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The Influence of High-Power Ultrasound and Bactofugation on Microbiological Quality of Milk

Running title: Influence of Ultrasound on Safety of Milk

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SUMMARY

Research background. The application of high power ultrasound, combined with a slightly increased temperature on raw whole cow milk, skimmed cow milk, and skimmed cow milk that passed the bactofugation process were analyzed. A combination of those techniques, ultrasound and the bactofugation of milk was conducted to achieve the microbiological accuracy that is usually achieved by the pasteurization process.

Experimental approach. The milk samples (200 mL) were treated for 2.5, 5, 7.5 and 10 minutes with high-power ultrasound (200 W and 400 W) with a frequency of 24 kHz. The treatments were conducted with a constant duty cycle of 100 %. Temperature levels during the treatments were 20 °C, and 55 °C. The count of somatic cells was analyzed for the aerobic mesophilic bacteria, as well the number of *Enterobacteriaceae, Escherichia coli,* and *Staphylococcus aureus* cells.

Results and conclusions. The best result from the perspective of the reduction of the total count of bacteria was achieved by high-power ultrasound with a power of 400 W treated for 10 minutes. High reduction of *Enterobacteriaceae*, *E. coli*, and *S. aureus* cells was achieved in ultrasound treatment of raw whole, skimmed, and skimmed cow milk with a power of 200 and 400 W regardless of a treatment time.

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Novelty and scientific contribution. High-power ultrasound with a combination of bactofugation as a pretreatment for milk and with a slightly increased temperature (up to 55 °C) is much more economical than the pasteurization process, while it preserves the sensory and physical-chemical properties of milk.

Key words: milk, high-power ultrasound, microbiological safety of milk, technology of milk bactofugation

INTRODUCTION

Milk is a biological fluid that deserves special attention as the most complete natural fluid (*1*). It is an ideal medium for the development of undesirable microorganisms (*2*). To ensure the safety of food, raw milk needs to be controlled by conducting chemical, and microbiological analyzes which determine the quality of milk. If the microbiological analysis reveals more than 10^5 CFU/ml microorganisms in raw milk, the result indicates a lack of hygienic conditions (*3*), and the count of somatic cells (SCC) in one milliliter must be ≤ 400 000 observed as a geometric average over three months.

The Food and Agriculture Organization of the United Nations and the World Health Organization define various thermal procedures that are carried out to reduce and remove the number of microorganisms from milk (*4*). The most common processes are continuous flow pasteurization, and UHT (ultra-high temperature) treatment. However, significant effects of heat treatment of milk are vitamin degradation, whey protein denaturation, Maillard reaction (due to protein and lactose reaction). Therefore, it is extremely important to use lower temperatures with the same or higher efficiency as pasteurization and/or sterilization.

Bactofugation is used to improve the bacteriological quality of raw milk. It belongs to mechanical processes, and is used in the production of pasteurized, and UHT milk. It reduces the primary number of heat-resistant microorganisms in milk before heat treatment, all to prolong the shelf life of milk using a milder temperature regime (*5*). The optimal temperature of bactofugation, at which the best results are achieved, is 55-60 °C.

Today, non-thermal methods as the high-power ultrasound (*6*,*7*) treatment with high hydrostatic pressure, pulsed electric and magnetic fields are often used in food industry. High hydrostatic pressure is commercially applied in food processing, and ultrasound is applied in homogenization, emulsification, and dispersion processes (*8*). New non-thermal methods can significantly save energy, and shorten the duration of the production process. The use of high-power ultrasound has shown several advantages over heat treatment by pasteurization, such as minimizing

taste loss in juices, greater homogeneity, and significant energy savings (*9*). During the high-power ultrasound processing acoustic energy transfer is instantaneous, and extends through the entire volume, which results in lower energy consumption (*6*). When using low-intensity ultrasound waves, the mechanism of microorganism inactivation is based on changing the metabolism of microorganism cells, while the mechanism of microorganism inactivation using high-power ultrasound waves is based on breaking cell membranes of microorganisms, and denaturation of enzymes (*10,11*).

Therefore, the aim of this paper is to examine the possibility of processing whole and skimmed milk using high-power ultrasound in combination with slightly elevated temperature, and pre-bactofuging technology of milk in order to achieve microbiological safety at the level achieved by pasteurization.

MATERIALS AND METHODS

Milk samples

Throughout the research, milk of different processing levels was used, excluded directly from production, where bactofuging is an integral part of the milk processing process.

All tests were performed on cow's milk that belonged to the same milk production batch from the dairy (Vindija, d.o.o. Varaždin, Croatia). The tests were performed on raw whole milk, skimmed milk, and skimmed bactofuged milk, and on pasteurized milk as a reference sample. Milk samples were aseptically taken into sterile vials at the sampling valves before the separator (whole milk, A), after the separator (skimmed milk, B), and after the bactofuging process (skimmed bactofuged milk, C). As a reference control sample pasteurized milk produced by the classical HTST (high temperature short time) process (D), (processing parameters 72 °C/15″) was taken.

