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' as used to manufacture Serra da Estrela Protected Designation of Origin cheese' was pre-treated by high pressure processing (HPP)' using previously optimized conditions (121 MPa for 30 min)' aiming 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 increased 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 no significant differences ($P > 0.05$) with respect to quantified microbial load or basic physicochemical quality parameters.

CONCLUSION: HPP appears to be a promising non-thermal treatment for ewes' milk to inactivate contaminant bacteria but with no negative effect on lactic acid bacteria' which is 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 Ori- gin (PDO) status, is made solely with milk from Bordaleira Serra da Estrela and/or Churra Mondegueira ewe's breeds, salt and car- doon flower (Cynara cardunculus L.) extract. The milk from these ewe breeds is known to give a good yield, comprising approxi-mately 1 kg ofcheese from 5.0–6.0 L of milk.[1] Different studies have indicated that the cheese yield can be increased by 4–23% through the non-thermal high pressure processing (HPP) pre-treatment of bovine milk.[2,3] Moreover, HPP can sub- stantially reduce the microbial pathogenic/spoilage microor- ganismsthatarepresentintherawmilkusedtoproduceSerra daEstrelacheese,thuspossiblyimprovingthesafetyofthistra- ditional dairy product.

In a previous study, a design of experiments (DoE) approach wasusedtoconstructexperimentallyefficientfactorscreening andoptimizationstudies,aimingtoidentifythebestHPPcon- ditions to be applied to raw ewes' milk for subsequent Serra daEstrelacheeseproduction,envisagingcheeseyieldimprove- ment and at least maintenance of the principal quality charac- teristics of the cheese.[4] The use of DoE revealed that 121 MPa for30 min comprisedthetreatmentthatenabledthemosteffi- cient maintenance of the beneficial microbiota responsible for the biochemical and sensory attributes of the cheese (lacto- cocci, lactobacilli and enterococci), at the same time causing inactivation of potential spoilage and pathogenic microorganisms such as Enterobacteriaceae, coliforms, Escheri- chia coli, staphylococci, yeasts and moulds. Several studies have used the application of more intense HPP conditions for milk pasteurisation (>345 MPa for 15 min), aiming to achieve highermicrobialinactivation,but,insuchcases,startercultures needed to be added to manufacture cheese.[5-7] In the present study,thefocuswasondefiningaHPPpre-treatmentofcheese milk, under HPP conditions that allow cheese manufacture without the need of the addition of starter cultures addition. Tothebestofourknowledge,therearenostudiesareavailable in the literature related to this aspect. Hence, the main goal of the present study was to determine the effects of the HPP pre- treatment (121 MPa for 30 min) on raw ewe's milk used to pro- duce Serra da Estrela cheese and on the 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 was collected, in the morning, from different dairy farms located in Serra da Estrela PDO region (Portugal), then pooled and transported to an arti- sanal 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 mixed well to ensure sample homogeneity. The bulk milk was then divided into two batches: 82 L was 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 pro- moted 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 (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), considered 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 was 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, sim- ilarly to control cheeses. All 69 cheeses manufactured from M_C and M_P milks, of approximately 500 g each, were ripened at 7 ± 2 ° C and 95% relative humidity for 15 days and then at 10 ± 2 ° C and 85% relative humidity, 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 number of 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-L capacity industrial scale high pressure equipment (model 55; Hiperbaric, Burgos, Spain) at 121 MPa for 30 min (as already noted above, this condition was selected based on a previous experimental design study, per- formed 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 tem-perature) and, taking into account that the literature reports an increase of 3-5 °C per 100 MPa, the expected temperature reached inside the high pressure vessel was around 11-13 °C considering the pressure used (121 MPa). The decompression time was no more than few seconds for the pressure used.

