Inactivation of Mesophilic Bacteria in Milk by Means of High Intensity Ultrasound Using Response Surface Methodology

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Abstract

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High-intensity ultrasound was used to investigate the inactivation of microorganisms in raw bovine milk. Raw bovine milk with 4% of milk fat was treated with ultrasonic probe that was 12 mm in diameter and with 20 kHz frequency immerged in milk directly. In the ultrasound treatment, three parameters were varied according to the statistical experimental design. The centre composite design was used to design and optimise the experimental parameters: temperature (20, 40, and 60°C), amplitude (120, 90, and 60 μm), and time (6, 9, and 12 min). All analyses were performed immediately after sonication and after 3 days and 5 days of storage under refrigeration at 4°C. The factors that seem to affect substantially the inactivation of microorganisms in using ultrasound are the amplitude of the ultrasonic waves, the exposure/contact time with the microorganisms, and the temperature of the treatment. The results achieved indicate a significant inactivation of microorganisms under longer periods of the treatment with ultrasonic probe, particularly in combination of higher temperature and amplitude. The output optimal value of total bacteria count was defined by Statgraphics where the lowest bacteria count was 3.688 log CFU/ml for the following specific ultrasound parameters: amplitude 120 μm, treatment time 9.84 min, and temperature 45.34°C.

Keywords: high power ultrasound; total mesophilic bacteria count; *D* values

Conventional thermal pasteurisation and sterilisation are the most common techniques currently used to inactivate microorganisms in food products; however, the demand for new methods with a reduced impact on the nutritional content and overall food quality is increasing. New preservation techniques have been developed that can eliminate microbial activity while significantly reducing or completely eliminating the amount of heat required. These processes are, for the most part, less energy-intensive and therefore more cost-efficient and environmentally friendly than the conventional thermal processing. Thermal processing kills vegetative microorganisms and some spores; however, its effectiveness depends on the treatment temperature and time. Unfortunately, the treatment time and process temperature are also proportional to the amount of the nutrient loss, development of undesirable flavours, and deterioration of functional properties of food products. Some of the common nonthermal alternatives to conventional thermal processing of foods include pulse-electric field inactivation, pulse-light inactivation, high pressure, and ultrasonication.

Ultrasonication is a rapidly growing field of research and development for the food industry, which can be classified mainly into two fields: high frequency low energy diagnostic ultrasound in the MHz range, and low frequency high-energy power ultrasound. The low-intensity ultrasound which uses very small power levels (typically less than 1 W/cm^2, with the frequency range of 5–10 MHz), causes no physical and chemical alterations in the properties of the treated material and thus can be used to measure the texture, composition, viscosity, or concentration of food. In contrast, the high-intensity ultrasound which uses much higher power levels (typically in the range of 10–1000 W/cm^2, with the frequency of 20–100 kHz), causes physical disruption of the material to which it is applied and promotes certain chemical reactions (Povey & Mason 1998). Various areas have been identified with a great potential for future development, e.g. modification of macromolecules, crystallisation, drying, degassing, extraction, filtration, homogenisation, meat tenderisation, oxidation, sterilisation, etc. (Mason 1990; Floros & Liang 1994; McClements 1995; Mason *et al.* 1996; Guerrero *et al*. 2001).

The investigation of ultrasound as a potential microbial inactivation method began in the 1960s, after it was discovered that the sound waves used in anti-submarine warfare killed fish (Allinger 1975). The mechanism of microbial killing is mainly due to the thinning of the cell membranes, localised heating, and production of free radicals (Earnshaw *et al.* 1995; Villamiel & De Jong 2000; Butz & Tauscher 2002; Piyasena *et al.* 2003; Cameron *et al.* 2008). During the sonication process, longitudinal waves are created when a sonic wave meets a liquid medium, thereby creating regions of alternating compression and expansion (Sala *et al.* 1995). These regions of pressure change cause cavitations to occur, and gas bubbles are formed in the medium. These bubbles have a larger surface area during the expansion cycle, which increases the diffusion of gas, causing the bubble to expand. A point is reached where the ultrasonic energy provided is not sufficient to retain the vapour phase in the bubble; therefore, rapid condensation occurs. The condensed molecules collide violently, creating shock waves. These shock waves create regions of very high temperature and pressure, the former reaching up to 5500°C while the peaks of pressure up to 50 000 kPa. It is estimated that these temperatures and pressures in the spots have a lifetime below 1 μs, and the liquid heating and cooling speed is in the range of 10^9°C/s. The high intensity ultrasound effects are dependent on the number and intensity of bubbles implosion per unit of time, the characteristics of the treatment, and the characteristics of the treatment media.