Milk processing with ultrasonic processor UP 400S

In this research, an ultrasonic processor model UP 400S, manufactured by "Dr. Hielscher ”GmbH, Teltow, Germany was used. The characteristics of this ultrasonic processor are opened system with an effective output power 400 W, current-voltage 230 V, 48-63 Hz, ultrasonic cycle 10-100 %, ultrasonic frequency 24 kHz, and amplitude 12-260 μm. A 7 mm diameter titanium probe was used in the work, and it was immersed at a depth of 2 cm in each milk sample. The treatments were performed in the Laboratory for Technological Operations, the Laboratory for Process Food Engineering, and in the Laboratory for Technical Thermodynamics of the Faculty of Food Technology and Biotechnology, University of Zagreb.

Determination of the count of somatic cells in milk

The count of somatic cells in milk, epithelial cells, and leukocytes, was determined by electronic cell counter using a cytometric method of fluoro-optoelectronic counting (instrument: Fossomatic 5000, Foss Electric A/S, Denmark) according to ISO standard (*12*).

Testing of microbiological correctness, hygienic quality of milk

To determine the achieved inactivation of the tested microorganisms, before the treatment on the raw whole, skimmed, and bactofuged milk, the initial number of tested microorganisms was determined. The achieved reduction of the logarithmic number of cells of microorganisms was calculated according to the formula:

$$log\frac{N_1}{N_0} = \log N_1 - logN_0 \qquad /1/$$

where N_0 is the total initial number of microorganisms before treatment and N_1 is the number of microorganisms after treatment.

Design of the experiment

The design of an experiment is marked with letters from A-D: (i) Experiment A: ultrasound power 200 W, frequency 24 kHz, temperature 20 °C; (ii) Experiment B: ultrasound power 200 W, frequency 24 kHz, temperature 55 °C; (iii) Experiment C: ultrasound power 400 W, frequency 24 kHz, temperature 20 °C and (iv) Experiment D: ultrasound power 400 W, frequency 24 kHz, temperature 55 °C.

Treatments were performed at four different times (2.5, 5, 7.5 and 10 min). Flowchart of the equipment and analyses is shown in Fig. S1. Each sample was analyzed three times and the results presented are the mean value of three measurements.

Preparation of a samples

Volume of 1 mL of prepared decimal dilution was added to a Petri dish and poured with a liquid nutrient medium. Furthermore, the samples were also inoculated on a prepared solid medium. Volume of 0.1 mL of a milk sample was added, and smear with a Drigalsky stick. Incubation of the samples was performed according to the ISO standard (*13*). Incubation of aerobic mesophilic bacteria was performed at (30±1) °C for (72±2) h.

Incubation of *Enterobacteriaceae* was done at (37±1) °C for (24±2) h. *Enterobacteriaceae* were inoculated on a VRBG agar (Crystal-violet neutral-red bille glucose agar, Merck, Germany) and then

coated with a cover layer of VRBG agar (15 mL) which, after solidification, prevented colony overgrowth and provided semi-anaerobic conditions (*14*).

E. coli cells were analyzed on a TBX agar (Tryptose were X-glucuronide agar for microbiology Chromocult, Merck, Germany). Incubation was performed at (44±1) °C for(21±3) h (*15*).

S. aureus cells were proved by inoculating the samples on Baird-Parker agar (Merck, Germany) with the addition of egg yolk and tellurite emulsion. Incubation was done at (37±1) °C for (24±2) h. Confirmation of colonies: when colonies appeared on the medium, they were confirmed by a positive catalase test. The test was performed using reagents bactident catalase (Merck, Darmstadt, Germany). The procedure was performed in such a way that a drop of reagent was applied directly to a randomly selected colony on medium. *S. aureus* colonies produce gas (*16*). If *S. aureus* colonies were not confirmed in the result section they were indicated as no found (n.f.). If the count of *S. aureus* cells were less than 10 colonies it was marked as >10, and if the number of cells was less than 100 it was marked as >100.

For the determination of *Enterobacteriaceae* and *E. coli* if the count of cells were less than 10 colonies it was marked as >10, and if the number of cells was less than 100 it was marked as >100. If the number of cells was higher than 100 the exact number of colonies was specified.

Determination of the total number of living cells of microorganisms and statistical data processing

Determination of the total number of bacteria (TVC) was performed based on the counted number of colonies multiplied by the degree of dilution (*17*). Increased colonies were counted on a counter (Colony counter, Bibi Sterlin, UK), and the number of living microorganism cells in the processed samples was expressed as the CFU value (Colony Forming Units). The number of units forming colonies was calculated according to the formula (2) and ISO standard (*12*):

$$\mathrm{CFU} = \frac{\text{number of grown colonies}}{\text{sample volume (inoculum)}} * \text{reciprocal value of sample dilution} \qquad /2/$$

escriptive statistics were used to show mean values, standard deviation (S.D.), and minimum and maximum values for each experiment (*18*). In order to connect, ie determine similarities and/or differences in a large data set for each observed characteristic (treatment, according to the experiment), multivariate statistical methods were applied (*19*). STATISTICS (data analysis software system) v. 8 was used for data processing (*20*).