Microbiological analysis

One milliliter milk samples were added to 9 mL of sterile 0.1% (w/v) aqueous peptone, subjected to appropriate serial decimal dilutions and then plated,in triplicate, on several types of suitable culture media. The pour plate method was used to enumerate Enterobacteriaceae, coliforms and E. coli o n violet red bile dextrose agar (Merck, Darmstadt, Germany)and chromocult coliform agar, respectively.The Miles and Misra technique was used forenumer- ation of total aerobic mesophiles on plate count agar, Enterococ- cus 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 tell- urite emulsion, and Pseudomonas spp. on pseudomonas agar base with glycerol and pseudomonas CFC supplement. Total aer- obic mesophiles, Lactobacillus spp., Lactococcus spp. and Pseudo- monas spp. media were incubated under aerobic conditions at 30 °C for 3 days and Enterobacteriaceae, coliforms, Enterococcus spp. and Staphylococcus spp. media were incubated at 37 °C for 24 h.Curd and cheese samples were aseptic allyhandled in a laminar flow cabinet, and homogenized for 4 min using a 2% (w/v) aqueous sodium citrate solution as extraction buffer in a Stom- acher Lab-Blender 400 (International PBI SpA, Milan, Italy), fol- lowed by serial decimal dilutions and plating as performed for milk samples.

Physicochemical analysis

The pHofthe Mcand MPand respective cheeses was measured at room temperature, at random points, using a properly calibrated pH/temperature penetration pH meter (Testo 205; Testo, Inc., NewJersey,USA).The titratable acidity was determined according to the AOAC 920.124 procedure using an automatic titrator with pH meter (Titromatic 1S with pH electrode 50 14; Crison, Barce- lona,Spain)bytitration to a pHend-point of8.9. Moisture content was determined bydrying approximately2.0 g ofcheese toa con- stant weight (approximately 24 h) at 105 °C using a laboratory drying oven equipment (Venticell, MMM Medcenter Einrichtun- gen GmbH, Munich, Germany). The total nitrogen content was determined via the micro-Kjeldahl procedure (AOAC 2001.14) using a Kjeltec system 1002Distilling unit (Tecator, Hoganas, Swe- den) and the crude protein content was determined by multiply- ing the total nitrogen content by 6.38.[10] Fat content was determined according to the Van Gulik method.[11]

Statistical analyies

All analytical results are presented as the mean ± SD. Student's t- test using SPSS, version 24.0 (IBM Corp., Armonk, NY, USA) was used to determine the significant differences, at a significance level of $P < 0.05$, between control milk (MC) 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(MC)and HPP pre-treated milk (MP) showed similar ($P > 0.05$) moisture and protein contents of approximately 808and 58.3 g kg^{-1},respectively(Table 1). Trujillo et al.[6] reported similar protein and fat contents in goat cheese produced from thermal pasteurized and HPP pre-treated milk (500 MPa for 15 min). Although statistically different ($P < 0.05$), the moisture, fat and protein contents of MP milk curd differed from those of MC milk curd by only approximately 1%. The literature reports a 5% lower moisture content for the curd from bovine milk treated at 100 or250 MPa compared to that from untreated milk.[3] Never- theless, in another study, raw whole bovine milk curd and HPP pre-treated milk curd (400 or 600 MPa for 10 min), revealed no significantdifferences in moisture,protein,fatand salt contents.[12]

The protein content of the whey obtained from the M_P milk cheese manufacture procedure was significantly($P < 0.05$) higher

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 654 ± 1.3 a
	Curd	666 ± 0.4 b	898 ± 0.2 a 403 ± 9.9 a
	Whey	904 ± 0.2 b	
	Cheese	412 ± 8.6 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 lowercase letters for the same analysis and product (milk, curd, whey, and cheese) indicate statistically significant differences (Student's t- test, $P < 0.05$).

(+19.7%) than that of the MC milk cheese counterpart (Table 1). An opposite behaviour has been reported in another study for HPP pre-treated bovine milk (250-600 MPa for 0-60 min), with the protein content ofwheyfrom HPP pre-treated bovine milkcheese decreasing progressively with an 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.[12] As such, considering that the present study was performed with raw ewes' milk, it can be speculated that the results need a more detailed evaluation because this is the first study in the literature covering the effects of high pres- sure pre-treatmentsforcheese manufacture usingewes' milk.Fur- thermore, MP milk had a higher calcium content than MC 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, as well as the 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 (MP) (0.302 kg_{curd} L_{milk}^{-1}) was increased 10.4% compared to that obtained with untreated milk (MC) (0.274 kg_{curd} L_{milk}^{-1}) (Fig. 1). This difference amplitude was basically maintained throughout ripening and, by 60 days of ripening, the MP milk cheeses achieved an 8.0% improved 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. By contrast, a study i n the literature reports that bovine milk cheese yield is not influenced by treatment at HPP < 250 MPa[3] and, to the best of our knowledge, there are

