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Effect of High Intensity Ultrasound Treatment on the Growth of Food Spoilage Bacteria

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Summary

The aim of this study is to determine the effect of high intensity ultrasound (amplitude, temperature and treatment time) on the inactivation of food spoilage bacteria *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30. The model suspensions of bacteria were treated with 12.7-mm ultrasonic probe operated at 600 W nominal power (ultrasonic treatment implemented at 20 kHz) and at amplitudes of 60, 90 and 120 μm. Also, treatment time of 3, 6 and 9 min and temperature of 20, 40 and 60 °C were used. The results were statistically processed with STATGRAPHICS Centurion computer program and response surface methodology. All three parameters studied seem to substantially affect the inactivation of bacteria in pure culture. The results also indicate increased inactivation of microorganisms under longer period of treatments, particularly in combination with higher temperature and/or amplitude. After ultrasonic treatment at 60 °C, 9 min and 120 μm, the viability of cells was not confirmed for *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064 and *Listeria monocytogenes* ATCC 23074. Under the mentioned conditions the highest inactivation (3.48 log CFU/mL) of *Bacillus cereus* 30 was obtained.

Key words: high intensity ultrasound, bacteria, inactivation, experimental design, optimization

Introduction

The investigation of ultrasonication as a potential microbial inactivation method began in the 1960s, after it was discovered that sound waves used in anti-submarine warfare killed fish (*1,2*). During the sonication process, longitudinal waves are created when a sonic wave meets a liquid medium, thereby creating regions of alternating compression and expansion. These regions of changes in pressure cause the occurrence of cavitations, 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, reaching up to 5500 °C, and peaks of pressure of 50 000 kPa. It is estimated that these temperatures and pressures in the bubbles have a life span lower than 1 μs, and the liquid heating and cooling speed is in the range of 10^9 °C/s. The effects of high intensity ultrasound are dependent on the number and intensity of bubble implosions per unit of time, the characteristics of the treatment and the characteristics of the treatment media (*3*).

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When microorganisms are present in the bulk liquid, microbial killing is believed to occur due to the thinning of cell membranes, localized heating and the production of free radicals (*4–12*). As an example, microbial inactivation using ultrasound has been investigated for application to a range of liquid foodstuffs. Levels of *E. coli* O157:H7 were reduced by 5 log CFU/mL with ultrasound and mild heating in apple cider (*13*) and the inactivation of *E. coli* K12 was enhanced using ultrasound at ambient temperatures (*14*). Dehghani (*15*) investigated the effectiveness of sonication as a disinfection method and found a strong influence of ultrasound on the disruption of *E. coli* in water. In milk, D'Amico *et al.* (*13*) found that ultrasound under mild heating reduces the levels of *Listeria monocytogenes* by 5 log CFU/mL. Knorr *et al.* (*16*) and Chemat *et al.* (*17*) evaluated the effects of continuous flow ultrasound combined with temperature treatment on bacterial decontamination (*E. coli* and *Lactobacillus acidophilus*) of model suspensions and various liquid food systems including milk, fruit juices and edible oil. The results were compared with conventional heating where it was shown that ultrasound-assisted thermal processing of liquid foods can be achieved at lower temperatures and result in further quality advantages (*17,18*).

Mathematical modelling is important in reducing energy consumption, and number of experiments, as well as for the analysis of interaction between the investigated parameters that cannot be considered using simple statistical analysis. Response surface methodology (RSM) may be employed to optimise critical processing parameters by estimating interactive and quadratic effects. A further benefit of using RSM is the reduction in the number of experiments needed as compared to a full experimental design (*19,20*). RSM has been successfully employed to optimise food processing operations by several investigators (*21–24*). The need to optimize processes demands more research to streamline processes by combining technologies, particularly with respect to the optimization of practical applications.

The purpose of this study is to determine the effect of high intensity ultrasound (amplitude, temperature and treatment time) on *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30. The effect of various parameters (treatment time, amplitude and temperature) on the inactivation of the bacteria was studied experimentally and according to the statistical experimental design.

Materials and Methods

Bacterial strains

Bacterial strains used in this study: *Escherichia coli* 3014, *Salmonella* sp. 3064, *Staphylococcus aureus* 3048, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30 were obtained from the Collection of Microorganisms of the Laboratory of General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb (Zagreb, Croatia). The cultures were stored on nutrient agar (Biolife, Milan, Italy) at 4 °C.