RESULTS AND DISCUSSION

The average values of the count of somatic cells, and their reduction in different experiments (A-D) are shown in Table 1, from which it is evident that between the reference samples row whole milk, and untreated skimmed samples (SP and SO) a lower count of somatic cells in SO compared to crude full-fat samples (SP) was observed. This is explained by the fact that the milk is collected in a centrifugal cream separator, and that part of the somatic cells ends up in the cream. To make it easier to monitor the impact of treatment on SCC, a reduction expressed in percentages was calculated. This means that the initial SCC from the reference whole milk sample (SP) was used to recalculate the T1-T4 treatment reduction, and the skimmed milk sample (SO) was used to recalculate the T4-T12 treatment reduction. Considering the used bactofuging technology of the samples, the expected decrease in the count of somatic cells in the reference bactofuged skimmed milk (BF) sample is noticeable in comparison with the reference untreated skimmed milk sample (SO). Since 80-90 % of the bacteria and 90-95 % of spores can be removed by milk bactofugation (*1*), it is evident that the process of milk bactofugation also removes somatic cells.

Experiments (A-D) have shown that high-power ultrasound, regardless of temperature, reduces the count of somatic cells, which explains the ultrasound mechanism. This effect creates high local temperatures, and pressures that cause cell wall rupture, and cell disintegration (*21,22*). In all experiments it was observed that the reduction of SCC is higher in all ultrasound treated samples (even at 2.5 min) than in pasteurized samples (in the pasteurization process, bactofugation was included as part of the process (Fig. 1a) with the statement of Povey and Mason (*23*) and Cameron (*24*) who found that ultrasound treatment of milk significantly reduces the count of somatic cells. However, reduction of somatic cells will not affect the improvement of milk quality due to high initial number.

For each experiment, the observed relationship of the pooled representations (Figs. 1a and 1b) with the corresponding Pearson correlation matrix table is shown (Table 2). The Pearson correlation matrix for the count of somatic cells and their reduction showed the expected negative correlation between the count of somatic cells and their reduction, which implies their inverse-proportional relationship depending on the experimental conditions (A-D). In the pooled SCC representations, and their reductions for a particular experiment versus the samples themselves that were treated differently, a grouping of bactofuged samples was seen in all experiments (Figs. 1a and 1b). The bactofuged samples were independently arranged in the first (Fig. 1a), and fourth quadrant (Fig. 1b). The analysis of the main components of the somatic cell count distribution, and their reduction before rotation is shown in Fig. 1a. The right side was occupied by treated milk samples (T8-T12, BF, P) in which the reduction was significantly higher than in samples located in the left part

of the coordinate system (T1-T7, SP, SO). Bactofuged samples (T9-T12, BF, pasteurized (P)) were placed in the first quadrant, and correlated with the reduction results of all experiments A-D (1st and 4th quadrant). The contribution of the first and second components is also important (F1=80.05 %; F2=13.62 %), and the dominance of the first component is visible. Precisely for this reason, rotation was used in the analysis of the principal components to distribute the influence of the principal components. Verimax rotation, which is often used in the food industry, was applied. The results after rotation are shown in Fig. 1b. According to the results shown in Fig. 1b, it was observed that the share of variations explained in this data set remains the same with a high 93.67 %, and the distribution of bactofugated samples grouped separately, but now the reduction data in the different experiments no longer spread through the 1st and 4th quadrants,. They were grouped only in the 4th quadrant, and the SCC content, depending on the experiments, was grouped into the 2nd quadrant. As expected, the ratio of SCC in milk, and their reduction still shows an inversely proportional ratio (Fig. 1b). The first main component explains the high 48% variations in SCC, and their reductions in experiments A, C, and D in which better reduction results were obtained, while the second main component explains the variations in less successful experiments B in terms of total SCC reduction (T1-T12, SO, BF and P).

The analysis of correspondence is presented through tables of significance,, and representation/contribution of values from rows, and columns in the form of a two-dimensional table (Table 3). Correspondence analysis (CA) is a method of data visualization that is applicable to contingency tables (*26*). It was performed with the aim of comparing differently treated samples in different experiments, and the efficiency of SCC reduction. Table 3 shows the change in the count of somatic cells, and their significance, and representation in the first two factors (F1 and F2). The significance column shows in which treatment the SCC 1/ml was highest where SP sample showed the highest count of somatic cells. In a bactofuged sample that was treated for 10 minutes, T12 (SCC count ranged from 2 000, experiment C, to 8 000 in experiment B). In Table 3, when observing SCC 1/ml, there are two columns showing the representation in the reduced number of factors (reduced to 2, ie F1 and F2) and the SP sample dominates in the factor F1, and T1 and T2 in the second factor, F2. Their dominance in factor F2 was associated with a high count of somatic cells.