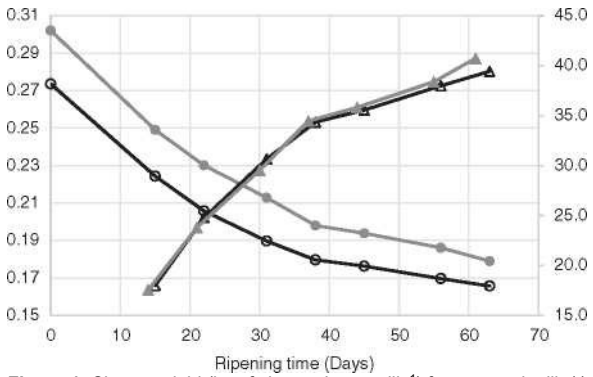

Figure 1. Chessesyield (kg of cheese L per $milk^{-1}$) from control milk ( ) and HPP pre-treated milk ( ), and weight loss percentage from control milk ( ) and madefrom HPP pre-treated milk ( ) (cheese production took place in a real artisanal dairy facility in accordance with the mandatory procedures ofthe Protected Designation ofOrigin, PDO).

no studies with an application of HPP pre-treatment at the range applied in the present study (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), with the effect being attributed to a higher moisture content,[5,15] possi- bly as a result of the formation of a finer structural network and the water-binding properties ofdenatured B-lactoglobulin, which was incorporated into the protein matrix.[15] However, in the pre- sent study, cheese moisture content was not affected by the milk pre-treatment (Table 1), although the cheese yield still increased by approximately 10%; other factors (protein, fat content) are possibly affecting the cheese yield.

Microbial composition of milk, curd and cheese

In MCewes milksamples, lactococci and lactobacilli were found at 7.02 and 2.38 log colony-forming units (CFU) mL^{-1}, total meso- philes 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 ewes'milkpre-treatment led to a reduction ofmicrobiota via- ble cell numbers,although this was one orderofmagnitude lower compared to that reported in literature for many ofthe microbial groups. Nonetheless, it must be recalled that the main aim in the presentstudywas toapplyHPP conditions thatmaymaximizethe inactivation of pejorative microorganisms at the same time as minimizing the reduction ofbeneficial microbiota viable cell num- bers, aiming 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 that reported for bovine milk treated at 100 and 200 MPa for 30 min (approximately 0.2–0.5 log cycle reduction).[15] High reductions were observed for more intensive HPP milk treatments; for example, HPP treatments at 586 MPa for 1 min and 500 MPa for 15 min in bovine and caprine milks revealed a viable cell number reduction of between 0.87 and 2.22 log cycles.[5,7] Minor numerical reductions in viable cell num- bers (< 1 log) (statistically not significant, $P > 0.05$) were observed for yeasts and moulds, total coliforms and Enterobacteriaceae in MP 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 for 1 min and 500 MPa for 15 min).[5,7] Gram-positive bacteria lactococci, lactobacilli, enterococci and staphylococci wereless affected in MPmilk,having been observed with less than 0.8log cycle reductions in viable cell numbers com- pared to M_C control values. More intense HPP treatments (500 MPa for 15 min and 600 MPa for 10 min) applied to caprine and bovine milks led to more than 2.36 log cycle reductions of lactobacilli viable cell numbers.[7,16]

In general,the curd samples obtained from MP milkdiffered sta- tistically in enterococci, staphylococci, coliforms and yeasts and moulds viable cell numbers relatively to curds obtained from MCmilk($P < 0.05$);however, the differences were once again very low, at less than 0.6 log units. Different studies have demon- strated that the decrease in viable cell numbers of microbial groups brought about by the HPP milk pre-treatment is subse- quently reflected in the curd microbiota, although for more

