Effects of high hydrostatic pressure on antimicrobial protein stability and the rheological and shelf-life properties of donkey milk

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SUPPLEMENTARY FILE

Supplementary materials and methods

Total nitrogen content analysis

Kjeldahl method was used to find the total nitrogen content of donkey milk samples. 5 mL of donkey milk samples were blended with Kjeldahl antifoaming agent tablets (Sigma, Germany), and 25 mL sulfiric acid, 98 % (Sigma, Germany) was added in the digestion tubes in a digestion unit (model K435; Büchi, Zürich, Switzerland). A boric acid solution, (Sigma, Germany) and 3-drops of methyl red (Sigma, Germany) were mixed in a conical flask and placed into a distillation unit (model 323; Büchi) where the analysis took place with sodium hydroxide solution (Sigma, Germany). From the distilled samples, pale-yellow solutions were obtained. Then, a hydrochloric acid solution (Sigma, Germany) was used to titrate the distilled donkey milk samples obtained from the distillation unit until a desired purple color was achieved. Accordingly, total protein contents of the milk samples were determined via the conversion factor of 6.38 (ISO 8968, 2001). All the experiments were performed in triplicate.

Fat content analysis

Gerber method was used to determine the fat contents of the milk samples (ISO 19662, 2018)). Sulfuric acid (sp. gr. 1.8) (Sigma, Germany) was added to milk samples to increase the temperature to the liquefying temperatures of milk fat. Subsequently, amyl alcohol (sp. gr.0,82) was also added to separate aqueous and fat phases of the samples. A Gerber centrifuge (Nova Safety; Funke

Gerber, Berlin, Germany) was used to separate fat and aqueous phases. Fat contents of the samples were determined by the readings from Gerber butyrometers (350xg), following centrifugation (1500 rpm, 5 mins).

Dry matter content analysis

Dry matter contents were determined with gravimetric method at 103 °C, according to the descriptions of ISO 6731 (2010).). Water is removed from the samples by a two-step procedure. Firstly, a part of water was evaporated from the samples that were placed in glass petri dishes in a water bath. The rest of the samples were subjected to evaporation in an oven at around 103 °C until the difference between two consecutive measurements is determined to be less than 0.0005.

pH and titratable acidity analysis

A pH meter (Mettler – Toledo MP 220, Schwerzenbach, Switzerland) was used to determine the pH values of the samples. The electrode of the instrument was directly inserted into the samples. The method reported by Bradley (1993) was used to perform titratable acidity measurements and expressed the results in lactic acid percentage (LA%).

Determination of lysozyme and lactoferrin content

The method described by Billakanti *et al.* (2010) was utilized to determine the lysozyme and lactoferrin activity of the samples. After HHP treatment, milk samples were heated to 45 °C and then pH values were adjusted to 4.6 with 1 M HCl in order to precipitate caseins. Samples were then centrifuged at 17,500 x g for 15 min (Sigma, K 3-18, Sartorius AG, Germany) to separate the precipitated caseins. The obtained supernatants were brought the pH of 7.0 with 1N NaOH. Before injecting into the high-performance-liquid-chromatography (HPLC) (Agilent 1100 HPLC system, CA, USA) equipped with a UV detector at 214 nm and a C18 column (4.6 cm x 250 mm x 5 µm), supernatants were filtered through 0.45 µm cellulose-membrane filters. The temperature of the

column was 45 °C and the injection volume was 50 µL. A calibration curve including various concentrations (10, 25, 50, 75 and 150 µg/mL) were prepared to quantitative determination of whey proteins. Standard solution of lysozyme from egg white and lactoferrin from human milk (Sigma-Aldrich, St. Louis, MO, USA) were used (Billakanti et al. 2010).

Table S1. Chemical composition of untreated, HHP-, and heat-treated donkey milk (Day 0)

	Untreated Milk	HHP-Treated Milk	Heat-Treated Milk
Total Protein Content (w/w %)	2.15±0.15 [a]	2.17±0.11 [a]	2.10±0.13 [a]
Fat Content (w/w %)	1.00±0.01 [a]	1.00±0.01 [a]	1.00±0.01 [a]
Dry Matter Content (w/w %)	8.40±0.41 [a]	8.46±0.39 [a]	8.37±0.49 [a]

*Results expressed as the mean ± standard deviation. Different lowercase letters in the same row indicate significant differences (P<0.05). 400 MPa-25 °C-5 min and 75 °C-2 min was given as HHP-treated milk and heat-treated milk, respectively, because there were no significant differences (P>0.05) within groups. All the experiments were performed in triplicate.