Analysis of the results of the total bacteria count and their reduction in milk samples is presented in Table 4. The antimicrobial effect of ultrasound is achieved by cavitation, ie the extremely rapid formation, and collapse of bubbles formed in the medium by the action of ultrasonic waves. These results occurred due to changes in pressure, and temperature, which can cause cell wall rupture and thinning of the cell membrane (*22*,*27*,*28*). Also, due to the action of free radicals DNA damage can occur (*22*,*27*,*28*). Table 4 shows the TVC (total number of bacteria), and the reduction

of their logarithmic number in milk samples according to different conditions of experiments A–D. Analysis of TVC reductions within the same experiment (regardless of power, frequency, and temperature parameters) shows that the best results are achieved by sounding bactofuged skimmed milk samples; experiment D (reduction of TVC from T9=2.31 to T12=2.68), experiment B (reduction of TVC from T9=2.14 to T11=2.44), experiment C (reduction of TVC from T12=2.06 to T9/10=2.20), experiment A (reduction of TVC from T10=1.94 to T12=2.04). From the analyzed data, a better result of TVC reduction was observed in experiments D (400 W, 55 °C) and B (200 W, 55 °C) in which milk was treated by high-power ultrasound. Cameron (*23*) states that in order to achieve better results for the inactivation of microorganisms in milk, it is recommended to combine high-power ultrasound with slightly elevated temperature, which can be confirmed in this work by achieving better results of TVC reduction by ultrasound treatment. Many other authors discussed in their papers that the inactivation of microorganisms exposed to the combination of ultrasound, and temperature is much higher, which is consistent with the results of this work (*29,30*). Also, the results of the number of bacteria proved to be satisfactory considering the recommendation from the Guide for microbiological criteria (*31*) ($m=10^3$ CFU/mL; $M=10^4$ CFU/mL, n=5, c=1) only in bactofuged treated samples of A-D experiments, and pasteurized reference samples (P). In Table 5 contribution of experiment A-D, their significance and representation in the first two factors for the changes in total number of bacteria (CFU/mL) are shown.

It is stated in accordance with the first part of Singh and Heldman's assumption (*32*) that the combination of ultrasound, and heat should result in a product with a longer shelf life, and that the required processing time could even be reduced leading to lower production cost. The main problem here, however, is the required processing time with ultrasound, which is much longer than the classical pasteurization method (7.5-10 min versus 0.25 min). One of the possibilities to solve the mentioned problem is to install ultrasonic probes in the process after bactofugation.

Herceg *et al*. (*25*) noted that the system of high-power ultrasound processing of milk in industrial production should be designed to allow maximum contact between milk, and the cavitation zone, and that it would be useful to explore the possibility of using multiple ultrasound probes. The action of a parallel series of ultrasound probes should be further demonstrated and confirmed. This would be in line with the suggestion by Ashokkumar *et al*. (*33*) who explained that in the dairy industry it would be interesting to add ultrasound as a new process function in terms of improving the functionality of the products themselves. Furthermore, Oliveira and Oliveirana (*34*) represented in their work that higher inactivation of microorganisms was obtained when using the sonication technique at 70 °C. Results achieved by Sala *et al*. (*35*) in the inactivation of microorganisms using the technique of thermal sonication of milk at a temperature of 70 °C, which is also used in standard

HTST procedure showed high reduction results. Sala *et al.* (*35*) consider that milk treated in this way would not contain vegetative cells, and that it would have a longer shelf life with minimal processing. Furthermore, the product could be similar, in terms of microorganism content, to ultra-high temperature (UHT) milk.

It is prescribed that the value of the number of *Enterobacteriaceae* must be within the following limits m=M≤10 CFU/mL for the sample to be considered satisfactory. "M" is the limit value below which all results are considered satisfactory, and "M" is the limit value above which the results are considered unsatisfactory. In the results shown in Table 6, it was observed that high-power ultrasound treatment of milk gives good results of *Enterobacteriaceae* inactivation. Satisfactory results of treatment of full-fat T1-T4 samples were observed in high-power ultrasound experiments B and D where power was 200 W and 400 W combined with an elevated temperature of 55 ° C. The same trend was observed in the selected samples T5-T8 of the same experiments B, D, and F.

The number of *Escherichia coli* bacteria in milk (Table 6) was also observed. Cameron *et al.* (*24*) believe that ultrasound cavitation destroys cells of undesirable contaminants such as *E. coli* bacteria, which is confirmed by the results in this work. As with *Enterobacteriaceae*, satisfactory results of treatment of full-fat T1-T4 samples were observed in high-power ultrasound samples of experiments B and D, and in samples F2 (5 min) and F4 (10 min). The same trend was observed in samples of T5-T8 experiments B, and D.

Bacterial species of *Staphylococcus aureus* according to the Guide to microbiological criteria (*31*) for food are among the recommended microorganisms whose presence is controlled in pasteurized milk, with satisfactory criterion M=10 CFU/mL (where M=limit value above which the results are considered unsatisfactory). Table 7, a common presentation of *Staphylococcus aureus* inactivation for all experiments, shows satisfactory results of T1-T4 whole milk treatments in all high-power ultrasound samples of experiments B, D, and F. The same trend is seen in high-power ultrasound samples of T5-T8 experiments B and D. In all experiments for reference samples BF and P, and treatments T9-T12, it was observed that the samples meet the criteria of *S. aureus* number (M=10 CFU/mL). For samples B9/10, D9/10, F9-F12, and P (for experiments B and D) it can be seen that the presence of bacteria was not proven/found (value nn CFU/mL), which means that these samples were additionally performed for the confirmation of the presence of bacteria of the *S. aureus* species which did not prove the presence of bacteria. Ordoñez *et al.* (*34*) investigated the inactivation of the *S. aureus* cells by high-power ultrasoud treatment. In their research they concluded that higher inactivation was obtained when using a combination of ultrasound with slightly elevated temperature compared to the results obtained when using only ultrasound for inactivation, which is in accordance with the obtained results of this work. Sherba *et al.* (*36*) studied the effect of ultrasound (24 kHz) on *S.*

aureus species, and concluded that ultrasound has a bactericidal effect on *S. aureus* species, and inactivation increases with time, and intensity of ultrasound. Thus, the reduction of *S. aureus* increased in their work with an increase in intensity from 1 to 3 W 1/cm^2 for 15 min from 22 % to 39 %, or with an extension of the ultrasound time (2-30 min, 3 W 1/cm^2) the reduction was 42-43 %.