Preparation of inocula

To prepare the inocula, the investigated bacteria were incubated on nutrient agar (Biolife) for 24/48 h at 37 °C, and then a loopful of young cells were suspended in sterile saline solution with 1 mL of glycerol.

The total viable cell count (TVC) was performed by standard dilution method on nutrient agar after incubation at 37 °C for 48 h. The bacterial colonies were counted and reported as log colony forming units per mL (log CFU/mL). The obtained cell counts (in log CFU/mL) were: 8.95 for *E. coli* 3014, 7.78 for *Salmonella* sp. 3064, 8.96 for *S. aureus* 3048, 9.28 for *L. monocytogenes* ATCC 23074 and 8.38 for *Bacillus cereus* 30.

Saline solution as basal medium (100 mL) was dispensed into 200-mL flasks. Each flask was inoculated with 0.1 mL of the initial bacterial suspension and the final counts (in log CFU/mL) were: 5.76 for *E. coli* 3014, 4.70 for *Salmonella* spp. 3064, 5.30 for *S. aureus* 3048, 5.96 for *L. monocytogenes* ATCC 23074 and 5.48 for *Bacillus cereus* 30 (Table 1, Sample A0). All samples were analyzed in triplicate and the given score is the mean value of three determinations. Control flasks and duplicate test flasks were used in the treatment with high intensity ultrasound probe of 12.7 mm.

Table 1. Amplitude (X_1), treatment time (X_2), temperature (X_3) and acoustic intensity (AI) during ultrasound treatments

Sample	X_1 / μm	X_2 / min	X_3 / °C	AI / W/cm^2
A0	–	–	–	–
A1	60	3	20	42.97–45.12
A2	120	3	20	40.71–42.63
A3	90	6	20	54.48–56.63
A4	60	9	20	61.59–65.12
A5	120	9	20	66.01–70.78
A6	90	3	40	38.54–44.86
A7	60	6	40	42.47–43.36
A8	90	6	40	49.63–55.74
A9	90	6	40	49.63–55.74
A10	120	6	40	43.36–46.01
A11	90	9	40	64.86–66.63
A12	60	3	60	38.05–39.82
A13	120	3	60	39.76–44.24
A14	90	6	60	48.26–53.09
A15	60	9	60	58.93–59.64
A16	120	9	60	60.17–68.43

Ultrasound treatments

Samples of bacterial suspension (100 mL) were placed in a sterile glass, which was used as the treatment vessel. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, CT, USA), set at 600 W, 20 kHz and 12–260 μm was used, and a 12.7-mm diameter probe was immersed in the sample (about 2 cm) and placed in the 'centre' of the sample. Ultrasonication was carried

out at amplitudes of 60, 90 and 120 μm. Samples were treated with ultrasounds for 3, 6 and 9 min at 20 °C. In the case of thermosonication before ultrasonic treatment, the samples were heated at 40 °C (8 s) and 60 °C (12 s) in a small tube heat exchanger. Overheating of the samples during ultrasound treatment was prevented by ice-water cooling of the treatment chamber during sonication. The final temperature of microorganism suspensions after sonication at 40 or 60 °C was ±1 °C. For this study, 16 samples of basal medium with a determined initial number of bacterial cells were ultrasonically treated (Table 1).

Determination of acoustic power and efficacy of ultrasonic treatments in terms of the elimination of microbes

The most widely accepted method for determining the power of an acoustic horn in an aqueous solution is the calorimetric technique described by Margulis and Margulis (*25*). This method involves taking a known volume of water and applying ultrasound (for approx. 3 min) while monitoring the change in the temperature with time at various ultrasonic amplitudes. The ultrasonic power *P* and the ultrasonic intensity AI can be readily determined from the following equations:

$$P = m \cdot C_p \cdot \frac{\Delta T}{\Delta t} \qquad /1/$$

$$\mathrm{AI} = P/A \qquad /2/$$

where *P* is the ultrasonic power (W), *m* is the mass of the sample (kg), C_p is the specific heat capacity of a sample (kJ/(kg·°C)), $\frac{\Delta T}{\Delta t}$ is temperature (°C) change in time (s), AI is the ultrasonic intensity (W/cm^2), and *A* is the surface area of the 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 Butz and Tauscher, 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 efficiency of ultrasonic treatment in terms of microbial reduction was measured by the decimal reduction time (*D*). *D* value is defined as the time (min) required to reduce the number of viable cells by one log cycle or the time required to kill 90 % of population at a given temperature and sonic wave amplitude. *D* values were calculated from the slope of the regression line obtained from the straight portion of the survival curve of the counts (CFU/mL). Only survival curves with a correlation coefficient (R_o) of ≥0.95 and with more than four values in the straight portion were used.

Based on the definition of decimal reduction time, the following equations of the first order reaction kinetics describe the survival curve during thermal processing (Eq. 3), ultrasound treatment (Eq. 4) and thermal processing and ultrasound treatment (Eq. 5):

$$\log \frac{N_t^T}{N_0} = -\frac{t}{D_T} \qquad /3/$$

$$\log \frac{N_t^S}{N_0} = -\frac{t}{D_S} \qquad /4/$$

$$\log \frac{N_t^{TS}}{N_0} = -\frac{t}{D_{TS}} \qquad /5/$$

Derivations of a mathematical model to determine the decimal reduction time during thermal processing and ultrasound treatment assume that ultrasound and temperature acted independently and that destruction of bacteria by heat and ultrasound were single reactions by first-order kinetics. In this way the logarithmic order of death of microorganisms can be expressed by the following equations (the developed model is based on the models of Págan *et al.* (*11*) and Raso *et al.* (*12*)):

$$\frac{N_t^{TS}}{N_0} = \frac{N_t^S}{N_0} \cdot \frac{N_t^T}{N_0} \qquad /6/$$

$$\log \frac{N_t^{TS}}{N_0} = \log\left(\frac{N_t^S}{N_0} \cdot \frac{N_t^T}{N_0}\right) \qquad /7/$$

$$\log \frac{N_t^{TS}}{N_0} = \log \frac{N_t^S}{N_0} + \log \frac{N_t^T}{N_0} \qquad /8/$$

$$-\frac{t}{D_{TS}} = -\frac{t}{D_S} - \frac{t}{D_T} \qquad /9/$$

$$\frac{1}{D_{TS}} = \frac{1}{D_S} + \frac{1}{D_T} \qquad /10/$$

$$\frac{1}{D_{TS}} = \frac{D_T + D_S}{D_S \cdot D_T} \qquad /11/$$

$$\frac{1}{D_{TS}} = \frac{D_T + D_S}{D_S \cdot D_T} \qquad /12/$$

where N_0 is the number of microorganisms before treatment, N_t^T is the number of microorganisms after time *t* and thermal processing, N_t^S is the number of microorganisms after time *t* and ultrasound treatment, N_t^{TS} is the number of microorganisms after time *t* and thermal processing and ultrasound treatment, D_T is the decimal reduction time during thermal processing (s), D_S is the decimal reduction time during ultrasound treatment (s), and D_{TS} is the decimal reduction time during thermal processing and ultrasound treatment (s).

Experimental methodology

In order to determine the influence of the operational parameters on the count of food spoilage bacteria (*Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30), central composite design (CCD; STATGRAPHICS Centurion, StatPoint Technologies, Inc, Richmond, VA, USA) and face-centred model were used (*24*). Because CCD requires the choice of operational parameters, the authors have chosen to study the effects of amplitude (μm), temperature (°C), and treatment time (min). 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, 16 experiments were conducted organized in a factorial design (including factorial points, axial points and centre point), and the remaining experiment involving the replication of the central point to get a good estimate of experimental error. Response (output) values were total count of food spoilage bacteria expressed as log CFU/mL.

The designs were based on a two-level full factorial design and augmented with centre and star points (*26–28*). The total number of experiments of the designs (N) was calculated as follows:

$$N=N_i+N_o+N_j \qquad /13/$$

where $N_i=2^n$ is the number of experiments of the two-level full factorial design, N_o is the number of centre points, and $N_j=2\cdot n$ is the number of star points.

