.8) in the ratio 1:1.5 (*v/v*) sample: buffer, followed by heating at 100 °C for five minutes. The chip-based separations were performed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with the Protein 80 Plus LabChip kit and the dedicated Protein 80 software assay on 2100 expert software. The analysis chips were prepared in accordance to Protein 80 LabChip kit protocol. According to the convention for SDS-PAGE (Torbica et al. 2010) smallest proteins are emerging first in the profiles at the bottom of the gel patterns and the fractioning is size-based. Bovine serum albumin was used as standard for quantification of the milk proteins. All samples were analysed in triplicate.

Determination of Calcium (Ca) content

The DM samples were mineralized using Milestone Ethos-10 microwave digestor, and content of Ca was determined by atomic absorption spectrometer (VARIAN, SpectrAA 10) after digestion (Šarić et al. 2014). All samples were analysed in triplicate.

Determination of pH value

The portable pH meter Testo 205 (Testo AG, USA) equipped with a combined penetration tip with temperature probe was used to measure pH value of tested DM samples.

Addition of $CaCl_2$ and EDTA

The appropriate amounts (276.93 mg and 553.87 mg) of anhydrous $CaCl_2$ (Centrohem, Serbia) were added to 1 L of the tested DM in order to increase the total Ca concentration in all samples by 100 and 200 mg/L, respectively. The required amounts of $CaCl_2$ were calculated on the basis of molecular mass of $CaCl_2$ and Ca. The positive control was nutrient broth (Himedia, India) with added $CaCl_2$. EDTA (Sigma-Aldrich, USA) was added to the tested samples in the concentration of 1.39 g/L. pH value of DM samples was adjusted to previous values using 0.1 mol/L NaOH. All samples were analysed in triplicate.

Antibacterial assay

DM samples were artificially contaminated with *Salmonella* Enteritidis ATCC 13076 and *Salmonella* Typhimurium ATCC 14028 at the level of contamination of 10^2 CFU/mL. Bacterial strains were kept frozen at –80 °C in separate cryoinstant vials with porous beads. Prior to use one bead was transferred into 10 mL of tryptone soya broth (TSB) (Himedia, India) and incubated at 37 °C for 24 h. After two consecutive transfers under the same conditions (37 °C, 24 h) nutrient agar (Himedia, India) was inoculated by tested strains separately and incubated at 37 °C for 24 h. After incubation well-isolated colonies of each test bacteria were selected and transferred with an inoculating loop to a tube of sterile saline and vortexed thoroughly. The DEN-1 densitometer (Biosan, Riga, Latvia) was used for adjusting the bacterial suspension turbidity to the 0.5 McFarland standard. Tenfold sequential dilutions of the bacterial suspensions made in sterile saline were used for the artificial contamination of selected milk samples. 25 mL of each artificially contaminated sample was placed into a sterile vessel and kept in a water bath (Raypa, Spain) at 38 °C during eight hours. Xylose lysine deoxycholate (XLD) agar (LABM Limited, UK) was used for enumeration of tested bacteria during the incubation period according to the international standard (ISO 2006). The count of tested *Salmonella* strains in DM samples with added $CaCl_2$ and EDTA was determinated after 8 hours of incubation at 38 °C. Non inoculated DM was used as negative control, while artificially contaminated NB (Himedia, India) was used as positive control. All samples were analysed in triplicate.

Statistical analysis

The data were processed statistically using the software package STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA). All determinations were made in triplicate, all data was averaged, and expressed by means ± standard deviation (SD). Analysis of variance (ANOVA) and Tukey's HSD test for comparison of means were used to analyse the variations of the load of *S.* Enteritidis and *S.* Typhimurium in DM and nutrient broth (NB), according to the incubation period (*t*) and pH value.

The content of LZ, LF and Ca were studied for the influence on the load of *S.* Enteritidis and *S.* Typhimurium during the incubation period, while $CaCl_2$ and EDTA content were studied for the influence on the load of *S.* Enteritidis and *S.* Typhimurium at the end of induction period. Second order polynomial (SOP) models in the following form were developed to relate responses (Y) and two process variables (X):

$$Y_k = \beta_{k0} + \sum_{i=1}^{l} \beta_{ki} \cdot X_i + \sum_{i=1}^{l} \beta_{kii} \cdot X_i^2 + \sum_{i=1}^{l} \sum_{j=i+1}^{l-1} \beta_{kij} \cdot X_i \cdot X_j, \quad k=1\text{-}2,\ l=5 \qquad (1)$$

where: β_{k0}, β_{ki}, β_{kii}, β_{kij} were constant regression coefficients; Y_k the load of *S.* Enteritidis and *S.* Typhimurium in DM (Y_1) or NB (Y_2), during the incubation period, while X_1 is the Ca content, X_2 is the LZ content, X_3 is the LF content, X_4 is pH value and X_5 is the induction period.