Table 2. Microbiota quantification in control and HPP pre-treated milk, and resulting curd, whey, and cheese with 60 days of ripening (cheese pro- duction 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	
Total mesophilic		6.06 ± 0.13 b	
	Milk		
	Curd	6.22 ± 0.08 a	5.34 ± 0.07 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 lowercase letters for the same microorganism indicate statistically significant differences between the milk, curd and cheese from control and HPP treated milk (Student's t-test, $P < 0.05$).

intensive treatments. For example, curds from HPP pre-treated goat milk (500 MPa for 5 or 30 min; 500 MPa for 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] whereas curd from HPP pre-treated bovine milk (400 MPa for 15 min) revealed 1.4 log cycle reductions of total microbiota viable cell numbers.[19]

At 60 days of ripening, cheeses manufactured from MC and MP milks showed no significant differences in lactococci, lactobacilli and enterococci viable cell numbers, with values of around 9 log CFU g^{-1} (Table 2). These values are close to those previously reported for Serra da Estrela cheese.[20,21] Also, in the traditional Pecorino di Farindola cheese (manufactured from raw ewes'milk), lactic acid bacteria and enterococci are the dominant microbiota, with values of around 7–9 log CFU g^{-1}.[22] Likewise, at 60 days of ripening, Buffa et al.[7] observed similar total bacteria, lactococci, lactobacilli and enterococci viable cell numbers for cheeses man- ufactured from HPP (500 MPa for15 min) treated and non-treated goat milk, except for Enterobacteriaceae viable cell numbers, which showed approximately 2 log cycle reductions in cheeses made from HPP pre-treated milk. For more intense HPP treat- ments, goat cheeses at 60 days of ripening, revealed reductions in viable cell numbers of total aerobic bacteria of approximately 3and 6 log cycles for500 MPa for5 and 30 min,[17]whereas, forlac- tobacilli, approximately 2 log cycle reductions were observed for 500 MPa for 15 min.[18] On the other hand, other studies observed a 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 for 10 min.[16]

According to the results ofthe present study, which, to the best of our knowledge, is one of the first to be performed on ewe's milk, a milder milk pre-treatment by HPP at 121 MPa for 30 min showed a tendency to cause slight reductions (approximately 1log cycle) in viable cell numbers ofpejorative microbiota in milk, at the same time as basically maintaining the beneficial micro- biota load; yet, by 60 days of ripening, similar values were found for cheeses produced from 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 because of the formation and consumption of lactic acid as a result of the metabolism of lactic acid bacteria and other microorganisms. In the present study, a statistically significant increase of pH was confirmed in MP milk relative to MC milk ($P < 0.05$), although the difference was only approximately 0.12 units. Even lower variations (< 0.05 pH units) were found for curd, whey and cheese produced from MC and MP 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 reported in a study for bovine milk treated by HPP (100, 250 and 400 MPa for 15 min).[24] These changes in pH caused by the HPP treatment were considered to be associated with the dissolution ofcolloidal calcium phosphate, possibly as a result of the weakening of hydrophobic and electrostatic interactions between sub-micelles. For curd, a small pH increment of 0.04 units was obtained with HPP (500 MPa for 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 for 10 min).[12] Regarding cheese, a small pH increment of 0.13 units was reported by Buffa et al.[18] for goat cheeses manufactured from HPP treated milk (500 MPa for 15 min).

As expected, HPP milk treatment showed no effect on the titrat- able acidity, with similar values being determined for milk, whey, curd and cheese ($P > 0.05$) (Table 1).

CONCLUSIONS

HPP milk pre-treatment led to a mild reduction of microbial load in milk, a small effect on curd and no significant differences in rip- ened cheese microbiota load. HPP pre-treated milk showed higher pH values, whereas cheese manufactured from HPP pre- treated milk had lower pH values; nevertheless, no significant dif- ferences in moisture and protein content were found. The present 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 the cheese yield and enable an improved micro- bial profile, which is important in terms ofboth safety and quality, thus contributing positively to the production of such an impor- tant cheese. At the microbial level, more detailed studies are desirable regarding the effect of HPP on the inoculated patho- genic organisms that may be found in raw milk cheeses (such as a combined surrogate cocktail of Staphylococcus aureus, Listeria innocua and Salmonella enterica), aiming to validate a possible improvement in food safety.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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