Response surface methodology

The experimental results were analyzed by response surface methodology (RSM) using the STATGRAPHICS Centurion software. Specifically, the RSM was used to study the effect of three different ultrasonic parameters; amplitude – X_1 (μm), treatment time – X_2 (min) and temperature – X_3 (°C). In order to optimize the ultrasonic treatment and to investigate the effects of the three independent variables on the count of of food spoilage bacteria, a central composite rotatable design with the variables at three levels was used in the experiments (Table 1). Design matrix for the experiment as well as the regression model proposed for the response are given by the following equation (*17,28*):

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i<j}^{4}\beta_{ij} X_i X_j \qquad /14/$$

where β_0 is the value of the fixed response at the central point of the experiment (point 0, 0, 0), and β_i, β_{ii} and β_{ij} are the linear, quadratic and cross-product coefficients, respectively (*27*). The model was fitted by multiple linear regressions (MLR). The validity of the quadratic empirical model was tested with the analysis of variance (ANOVA) with the confidence level of 95 %. Durbin-Watson statistical analysis was also conducted, which enabled autocorrelation and prediction errors after the statistical regression analysis was completed. The results of Durbin-Watson statistics are always between 0 and 4. A value of 2 means that there is no autocorrelation in the sample. Values approaching 0 indicate positive autocorrelation and values towards 4 indicate negative autocorrelation.

Results and Discussion

Previous research shows that the use of ultrasound alone has no significant effect on the inactivation of food spoilage bacteria. However, the combination of high temperature and ultrasound exhibited a significant effect on the inactivation of food spoilage bacteria. The effect of ultrasound at temperatures up to 50 °C was not effective for the inactivation of bacteria, while temperatures above 50 °C recorded a significant effect on the inactivation of microorganisms (*6,10,16,22–24*). These findings are not consistent with the results obtained during this study since a significant inactivation of food spoilage bacteria occurred when processing with high-power ultrasound at a temperature of 40 °C (Table 2 and Fig. 1).

When compared with Gram-positive bacteria, Gram-negative bacteria in sample A11 (40 °C, 9 min, 90 μm) showed higher susceptibility to the combination of high temperature and ultrasound. The highest inactivation was observed against *Salmonella* sp. 3064 with reduction of 3.40 log CFU/mL, followed by *Escherichia coli* 3014 (2.58 log CFU/mL), *Listeria monocytogenes* ATCC 23074 (2.48 log CFU/mL), *Staphylococcus aureus* 3048 (2.00 log CFU/mL), and *Bacillus cereus* 30 (1.97 log CFU/mL) (Tables 1 and 2). Under the same processing conditions (A6), but shorter holding time (3 min), the smallest reduction in the number of bacteria was observed. This reduction ranged from 1.28 log CFU/mL for *Salmonella* sp. 3064 to 0.78 log CFU/mL for *Bacillus cereus* 30 (Table 2 and Fig. 1).

Also, some authors have suggested that the efficiency of the ultrasonic treatment for killing bacteria by cavitational effects could be reduced with an increase in temperature (*10–12*). This trend could be the result of an increased thermal effect that either hinders the effect of sonication or decreases the violence of the bubble implosion due to the increased vapour pressure at higher temperatures (*9,29*). However, the predominant effect on the inactivation of microorganisms at 60 °C is that of temperature, while the effect of ultrasound is reduced. The synergy between heat and ultrasound disappears at 60 °C, turning into a cumulative effect. The data presented in Table 2 clearly show that in sample A16 (ultrasound treatment at 120 μm, 9 min, 60 °C), *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064 and *Listeria monocytogenes* ATCC 23074 were not determined, while *Bacillus cereus* 30 achieved the largest reduction of 3 log units. In sample A15 (ultrasound treatment at 60 μm, 9 min, 60 °C), maximal reduction of *Escherichia coli* 3014, *Staphylococcus aureus* 3048 and *Salmonella* sp. 3064 was also achieved, while the reduction of bacteria *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30 was between 3.96 and 3.22 log CFU/mL, respectively. Also, a maximal reduction of *Salmonella* sp. 3064 was obtained when the bacterial suspensions were treated with ultrasound at amplitude of 90 μm for a period of 6 min and a temperature of 60 °C (A14) (Table 2 and Fig. 1).

