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Effect of high pressure pre-treatment on raw ewes' milk and on subsequently produced cheese throughout ripening

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Abstract

BACKGROUND: Raw ewe's milk, used to manufacture *Serra da Estrela* Protected Designation of Origin (PDO) cheese, was pre-treated by high pressure processing (HPP), using previously optimized conditions (121 MPa/30 min), in order to evaluate its effect on milk technological properties for subsequent cheese production, namely the impact on resulting curd, whey, and cheese throughout ripening.

RESULTS: The cheese yield increase 10.4% as a result of milk pre-treated by HPP, which also yielded inactivation of beneficial microbial groups. After 60 days of ripening, both treated and control cheeses showed neither significant differences ($P \geq 0.05$) in quantified microbial load nor in basic physicochemical quality parameters.

CONCLUSION: HPP seems to be a promising non-thermal treatment for ewes' milk to inactivate contaminant bacteria yet with no negative effect on lactic acid bacteria, which are very important for the unique characteristics of *Serra da Estrela* cheese.

Keywords: *Serra da Estrela* cheese; raw ewe's milk; yield; microbial evolution; safety, scale-up

INTRODUCTION

Serra da Estrela cheese, holding a Protected Designation of Origin (PDO) status, is made solely with milk from Bordaleira *Serra da Estrela* and/or Churra Mondegueira ewe's breeds, salt and cardoon flower (*Cynara cardunculus* L.) extract. The milk from these ewe breeds is known to give a good yield, about 1 kg of cheese from 5.0 to 6.0 L of milk.[1] Different literature reports have indicated that cheese yield can be increased in 4 – 23 % through a non-thermal high pressure processing (HPP) pre-treatment of bovine milk.[2,3] Moreover, HPP can substantially reduce the microbial pathogenic/spoilage microorganisms present in the raw milk used to produce *Serra da Estrela* cheese, thus possibly improving the safety of this traditional dairy product.

In a previous work, a design of experiments (DoE) approach was used to construct experimentally efficient factor screening and optimization studies, in order to identify the best HPP conditions to be applied to raw ewes' milk for subsequent *Serra da Estrela* cheese production, envisaging cheese yield improvement and at least maintenance of the principal quality characteristics of the cheese.[4] The use of DoE allowed concluding that 121 MPa for 30 min was the treatment that enabled the most efficient maintenance of the beneficial microbiota responsible for biochemical and sensory attributes of the cheese (lactococci, lactobacilli and enterococci), while causing inactivation of potential spoilage and pathogenic microorganisms such as *Enterobacteriaceae*, coliforms, *Escherichia coli*, staphylococci, yeasts and moulds. Several studies have used the application of more intense HPP conditions for milk pasteurisation (>345 MPa/15 min) in order to cause higher microbial inactivation, but, in such cases, starter cultures needed to be added to manufacture cheese.[5–7] In this present study, focus is on defining a HPP pre-treatment of the cheese milk, at HPP conditions that will allow cheese manufacture therewith without the need of starter cultures addition. To the best of our knowledge, there is no study available in literature related with this aspect. Hence, the main goal of the present research was to understand the effects of the HPP pre-treatment

(121 MPa for 30 min) on raw ewe's milk used to produce *Serra da Estrela* cheese and on subsequently produced curd, whey, and ripened cheese.

MATERIALS AND METHODS

Milk supply, cheese manufacture and yield determination

One hundred and seventy litres of raw ewe's milk were collected, in the morning, from different dairy farms located in *Serra da Estrela* PDO region (Portugal), pooled and transported to an artisanal dairy facility that produces commercial *Serra da Estrela* cheese, according to the PDO procedure. The bulk milk was kept in a cooling tank until use and prior to sampling, milk was well mixed to ensure sample homogeneity. The bulk milk was then divided into two batches: 82 L were used, in the same morning, to manufacture 35 cheeses according to the PDO procedure,[1] considered as milk control cheeses (M_C). Milk coagulation is promoted by the addition of an aqueous extract of *C. cardunculus* L. thistle flower (cardoon) (0.3 g L^{-1}).[8] After coagulation, cutting and pressing of the curd, samples of control curd and control whey were collected (1.5 h after milk coagulation initiated).[9] The remaining milk was packaged in portions of 8 L into polyamide-polyethylene (PA-PE, Plásticos Macar, Indústria de Plásticos Lda, Santo Tirso, Portugal) bags that were heat sealed and stored under refrigeration (4 °C) before HPP pre-treatment (121 MPa for 30 min), as milk pre-treated (M_P), which occurred in the afternoon of the same day. The next day, in the morning, 77 L of the pre-treated milk were used to produce 34 cheeses, according to the PDO procedure. Samples of curd and whey were collected from the cheese manufacture process with M_P milk similarly to that of control cheeses. All 69 cheeses manufactured from M_C and M_P milks, of about 500 g each, were ripened at 7 ± 2 ºC and 95% relative humidity (RH) for 15 days and then at 10 ± 2º C and 85% RH, for 60 days at the artisanal dairy. During the ripening period, the cheeses were washed and weighed periodically/weekly (according to the procedures implied by the PDO Regulations). Cheese yield and percentage weight loss was determined weekly taking into account the litres used in the manufacture of each batch and the cheese weight upon surface cleaning.