The similar formula was used for evaluation of the load of *S.* Enteritidis and *S.* Typhimurium in DM (Y_1) or NB (Y_2), after the induction period, where β_{k0}, β_{ki}, β_{kii}, β_{kij} were constant regression coefficients; Y_k the load of *S.* Enteritidis and *S.* Typhimurium in DM (Y_1) or NB (Y_2), while X_1 is the Ca content, X_2 is the lysozime content, X_3 is the LF content, X_4 is pH value, X_5 is $CaCl_2$ content, X_6 is EDTA content, and l=6.

ANOVA was conducted to show the significant effects of independent variables to the responses, and to show which of responses were significantly affected by the varying treatment combinations.

The experimental database is randomly divided into three groups for ANN model developing: training data (60%), cross-validation (used to test the performance of the network while training) (20%) and testing data (used to examine the network generalization capability) (20%). A multi-layer perceptron model (MLP) consisted of three layers (input, hidden and output), which is the most common, flexible and general-purpose kind of the ANN was used, (Arsenović et al. 2013), giving the reason for choosing it in this study. The MLP neural network learns using an algorithm called „backpropagation". Levenberg–Marquardt algorithm is proved to be the fastest and particularly adapted for networks of moderate size. During this iterative process, input data are repeatedly presented to the network (Grieua et al. 2011).

Results

Antibacterial assay

The determined contents of Ca, LZ and LF as well as pH values of examined DM are summarized in Table 1. The obtained results of antibacterial assay clearly indicate different antibacterial potential of tested DM samples toward *S.* Enteritidis (Table 2). The highest decrease in *S.* Enteritidis count was observed in samples 1, 2, 3, 5, 6, 7 and 8, where tested bacteria was not present after 2, 3 or 4 h of incubation. On the contrary, *S.* Enteritidis count was found at a level of 2.80 - 3.50 log CFU/mL in other milk samples, at the end of incubation (Table 2). At 38 °C, the *S.* Typhimurium count showed a decreasing trend with differences in the final value of bacterial counts (Table 3). Samples 1, 2, 5, 7 and 8 exhibited the strongest antibacterial activity toward tested bacteria, since the count of this pathogen was reduced under detectable level after 1, 2 or 3 h of incubation. These samples also had the highest calcium content ranged from 961.25 to 1127.5 mg/L. Slightly weaker antibacterial activity was observed for samples 6 and 4, where the presence of this bacterial strain was not detectable after 5 and 7 h,

respectively. At the end of incubation, *S.* Typhimurium was detected only in the samples with low calcium content (508.12 – 620.15 mg/L) (Table 3). During the incubation at 38 °C in positive controls the count of *S.* Enteritidis and *S.* Typhimurium increased constantly reaching, after 8 h, the values of 8.09 ± 0.04 and 8.05 ± 0.05 log CFU/mL, respectively. On the other hand, the presence of these pathogens was not detected in negative controls. The significance of the calcium ion concentrations in antibacterial activity of DM was demonstrated by the addition of $CaCl_2$ and EDTA. The inclusion of the higher concentration of $CaCl_2$ in DM appeared to improve its antibacterial properties toward both tested bacteria (Tables 4 & 5). After 8 h of incubation the count of *S.* Enteritidis in nutrient broth with added $CaCl_2$ reached the values of 8.11 ± 0.03 and 8.12 ± 0.02 log CFU/mL, while observed counts of *S.* Typhimurium were 8.04 ± 0.04 and 8.07 ± 0.03 log CFU/mL, respectively. The weakening of the antibacterial activity toward *S.* Typhimurium was demonstrated in DM samples containing EDTA (Table 5). In the case of *S.* Enteritidis, the addition of EDTA slightly increased the load of viable cells after 8 hours of incubation (Table 4).

