Italian Journal of Food Safety

https://www.pagepressjournals.org/index.php/ijfs/index

eISSN 2239-7132

Please cite this article as:
Lauteri C, Pennisi L, Di Clerico D, et al. **Low-frequency focused thermosonication for *Salmonella typhimurium* inactivation: an *in vitro* study.** *Ital J Food Saf* doi:10.4081/ijfs.2024.12217

Submitted: 21-12-2023
Accepted: 18-04-2024

Low-frequency focused thermosonication for *Salmonella typhimurium* inactivation: an *in vitro* study

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Key words: *Salmonella typhimurium*, low frequency focused ultrasound, thermosonication, *Salmonella* inactivation in food matrix.

Contributions: all the authors made a substantial intellectual contribution, read and approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare no potential conflict of interst.

Ethics approval and consent to participate: not applicable.

Funding: none.

Availability of data and materials: data and materials are available from the corresponding author upon request.

Conference presentation: this paper was presented at the XXXII National Conference of the Italian Association of Veterinary Food Hygienists (AIVI), September 13-15, 2023, Maierato, Italy.

Abstract
Customer requests are addressed to safe products that best express their characteristics of "naturalness" and "freshness" for the entire shelf life; therefore, scientific research has been exploring the use of "non-thermal technologies". Thermosonication using low-frequency focused ultrasound determines bacterial inactivation through the phenomenon of "cavitation", guaranteeing high-quality standards of safety, nutrition, and freshness of the products. The present work aims to evaluate the effectiveness of inactivation of *Salmonella typhimurium* in culture broth by low-frequency focused thermosonication with two different operational parameters: sublethal temperature (40°C, 50°C) and treatment time (5, 10, and 15 minutes). Treatment determined a bacterial load reduction compared to the negative control (untreated inoculum), which was statistically significant at the *T*-test ($p<0.05$). Average decreases of 1.5 log and 3.5 CFU/mL were observed, respectively, after treatment and after 24 hours of storage at +4°C. Treatment at 50°C for 15 minutes was the most effective (average value: 3.06 log CFU/mL; minimum value: 2.13 log CFU/mL; maximum value: 4.59 log CFU/mL).
However, strains have shown markable variability: one of them even showed an increase in the microbial load 24 hours after treatment at 40°C for 5 minutes (-0.20 log CFU/mL); however, the same treatment showed a reduction of bacterial charge in all the other strains (average value: 1.05 log CFU/mL; minimum value: -0.20 log CFU/mL; maximum value: 2.28 log CFU/mL).
The study poses numerous perspectives on the use of low-frequency focused thermosonication treatment in the food industry as a sustainable and safe alternative to classic thermal treatments.

Introduction
Every year, millions of people suffer from foodborne diseases; hence food safety is an imperative topic to hinder this trend (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). Traditionally, thermal treatments have been used to contrast microbiological hazards, but they can cause the loss of important compounds. Sanitization techniques called “non thermal technologies” (*i.e.*, ultrasound, hydrostatic high pressures, cold plasma, *etc*.) have allowed a better retention of flavors, texture, color, and nutrient of foods than conventional thermal treatments (Beitia *et al*., 2023; Ferri *et al*., 2023). Among these techniques, high-intensity ultrasounds (<100 KHz) have attracted considerable interest because they are cheap, safe, “green friendly”, sustainable, and versatile (Lauteri *et al*., 2023). When ultrasounds propagated in liquid media, cavitation phenomena are generated (Huang *et al*., 2017). This phenomenon is the formation, growth, and collapse of microgas bubbles. Due to the pressure shocks up to several 100 MPa, the strong shock waves with 400 km/h microjets, and the production of hot spots with a 5500°C, this occurrence improves microbial inactivation through severe damage to cell wall (Suslick, 1988; Pokhrel *et al*., 2017).
Salmonellosis is the second foodborne pathogen in Europe and poultry and swine food chains are the major pathways (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). It is pivotal an early elimination of pathogenic microflora from food hindering this trend. Ultrasounds employment, as an alternative method of food decontamination, are largely investigated in different food matrices: poultry skin (Musavian *et al*. 2014), meat and meat products (Wang *et al*., 2019), eggs (Bi *et al*., 2020), milk (Herceg *et al*. 2012) as well as in fruit and vegetable processing (Cruz-Cansino et al., 2016).
Research results suggest that the effectiveness of ultrasound technology is influenced by frequency, intensity, treatment time and treatment alone or combined with other technologies should be optimized for each food type. Indeed, the combined action of different technologies is more efficient than the use of ones allowed by the “hurdle technology method” (Lauteri *et al*., 2023).
The present study aimed to better understand the *Salmonella typhimurium* response against thermosonication treatment in different sublethal temperatures and time of treatments.