High Pressure Processing

HPP treatments were performed in a 55-liter capacity industrial scale high pressure equipment (model 55, Hiperbaric, Burgos, Spain) at 121 MPa for 30 min (as already explained above, this condition was selected based on a previous experimental design study, performed to find the optimum HPP pre-treatment to be applied to ewes milk for cheese production), with the initial temperature of the water used as transmitting fluid being 8 °C (water inlet temperature) and considering that the literature reports an increase of 3-5 ºC per 100 MPa, the expected temperature reached inside the high pressure vessel was around 11-13 ºC considering the pressure used (121 MPa). The decompression time was no more than few seconds for the pressure used.

Microbiological analyses

One mL milk samples of were added to 9 mL of sterile 0.1% (w/v) aqueous peptone, subjected to appropriate serial decimal dilutions and plated, in triplicate, on several suitable culture media. The pour plate method was used to enumerate *Enterobacteriaceae*, coliforms and *E. coli* on violet red bile dextrose agar (Merck, Germany) and chromocult coliform agar respectively. The Miles and Misra technique was used for enumeration of: total aerobic mesophiles on plate count agar, *Enterococcus* spp. on kanamycin aesculin azide agar base, *Lactobacillus* spp. on Man, Rogosa and Sharpe agar, *Lactococcus* spp. on M17 agar, *Staphylococcus* spp. on Baird-Parker agar with egg-yolk tellurite emulsion and *Pseudomonas* spp. on pseudomonas agar base with glycerol and pseudomonas CFC supplement. Total aerobic mesophiles, *Lactobacillus* spp., *Lactococcus* spp. and *Pseudomonas* spp. media were incubated under aerobic conditions at 30 ºC, for 3 d and *Enterobacteriaceae*, coliforms, *Enterococcus* spp. and *Staphylococcus* spp. media were incubated at 37 ºC for 24 h. Curd and cheese samples were aseptically handled in a laminar flow cabinet, and homogenized for 4 min using a 2 % (w/v) aqueous sodium citrate solution as extraction buffer in a Stomacher Lab-Blender 400 (Milano, Italy), followed by serial decimal dilutions and plating as performed for milk samples.

Physicochemical analyses

The pH of the M_c and M_P and respective cheeses was measured at room temperature, at random points using a properly calibrated pH/temperature penetration pH meter (Testo 205, Testo, Inc., New Jersey, USA). The titratable acidity was determined according to the AOAC 920.124 procedure using an automatic titrator with pH meter (Crison – Titromatic 1S with pH electrode 50 14, Barcelona, Spain), by titration to a pH end-point of 8.9. Moisture content was determined by drying approximately 2.0 g of cheese to a constant weight (ca. 24 h) at 105 °C using a laboratory drying oven equipment (Venticell, MMM Medcenter Einrichtungen GmbH, Munich, Germany). The total nitrogen (TN) content was determined by the micro-Kjeldahl procedure (AOAC 2001.14) using a Kjeltec system 1002 Distilling unit (Tecator, Sweden) and the crude protein content was determined by multiplying the total nitrogen content by 6.38.[10] Fat content was determined according to Van Gulik method. [11]

Statistical analyses

All analytical results are presented as average values with the standard deviation. The *t*-student test using SPSS software, version 24.0 (SPSS Inc., Chicago, IL, USA) was used to determine the significant differences, at significance level of $P < 0.05$, between control milk (M_C) and HPP pre-treated milk (M_P) and resulting curd and cheese samples.