The ANOVA test

ANOVA exhibits the significant independent variables as well as the interactions of these variables. The ANOVA test shows the significant effects of the independent variables to the responses and which of responses were significantly affected by the varying treatment combinations (Table 6). The evaluation of the load of *S.* Enteritidis and *S.* Typhimurium in DM, during the incubation period was mostly affected by the linear term of Ca content, the linear term of induction time and the interchange term of Ca × t, statistically significant at $p<0.01$ level. SOP models representing the load of *S.* Enteritidis and *S.* Typhimurium in DM during the experiments had an insignificant lack of fit tests. The influences of CA, LF, pH and it were found statistically significant, while predicted and observed responses correspond well, with coefficients of determination were 0.904 and 0.793 for the load of *S.* Enteritidis and *S.* Typhimurium in DM during the experiments, respectively.

The ANOVA test shows the significant effects of the independent variables to the responses at the end of the incubation period (experiments with added $CaCl_2$ and EDTA) (Table 7). The evaluation of the load of *S.* Enteritidis in DM at the end of the incubation period (experiment with added $CaCl_2$ and EDTA) was mostly affected by the linear term of Ca content, the linear term of $CaCl_2$, the interchange term of Ca × $CaCl_2$, as well as the interchange terms LZ × LF and LZ × $CaCl_2$, statistically significant at $p<0.01$ level. The evaluation of the load of *S.* Typhimurium in DM was mostly affected by the linear term of EDTA, as well as the interchange terms Ca × $CaCl_2$ and Ca × EDTA, statistically significant at $p<0.01$ level. SOP models representing the load of *S.* Typhimurium in DM at the end of induction period had an insignificant lack of fit tests. The influences of Ca,

$CaCl_2$ and EDTA, as well as the combined pH, LZ and LF influences were found statistically significant, while predicted and observed responses correspond well, with coefficients of determination were 0.937 and 0.965 for the load of *S.* Enteritidis and *S.* Typhimurium in DM during the experiments, respectively.

ANN model

Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm, implemented in StatSoft Statistica's evaluation routine, was used for ANN modelling. The optimization procedures to minimize the error function between network and experimental outputs was used during ANN training cycle (Pezo et al. 2013), and the sum of squares (*SOS*) was evaluated according to the BFGS algorithm, to speed up and stabilize convergence of the results (Basheer and Hajmeer 2000). The training process was repeated several times in order to get the best performance of the ANN, due to a high degree of variability of parameters. It was accepted that the successful training was achieved when learning and cross-validation curves (*SOS* vs. training cycles) approached zero. Coefficient of determination (r^2) and *SOS* were used as parameters to check the performance (i.e. the accuracy) of the obtained ANN. The optimum number of hidden neurons was chosen upon minimizing the difference between predicted ANN values and desired outputs, using *SOS* during testing as a performance indicator. According to ANN performance (sum of r^2 and SOSs for all variables in one ANN), it was noticed that the optimal number of neurons in the hidden layer for the load of *S.* Enteritidis and *S.* Typhimurium during incubation period is 11 (network MLP 5-11-2), with observed training performance 0.983 and training error $4.57 \cdot 10^{-3}$, while the optimal network for the load of *S.* Enteritidis and *S.* Typhimurium was MLP 6-7-2, training performance was 0.996 and training error was 0.001. It can be seen that these r^2 values for SOP models (Table 6) are very much alike to those associated with the ANN model. This agrees with other authors (Basheer and Hajmeer 2000; Pezo et al. 2013). ANN models performed a bit better because of the high nonlinearity of the developed system (Pezo et al. 2013).

Discussion

S. Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028 have been used in this study as commercial representative of the most frequently isolated ubiquitous serotypes *S.* Enteritidis and *S.* Typhimurium, which affect both man and animals, generally causes gastrointestinal infections. In 2001 more than 70% of human cases registered in France were caused by just three serotypes: *S.* Enteritidis (33%), *S.* Typhimurium (32%), and *S.* Hadar (6%). *S.* Enteritidis was caused 39.5% of the total of 82,694 laboratory-confirmed *salmonellosis* outbreaks reported in Europe in 2013 (Velge et al. 2005). Literature sources about the antibacterial activity of DM toward *Salmonella* species are quite limited. However, there are few reports about strong antibacterial potential of this milk against some *Salmonella* species. Zhang et al. (2008) reported that *Salmonella* Choleraesuis was the most

sensitive tested strain towards the antimicrobial activity of donkey milk obtained from an autochthonous Chinese breed. Beside *S.* Enteritidis and *S.* Typhimurium previous investigations performed on Domestic Balkan donkey milk showed strong antibacterial activity of this milk toward *Salmonella* Livingstone. The serious damage of bacterial cell walls and the leak of the cellular content was observed by scanning electron microscopy (Šarić et al. 2014).