Materials and Methods

Bacterial strains

A total of 16 *S. typhimurium* strains, belonging to the biobank of the unit of Food Inspection and Control of Animal Origin Food of the Department of Veterinary Medicine of the University of Teramo (Italy) were included in this study (Table 1). Inoculum was prepared in Mueller-Hinton broth (MH, Oxoid Thermo Fisher Scientific, Rodano, Milan, Italy) and incubated at 37°C for 18 hours until early stationary phase. Cells were then harvested by centrifugation and washed three times with phosphate buffer saline (PBS) (Liofichem, Roseto degli Abruzzi, Teramo, Italy) 50 mM, pH 7.4. Inoculum was standardized to OD_{620nm}0.1-0.2 (10×10^7 cells/mL) and then diluted to 10×10^6 cells/ml.

Test site

Waveco® (Next Cooking Generation, Milano, Italy), an ultrasonic bath with a capability of 30 L, was performed for focused ultrasound treatments. All samples were processed at 40 kHz with 100% amplitude with focused ultrasonic wave according to the patent (International Application No.: Patent EP17733039.6). A special construction of the cover equipped with coils enabled the control and adjustment of the temperature of the medium.

Ultrasound treatment

The test involved a total of 16 *S. typhimurium* strains. More in detail, 9 mL of PBS (Liofichem, Roseto degli Abruzzi, Teramo, Italy) with an addition of 1 ml of 18–20 h *S. typhimurium* culture. Thermosonication antibacterial effect against *S. typhimurium* was performed by various ultrasound parameters: the frequencies of 40 kHz, the power of 800 W, the time of 5, 10, and 15 minutes, temperature 40°C and 50°C. The temperature of the treatment medium was monitored during the whole process.

Microbiological analysis

Storage tests were conducted on all samples immediately after treatments and after storage at 4°C for 24 hours by plating out onto xylose lysine deoxycholate (Liofichem, Roseto degli Abruzzi, Teramo, Italy) agar plates. One sample untreated (sample control) of every single strain was included in the analysis. Every strain was performed three times.

Statistical analysis

To evaluate the significant effect of ultrasound parameters, temperature, and treatment time on reducing number of *S. typhimurium*, one-way analysis of variance (ANOVA between subjects) and *t*-test were applied with Tukey-HSD test at $p<0.05$ significance level. Normality of variance was tested by Shapiro-Wilk test; homogeneity of variances was tested by the Levene test (XLSTAT 2014 software, Redmond, WA, United States) (Jamovi project 2022).

Results and Discussion

Tables 2 and 3 showed the descriptive statistical result of *S. typhimurium* after treatment and after 24 hours from treatments.

In response to thermosonication treatments average bacterial count decreases and this reduction becomes more pronounced with temperature increment and prolonged time in accordance with Liu *et al.* (2021). Horwood *et al.* (1951) found that there was a relationship between initial bacterial count and ultrasound inactivation capacity: they noticed that there was a better efficiency of treatment when bacterial load was low.

T-test showed that the control group was significantly ($p<0.05$) distinguishable from the treated groups. The best effective treatment was at 50°C for 15 minutes *in vitro* essay where the reduction was found to average 1.42 log after treatment and 3.96 log after 24 hours. ANOVA test showed that this treatment was significantly ($p<0.05$) distinguishable from the control group. Tukey test supports

the assumption that the applied ultrasound power and treatment duration have a significant effect on the decrease of *Salmonella* count, compared to the control group.
Similar inhibition results were previously reported by Bi *et al.* (2020) and Liu *et al.* (2021), where they found a 3.31 log reduction in *S. typhimurium* in liquid eggs after treatment at 968 W/cm^2 and 35°C for 20 minutes, a the decrease of 2 log of *Salmonella* in sprouts exposed to 200 W and 26 kHz for 5 minutes, respectively. It is important to underline that in the present study, PBS was used as a solution which is different from food matrices and may attenuate the ultrasound effect as reported by Luo *et al.* (2022).
As showed in Tables 4 and 5, there was a markable variability from different strains after treatment and after storage test.
Effectiveness evaluation showed that after the first treatment (40°C 5'), three strains (3/16, 18,75%) increased their bacterial count. The same increment was observed after treatments at 40°C 10' (6 strains, 6/16, 37,50%), 40°C 15' in (three strains, 3/16, 18,75%) and 50°C 5' and 10'(one strain, 1/16, 6,25%). In the evaluation of 24 hours post-treatment, only one strain showed an increment of bacterial count (40°C 5'). After 24 hours, there was a better efficacy in the other treatments analyzed. Indeed, thermosonication technology causes wall damage to the bacterial cell, and after 24 hours, there was more sensible data than after treatments (Baumann *et al*. 2005; Pennisi *et al*., 2020).
Luo *et al.* (2022) studied ultrasound-resistant *Salmonella* and noticed that key enzymes of the tricarboxylic acid cycle were significantly downregulated, which led to a reduced adenosine triphosphate (ATP) content, although ATPase activity was augmented. *Salmonella* tolerated ultrasound stress by upregulating their environmental sensing, chemiotaxis, substance uptake, and ATP production.