CONCLUSIONS

This work deals with the possibility of processing whole and skimmed cow's milk using high-power ultrasound in combination with slightly elevated temperature and pre-bactofuging technology of milk in order to achieve microbiological safety. This is achieved by optimizing the processes of bactofuging, ultrasound treatment (frequency, power, and amplitude of ultrasound), and slightly elevated temperatures up to 55 °C, with emphasis on the microbiological quality of milk, in accordance with legislation. In bactofuged milk processed by high-power ultrasound, high inactivation of the total number of bacteria was observed. This suggests that bactofuged milk in combination with ultrasound could be an alternative to the pasteurization process in terms of reducing the total number of bacteria. The findings suggest that there is a possible application of new technologies in food processing as an effective replacement for thermal treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr

AUTHORS' CONTRIBUTION

E. Juraga collected, analyzed, and interpreted data. V. Stulić made a draft and participated in the writing of the article. T. Vukušić Pavičić analyzed data and interpreted the results. J. Gajdoš Kljusurić analyzed, and interpreted data. M. Brnčić did the conceptualization, and Z. Herceg as a project administrator made a conceptualization.

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Table 1. The count of somatic cells (SCC), and the percentage of their reduction (SCC reduction/%) in milk samples.

Samples	Treatment							
	A (200W, 24 kHz, 20 °C)		B (200W, 24 kHz, 55 °C)		C (400W, 24 kHz, 20 °C)		D (400W, 24 kHz, 55 °C)	
	SCC	SCC reduction/ %	SCC	SCC reduction/ %	SCC	SCC reduction/ %	SCC	SCC reduction /%
SP	320 000	0	365 000	0	278 000	0	347 000	0
T1	227 000	29	329 000	10	36 000	87	178 000	49
T2	132 000	59	280 000	23	19 000	93	110 000	68
T3	134 000	58	130 000	64	17 000	94	54 000	84
T4	84 000	74	160 000	56	14 000	95	63 000	82
SO	262 000	0	192 000	0	264 000	0	201 000	0
T5	130 000	50	120 000	38	119 000	55	140 000	30
T6	50 000	81	95 000	51	67 000	75	106 000	47
T7	37 000	86	84 000	56	37 000	86	68 000	66
T8	21 000	92	76 000	60	21 000	92	24 000	88
BF	19 000	93	17 000	91	9 000	97	24 000	88
T9	11 000	96	16 000	92	7 000	97	10 000	95
T10	8 000	97	12 000	94	4 000	98	7 000	97
T11	6 000	98	10 000	95	2 000	99	4 000	98
T12	4 000	98	8 000	96	2 000	99	3 000	99
P	22 000	92	24 000	88	7 000	97	18 000	91

SCC=somatic cell count. Reference samples: SP=raw whole milk; SO=untreated skimmed milk; BF=bactofuged skimmed milk; P=pasteurized milk. Treatments T1-T4 denote whole milk treated 2.5 (T1); 5 (T2); 7.5 (T3) and 10 (T4) minutes, T5-T8 treatments denote skimmed milk treated with 2.5 (T5); 5 (T6); 7.5 (T7) and 10 (T8) minutes and T9-T12 treatments indicate bactofuged skimmed milk treated with 2.5 (T9); 5 (T10); 7.5 (T11) and 10 (T12) minutes.

Table 2. Pearson correlation matrix for somatic cell number (SCC), and their reduction, depending on the treatment with a significance level of 5 %

Observed	SCC (_s)				SCC reduction% (_r)			
	A_s	B_s	C_s	D_s	A_r	B_r	C_r	D_r
A_s	1.00	0.90	0.82	0.94	-0.99	-0.92	-0.81	-0.87
B_s	0.90	1.00	0.58	0.87	-0.85	-0.92	-0.57	-0.72
C_s	0.82	0.58	1.00	0.88	-0.86	-0.75	-1.00	-0.93
D_s	0.94	0.87	0.88	1.00	-0.93	-0.90	-0.87	-0.93
A_r	-0.99	-0.85	-0.86	-0.93	1.00	0.92	0.86	0.91
B_r	-0.92	-0.92	-0.75	-0.90	0.92	1.00	0.75	0.89
C_r	-0.81	-0.57	-1.00	-0.87	0.86	0.75	1.00	0.93
D_r	-0.87	-0.72	-0.93	-0.93	0.91	0.89	0.93	1.00

A=high-power ultrasound 200 W, 24 kHz, 20 °C
B=high-power ultrasound 200 W, 24 kHz, 55 °C
C=high-power ultrasound 400 W, 24 kHz, 55 °C
D=high-power ultrasound 400 W, 24 kHz, 55 °C

Table 3. The contribution of the count of somatic cells (SCC), and the share of their reduction, significance, and representation in the first two factors for different treatments.