Results and discussion

Milk, curd and whey composition

Raw ewe's milk (M_C) and HPP pre-treated milk (M_P) showed similar ($P \geq 0.05$) moisture and protein contents, about 808 g kg^{-1} and 58.3 g kg^{-1}, respectively (**Table 1**). Trujillo et al (1999)[6] found similar protein and fat contents in goat cheese produced from thermal pasteurized and HPP pre-treated milk (500 MPa/15 min). Although statistically different ($P < 0.05$), the moisture, fat and protein contents of M_P milk curd differed only around 1% from those of M_C milk curd. Literature reports 5 % lower moisture content for the curd from bovine milk treated at 100 or 250 MPa compared to that from untreated milk.[3] Nevertheless, in another study, raw whole bovine milk curd and HPP pre-treated milk curd (400 or 600 MPa/10 min), revealed no significant differences in moisture, protein, fat, and salt contents.[12]

The protein content of the whey obtained from the M_P milk cheese manufacture procedure was significantly ($P < 0.05$) higher (+19.7 %) than that of the M_C milk cheese counterpart (**Table 1**). An opposite behaviour has been reported in another study for HPP pre-treated bovine milk (250-600 MPa/0-60 min), having the protein content of whey from HPP pre-treated bovine milk cheese decreased progressively with increasing treatment pressure;[3] on the other hand, no changes in whey obtained from cheeses production with HPP pre-treated bovine milk at 400 MPa were reported by Voigt et al (2012).[12] As such, considering that this study was performed with raw ewes' milk, it can be speculated that the results presented need further and deeper evaluation, as this is the first study in the literature covering the effects of high pressure pre-treatments for cheese manufacture using ewes' milk. Furthermore, M_P milk had a higher calcium content than M_C milk ($P < 0.05$), which can be the result of the effect of HPP on the weakening of hydrophobic and electrostatic interactions between sub-micelles leading to the dissolution of colloidal calcium phosphate, and consequent solubilization of calcium in the media.[13,14]

Curd and cheese yield and weight loss along ripening

The curd yield obtained from HPP treated milk (M_P) of 0.302 kg_{curd} L_{milk}^{-1}, increased 10.4 % in comparison to that obtained with untreated milk (M_C), 0.274 kg_{curd} L_{milk}^{-1} (**Fig. 1**). This difference amplitude was basically maintained throughout ripening and by 60 days ripening, the M_P milk cheeses allowed 8.0 % more cheese yield. All cheeses revealed a similar weight loss trend throughout ripening as illustrated in **Fig. 1**. According to these results, a yield improvement is achieved by HPP milk pre-treatment. In contrast, a study in literature refers that bovine milk cheese yield is not influenced by treatment at HPP < 250 MPa,[3] and to the best of our knowledge there are no publications with application of HPP pre-treatment at the range applied in this work (121 MPa for 30 min) on ewes milk. Higher cheese yields from HPP pre-treated cow milk were reported for more intensive HPP treatments (> 250 MPa), being the effect attributed to a higher moisture content, [5,15] possibly due to the formation of a finer structural network and due to the water-binding properties of denatured ß-lactoglobulin, which was incorporated into the protein matrix.[15] However, in the present study, cheese moisture content was not affected by the milk pre-treatment (**Table 1**), but still, cheese yield increased about 10 %; other factors (protein, fat content) are possibly affecting the cheese yield.

Microbial composition of milk, curd and cheese

In M_C ewes milk samples, lactococci and lactobacilli were found at 7.02 and 2.38 log cfu mL^{-1}, total mesophiles at 6.06 log cfu mL^{-1}, enterococci at 4.43 log cfu mL^{-1} and *Enterobacteriaceae,* total coliforms and staphylococci were all found at similar levels of 5.51, 5.57 and 5.06 log cfu mL^{-1}, respectively (**Table 2).** *Escherichia coli*, and yeasts and moulds were detected at 4.34 and 4.10 log cfu mL^{-1}, respectively. The HPP ewe milk pre-treatment led to a reduction of microbiota viable cell numbers although one order of magnitude lower than that reported in literature for many of the microbial groups. Nonetheless, it must be recalled that the main aim in this study is to apply such HPP conditions that may maximize the inactivation of pejorative microorganisms while minimizing the reduction of beneficial microbiota viable cell numbers in order to produce *Serra da Estrela* Cheese with a higher yield, maintaining as much as possible the characteristics of this cheese. [4]