Wide range of Ca concentrations (508.12 - 1127.5 mg/L) determined in this study could be explained by different lactation periods of the milking animals. Fantuz et al. (2012) reported that calcium content in DM decreased by approximately 30% to 116 - 142 days pp. Similarly, the substantial variations observed for LZ and LF content in DM (Table 1) could be due to the influence of lactation stage during which the samples were collected. Salimei and Fantuz (2012) suggested that different reports on the level of LZ in DM can also be attributed to different analytical methods applied. The observed calcium – dependant antimicrobial activity of tested DM samples might be related to calcium-binding properties of DM LZ. The DM LZ and mare LZ have similar structure, since both belong to g-type of LZs characteristics for genus *Equus* (Wilhelm 2009; Bruhn et al. 2011). The calcium-binding capability of more thoroughly studied equine LZ (Nitta et al. 1987; Tsuge et al. 1991) actively participates in its formation of linear or ring-shaped forms (Lyster 1992; Malisauskas et al. 2003). The ring-shaped structures were present in large quantities only in the absence of calcium ions, while the amount of linear forms was increased with increasing of calcium ion concentration. At neutral pH (Table 1), the self-assembly of g-type of LZs into linear forms is probably caused by the neutralization of negative charges on LZ molecule with calcium ions (Malisauskas et al. 2003). It has been reported that the linear form of mare LZ possesses improved antibacterial activity against *E. coli* (Bruhn et al. 2011). Sarwar et al. (2001) also showed high correlation between LZ activity and calcium content in the mare's milk, while Šarić et al. (2014) reported significant role of calcium content in antibacterial activity of DM toward *E. coli*. The higher count of *S.* Typhimurium obtained in milk samples after the addition of EDTA (Table 4) can be explained by the fact that calcium ions, through the interaction with EDTA (Christensen et al. 2003), became unavailable for DM LZ and the formation of its linear structures. In the case of *S.* Enteritidis, the addition of EDTA slightly increased the number of viable cells after 8 hours of incubation (Table 4). The binding capacity of EDTA (1.39 g/L) calculated on the basis of molecular mass of Ca, EDTA and their binding ratio was approximately 190 mg/L of Ca. It can be concluded that the binding of calcium ions in this concentration had no significant effect on the antimicrobial activity of DM towards *S.* Enteritidis, which has proved to be more resistant to the antimicrobial activity of DM in comparison to *S.* Typhimurium. This is also supported by the lower reduction in the number of cells after the

addition of $CaCl_2$ (Table 4) in comparison to *S.* Typhimurium, which indicates that the DM antibacterial activity is also dependant on the bacterial strain.

The additional synergistic activity of LZ and LF toward tested pathogens also cannot be excluded. LF could interact with the LPS layer of the outer membrane of Gram negative bacteria and thus enable direct access to molecules of LZ to target places on peptidoglicane in cell wall (Ellison and Giehl 1991). The synergistic antibacterial activity of LZ and LF to the "smooth" bacterial strains has already been proven in earlier studies by other authors (Ellison and Giehl 1991; Jenssen and Hancock 2009). Ellison and Giehl (1991) reported a synergistic bactericidal effect of these two proteins to the "smooth" strains of *S.* Typhimurium and *E. coli*. The higher susceptibility of *S.* Typhimurium to the antibacterial activity of DM in comparison to *S.* Enteritidis could be the result of differences in their cell structures, since both tested pathogens belong to the "smooth" strains. *S.* Typhimurium fall into group B, with O-antigen factor 4, while *S.* Enteritidis fall into group D with O-antigen factor 9 (Lindberg et al. 1993). According to the Appelmelk et al. (1994) elongation of the chain of the oligosaccharide core of the LPS layer of the outer membrane of Gram negative bacteria inhibits interaction between LF and LPS layer. In this study, direct correlation between the antibacterial activity of DM samples and their LZ concentration was not established. However, the certainty that the DM samples, containing low concentrations of LF, as well as those samples in which this protein was less than the detection limit of the method (samples 3, 5, 11) still showed antibacterial activity (Table 1) could be explained by the fact that the saturation of the lipid A in LPS layer requires small concentrations of LF (Appelmelk et al. 1994).