These differences, among others (varying morphological and biochemical properties), are due to the sensitivity of cell membranes to mechanical damage caused by ultrasound, also Gram-negative bacteria contain very low levels of peptidoglycan so are more susceptible to mechanical damage than Gram-positive ones.

According to the equation given by Margulis and Margulis (*25*), the value of ultrasonic intensity AI (defined as the power of the probe per unit of the probe area, W/cm^2) can be used to determine the effect of the cavitation and microstreaming on the inactivation of food spoilage bacteria. Thus, by the comparison of the acoustic intensity (AI) values in Table 1 with the results given in Table 2 and Fig. 1, direct correlation between the extent of deactivation and the ultrasonic intensity

Table 2. Influence of ultrasound treatment on the count of Gram-negative (*Salmonella* sp. 3064 and *Escherichia coli* 3014) and Gram-positive bacteria (*Staphylococus aureus* 3048, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30) after ultrasound treatments

Sample	*E. coli* 3014	*Salmonella* sp. 3064	*S. aureus* 3048	*L. monocytogenes* ATCC 23074	*B. cereus* 30
	N/(log CFU/mL)				
A0	5.76±0.05	4.70±0.04	5.30±0.06	5.76±0.07	5.48±0.06
A1	4.93±0.06	3.78±0.05	5.11±0.03	5.48±0.02	5.00±0.05
A2	4.68±0.04	3.60±0.04	5.04±0.05	5.20±0.03	4.95±0.02
A3	4.53±0.06	3.32±0.02	5.00±0.03	5.18±0.05	4.78±0.03
A4	4.46±0.07	2.60±0.05	4.78±0.01	5.11±0.04	4.58±0.06
A5	3.41±0.02	2.48±0.02	4.75±0.03	4.75±0.05	4.51±0.01
A6	4.51±0.03	3.32±0.02	4.93±0.03	5.15±0.04	4.70±0.02
A7	4.59±0.05	1.90±0.04	3.70±0.05	4.34±0.05	4.26±0.05
A8	3.58±0.03	1.78±0.05	3.60±0.04	4.30±0.02	4.18±0.04
A9	3.58±0.03	1.78±0.03	3.60±0.05	4.30±0.05	4.18±0.01
A10	3.41±0.02	1.60±0.05	3.48±0.02	4.26±0.04	3.72±0.02
A11	3.18±0.01	1.30±0.01	3.30±0.01	3.48±0.05	3.51±0.03
A12	3.04±0.02	2.30±0.03	3.81±0.03	4.59±0.05	4.34±0.05
A13	4.00±0.02	2.00±0.01	3.78±0.02	4.41±0.03	4.32±0.03
A14	2.00±0.01	N/A	2.30±0.01	2.65±0.01	3.26±0.04
A15	N/A	N/A	N/A	2.00±0.01	2.26±0.02
A16	N/A	N/A	N/A	N/A	2.00±0.01

N/A=viability of cells not confirmed after ultrasonic treatment
All values are expressed as mean of three repetitions±standard deviation