Microbial inactivation by ultrasound depends on several factors that can be classified under the treatment conditions, microbial characteristics, and environmental factors (Raso *et al.* 1998; Lopez-Malo *et al*. 1999; Pagan *et al.* 1999; Villamiel & De Jong 2000; Mason *et al.* 2003; Cameron *et al.* 2008). If ultrasound was to be used in any practical application, it would most likely have to be used in conjunction with the pressure treatment (manosonication), heat treatment (thermosonication), or both (manothermosonication). The enhanced mechanical disruption of cells is the reason for the enhanced killing when ultrasound is combined with heat or pressure.

The objective of this work was, therefore, to investigate the effect of high intensity ultrasound on total mesophilic bacterial counts in milk, immediately after ultrasonic treatment and after 3 days and 5 days of storage, using a frequency of 20 kHz under various conditions (treatment time, amplitude, and temperature).

MATERIAL AND METHODS

Milk samples. Raw cow's milk with 4% of milk fat was kept under refrigeration at 4°C until used. The pH values of milk were determined using a pH-meter(Knick, type 647-1, Knickelektronische Massgarate GmbH, Berlin, Germany), and titratable acidity (°SH) was determined by the Soxhlet-Henkel method. The initial microbial load of mesophilic bacteria was tested in all milk samples – raw milk (R), sonicated (US – 20°C and amplitude 120 μm), and thermosonicated (TS – 60°C and amplitude 120 μm). All analyses were performed immediately after sonication or thermosonication and after 3 days and 5 days of storage in a refrigerator at +4°C.

Microbiological analysis. Serial dilutions were made in peptone water (0.1%) with samples taken from raw milk, sonicated and thermo-sonicated milk to evaluate the microbial load. Afterwards, the serial dilutions of the samples were pour-plated for mesophiles in Plate Count Agar (30°C/72 h) (ISO

4833:2003). Microbiological analyses were performed after ultrasonic or thermoultrasonic treatment and after 3 days and 5 days of storage in a refrigerator at 4°C. All microbiological analyses were conducted at least in triplicate for each experiment.

Ultrasound treatments. Raw milk (200 ml) was placed in a double-walled vessel (200 ml), which served as the treatment chamber. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, USA), set at 600 W, 20 kHz, 12-260 μm with a 12 mm diameter probe, was introduced into the vessel. Ultrasonication was carried out with 60, 90, and 120 μm amplitude. The raw milk samples were treated by ultrasonic for 6, 9, and 12 minutes and then transferred to tubes with peptone water to perform microbiological analysis. In the case of thermosonication, before the ultrasonic treatment the samples were heated at 20, 40, and 60°C. Overheating of the samples was prevented by water cooling of the treatment chamber. Each experiment was conducted at least in triplicate. For this study, 26 samples were ultrasonically treated (Table 1).

Determination of acoustic power and efficacy of ultrasonic treatments in terms of eliminating microbes. The most widely accepted method for determining the power from an acoustic horn into an aqueous solution is the calorimetric technique described by Marguilis and Maltsev (2003). This method involves taking a known volume of water and applying ultrasound (for ca. 3 min) while monitoring the change in temperature with time for various ultrasonic amplitudes.