When LZ amount was considered independently of the calcium concentration in DM, the correlation between the antibacterial activity of DM and its LZ content was not visible. As an example, the samples 1, 3 and 5 showed almost the same antibacterial properties against *S.* Enteritidis (Table 2), although LZ content in these samples was 3.89, 1.88 and 2.70 g/L, respectively. On the other hand, after addition of $CaCl_2$ to DM, the stronger reduction of *S.* Enteritidis count (Table 3) was identified in samples with higher LZ content (Table 4), possibly due to the higher number of calcium ion receptors present.

Conclusions

Observed calcium-dependant antibacterial activity of DM against both tested *Salmonella* strains is the likely result of the ability of DM lysozyme to bind calcium ions and transform into the filamentous structure. The determined relationship between Ca content in examined samples and the intensity of reduction in the tested *Salmonella* strains counts was confirmed by the addition of $CaCl_2$ and EDTA to DM. *S.* Typhimurium proved to be more sensitive to this antibacterial activity compared to *S.* Enteriditis. Lysozyme might be marked as the main

antibacterial agent with the most probable nonenzymatic mode of action against tested *Salmonella* strains. The additional synergistic activity of LZ and LF toward tested pathogens also cannot be excluded, since *S.* Typhimurium as well as *S.* Enteriditis belong to the "smooth" bacterial strains.

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Table

Table 1. Lysozyme (LZ), lactoferrin (LF), calcium (Ca) content and pH value in donkey

	DM samples														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Ca	969.0 k (5.00)	1127.5 m (2.50)	902.5 j (0.00)	765.0 h (0.75)	961.2 k (3.75)	877.5 i (2.50)	962.5 k (5.00)	986.2 l (13.75)	637.2 g (0.27)	630.0 fg (12.50)	617.5 ef (12.00)	597.1 d (1.36)	608.0 de (24.5)	544.2 b (9.75)	566. c (23.7
LZ	3.89 m (0.04)	3.74 kl (0.05)	1.88 d (0.08)	3.46 j (0.06)	2.70 h (0.06)	1.92 d (0.03)	3.84 m (0.05)	2.47 g (0.06)	2.08 e (0.08)	3.67 k (0.05)	3.03 i (0.06)	1.44 b (0.04)	3.05 i (0.05)	1.73 c (0.09)	3.14 i (0.14
LF	32.8 i (1.80)	11.15 e (0.05)	n.d. a	54.3 k (0.40)	n.d. a	26.8 h (1.95)	4.1 b (1.14)	22.8 g (2.80)	40.06 j (0.30)	7 cd (0.05)	n.d. a	7.6 d (0.30)	15.4 f (0.10)	5.4 bc (0.40)	7.5 d (0.50
pH	7.12 ab (0.01)	7.12 ab (0.02)	7.14 abcd (0.02)	7.15 bcde (0.02)	7.19 fg (0.03)	7.16 cdef (0.01)	7.13 abc (0.01)	7.17 def (0.02)	7.15 bcde (0.01)	7.21 g (0.02)	7.19 fg (0.02)	7.13 abc (0.02)	7.18 efg (0.01)	7.11 a (0.00)	7.17 def (0.02

[1] Results are expressed in g/L (LZ) and mg/L (LF, Ca). Each value is the mean of three replicates. Standard deviation values are g
with different superscript letters are statistically different ($p<0.05$).
[2]Abbreviations are: DM, donkey's milk, n.d., not detected.