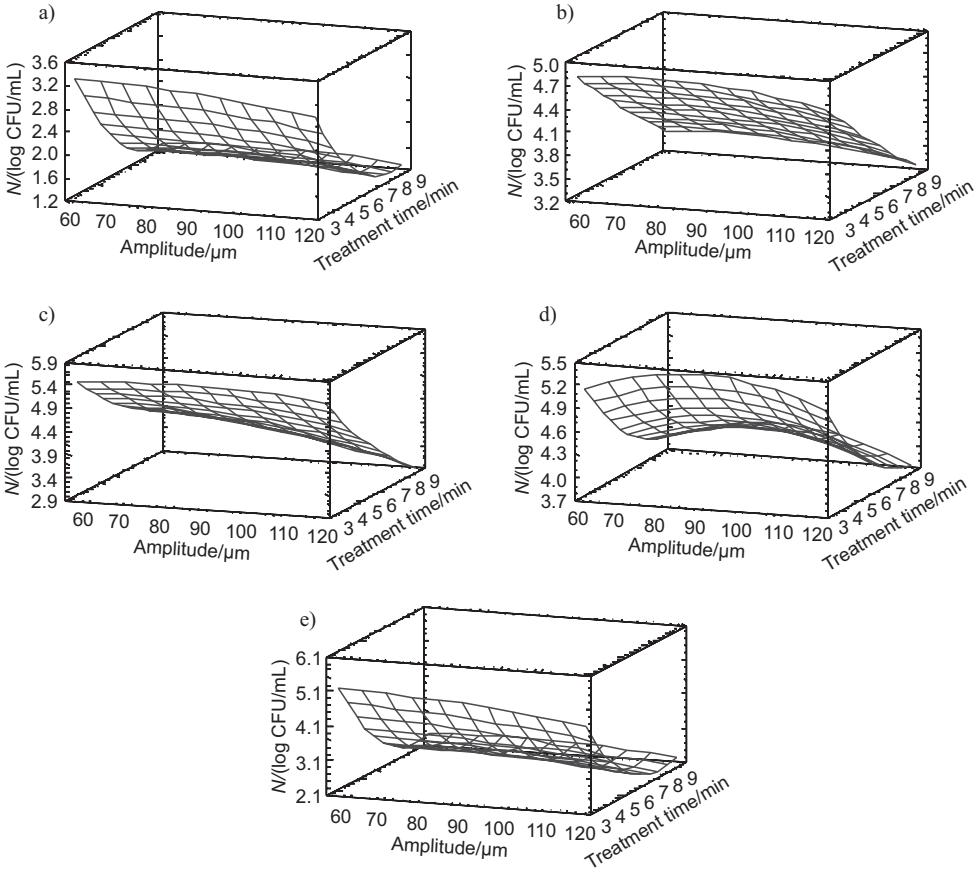

Fig. 1. Surface plots for: a) *Salmonella* sp. 3064, b) *Bacillus cereus* 30, c) *Escherichia coli* 3014, d) *Listeria monocytogenes* ATCC 23074, and e) *Staphylococcus aureus* 3048 (log CFU/mL) count at the temperature of 40 °C

can be seen. Similarly, the comparison of the AI values with the *D* values given in Table 3 demonstrates that the decimal reduction time (D_{TS}) at the specific amplitude (60, 90 and 120 μm) of ultrasound is proportional to the applied intensity. Also, it was observed that D_{TS} values of all investigated bacteria decrease with the increase in ultrasound amplitude between 60 and 120 μm (Table 3). Very little information is found in the literature on the influence of the wave amplitude on microorganism inactivation. Nevertheless, it has been reported that the intensity of ultrasound is directly related to the amplitude: when ultrasound amplitude increases, the zone undergoing cavitation increases, leading to more inactivation (*8*,*24*,*30*,*31*).

Table 3. Decimal reduction time (*D*) values after ultrasound treatments at amplitudes of 60, 90 and 120 μm for *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30

Microorganism	Temperature	$D_{60\,\mu m}$	$D_{90\,\mu m}$	$D_{120\,\mu m}$
	°C		s	
Escherichia coli 3014	20	433	431	414
	40	217	199	214
	60	131	129	116
Staphylococus aureus 3048	20	572	535	519
	40	246	254	239
	60	154	157	133
Salmonella sp. 3064	20	388	361	363
	40	209	201	193
	60	129	122	91
Listeria monocytogenes ATCC 23074	20	718	711	677
	40	246	223	225
	60	194	187	179
Bacillus cereus 30	20	2548	2580	2521
	40	851	837	800
	60	499	486	459

In this paper a mathematical model was derived in order to determine the decimal reduction time during the combined processing by ultrasound and heat of the suspension of microorganisms. The model was based on the assumption that ultrasound and temperature acted independently, and that heat and ultrasound destruction of bacteria were single reactions by first-order kinetics. This model was developed based on the model developed by Raso *et al.* (*12*).