In M_P ewes milk total mesophiles suffered a reduction of 0.66 log cycles, similar to what was reported for bovine milk treated at 100 and 200 MPa/30 min (about 0.2-0.5 log cycles reduction).[15] High reductions were observed for more intensive HPP milk treatments, *e.g.* HPP treatments at 586 MPa/1 min and 500 MPa/15 min in bovine and caprine milks revealed viable cell numbers' reduction between 0.87 and 2.22 log cycles.[5–7] Minor numerical reductions in viable cell numbers (< 1 log), statistically not significant ($P \geq 0.05$), were observed for yeasts and moulds, total coliforms, and *Enterobacteriaceae* in M_P milk. Higher reductions in viable cell numbers of coliforms and *Enterobacteriaceae* (> 1.32 log units) have been reported in caprine and bovine milks that underwent far more intensive HPP treatments (586 MPa/1 min and 500 MPa/15 min).[5–7] Gram-positive bacteria lactococci, lactobacilli, enterococci and staphylococci were less affected in M_P milk, having been observed less than 0.8 log cycle reductions in viable cell numbers in comparison to M_C control values. More intense HPP treatments (500 MPa/15 min and 600 MPa/10 min) applied to caprine and bovine milks, led to more than 2.36 log cycles reductions of lactobacilli viable cell numbers.[7,16]

In general, the curd samples obtained from M_P milk differed statistically in enterococci, staphylococci, coliforms and yeasts and moulds viable cell numbers relatively to curds obtained from M_C milk ($P < 0.05$), however the differences were, once again very low, less than 0.6 log units. Different studies have demonstrated that the decrease in microbial groups' viable cell numbers brought about by the HPP milk pre-treatment is subsequently reflected in the curd microbiota, but for more intensive treatments. For example, curds from HPP pre-treated goat milk (500 MPa/5 or 30 min; 500 MPa/15 min) showed 1.8-2.0 log cycle reductions of total aerobic bacteria,[17] 2.46 log cycle reductions of *Enterobacteriaceae* and approximately 3 log cycle reductions of lactobacilli viable cell numbers,[18] while curd from HPP pre-treated bovine milk (400 MPa/15 min) revealed 1.4 log cycle reductions of total microbiota viable cell numbers.[19]

At 60 days of ripening, cheeses manufactured from M_C and M_P milks showed no significant differences in lactococci, lactobacilli and enterococci viable cell numbers, with values around 9 log cfu g^{-1} (**Table 2**). These values being close to those previously reported for *Serra da Estrela* cheese.[20,21] Also the traditional Pecorino di Farindola cheese (manufactured from raw ewe milk) reports that lactic acid bacteria and enterococci are the dominant microbiota with values around 7-9 log cfu g^{-1}.[22] Likewise, at 60 days of ripening, Buffa et al (2006)[7] observed similar total bacteria, lactococci, lactobacilli and enterococci viable cell numbers for cheeses manufactured from HPP (500 MPa/15 min) treated and non-treated goat milk, except for *Enterobacteriaceae* viable cell numbers that showed about 2 log cycle reductions in cheeses made from HPP pre-treated milk. For more intense HPP treatments, goat cheeses at 60 days of ripening, revealed reductions in viable cell numbers of total aerobic bacteria of about 3 and 6 log cycles for 500 MPa/5 and 30 min,[17] while for lactobacilli approximately 2 log cycle reductions were observed for 500 MPa/15 min.[18] On the other hand, other authors observed less than 1 log cycle reduction in lactobacilli viable cell numbers after 60 days of ripening for bovine cheese produced from HPP milk pre-treated at 400 and 600 MPa/10 min.[16]

According to the results of the present study, one of the first to be done on ewe's milk to the best of our knowledge, a milder milk pre-treatment by HPP at 121 MPa for 30 min showed a tendency to cause slight reductions (ca. 1 log cycle) in viable cell numbers of pejorative microbiota in milk, while basically maintaining the beneficial microbiota load; yet by 60 days of ripening similar values were found for cheeses produced form control and HPP pre-treated milk, independently of the microbial group.