Milk samples	SCC/mL			SCC reduction/%		
	Order significance	F1	F2	Order significance	F1	F2
SP	1	0.275	0.010	15	0.000	0.000
T1	3	0.001	0.283	14	0.098	0.521
T2	4	0.043	0.162	12	0.105	0.123
T3	7	0.050	0.017	7	0.029	0.007
T4	6	0.101	0.010	9	0.073	0.001
SO	2	0.230	0.097	15	0.000	0.000
T5	5	0.013	0.071	13	0.029	0.052
T6	8	0.017	0.099	11	0.079	0.148
T7	9	0.075	0.105	10	0.089	0.084
T8	10	0.173	0.124	8	0.118	0.062
BF	12	0.011	0.004	5	0.068	0.000
T9	13	0.001	0.001	4	0.064	0.000
T10	14	0.000	0.002	3	0.064	0.000
T11	15	0.001	0.001	2	0.062	0.000
T12	16	0.004	0.001	1	0.061	0.000
P	11	0.003	0.012	6	0.063	0.003

Treatments T1-T4 denote whole milk treated 2.5 (T1); 5 (T2); 7.5 (T3) and 10 (T4) minutes, T5-T8 treatments denote skimmed milk treated with 2.5 (T5); 5 (T6); 7.5 (T7) and 10 (T8) minutes and T9-T12 treatments indicate bactofuged skimmed milk treated with 2.5 (T9); 5 (T10); 7.5 (T11) and 10 (T12) minutes.

Table 4. Influence of different experimental treatments on total number of bacteria, and reduction of logarithmic number in milk samples

Observed	Treatment											
	A (200 W, 24 kHz, 20 °C)			B (200 W, 24 kHz, 55 °C)			C (400 W, 24 kHz, 20 °C)			D (400 W, 24 kHz, 55 °C)		
	TVC (CFU/ml)	LCN (Log_{10} CFU/ml)	Reduction of LCN	TVC (CFU/ml)	LCN (Log_{10} CFU/ml)	Reduction of LCN	TVC (CFU/ml)	LCN (Log_{10} CFU/ml)	Reduction of LCN	TVC (CFU/ml)	LCN (Log_{10} CFU/ml)	Reduction of LCN
SP	450 000	5.65	0.00	1 050 000	6.02	0.00	400 000	5.60	0.00	580 000	5.76	0
T1	110 000	5.04	0.61	142 000	5.15	0.87	72 000	4.86	0.75	31 000	4.49	1.27
T2	57 600	4.76	0.89	126 000	5.10	0.92	40 000	4.60	1.00	18 000	4.26	1.51
T3	48 000	4.68	0.97	24 000	4.38	1.64	25 000	4.40	1.20	4 000	3.60	2.16
T4	76 800	4.89	0.77	16 000	4.20	1.82	44 000	4.64	0.96	5 000	3.70	2.06
SO	438 000	5.64	0.00	656 000	5.82	0.00	160 000	5.20	0.00	408 000	5.61	0.00
T5	69 000	4.84	0.80	100 000	5.00	0.82	32 000	4.51	0.70	78 000	4.89	0.72
T6	100 000	5.00	0.64	120 000	5.08	0.74	23 000	4.36	0.84	58 000	4.76	0.85
T7	96 000	4.98	0.66	140 000	5.15	0.67	29 000	4.46	0.74	60 000	4.78	0.83
T8	62 000	4.79	0.85	29 000	4.46	1.36	20 000	4.30	0.90	8 800	3.94	1.67
BF	11 000	4.04	1.60	17 800	4.25	1.57	4 080	3.61	1.59	11 000	4.04	1.57
T9	4 560	3.66	1.98	4 800	3.68	2.14	1 000	3.00	2.20	2 000	3.30	2.31
T10	5 000	3.70	1.94	4 200	3.62	2.19	1 000	3.00	2.20	1 100	3.04	2.57
T11	4 160	3.62	2.02	2 400	3.38	2.44	1 200	3.08	2.13	1 300	3.11	2.50
T12	4 000	3.60	2.04	2 600	3.42	2.04	1 400	3.15	2.06	860	2.93	2.68
P	336	2.53	3.12	100	2	3.82	100	2.00	3.20	920	2.96	2.65

TVC=total count of bacteria, LNC=logarithm of total count of bacteria. Reference samples: SP=raw whole milk; SO=untreated skimmed milk; BF=bactofuged skimmed milk; P=pasteurized milk. Treatments T1-T4 denote whole milk treated 2.5 (T1); 5 (T2); 7.5 (T3) and 10 (T4) minutes, T5-T8 treatments denote skimmed milk treated with 2.5 (T5); 5 (T6); 7.5 (T7) and 10 (T8) minutes and T9-T12 treatments indicate bactofuged skimmed milk treated with 2.5 (T9); 5 (T10); 7.5 (T11) and 10 (T12) minutes

Table 5. Contribution of experiment A-D, their significance, and representation in the first two factors for the changes in total number of bacteria

Experiment	Significance	F1	F2
A	4	0.001	0.031
B	3	0.339	0.013
C	6	0.031	0.235
D	5	0.272	0.059