The ultrasonic power can be readily determined from the following equation:

$$P = \frac{dT}{dt} \times m \times C_p \quad (1)$$

$$AI = P/A \quad (2)$$

where:

P – ultrasonic power (W)

m – mass of the sample (kg)

C_p – specific heat capacity of milk (kJ/(kgK))

dT/dt – initial slope of the graph of temperature of the sample versus the time of exposure to ultrasound

AI – ultrasonic intensity (W/cm^2)

A – surface of probe (cm^2)

A common problem in the sonochemical literature is that the power delivered to the system (as quoted by the manufacturer) is mentioned, but the actual power dissipated (P) in the treated system is rarely reported. One of the most common methods of measuring P, introduced by Mason *et al.* (1996), is to use Eq. (1). This equation is based on the use of calorimetry and assumes that all of the power entering the system is dissipated as heat.

This simple equation has been widely used throughout the sonochemistry literature.

The efficacy of ultrasonic treatments in terms of eliminating microbes was measured by their decimal reduction time (D). D value was calculated as the time (min) required to reduce the number of viable cells by one log cycle or to kill 90% of population at the given temperature, time of ultrasonic treatment, and sonic wave amplitude. D values were calculated from the slope of the regression line plotted with the counts (CFU/ml) of the straight portion of the survival curve. In this study, the D value at 20 kHz was abbreviated as D_{US}:

$$\log \frac{N_1}{N_0} = -\frac{t}{D_{US}} \quad (3)$$

where:

N_0 – number of total mesophilic bacteria before ultrasound treatment

N_1 – number of total mesophilic bacteria after ultrasound treatment at time t

D_{US} – decimal reduction time (min)

Samples marked A2, A4, A5, A6, A7, A11, A19, A21, and A24 were chosen for the calculation of D values because in these samples were microbiological analyses done immediately after sonication or thermosonication. In other samples were microbiological analyses done after storage for 3 days or 5 days in a refrigerator at +4°C.

Experimental methodology. Multivariate methods provide advantages over more traditional univariate optimisation designs including the fact that a smaller number of experiments produces more information and allows to identify the interactions between variables. Response surface methodology includes four major steps, which are the experimental design, model fitting, model validation, and condition optimisation (Montgomery 2001). Experimental designs such as Central Composite Designs (CCD) are useful for RSM because they do not require an excessive number of experimental runs. Response surface methodology (RSM), a statistical method, uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations (Myers & Montgomery 2002). It is a collection of statistical techniques for

Table 1. Ultrasonic treatments (treatment time, amplitude, temperature) and duration of storage

Samples	Treatment time (min)	Amplitude (μm)	Temperature (°C)	Duration of storage (days)
R	–	–	–	1, 3, 5
A1	9	90	40	3
A2	12	120	60	1
A3	6	90	40	3
A4	6	120	60	1
A5	6	60	60	1
A6	12	120	20	1
A7	12	60	60	1
A8	12	60	60	5
A9	6	120	60	5
A10	9	90	40	3
A11	12	60	20	1
A12	12	90	40	3
A13	6	120	20	5
A14	12	120	20	5
A15	12	60	20	5
A16	9	60	40	3
A17	9	90	40	5
A18	9	90	60	3
A19	6	60	20	1
A20	6	60	20	5
A21	6	120	20	1
A22	9	90	20	3
A23	6	60	60	5
A24	9	90	40	1
A25	12	120	60	5
A26	9	120	40	3

designing experiments, building models, evaluating the effects of factors, and analysing optimum conditions of factors for desirable responses.

A general factorial design (STATGRAPHICS Centurion, StatPonit Technologies, Inc., Warrenton, USA) consists of 26 experimental trials which have been designed and chosen to obtain general observation of the ultrasound treatment of bacteria count. In order to determine the influence of each factor on the total count of mesophilic bacteria, central composite design (CCD) and face centred model were chosen. The ultrasound factors of amplitude (μm), temperature (°C), treatment time (min), and the factor the day of storage were studied. The analysis of variance (ANOVA) was carried out to determine any significant differences ($P < 0.05$) among the applied treatments.