pH variation in milk, curd and cheese

The pH variations in cheese are mainly due to the formation and consumption of lactic acid, due to the metabolism of LAB and other microorganisms. In this work, a statistically significant increase of pH was verified in M_P milk relatively to M_C milk ($P < 0.05$), although the difference was only about 0.12 units. Even lower variations (≤ 0.05 pH units) were verified for curd, whey and cheese produced from M_C and M_P milks. The cheese pH values observed in the present study were within the range of those reported in the literature (4.82-5.66).[21,23] Similar low variations in pH were observed in a study with bovine milk treated by HPP (100, 250 and 400 MPa/15 min).[24] These changes in pH caused by the HPP treatment were associated by the authors to dissolution of colloidal calcium phosphate (CCP), possibly due to the weakening of hydrophobic and electrostatic interactions between sub micelles. For curd, a small pH increment of 0.04 units was found when obtained with HPP (500 MPa/5 min) treated goat milk.[25] Higher differences (0.16-0.36 pH units decrease) were reported for curd from HPP treated bovine milk (400 and 600 MPa/10 min).[12] Concerning cheese, a small increment of 0.13 pH units was verified by Buffa and colleagues[18] for goat cheeses manufactured from HPP treated milk (500 MPa/15 min).

As expected, HPP milk treatment showed no effect on the titratable acidity, being determined similar values for milk, whey, curd and cheese ($P \geq 0.05$) (**Table 1**).

CONCLUSION

HPP milk pre-treatment led to a mild reduction of microbial load in milk, a small effect on curd and without significant differences in ripened cheese microbiota load. HPP pre-treated milk showed higher pH-values, while cheese manufactured from HPP pre-treated milk had lower pH values; nevertheless no significant differences in moisture and protein content were verified. This study has been able to demonstrate, for the first time, that HPP treatment of raw ewes' milk prior to cheese manufacture can be used to increase cheese yield and to enable an improved microbial profile which is important from a safety and quality point of view, thus contributing positively to the production of such an important cheese. At the microbial level, deeper studies are desirable on the effect of HPP on inoculated pathogenic organisms that may be found in raw milk cheeses (like a combined surrogate cocktail of *Staphylococcus aureus*, *Listeria innocua* and *Salmonella enterica*) in order to validate a possible food safety improvement.

Conflict of interest

The authors of this research paper declare no conflict of interest.

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List of figures and tables

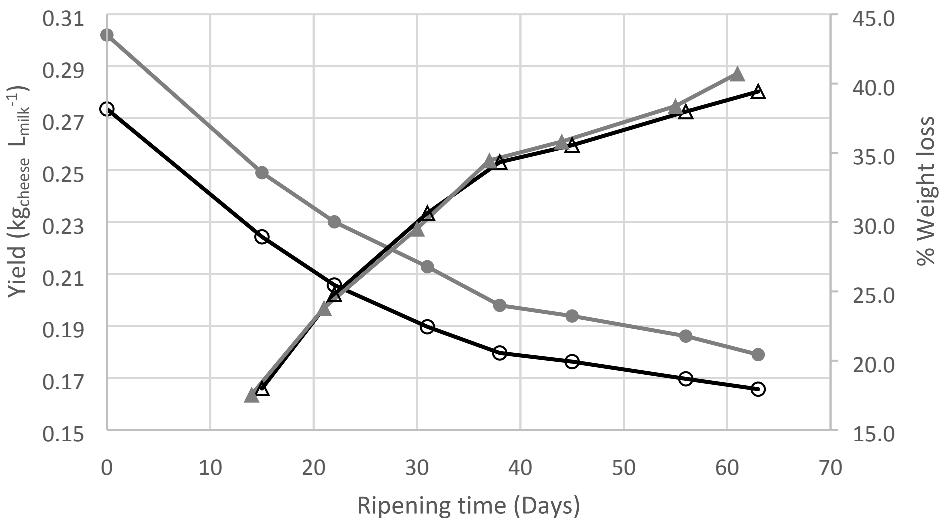

Figure 1. Chesses' yield (kg of cheese per L of milk) from control milk (○) and HPP pre-treated milk (●), and weight loss percentage from control milk (△) and made from HPP pre-treated milk (▲) (cheese production took place in a real artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).

Table 1. Average values for moisture, fat, protein and calcium contents, pH and titratable acidity of control and HPP pre-treated milk, and resulting curd, whey, and cheese with 60 days of ripening (cheese production took place in an artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).