Compared to the *D* values obtained in previous studies of thermal treatment at 60 °C (*E. coli* 3014, *D*=168 s; *Salmonella* sp. 3064, *D*=158 s; *Staphylococcus aureus* 3048, *D*=203 s; *Listeria monocytogenes* ATCC 23074, *D*=219 s and *Bacillus cereus* 30, *D*=597 s), ultrasound in combination with high temperatures significantly reduces the decimal reduction (Table 3). In this table, it can be seen that when applying the ultrasound at 60 °C, the inactivation is not due solely to the ultrasound, but also to heat. At 60 °C, the synergy between heat and ultrasound disappears, turning into a cumulative effect. It may also be noted that the Gram-negative bacteria (*Salmonella* sp. 3064 and *Escherichia coli* 3014) are significantly more sensitive to ultrasound than Gram-positive bacteria (*Staphylococcus aureus* 3048, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30). Many authors did not find any differences in the resistance to ultrasound between the Gram-negative, rod-shaped bacteria and Gram-positive, coccus-shaped bacteria (*S. aureus*) (*5*,*29*). In contrast, some authors have suggested that Gram-negative bacteria are more sensitive than Gram-positive ones (*7*,*10*,*23*,*24*). The results of this study demonstrate that Gram-negative bacteria with *D* values for *E. coli* of $D_{120\ \mu m}$=116 s and *Salmonella* sp. of $D_{120\ \mu m}$=91 s are more susceptible to the ultrasonic treatment at 60 °C than Gram-positive bacteria (*S. aureus* 3048 $D_{120\ \mu m}$=133 s; *Listeria monocytogenes* ATCC 23074 $D_{120\ \mu m}$=179 s; *Bacillus cereus* 30 $D_{120\ \mu m}$=459 s) (Table 3). Gram-positive bacteria usually have a thicker and more tightly adherent layer of peptidoglycan than Gram-negative bacteria, and this morphological feature does seem to be a differentiating factor in ranking the microorganisms according to the percentage of bacteria killed by ultrasonic treatment (*6*).

Table 4. The predicted mathematical model for the count of *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30 after ultrasound treatments

Microorganism	Polynomial*
Escherichia coli 3014	$2.90955+0.0478034\cdot X_1+0.120023\cdot X_2+0.0816784\cdot X_3-0.000150575\cdot X_1^2-0.00227778\cdot X_1\cdot X_2-0.000379167\cdot X_1\cdot X_3+0.024387\cdot X_2^2\cdot 0.013583\cdot X_2\cdot X_3-0.000426293\cdot X_3^2$
Staphylococcus aureus 3048	$8.27052+0.0136345\cdot X_1-1.14119\cdot X_2+0.032397\cdot X_3-0.000116858\cdot X_1^2+0.00134722\cdot X_1\cdot X_2-0.000185417\cdot X_1\cdot X_3+0.103314\cdot X_2^2-0.0156875\cdot X_2\cdot X_3-0.000125431\cdot X_3^2$
Salmonella sp. 3064	$6.26786+0.00925747\cdot X_1-0.907006\cdot X_2-0.00125086\cdot X_3-0.00008735\cdot X_1^2+0.000861111\cdot X_1\cdot X_2-0.0000541667\cdot X_1\cdot X_3+0.0601533\cdot X_2^2-0.00470833\cdot X_2\cdot X_3-0.000271552\cdot X_3^2$
Listeria monocytogenes ATCC 23074	$7.36507+0.0659046\cdot X_1-0.457164\cdot X_2-0.14345\cdot X_3-0.000350766\cdot X_1^2-0.000319444\cdot X_1\cdot X_2-0.00006875\cdot X_1\cdot X_3+0.0327011\cdot X_2^2-0.0029375\cdot X_2\cdot X_3+0.00154828\cdot X_3^2$
Bacillus cereus 30	$4.19528+0.0204517\cdot X_1+0.0263448\cdot X_2+0.0235767\cdot X_3-0.000114176\cdot X_1^2-0.0000555556\cdot X_1\cdot X_2-0.0000791667\cdot X_1\cdot X_3+0.0058046\cdot X_2^2-0.00783333\cdot X_2\cdot X_3-0.0000818966\cdot X_3^2$

*X_1=amplitude (μm), X_2=treatment time (min), X_3=temperature (°C)

The inactivation of food spoilage bacteria after ultrasonic treatment was analyzed by response surface methodology (RSM) using the STATGRAPHICS Centurion software. Calculations were done at 95 % confidence level. According to the RSM model, the inactivation of food spoilage bacteria can be described by a predicted mathematical model for the count of *Escherichia coli* 3014, *Staphylococcus aureus* 3048, *Salmonella* sp. 3064, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30 (Table 4). Durbin-Watson statistics (Tables 5 and 6) showed that the Durbin-Watson number in all tested bacteria was between 1 and 2, which shows that there was a positive correlation, as well as high probability of the accuracy of mathematical models for the inactivation of food spoilage bacteria after ultrasonic treatment (Table 4).