Table S2. pH values of untreated, HHP-treated (400 MPa-25 °C-5min), and heat-treated (75 °C - 2 min) donkey milk during shelf-life

4 ℃						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	7.00±0.09 ^{a,A,B}	7.16±0.15 ^{a,A}	7.09±0.08 ^{a,A,B}	6.25±0.06 ^{b,F,G}	6.13±0.06 ^{b,G}	5.23±0.04 ^{c,I,J}
Heat-Treated	7.21±0.18 ^{a,A}	7.19±0.16 ^{a,A}	7.00±0.08 ^{a,A,B}	7.00±0.06 ^{a,A,B}	7.00±0.10 ^{a,A,B}	6.64±0.07 ^{a,D,E}
HHP-Treated	7.00±0.13 ^{a,A,B}	7.06±0.08 ^{a,A,B}	7.08±0.11 ^{a,A,B}	6.94±0.07 ^{a,A,B,C}	7.00±0.08 ^{a,A,B}	6.66±0.11 ^{a,C,D,E}
25 ℃						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	7.00±0.09 ^{a,A,B}	7.08±0.10 ^{a,A,B}	5.37±0.05 ^{a,H,I}	4.97±0.06 ^{b,J}	5.01±0.09 ^{b,J}	4.51±0.06 ^{c,K}
Heat-Treated	7.21±0.18 ^{a,A}	6.98±0.11 ^{a,A,B}	6.48±0.06 ^{a,b,E,F}	6.10±0.10 ^{b,c,G}	5.48±0.07 ^{e,H,I}	5.62±0.05 ^{d,e,H}
HHP-Treated	7.00±0.13 ^{a,A,B}	7.00±0.07 ^{a,A,B}	6.83±0.08 ^{a,B,C,D}	6.57±0.10 ^{a,K}	6.43±0.07 ^{b,K,L}	6.21±0.05 ^{b,L}

*Results expressed as the mean ± standard deviation. Different lowercase letters in the same row indicate significant differences (P<0.05). Different uppercase letters indicate significant differences between samples (P<0.05). All the experiments were performed in triplicate.

Table S3. Titratable acidity values (LA%) of untreated, HHP-treated (400 MPa-25 °C-5min), and heat-treated (75 °C -2 min) donkey milk samples during shelf-life analysis.

4 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	0.036±0.002 ^{d,O,P}	0.036±0.004 ^{d,O,P}	0.036±0.002 ^{d,O,P}	0.126±0.002 ^{c,K}	0.189±0.003 ^{b,I}	0.219±0.005 ^{a,H}
Heat-Treated	0.027±0.002 ^{b,P}	0.027±0.001 ^{b,P}	0.027±0.001 ^{b,P}	0.036±0.002 ^{b,O,P}	0.063±0.004 ^{b,M}	0.081±0.005 ^{a,L}
HHP-Treated	0.036±0.004 ^{c,O,P}	0.036±0.001 ^{c,O,P}	0.036±0.005 ^{c,O,P}	0.045±0.004 ^{c,N,O}	0.045±0.004 ^{b,N,O}	0.075±0.003 ^{a,L}
25 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	0.036±0.002 ^{d,O,P}	0.045±0.003 ^{d,N,O}	0.306±0.002 ^{c,F}	0.387±0.005 ^{b,C}	0.369±0.003 ^{b,D}	0.421±0.004 ^{a,A}
Heat-Treated	0.027±0.002 ^{e,P}	0.054±0.001 ^{e,M,N}	0.153±0.004 ^{d,J}	0.180±0.003 ^{c,I}	0.261±0.003 ^{b,G}	0.351±0.006 ^{a,E}
HHP-Treated	0.036±0.004 ^{f,O,P}	0.045±0.003 ^{e,N,O}	0.252±0.003 ^{d,G}	0.306±0.002 ^{c,F}	0.342±0.005 ^{b,E}	0.398±0.006 ^{a,B}

*Results expressed as the mean ± standard deviation. Different lowercase letters in the same row indicate significant differences (p<0.05). Different uppercase letters indicate significant differences between samples (p<0.05). All the experiments were performed in triplicate.