The operating variables were considered at three levels, namely low (–1), central (0), and high (1). Accordingly, 26 experiments were conducted with experiments organised in a factorial design (including factorial points, axial points, and center point) and the remaining involving the replication of the central point to get a good estimate of experimental error. Repetition experiments were carried out after other experiments followed by the order of runs designed by the program. The response (output) values were total bacteria count in (log CFU/ml).

The designs were based on two-level full factorial design, which was augmented with centre and star points (Kuehl 2000). The total number of experiments of the designs (N) can be calculated as follows:

$$N = N_i + N_o + N_j \quad (4)$$

where:

$N_i = 2^n$ – number of experiments of the two level full factorial design

N_o – number of centre points

$N_j = 2 \times n$ – number of star points

CCD designs are also rotatable, which means that the responses can be predicted equally well in all equidistant directions from the centre point. These two desirable properties allow accurate calculation of all the model terms (including the quadratic terms) and therefore, more accurate estimation of the shape of the response surface under investigation.

Response surface methodology. The experimental results were analysed by the response surface methodology (RSM) using the software STATGRAPHICS Centurion (StatPonit Technologies, Inc., Warrenton, USA). The calculations were done at 95% confidence level. The ultrasound factors of amplitude – X_1 (μm), temperature – X_2 (°C), treatment time – X_3 (min), and factor the day of storage – X_4 were studied using RSM. In order to optimise the ultrasound treatment and investigate the effects of the above independent variables on the total mesophilic bacteria count, a central-composite rotary design with the variables at three levels was used in the experiments (Table 1). The design matrix for the experiment and the regression model proposed for the response are given below (Khuri & Cornell 1996):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i X_i^2 + \sum_{i<j}^{4} \beta_j X_i X_j \quad (5)$$

where:

β_0 – value of the fixed response at the central point of the experiment which is the point (0, 0, 0)

β_i, β_{ii}, β_{ij} – linear, quadratic, and cross-products coefficients

While demonstrating the significant effects, 3-dimensional fitted surfaces were drawn (Lu *et al.* 2008). The model was fitted by multiple linear regression (MLR). The validity of the quadratic empirical model was tested using the analysis of variance (ANOVA). The confidence level used was 95%.

RESULTS AND DISCUSSION

The heterogeneous nature of food with the inclusion of particulate and other interfering substances severely curtails the singular use of ultrasound as a preservation method. Although these limitations make the current probability of commercial development low, the combination of ultrasound with other preservation processes (mild heat or pressure) appears to have the greatest potential for industrial applications (USDA 2000).

In this work, high-intensity ultrasound was used to investigate the inactivation of microorganisms in raw milk with 4% milk fat. The total mesophilic bacteria counts in milk after ultrasonic treatment were analysed by response surface methodology (RSM) using the software STATGRAPHICS Centurion (Table 1).

The predicted model can be described by the polynom given below:

$$\text{TBC} = 6.47663 - 0.424812 \times TT + 0.0167669 \times \text{A} - 0.0759344 \times T + 0.979472 \times \text{DS} + 0.0273333 \times TT^2 - 0.001775 \times TT \times \text{A} + 0.000802083 \times TT \times T - 0.0626042 \times \text{TT} \times \text{DS} + 0.0000656 \times \text{A}^2 - 0.00013375 \times \text{A} \times \text{T} + 0.0011875 \times \text{A} \times \text{DS} + 0.0008775 \times \text{T}^2 - 0.00498438 \times \text{T} \times \text{DS} + 0.004 \times \text{DS}^2$$

where:

TBC – total bacteria count

TT – treatment time

T – temperature

A – amplitude

DS – days of storage

All analyses were performed immediately after ultrasonic or termoultrasonic treatments and after 3 days and 5 days of storage in refrigeration at 4°C. The initial mesophilic bacterial counts before milk processing were 6.34 log CFU/ml. According to the national sanitary standards, the acceptable amount of total mesophilic bacteria count in pasteurised milk is less than 4.699 log CFU/ml (5×10^4 CFU/ml) for pasteurised milk in bottles and packages (Commission Directive 89/362EEC; Council Directive 92/46/EEC; NN 20/2001; Hillerton & Berry 2004).