		Control		HPP pre-treated milk	
Moisture Content ($g\ kg^{-1}$)	Milk	808 ± 0.4	a	809 ± 0.4	a
	Curd	666 ± 0.4	b	654 ± 1.3	a
	Whey	904 ± 0.2	b	898 ± 0.2	a
	Cheese	412 ± 8.6	a	403 ± 9.9	a
Fat content ($g\ kg^{-1}$)	Milk	79.3 ± 0.2	b	75.3 ± 0.0	a
	Curd	138 ± 0.2	a	149 ± 1.0	b
	Whey	8.5 ± 0.3	a	9.1 ± 0.1	a
Protein content ($g\ kg^{-1}$)	Milk	58.3 ± 0.5	a	58.4 ± 0.4	a
	Curd	114 ± 0.5	a	119 ± 0.8	b
	Whey	13.2 ± 0.4	a	15.8 ± 0.3	b
	Cheese	239 ± 3.6	a	222 ± 4.7	a
Calcium content ($g\ kg^{-1}$)	Milk	1.2 ± 0.1	a	1.3 ± 0.0	b
pH-values	Milk	6.46 ± 0.01	a	6.58 ± 0.02	b
	Curd	6.37 ± 0.01	a	6.41 ± 0.01	b
	Whey	6.33 ± 0.01	a	6.37 ± 0.03	b
	Cheese	5.16 ± 0.01	b	5.11 ± 0.03	a
Titratable acidity ($g_{lactic\ acid}\ kg^{-1}$)	Milk	0.33 ± 0.01	a	0.32 ± 0.01	a
	Curd	4.60 ± 0.02	a	4.70 ± 0.64	a
	Whey	2.60 ± 0.20	a	2.50 ± 0.03	a
	Cheese	13.8 ± 0.44	a	13.1 ± 0.45	a

Different letters for the same analysis and product (milk, curd, whey, and cheese) indicate statistically significant differences (*t*-Student test $P < 0.05$).

Table 2. Microbiota quantification in control and HPP pre-treated milk, and resulting curd, whey, and cheese with 60 days of ripening (cheese production took place in an artisanal dairy facility following the mandatory procedures of the Protected Designation of Origin, PDO).

		Control		**HPP**	
		Log cfu mL^{-1} or g^{-1}		Log cfu mL^{-1} or g^{-1}	
Total mesophilic	Milk	6.06 ± 0.13	b	5.34 ± 0.07	a
	Curd	6.22 ± 0.08	a	6.66 ± 0.31	a
	Cheese	8.33 ± 0.16	a	8.48 ± 0.41	a
Lactococci	Milk	7.02 ± 0.45	b	6.26 ± 0.08	a
	Curd	7.09 ± 0.41	a	6.42 ± 0.34	a
	Cheese	9.10 ± 0.16	a	9.11 ± 0.12	a
Lactobacilli	Milk	2.38 ± 0.03		< 2.00	
	Curd	< 3.00		3.84 ± 0.07	
	Cheese	8.62 ± 0.18	a	9.03 ± 0.17	a
Enterococci	Milk	4.33 ± 0.09	a	4.22 ± 0.06	a
	Curd	5.46 ± 0.05	b	4.79 ± 0.07	a
	Cheese	8.31 ± 0.08	a	8.32 ± 0.13	a
Staphylococci	Milk	5.06 ± 0.13	b	4.43 ± 0.19	a
	Curd	5.65 ± 0.16	a	6.24 ± 0.09	b
	Cheese	7.68 ± 0.06	b	7.51 ± 0.08	a
Enterobacteriaceae	Milk	5.51 ± 0.14	b	4.85 ± 0.13	a
	Curd	5.75 ± 0.11	a	5.77 ± 0.07	a
	Cheese	5.78 ± 0.08	a	6.06 ± 0.22	b
Coliforms	Milk	5.57 ± 0.12	b	4.86 ± 0.07	a
	Curd	6.06 ± 0.09	a	6.58 ± 0.38	b
	Cheese	7.32 ± 0.00	b	7.15 ± 0.00	a
Escherichia coli	Milk	4.34 ± 0.11	a	4.10 ± 0.11	a
	Curd	4.20 ± 0.51	a	4.79 ± 0.45	a
	Cheese	5.59 ± 0.10	a	5.76 ± 0.28	a
Yeasts and moulds	Milk	4.10 ± 0.19	a	3.58 ± 0.20	a
	Curd	4.06 ± 0.13	b	3.84 ± 0.13	a
	Cheese	4.24 ± 0.18	a	4.62 ± 0.38	b

Different letters for the same microorganism indicate statistically significant differences between the milk, curd and cheese from control and HPP treated milk (*t*-student test $P < 0.05$)