Table S4. Flow consistency index values (K) of untreated, HHP-treated (400 MPa-25 °C-5min), and heat-treated (75 °C -2 min) donkey milk samples during shelf-life analysis

4 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	0.028 ±0.04 ^{f,X}	0.034±0.06 ^{e,X}	0.041 ±0.05 ^{d,X}	0.060 ±0.04 ^{c,V,W,X}	0.082 ±0.05 ^{b,V,W}	1.233 ±0.07 ^{a,U}
Heat-Treated	0.818 ±0.05 ^{f,Q}	0.931 ±0.05 ^{e,P}	1.232 ±0.05 ^{d,N}	2.204 ±0.03 ^{c,I}	4.042 ±0.06 ^{b,E}	7.474 ±0.09 ^{a,C}
HHP-Treated	0.455 ±0.06 ^{f,T}	0.523 ±0.04 ^{e,S}	0.588 ±0.04 ^{d,R}	0.895 ±0.05 ^{c,P}	1.395 ±0.03 ^{b,M}	3.035±0.06 ^{a,H}
25 °C						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	0.028 ±0.04 ^{f,X}	0.052 ±0.07 ^{e,W,X}	0.063 ±0.07 ^{d,V,W,X}	0.092 ±0.07 ^{c,U,W}	1.25±0.03 ^{b,U}	4.20-±0.04 ^{a,T}
Heat-Treated	0.818 ±0.05 ^{f,Q}	1.471 ±0.07 ^{e,L}	2.034 ±0.02 ^{d,J}	3.867±0.04 ^{c,F}	7.615±0.03 ^{b,B}	10.492 ±0.82 ^{a,A}
HHP-Treated	0.455 ±0.06 ^{f,T}	0.818 ±0.06 ^{e,Q}	1.029 ±0.06 ^{d,O}	1.819 ±0.06 ^{c,K}	3.403 ±0.04 ^{b,G}	6.145 ±0.07 ^{a,D}

*Results expressed as the mean ± standard deviation. Different lowercase letters in the same row indicate significant differences (P<0.05). Different uppercase letters indicate significant differences between samples (P<0.05). All the experiments were performed in triplicate.

Table S5. Flow behaviour index values (n) of untreated, HHP-treated (400 MPa-25 °C-5min), and heat-treated (75 °C -2 min) donkey milk samples during shelf-life analysis.

4 ℃						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	1.141±0.006 ^{a,A}	1.102±0.006 ^{b,B}	1.057±0.005 ^{c,E,F}	0.960±0.007 ^{d,I,J}	0.901±0.006 ^{e,K,L}	0.882±0.008 ^{e,M}
Heat-Treated	0.989±0.006 ^{a,H,I}	0.893±0.002 ^{b,L,M}	0.823±0.002 ^{c,O,P}	0.812±0.001 ^{c,d,PQ}	0.792±0.005 ^{d,Q,R}	0.759±0.006 ^{e,S}
HHP-Treated	1.089±0.006 ^{a,B,C}	1.083±0.002 ^{a,b,C,D}	1.080±0.007 ^{a,b,C,D,E}	1.065±0.003 ^{b,D,E,F}	1.019±0.007 ^{c,G}	0.970±0.005 ^{d,I}
25 ℃						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
Untreated	1.141±0.006 ^{a,A}	1.069±0.006 ^{b,C,D,E,F}	0.998±0.005 ^{c,G,H}	0.912±0.006 ^{d,K}	0.822±0.005 ^{e,O,P}	0.775±0.006 ^{f,R,S}
Heat-Treated	0.989±0.006 ^{a,H,I}	0.859±0.006 ^{b,,N}	0.825±0.004 ^{c,O,P}	0.803±0.005 ^{c,P,Q}	0.779±0.007 ^{d,R,S}	0.732±0.004 ^{e,T}
HHP-Treated	1.089±0.006 ^{a,B,C}	1.056±0.004 ^{b,F}	0.983±0.005 ^{c,H,I}	0.949±0.006 ^{d,J}	0.924±0.003 ^{e,K}	0.840±0.005 ^{e,N,O}

*Results expressed as the mean ± standard deviation. Different lowercase letters in the same row indicate significant differences (p<0.05). Different uppercase letters indicate significant differences between samples (p<0.05). All the experiments were performed in triplicate.