After milk treatment by ultrasound at 20°C, the plate count was reduced to 5.69 (sample A19) and 4.74 log CFU/ml (sample A21), respectively (Table 2). The significant difference in the reduction of the plate count of aerobic mesophilic bacteria (AMB-a) is a consequence of different amplitudes of the applied ultrasound. This research demonstrates that ultrasound treatment at ambient temperature (20°C) for 6 min is not sufficient for reaching the criteria for the count of aerobic mesophilic bacteria determined by the Regulation for

Table 2. Acidity and total bacteria count of raw milk (R) and milk after ultrasound treatment

Samples	pH	°SH	Mesophilic bacteria (log CFU/ml)
R	6.67	6.09	6.34
A1	6.77	6.0	4.58
A2	6.76	5.8	3.99
A3	6.39	8.9	5.21
A4	6.76	6.0	4.30
A5	6.76	6.0	4.40
A6	6.52	6.8	4.12
A7	6.77	5.8	4.04
A8	6.75	6.2	4.08
A9	6.76	6.2	5.72
A10	6.77	6.0	4.58
A11	6.53	7.0	4.38
A12	6.76	6.1	4.25
A13	6.19	10.3	7.87
A14	6.75	6.2	5.26
A15	6.75	6.2	5.78
A16	6.77	6.0	4.47
A17	6.76	6.1	4.72
A18	6.77	6.0	4.30
A19	6.56	7.0	5.69
A20	6.22	10.1	7.23
A21	6.66	7.0	4.74
A22	6.76	6.4	5.37
A23	6.76	6.2	6.93
A24	6.52	6.8	4.28
A25	6.75	6.2	4.12
A26	6.52	6.8	4.56

quality of milk and milk products (NN 20/2001). However, in samples A4 and A5 (Table 2) the plate counts of aerobic mesophilic bacteria were 4.3 log CFU/ml and 4.4 log CFU/ml, respectively, which is bellow maximal acceptable limit determined by the Regulation NN 20/2001**??**. The mentioned samples were treated at 60°C/6 minutes. It was also established that after a longer ultrasound treatment (12 min) at ambient temperature (20°C) the reduction of aerobic mesophilic bacteria was significant. Sample A6 was treated at ultrasound amplitude 120 μm and plate count was 4.12 log CFU/ml while in sample A11 treated at amplitude 60 μm the count of aerobic mesophilic bacteria was 4.38 log CFU/ml (Table 2). Higher temperature of the milk treatment increases the reduction of aerobic mesophilic bacteria. In sample A24 (treatment time 9 min, amplitude 90 μm at 40°C) a higher reduction of aerobic mesophilic bacteria (4.28 log CFU/ml) was demonstrated. Maximal inactivation of bacteria in milk was obtained after ultrasound treatment at amplitude 120 μm for 12 min at 60°C (sample A2) (Table 2). After the milk treatment with ultrasound, the differences in pH and titratable acidity of milk were not notable among all milk samples, while the increase in titratable acidity after 5 days of storage was higher in the samples treated with ultrasound at 20°C (A13 and A20) (Table 2).