A=high-power ultrasound 200 W, 24 kHz, 20 °C
B=high-power ultrasound 200 W, 24 kHz, 55 °C
C=high-power ultrasound 400 W, 24 kHz, 55 °C
D=high-power ultrasound 400 W, 24 kHz, 55 °C

Table 6. Influence of treatments (A-D) on total number of *Enterobacteriaceae* (E), and number of *Escherichia coli* (Ec) cells

Observed	Treatment							
	A (200 W, 24 kHz, 20 °C)	B (200 W, 24 kHz, 55 °C)	C (400 W, 24 kHz, 20°C)	D (400 W, 24 kHz, 55 °C)	A (200 W, 24 kHz, 20 °C)	B (200 W, 24 kHz, 55 °C)	C (400 W, 24 kHz, 20°C)	D (400 W, 24 kHz, 55 °C)
	Enterobacteriaceae (E)				*Escherichia coli (Ec)*			
	CFU/mL	CFU/mL	CFU/mL	CFU/mL	CFU/mL	CFU/mL	CFU/mL	CFU/mL
SP	900	510	200	910	500	390	210	730
T1	840	10	140	<10	410	10	90	<10
T2	720	<10	110	<10	100	<10	100	<10
T3	1.000	<10	140	<10	500	<10	50	<10
T4	360	<10	160	<10	<100	<10	20	<10
SO	200	200	130	620	180	110	100	100
T5	100	n.n.	<100	<10	100	<10	<100	<10
T6	<100	<10	<100	<10	<100	<10	<100	<10
T7	<100	<10	<100	<10	100	<10	<100	<10
T8	<100	<10	<100	<10	<100	<10	<100	<10
BF	<10	<10	15	20	<10	<10	13	10
T9	<10	<10	10	<10	<10	<10	10	<10
T10	<10	<10	10	<10	<10	<10	10	<10
T11	<10	<10	<10	<10	<10	<10	<10	<10
T12	<10	<10	10	<10	<10	<10	10	<10
P	<10	<10	<10	<10	<10	<10	<10	<10

Reference samples: SP=raw whole milk; SO=untreated skimmed milk; BF=bactofuged skimmed milk; P=pasteurized milk. Treatments T1-T4 denote whole milk treated 2.5 (T1); 5 (T2); 7.5 (T3) and 10 (T4) minutes, T5-T8 treatments denote skimmed milk treated with 2.5 (T5); 5 (T6); 7.5 (T7) and 10 (T8) minutes and T9-T12 treatments indicate bactofuged skimmed milk treated with 2.5 (T9); 5 (T10); 7.5 (T11) and 10 (T12) minutes