Table

Table 2. *S.* Enteritidis count in DM during 8 h at 38 °C

Incubation (h)	DM samples														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
0	2.50^{d} (0.04)	2.50^{d} (0.04)	2.50^{d} (0.04)	2.50^{g} (0.04)	2.50^{d} (0.04)	2.50^{e} (0.04)	2.50^{c} (0.04)	2.50^{c} (0.04)	2.50^{b} (0.04)	2.50^{e} (0.04)	2.50^{b} (0.04)	2.50^{b} (0.04)	2.50^{a} (0.04)	2.50^{b} (0.04)	2.50^{c} (0.04)
1	2.12^{c} (0.07)	1.82^{c} (0.04)	2.40^{c} (0.05)	1.87^{f} (0.01)	2.36^{c} (0.04)	2.10^{d} (0.05)	1.20^{b} (0.06)	1.00^{b} (0.00)	2.75^{c} (0.05)	2.81^{cd} (0.02)	3.01^{a} (0.00)	2.49^{b} (0.02)	2.50^{a} (0.04)	2.47^{b} (0.06)	2.55^{c} (0.04)
2	1.30^{b} (0.05)	1.52^{b} (0.07)	1.50^{b} (0.06)	1.72^{b} (0.01)	1.46^{b} (0.15)	1.82^{c} (0.05)	n.d.^{a}	n.d.^{a}	2.88^{d} (0.02)	2.83^{d} (0.01)	3.00^{a} (0.06)	2.32^{d} (0.02)	2.29^{c} (0.04)	2.60^{f} (0.04)	2.66^{d} (0.05)
3	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.69^{b} (0.01)	n.d.^{a}	1.26^{b} (0.04)	n.d.^{a}	n.d.^{a}	3.06^{e} (0.01)	2.70^{a} (0.06)	3.00^{a} (0.07)	2.41^{e} (0.01)	2.44^{a} (0.05)	3.00^{c} (0.00)	2.90^{b} (0.02)
4	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.54^{e} (0.03)	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.14^{a} (0.06)	2.72^{ab} (0.02)	3.02^{a} (0.00)	2.66^{c} (0.03)	2.60^{d} (0.02)	3.05^{ac} (0.04)	2.94^{b} (0.05)
5	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.33^{d} (0.02)	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.18^{a} (0.00)	2.71^{ab} (0.02)	2.98^{a} (0.04)	2.67^{c} (0.03)	2.71^{e} (0.04)	3.08^{a} (0.05)	2.98^{ab} (0.06)
6	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.20^{c} (0.05)	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.16^{a} (0.04)	2.76^{bc} (0.01)	2.98^{a} (0.04)	2.88^{a} (0.01)	2.80^{b} (0.03)	3.11^{ad} (0.03)	3.04^{a} (0.05)
7	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.15^{a} (0.03)	2.72^{ab} (0.02)	2.82^{c} (0.09)	2.87^{a} (0.01)	2.85^{b} (0.04)	3.18^{de} (0.05)	3.03^{a} (0.04)
8	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	3.20^{a,E} (0.03)	2.80^{cd,B} (0.02)	3.17^{d,E} (0.01)	2.89^{a,C} (0.01)	2.93^{f,C} (0.05)	3.20^{e,E} (0.04)	3.05^{a,D} (0.06)

[1]Results are expressed in log CFU/mL. Each value is the mean of three replicates. Standard deviation values are given in parenthe
different superscript letters between rows are statistically different ($p<0.05$). Means in the same line with different superscript capital lett
[2]Abbreviations are: NB, nutrient broth; n.d., not detected

Table

Table 3. *S.* Typhimurium count in DM during 8 h at 38 °C

Incubation (h)	DM samples														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
0	2.48^{c} (0.02)	2.48^{b} (0.02)	2.48^{b} (0.02)	2.48^{d} (0.02)	2.48^{c} (0.02)	2.48^{e} (0.02)	2.48^{c} (0.02)	2.48^{d} (0.02)	2.48^{a} (0.02)	2.48^{g} (0.02)	2.48^{g} (0.02)	2.48^{f} (0.02)	2.48^{g} (0.02)	2.48^{a} (0.02)	2.48^{c} (0.02)
1	1.32^{b} (0.04)	n.d.^{a}	2.74^{d} (0.06)	2.48^{d} (0.01)	2.75^{d} (0.01)	2.69^{f} (0.03)	1.28^{b} (0.02)	2.00^{c} (0.03)	2.54a^{b} (0.04)	2.78^{a} (0.00)	2.74^{ab} (0.01)	2.69^{b} (0.04)	2.71^{b} (0.06)	2.62^{c} (0.03)	2.68^{b} (0.04)
2	n.d.^{a}	n.d.^{a}	2.49^{b} (0.04)	1.30^{b} (0.00)	2.19^{b} (0.10)	2.41^{d} (0.05)	n.d.^{a}	1.40^{b} (0.05)	2.68^{b} (0.05)	2.71^{a} (0.01)	2.80^{b} (0.06)	2.72^{b} (0.05)	2.75^{bc} (0.03)	2.60b^{c} (0.05)	2.65^{ab} (0.06)
3	n.d.^{a}	n.d.^{a}	1.26^{c} (0.24)	1.98^{c} (0.03)	n.d.^{a}	1.48^{c} (0.03)	n.d.^{a}	n.d.^{a}	2.66^{b} (0.06)	2.58^{h} (0.03)	2.97^{cd} (0.03)	2.91^{a} (0.04)	2.88^{a} (0.05)	2.72^{d} (0.04)	2.66^{ab} (0.04)
4	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.84^{c} (0.06)	n.d.^{a}	1.20^{b} (0.00)	n.d.^{a}	n.d.^{a}	2.48^{a} (0.01)	2.29^{f} (0.03)	3.06^{e} (0.00)	2.93^{a} (0.00)	2.86^{a} (0.05).	2.73^{d} (0.05)	2.59^{ad} (0.04)
5	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.26^{b} (0.24)	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	2.43^{a} (0.04)	2.08^{e} (0.04)	3.00^{de} (0.07)	2.92^{a} (0.06)	2.80^{ac} (0.04)	2.51^{ab} (0.06)	2.53^{cd} (0.05)
6	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.00^{e} (0.00)	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	2.07^{e} (0.06)	1.46^{d} (0.15)	2.93^{c} (0.03)	2.23^{e} (0.06)	2.18^{f} (0.04)	2.00^{g} (0.04)	2.21^{g} (0.05)
7	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.30^{d} (0.24)	1.00^{e} (0.00)	2.73^{a} (0.02)	1.43^{d} (0.05)	1.39^{e} (0.03)	1.67^{f} (0.05)	1.48^{f} (0.05)
8	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{a,A}	n.d.^{c,A}	n.d.^{b,A}	1.23^{f,CD} (0.03)	1.30^{e,DE} (0.07)	1.20^{d,C} (0.08)	1.35^{e,E} (0.10)	1.25^{e,CD} (0.05)