Some authors have suggested that the efficacy of ultrasonic treatment in killing bacteria by cavitational effects could be minimised with an increase in temperature. This fact could be probably the result of an increased thermal effect that would masque the effect of sonication, and/or a decrease in the violence of implosion due to the increased vapour pressure at higher temperatures. More bubbles are formed but these are smaller and the violence of implosion decreases (Allinger 1975; Sala *et al.* 1995; Guerrero *et al.* 2001). This behaviour is not in agreement with our results. Although the cavitation effect could be minimised by the increase of temperature, in the case of milk the concentration of solids in suspension could play an important role and improve the cavitation intensity (Sala *et al.* 1995; Villamiel & De Jong 2000). Garcia *et al.* (1989) observed at high temperatures that the advantages of thermoultrasonication for killing bacteria were maintained in milk since the z-values of thermo-ultrasonication and thermal destruction were very similar. Although the mechanism is not clear, they attributed these results to the concentration of solids present in milk. Ciccolini *et al.* (1997) studied the survival of *S. cerevisiae* suspended in water at 45, 50, and 55°C at different ultrasonic powers, and found that the application of ultrasonic waves at non-lethal temperature (45°C) did not display a deactivation action while synergy between ultrasound and heat was confirmed at the higher temperatures.

The efficacy of the cavitations phenomena and micro streaming for bacteria inactivation in milk is possible to monitor as ultrasound intensity by Marguillis and Maltsev (2003) equation where the intensity of the ultrasound applied is represented as the power of the probe per square unit

Table 3. Resume of the ultrasound treatment (intensity and energy) applied on the nine samples and decimal reduction time (*D*) for this samples

Samples	Ultrasonic intensity (W/cm^2)	Energy (J)	Decimal reduction time (min)	Reduction (log CFU/ml)
A2	54.59	60.125	3.941	3.35
A4	38.97	19.145	5.106	3.04
A5	26.06	11.436	5.217	2.93
A6	32.26	34.423	5.405	3.30
A7	57.49	64.348	4.092	3.22
A11	33.98	38.548	5.122	2.96
A19	24.99	12.763	9.231	1.65
A21	37.84	21.521	8.550	2.59
A24	49.85	36.836	4.369	3.06

(W/cm^2) or as emitted energy in J (Table 3). As can be seen in Table 3, it is obvious that the time for decimal reduction (*D*) and also log of reduction for specific amplitude (60, 90, and 120 μm) of ultrasound are in proportion to the accepted energy, respectively applied intensity. At the lowest temperature (20°C), the *D* values were between 5.122 and 9.231 depending on the applied wave amplitude (60 μm or 120μm). When the temperature of treatment was increased to 40°C, *D* values for the sonification treatment decreased by as much as approximately 20% as compared with the corresponding values at 20°C, depending on the wave amplitude. In samples A2 and A7, the time of decimal reduction was the smallest ($D_{120\mu m}$) 3.941 min, respectively 4.092 min where the intensity of the applied ultrasound was maximal, i.e. 54.59 W/cm^2, respectively 57.49 W/cm^2 but the delivered energy was 60.125 J, respectively 64.348 J (Table 3). Scarce information is found in the literature on the influence of the wave amplitude on microorganism inactivation. It has been reported that the intensity of the ultrasound effect is directly related to the amplitude: when ultrasound amplitude increases, the zone undergo-

Table 4. Analysis of Variance for total bacteria count

Source	*F*-Ratio	*P*-Value
A – treatment time	82.66	0.0000
B – amplitude	9.14	0.0116
C – temperature	41.29	0.0000
D – days of storage	81.28	0.0000
AA	1.61	0.2308
AB	2.94	0.1142
AC	0.38	0.5477
AD	23.44	0.0005
BB	0.04	0.8364
BC	0.74	0.4071
BD	0.59	0.4602
CC	3.28	0.0977
CD	6.60	0.0261
DD	0.01	0.9357

R-squared = 96.0665%; R-squared (adjusted for d.f.) = 91.0601%; Standard Error of Est. = 0.310323; mean absolute error = 0.161532