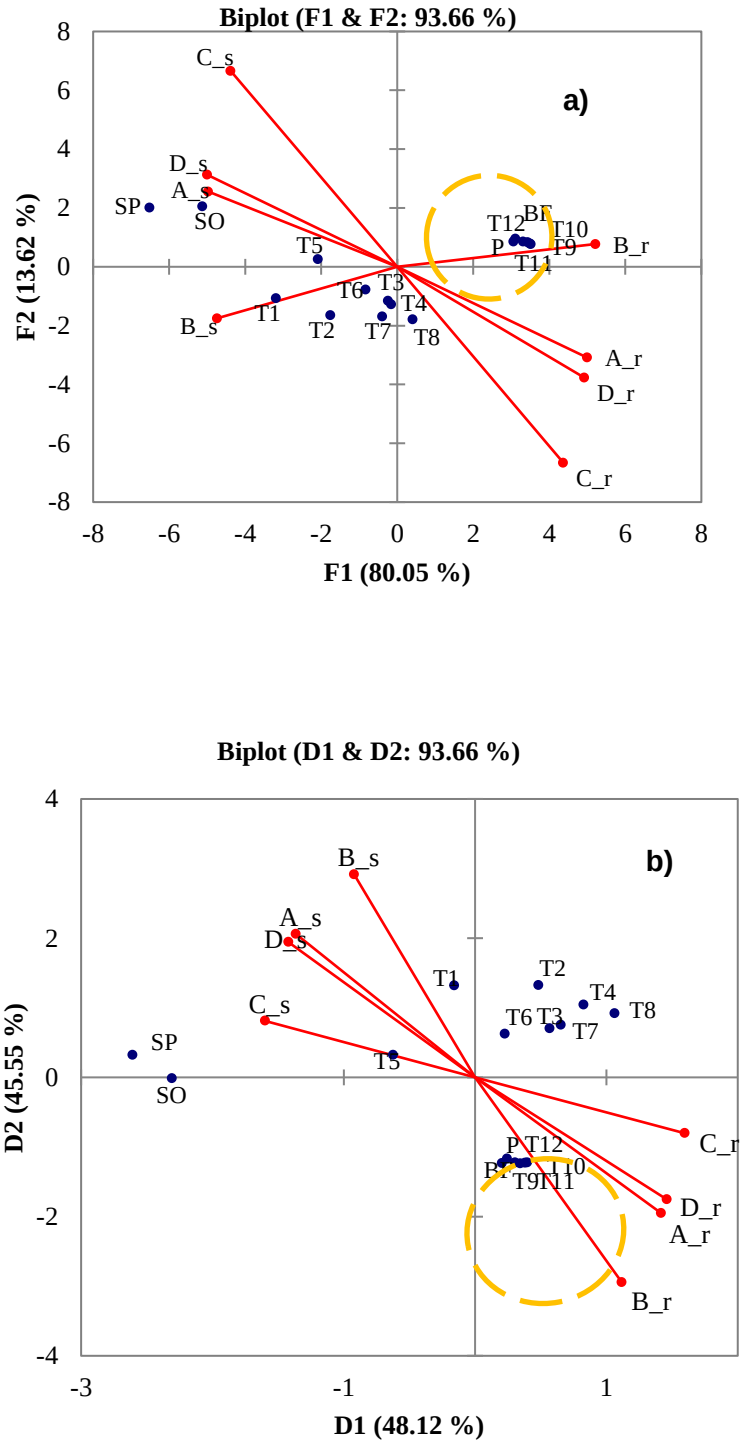

Fig. 1. Principal component analysis of somatic cell distribution (SCC), and their reduction without rotation (a), and after Verimax rotation (b)

Table 7. Inflence of tretatments (A-D) on total number of *Staphylococcus aureus* (Sa) cells.

	Treatment			
	A (200 W, 24 kHz, 20 °C)	B (200 W, 24 kHz, 55 °C)	C (400 W, 24 kHz, 20°C)	D (400 W, 24 kHz, 55 °C)
	Staphylococcus aureus (Sa)			
	CFU/mL	CFU/mL	CFU/mL	CFU/mL
SP	200	350	150	100
T1	160	10	180	<10
T2	170	n.f.	120	<10
T3	110	<10	120	<10
T4	140	<10	110	<10
SO	120	130	<100	100
T5	<100	n.f.	<100	<10
T6	<100	n.f.	<100	n.f.
T7	<100	<10	100	<10
T8	<100	<10	<100	<10
BF	<10	<10	<10	<10
T9	10	n.f.	<10	n.f.
T10	<10	n.f.	<10	n.f.
T11	<10	<10	<10	<10
T12	<10	<10	<10	<10
P	<10	n.f.	<10	n.f.

Reference samples: SP-raw whole milk; SO-untreated skimmed milk; BF-bactofuged skimmed milk; P-pasteurized milk; n.f.- no bacteria was found / proven .; (-) sample not analyzed

Treatments T1-T4 denote whole milk treated 2.5 (T1); 5 (T2); 7.5 (T3) and 10 (T4) minutes, T5-T8 treatments denote skimmed milk treated with 2.5 (T5); 5 (T6); 7.5 (T7) and 10 (T8) minutes and T9-T12 treatments indicate bactofuged skimmed milk treated with 2.5 (T9); 5 (T10); 7.5 (T11) and 10 (T12) minutes.

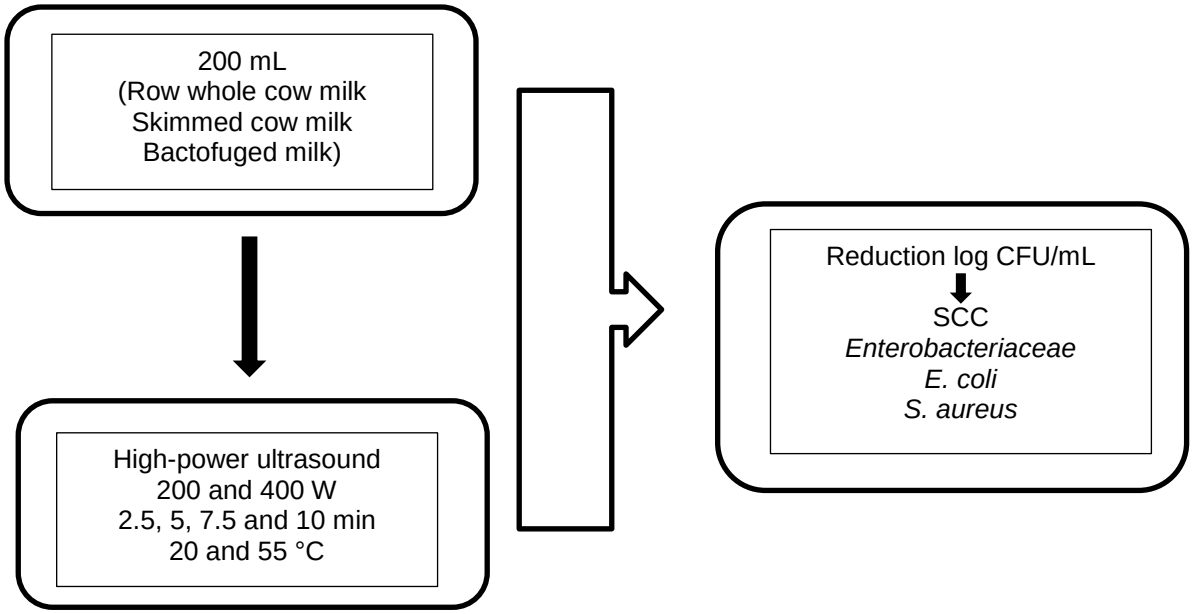

Fig.S1. Flowchart of the equipment and analyses