[1]Results are expressed in log CFU/mL. Each value is the mean of three replicates. Standard deviation values are given in parenthe
different superscript letters between rows are statistically different (p<0.05). Means in the same line with different superscript capital lett
[2]Abbreviations are: NB, nutrient broth; n.d., not detected

Table

Table 4. *S.* Enteritidis count in DM containing $CaCl_2$/EDTA after 8 h at 38 °C

Sample	DM	I	II	III
1	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
2	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
3	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
4	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
5	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
6	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
7	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
8	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
9	3.20[e] (0.03)	2.54[h] (0.05)	1.28[b] (0.04)	3.26[g] (0.04)
10	2.80[b] (0.02)	1.20[d] (0.05)	n.d.[a]	2.95[c] (0.02)
11	3.17[e] (0.01)	2.20[f] (0.00)	1.77[d] (0.07)	3.24[g] (0.04)
12	2.89[c] (0.01)	2.68[i] (0.02)	2.64[f] (0.00)	3.00[d] (0.00)
13	2.93[c] (0.05)	1.10[c] (0.06)	n.d.[a]	3.00[d] (0.02)
14	3.20[e] (0.04)	2.77[j] (0.05)	2.55[e] (0.04)	3.25[g] (0.05)
15	3.05[d] (0.06)	0.90[b] (0.05)	n.d.[a]	3.09[e] (0.04)
16	2.80[b] (0.05)	2.35[g] (0.03)	1.65[c] (0.00)	2.82[b] (0.06)
17	3.16[e] (0.02)	1.35[e] (0.06)	n.d.[a]	3.17[f] (0.04)
18	3.50[f] (0.06)	3.30[k] (0.05)	3.22[g] (0.04)	3.52[h] (0.03)

[1]Results are expressed in log CFU/mL. Each value is the mean of three replicates. Standard deviation values are given in parentheses. Means in the same line with different superscript letters are statistically different (p<0.05).
[2]Abbreviations are: DM, donkey's milk; I, donkey's milk with $CaCl_2$ (276.93 mg/L); II, donkey's milk with $CaCl_2$ (553.87 mg/L); III, donkey's milk with EDTA (1.39 g/L); n.d., not detected

s

Table

Table 5. *S.* Typhimurium count in DM containing $CaCl_2$/EDTA after 8 h at 38 °C