Conclusions

Ultrasound is an increasing field of research, particularly in food industry, and it is effective against *S. typhimurium*. However, it is important to better understand the variability response to treatment: bacteria could show adaption to stress like in antibiotic resistance (Ferri *et al*., 2022). It is also pivotal to better quantify the cell injury after treatment and evaluate the effectiveness in different *Salmonella* serovar. The ultrasound applicability combined with other methods in a synergic antimicrobial effect needs to be more investigate. To promote ultrasounds application of food industry it is warranted to explore the interactions between acoustic energy and food matrix.

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Table 1. *Salmonella typhimurium* strains under this study.

Serovar	ID Strain	Origin
S. typhimurium	114	Meat product (sausage)
S. typhimurium	115	Meat product (sausage)
S. typhimurium	117	Meat product (sausage)
S. typhimurium	118	Meat product (sausage)
S. typhimurium	669	Cecal sample
S. typhimurium	670	Pig carcass
S. typhimurium	685	Pig carcass
S. typhimurium	686	Pig carcass
S. typhimurium	687	Cecal sample
S. typhimurium	689	Slaughtering environments
S. typhimurium	690	Slaughtering environments
S. typhimurium	691	Slaughtering environments
S. typhimurium	693	Pig carcass
S. typhimurium	694	Pig carcass
S. typhimurium	695	Pig carcass
S. typhimurium	785	Slaughtering environments

Table 2. Bactericidal effect of thermosonication treatments on *Salmonella typhimurium.*

Descriptive value after treatments							Shapiro-Wilk	
	Mean*	SE	Median	SD	Min	Max	W	p
Control	6.50	0.174	6.54	0.698	5.00	7.85	0.980	0.960
40° C 5' vitro	6.02	0.249	5.80	0.995	4.30	7.78	0.967	0.786
40° C 10' vitro	6.17	0.217	5.95	0.867	4.30	7.78	0.908	0.108
40° C 15' vitro	6.04	0.261	6.15	1.043	4.00	7.54	0.925	0.200
50° C 5' vitro	5.83	0.249	5.85	0.995	4.30	7.60	0.917	0.153
50° C 10' vitro	5.78	0.241	5.90	0.964	4.00	7.73	0.921	0.173
50° C 15' vitro	5.08	0.214	5.00	0.856	4.00	6.71	0.922	0.183
***log CFU/mL**								

SE, standard error; SD, standard deviation.

Table 3. Bactericidal effect of thermosonication treatments on *Salmonella typhimurium* after 24 of storage.

Descriptive value after treatment							Shapiro-Wilk	
	Mean*	SE	Median	SD	Min	Max	W	p
Control vitro 24 h	6.71	0.175	6.69	0.700	5.24	8.09	0.971	0.850
24 h 40°C 5' *vitro*	5.66	0.260	5.38	1.038	3.91	7.53	0.960	0.670
24 h 40°C 10' *vitro*	5.20	0.215	4.95	0.859	3.30	6.86	0.900	0.082
24 h 40°C 15' *vitro*	5.30	0.216	5.33	0.864	3.00	6.54	0.920	0.171
24 h 50°C 5' *vitro*	4.71	0.252	4.78	1.010	3.10	6.54	0.926	0.211
24 h 50°C 10' *vitro*	4.47	0.238	4.68	0.953	2.70	6.43	0.920	0.168
24 h 50°C 15' *vitro*	3.65	0.205	3.65	0.819	2.50	5.21	0.937	0.318
***log CFU/mL**								

h, hours; SE, standard error; SD, standard deviation.

Table 4. Overview of *Salmonella typhimurium* response after treatments.