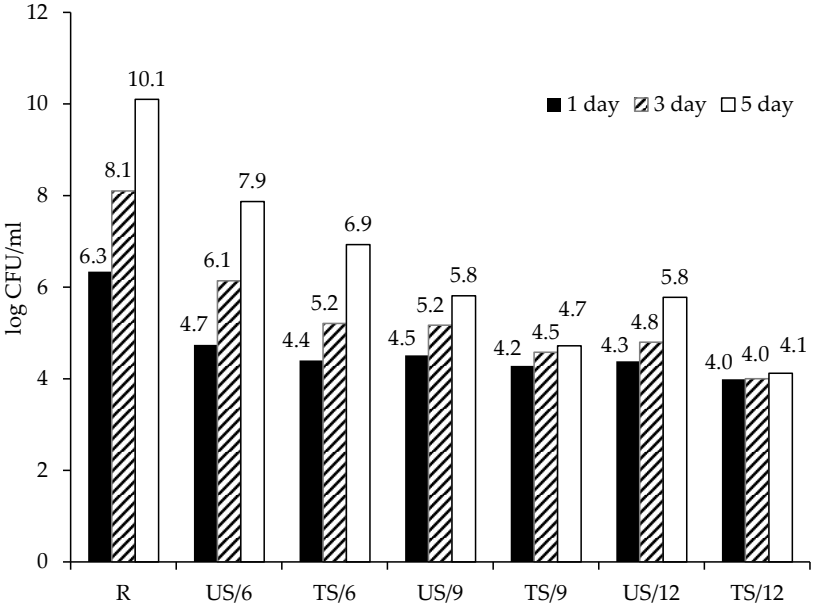

Figure 1. Influence of sonification and termosonification (60°C) at amplitude 120 μm on total count mesophilic bacteria inactictivation in milk during 5 days of storage

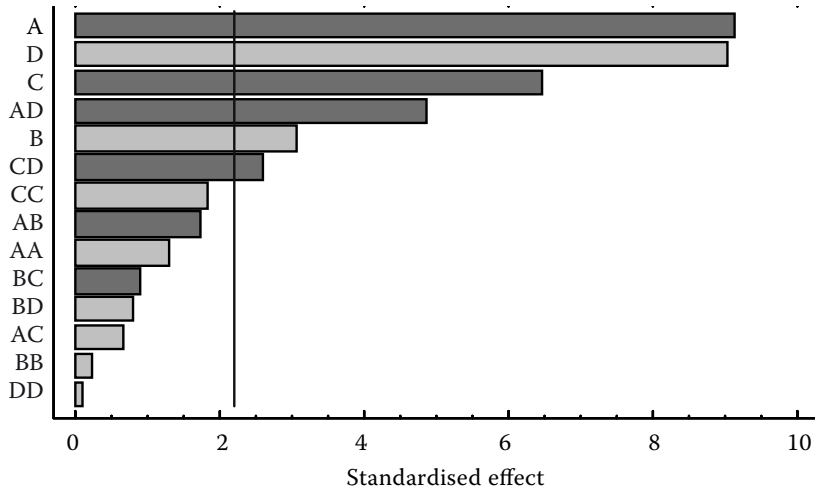

Figure 2. Standardized Pareto Chart for total bacteria count after ultrasound treatment and after 5 days of storage

+; –; A – temperature time; D – days of storage; C – temeprature; B – amplitude

ing cavitation increases, leading to more extensive inactivation (Guerrero *et al.* 2001; Patist & Bates 2008; Noci *et al.* 2009).

The shelf life of milk processed by ultrasound or thermoultrasound tratments was evaluated and the results are shown in Figure 1. During the storage period, the increase of bacterial counts was higher in milk samples treated with ultrasonund at ambient temperature (20°C) (samples marked as US) as compared with the corresponding values at the higher temperature 60°C (samples marked as TS), as shown in Figure 1. As presented in Figure 1, it is clear that milk treated at 20°C (samples US/6 min, US/9 min and US/12 min) already contained after 3 days of storage at the temperature 4°C a higher count of aerobic mesophilic bacteria, which was over maximal level accepted by the national sanitary standards for milk (4.699 log CFU/ml) (NN 20/2001). Sample TS/6 treated at 60°C met the criteria of the national sanitary standards for milk (NN 20/2001), however, after 3 days of storage the count of bacteria increased to 5.21 log CFU/ml. Milk treated with combined temperature and ultrasound for 9 min (TS/9) was not suitable for consumption after 5 days. However, ultrasound application for 12 min at 60°C puts 5 days shelf life of milk in the frame of sanitary standards.