Sample	DM	I	II	III
1	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.27^{d} (0.06)
2	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.20^{cd} (0.05)
3	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.40^{e} (0.00)
4	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.05^{a} (0.05)
5	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.10^{ab} (0.04)
6	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.17^{bc} (0.05)
7	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.35^{e} (0.04)
8	n.d.^{a}	n.d.^{a}	n.d.^{a}	1.22^{cd} (0.06)
9	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.14^{ijk} (0.06)
10	n.d.^{a}	n.d.^{a}	n.d.^{a}	3.02^{fg} (0.04)
11	1.23^{cd} (0.03)	0.80^{d} (0.00)	n.d.^{a}	3.05^{fgh} (0.07)
12	1.30^{de} (0.07)	0.90^{e} (0.04)	0.40^{b} (0.06)	3.00^{f} (0.05)
13	1.20^{c} (0.08)	0.70^{c} (0.05)	n.d.^{a}	3.10^{ghij} (0.05)
14	1.35^{e} (0.10)	0.90^{e} (0.05)	0.50^{c} (0.00)	3.08^{fghi} (0.04)
15	1.25^{cd} (0.05)	0.60^{b} (0.07)	n.d.^{a}	3.15^{ijk} (0.03)
16	1.00^{b} (0.00)	n.d.^{a}	n.d.^{a}	3.12^{hijk} (0.06)
17	1.46^{f} (0.15)	1.10^{f} (0.05)	0.60^{d} (0.00)	3.18^{jk} (0.00)
18	1.60^{g} (0.04)	1.15^{f} (0.10)	0.70^{e} (0.05)	3.20^{k} (0.05)

[1]Results are expressed in log CFU/mL. Each value is the mean of three replicates. Standard deviation values are given in parentheses. Means in the same line with different superscript letters are statistically different ($p<0.05$).
[2]Abbreviations are: DM, donkey's milk; I, donkeys' milk with $CaCl_2$ (276.93 mg/L); II, donkey's milk with $CaCl_2$ (553.87 mg/L); III, donkey's milk with EDTA (1.39 g/L); n.d., not detected

Table

Table 6. ANOVA calculation of the load of *S.* Enteritidis and *S.* Typhimurium during incubation period

	df	*S.* Enteritidis	*S.* Typhimurium
Ca	1	28.283^{+}	13.192^{+}
Ca^2	1	0.000	0.044
LZ	1	0.554**	0.297
LZ^2	1	0.526**	0.639
LF	1	0.049	0.546
LF^2	1	1.677^{+}	0.000
pH	1	2.103^{+}	0.195
pH^2	1	0.473**	0.413
t	1	16.992^{+}	43.236^{+}
t^2	1	4.062^{+}	0.207
Ca × LZ	1	0.008	0.003
Ca × LF	1	0.201	0.014
Ca × pH	1	1.578^{+}	0.408
Ca × t	1	30.211^{+}	2.911^{+}
LZ × LF	1	0.939*	0.180
LZ × pH	1	0.083	1.586*
LZ × t	1	0.152	0.133
LF × pH	1	0.034	0.096
LF × t	1	1.318^{+}	0.735
pH × t	1	0.158	0.000
Error	141	23.945	42.561
r^2		0.904	0.793

$^{+}$Significant at $p<0.01$ level, *Significant at $p<0.05$, **Significant at $p<0.10$, error terms have been found statistically insignificant, df - degrees of freedom

Table

Table 7. ANOVA calculation of the load of *S.* Enteritidis and *S.* Typhimurium at the end of incubation period (experiments with added $CaCl_2$ and EDTA)

	df	*S.* Enteritidis	*S.* Typhimurium
Ca	1	8.746^{+}	1.356*
Ca^2	1	0.004	0.951*
LZ	1	0.020	0.140
LZ^2	1	0.294	0.023
LF	1	0.955*	0.017
LF^2	1	1.162*	0.148
pH	1	0.484	0.081
pH^2	1	0.092	0.012
$CaCl_2$	1	1.448^{+}	0.294*
$CaCl_2^2$	1	0.085	0.001
EDTA	1	0.003	12.421^{+}
Ca × LZ	1	0.068	0.009
Ca ×LF	1	0.507	0.123
Ca × pH	1	0.730**	0.026
Ca × $CaCl_2$	1	4.724^{+}	1.283^{+}
Ca × EDTA	1	0.022	1.017^{+}
LZ × LF	1	6.394^{+}	0.090
LZ × pH	1	0.579**	0.339*
LZ × $CaCl_2$	1	2.444^{+}	0.124
LZ × EDTA	1	0.001	0.270*
LF × pH	1	0.419	0.004
LF × $CaCl_2$	1	0.001	0.300*
LF × EDTA	1	0.004	0.037
pH × $CaCl_2$	1	1.139*	0.018
pH × EDTA	1	0.019	0.056
Error	46	9.139	2.814
r^2		0.937	0.965

$^{+}$Significant at $p<0.01$ level, *Significant at $p<0.05$, **Significant at $p<0.10$, error terms have been found statistically insignificant, df - degrees of freedom