ID	C	SD	T1-1	SD	T1-2	SD	T1-3	SD	T2-1	SD	T2-2	SD	T2-3	SD
114	6.64	0.23	6.46	0.14	5.95	0.45	6.53	0.71	6.63	0.53	6.15	0.79	6.18	0.63
115	7.55	1.03	7.65	0.63	7.60	0.03	7.54	0.68	7.60	0.98	7.30	0.92	5.00	0.29
117	6.52	0.59	5.30	0.85	5.78	0.89	5.60	0.49	5.85	0.53	6.00	0.92	4.30	0.56
118	7.07	0.86	6.54	0.84	6.41	0.64	6.15	0.14	5.78	0.56	4.00	0.34	5.00	0.55
669	6.74	1.01	5.70	0.10	5.70	0.90	4.00	0.63	5.85	0.32	5.78	0.45	4.00	0.33
670	5.95	0.95	5.90	0.53	6.08	0.93	5.60	0.44	4.30	0.33	5.85	0.13	4.30	0.26
685	6.56	0.19	5.30	0.89	6.15	0.54	6.15	0.81	5.78	0.55	6.23	0.12	5.60	0.18
686	6.15	0.83	5.70	1.02	5.70	0.86	5.70	1.22	5.48	0.54	5.48	0.01	5.30	0.34
687	5.78	0.02	5.00	0.40	5.95	0.23	6.30	0.51	4.30	0.82	6.04	0.98	4.30	0.71
689	6.61	1.03	5.48	0.38	5.70	0.88	6.36	1.08	6.41	0.59	6.00	0.12	5.48	0.33
690	7.10	0.59	6.98	0.89	7.27	0.34	7.20	0.72	5.90	0.33	5.00	0.54	6.71	0.29
691	6.38	0.64	7.06	0.86	6.91	0.41	6.87	0.12	6.15	0.84	5.95	0.66	5.00	0.44
693	6.18	0.01	6.23	1.21	5.90	0.36	5.60	0.77	6.04	0.37	5.60	0.59	5.30	0.31
694	5.00	0.95	5.00	0.53	5,60	1.03	4.00	0.63	5.30	0.12	4.00	0.72	4.00	0.04
695	7.85	0.19	7.78	0.89	7.78	0.81	7.54	1.09	7.60	0.32	7.73	0.72	6.43	0.81
785	5.95	0.34	4.30	0.65	4.30	0.91	5.48	1.15	4.30	0.54	5.30	0.27	4.30	0.33

ID, ID strains; C, control; SD, standard deviation; T1-1, 40°C - 5 minutes; T1-2, 40°C - 10 minutes; T1-3, 40°C - 15 minutes; T2-1, 50°C - 5 minutes; T2-2, 50°C - 10 minutes; T2-3, 50°C - 15 minutes.

Table 5. Overview of *Salmonella typhimurium* response after 24 hours from treatments.

ID	C	SD	T1-1	SD	T1-2	SD	T1-3	SD	T2-1	SD	T2-2	SD	T2-3	SD
114	6.88	0.13	6.07	0.24	4.95	0.15	5.68	0.21	5.43	0.13	4.83	0.99	4.68	0.32
115	7.79	0.23	7.23	0.34	6.40	0.25	6.54	0.18	6.45	0.28	5.87	0.12	3.20	0.73
117	6.76	0.53	5.05	0.86	4.86	0.85	4.60	0.49	4.78	0.43	4.70	0.95	2.80	0.32
118	7.31	0.36	6.30	0.39	5.50	0.44	5.15	0.54	4.71	0.56	2.70	0.35	3.50	0.88
669	6.40	0.51	5.45	0.77	4.78	0.70	5.52	0.63	4.78	0.62	4.48	0.01	4.00	0.64
670	6.19	0.92	4.75	0.63	5.04	0.53	5.30	0.44	3.23	0.43	4.74	0.10	2.80	0.32
685	6.80	0.69	4.91	0.39	5.15	0.44	5.30	0.61	4.58	0.35	4.91	0.37	4.10	0.41
686	6.39	0.93	5.31	1.72	4.70	0.82	4.85	1.52	4.28	0.74	4.16	0.21	3.80	0.77
687	6.02	0.37	4.61	0.30	4.95	0.24	5.45	0.41	3.10	0.32	4.72	0.99	2.80	0.42
689	6.85	0.03	5.23	0.18	4.78	0.77	5.36	1.38	5.35	0.29	4.70	0.25	3.98	0.33
690	7.34	0.93	6.73	0.29	6.35	0.91	6.20	0.82	4.84	0.03	3.70	0.57	5.21	0.02
691	6.62	0.64	6.82	0.86	5.99	0.01	5.87	0.22	5.08	0.74	4.66	0.85	3.50	0.74
693	6.42	0.41	5.84	1.01	4.90	0.26	4.75	0.47	4.84	0.27	4.28	0.76	3.80	0.64
694	5.24	0.82	4.75	0.63	4.68	1.03	3.00	0.43	4.23	0.22	2.70	0.66	2.50	0.01
695	8.09	0.77	7.53	0.59	6.86	0.61	6.54	0.09	6.54	0.21	6.43	0.42	4.93	0.82
785	6.19	0.31	3.91	0.35	3.30	0.13	4.63	0.15	3.10	0.51	3.98	0.31	2.80	0.21

ID, ID strains; C, control; SD, standard deviation; T1-1, 40°C - 5 minutes; T1-2, 40°C - 10 minutes; T1-3, 40°C - 15 minutes; T2-1, 50°C - 5 minutes; T2-2, 50°C - 10 minutes; T2-3, 50°C - 15 minutes.