The estimated effects of each operating variable and an analysis of variance for the model are presented in Tables 5 and 6. According to the ANOVA results, the fitted model was significant at the considered confidence level since the F-value was more than three times higher than that of the listed F-value. In order to determine the significance of the effect, the p-values in Tables 5 and 6 need to be observed. Indeed, the p-value is lower than 0.05, which indicates that the considered factor is significant for the count of food spoilage bacteria.

Table 5. Analysis of variance (ANOVA) for ultrasound treatments and viability of Gram-negative bacteria (*Escherichia coli* 3014 and *Salmonella* sp. 3064)

Source	*Escherichia coli* 3014		*Salmonella* sp. 3064	
	F-value	p-value	F-value	p-value
X_1=amplitude	3.09	0.1293	0.66	0.4484
X_2=treatment time	111.99	0.0001	30.76	0.0015
X_3=temperature	119.64	0.0001	49.93	0.0004
X_1:X_1	0.81	0.4016	0.42	0.5392
X_1:X_2	0.50	0.5053	0.10	0.7682
X_1:X_3	1.53	0.2623	0.02	0.8992
X_2:X_2	0.64	0.4558	1.85	0.2229
X_2:X_3	45.64	0.0005	14.03	0.0096
X_3:X_3	4.08	0.0900	4.70	0.0733

Durbin-Watson statistics (*Escherichia coli* 3014)=1.85878
Durbin-Watson statistics (*Salmonella* sp. 3064)=1.95652

Conclusion

The results described in this work suggest that the combination of high temperature and ultrasonic treatment had a cumulative effect and showed more inhibitory activity than the use of ultrasonic treatment or temperature alone. The parameters that seem to substantially affect the inactivation of food spoilage bacteria are the exposure/contact time with the microorganisms and the temperature of the treatment. It was found that Gram-negative bacteria (*Escherichia coli* 3014 and *Salmonella* sp. 3064) are more susceptible to the ultrasonic treatment than the Gram-positive ones (*Staphylococcus aureus* 3048, *Listeria monocytogenes* ATCC 23074 and *Bacillus cereus* 30). In this paper a mathematical model, based on the assumption that ultrasound and temperature acted independently, was derived in order to determine the decimal reduction time during the combined processing by ultrasound and heat of the suspension of microorganisms. Also, according to the response surface methodology (calculations were done at 95 % of confidence level), the inactivation of food spoilage bacteria can be described by a predicted mathematical model for the bacterial count.

Table 6. Analysis of variance (ANOVA) for ultrasound treatments and viability of Gram-positive bacteria (*Staphylococcus aureus* 3048, Bacillus cereus 30 and *Listeria monocytogenes* ATCC 23074)

Source	*Staphylococcus aureus* 3048		*Bacillus cereus* 30		*Listeria monocytogenes* ATCC 23074	
	F-value	p-value	F-value	p-value	F-value	p-value
X_1=amplitude	2.22	0.1865	6.99	0.0383	4.88	0.0692
X_2=treatment time	179.77	0.0001	116.62	0.0001	3.69	0.1030
X_3=temperature	295.76	0.0001	196.70	0.0001	40.41	0.0007
X_1:X_1	3.64	0.1051	3.37	0.1159	0.27	0.6193
X_1:X_2	2.01	0.2062	5.31	0.0607	1.11	0.3317
X_1:X_3	1.49	0.2673	3.62	0.1056	6.15	0.0478
X_2:X_2	3.10	0.1287	3.40	0.1147	0.70	0.4352
X_2:X_3	121.07	0.0001	42.42	0.0006	0.65	0.4502
X_3:X_3	1.45	0.2733	0.85	0.3910	0.08	0.7925

Durbin-Watson statistics (*Staphylococcus aureus* 3048)=1.69799
Durbin-Watson statistics (*Bacillus cereus* 30)=1.77351
Durbin-Watson statistics (*Listeria monocytogenes* ATCC 23074)=1.90853

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