The factors that seem to affect substantially the inactivation of microorganisms in using ultrasound are the amplitude of the ultrasonic waves, exposure/contact time with the microorganisms, and temperature of the treatment. The estimated effects of each variable and analysis of variance for the model are presented as Pareto chart in Figure 2. According to the ANOVA table, the fitted model was significant at the considered confidence level since the *F*-value was more than three times higher than that of the *F*-value listed

Table 5. Optimised values of specific ultrasound parameters defined by Statgraphics where lowest bacteria count was found

Factor	Low	High	Optimum	Optimum (lowest) value of total bacteria count (log CFU/ml)
Treatment time (min)	6.0	12.0	9.84942	
Amplitude (μm)	60	120	120.00086	3.688
Temperature (°C)	20.0	60.0	45.3458	

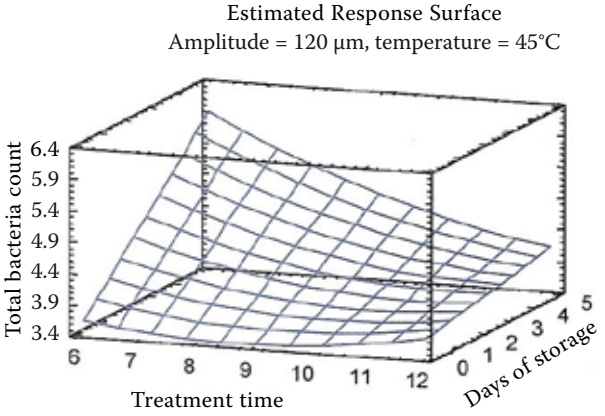

Figure 3. Surface plot for total bacteria count at optimise values of amplitude (120 μm) and temperature (45°C)

(Table 4). In order to determine whether or not an effect is significant, we can just observe the values of the column *P*-value in Table 4. Indeed, a *P*-value below 0.05 indicates that the considered factor is significant for the count of mesophilic bacteria in milk. As one can see from the chart, the lines below the vertical blue line are statistically significant factors influencing total bacteria count, including linear (A, B, C, D) and quadratic factors (AD, CD).

The surface plot for total bacteria count is given in Figure 3. At fixed (optimised) values of amplitude and temperature, the surface plot as function of the treatment time and days of storage is given. The plot shows that total bacteria count is lowest as optimised for the treatment time of 9.84 min (Table 5). For the shorter treatment time and more storage days, total bacteria count is the highest. This is very logical because for shorter treatment there is not enough cavitation phenomena and micro streaming that can break cell walls of the bacteria and kill them in that way. The output optimal value of total bacteria count has been defined by Statgraphics where the lowest bacteria count (3.688 log CFU/ml) appears with the following specific ultrasound parameters: amplitude of 120 μm, treatment time for 9.84 min, and temperature of 45.34°C.

CONCLUSION

The aim of the simultaneous use of ultrasound and temperature on bacterial inactivation was to reduce the temperature and/or the process time of the sterilisation processes. The results of this investigation of the combined effects of ultrasound and heat treatment versus temperature or ultrasound treatment alone on the inactivation of mesophilic bacteria in milk also clearly indicate improved inactivation of total count of mesophilic bacteria by this procedure. The factors that seem to affect substantially the inactivation of mesophilic bacteria in milk using ultrasound are the amplitude of the ultrasonic waves, the exposure/contact time with the microorganisms, and the temperature of the treatment. The results achieved indicate significant inactivation of microorganisms at longer periods of the treatment with ultrasonic probe, particularly in combination with higher temperature and amplitude. The output optimal value of total bacteria count has been defined by Statgraphics with the lowest bacteria count (3.688 log CFU/ml) having been obtained with the following specific ultrasound parameters: amplitude of 120 μm, treatment time for 9.84 min, and temperature of 45.34°C.

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