ORIGINAL ARTICLE

Inactivation of *E. faecalis* under microwave heat treatment and ultrasound probe

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ABSTRACT

Background and aims: The Weibull model was fitted to survival curves in order to describe inactivation kinetics, and the effect of combined microwave (MW) and ultrasound (US) treatments was evaluated. **Methods:** *Enterococcus faecalis* ATCC 29212 present in 40 mL of sterile physiological water was treated with microwaves at 300W, 600W, and 900W and/or ultrasonic probes (amplitude 60 %, 80 % and 100 %, pulse (3s continuous, 3s discontinuous). **Results:** The use of an ultrasonic probe at 20 kHz displayed no significant impact on the patients' ability to survive. At 600 W and 300 W of MW treatment, a decrease of 3.96 log and 0.90 log, respectively, was obtained. Total destruction was accomplished in 70 seconds when 900 W of microwave therapy was used. Additionally, it was shown that the effectiveness of WM and US increased with increasing power and exposure duration. This was the case even when microwave or ultrasonic technology was utilized independently. In addition, the treatment that included both microwaves and ultrasound showed a significantly better effect than the treatment that only involved microwaves, but there were no significant differences between the coupled treatment and the microwave treatment given for 30 seconds. **Conclusions:** The results of the current study show that the inactivation of *Enterococcus faecalis* by ultrasound followed by microwave treatment was significantly higher than that obtained by microwave treatment followed by ultrasound.

Keywords: *Enterococcus faecalis,* ultrasound, microwave, inactivation.

ARTICLE INFORMATION

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1 Introduction

Several techniques have been developed to destroy pathogenic bacteria present in food products [1-3] or industrial waste [4-6]. Several writers have brought attention to the use of microwave technology, which is an application of green chemistry that possesses a high sterilizing capacity and can efficiently deactivate bacteria and enzymes [7, 8]. It is a well-known method of drying and heating that may be used for a variety of purposes, including those in the home and the workplace. Microwave radiation or pretreatment enhances the removal of harmful bacteria from the medium, which causes the product's volume to be heated and starts the thermal pretreatment process. Microwaves also expedite the heating of the product [9].

Despite the fact that the microwave method has certain benefits, it also presents some drawbacks, the most significant of which is the significant amount of electrical energy that is wasted during the process of being turned into heat. In fact, since water has such a high specific thermal capacity, the option to treat food or waste with microwaves entails not only a high level of energy consumption but also a high level of cost associated with running the system; both of these aspects restrict the usage of microwaves. Although the standard microwave ovens that are now on the market provide a high degree of disinfection, this is not sufficient to render them suitable for use in the wastewater sterilization [10].

Thus, ultrasonic disinfection has been studied extensively. There are physical and chemical effects of ultrasound (US), including cavitation, mechanical effects, and micromechanical shocks [11-12]. Ultrasonic irradiation must be high-intensity or long-term to reduce bacteria logarithmically. These cost reasons hinder the use of large-scale disinfection technology. Industry may now use microwave and ultrasonic technologies to minimize energy. Leonelli and Mason [13] suggest optimizing green procedures and technology for industrial microwave and ultrasound reactor manufacturing. Thus, ultrasound and microwaves synergistically inactivate microbes and save energy [14].

The primary objective of this research is, in fact, to use this method in conjunction with the process of inactivating *Enterococcus faecalis* ATCC 29212, a pathogenic opportunistic bacterium [15] that is thought to be the most thermoresistant of the vegetative forms [16].

2 Material and Methods

2.1 Bacterial strain and culture conditions

The Pasteur Institute's strain *Enterococcus faecalis* ATCC 29212 was used in experiments (Algiers, Algeria). Up to usage, the strain was kept on tryptone soy agar (TSA; Conda Pronadisa, Spain). Colonies were suspended in Tryptone Soy Broth (TSB, Conda Pronadisa, Spain) to create young cultures, which were then incubated at 37 °C for 18 hours. Centrifugation (4000 g for 15 min at 4 °C) was then used to recover bacteria [1].

A final dosage of 1.5 10^8 colony forming units (UFC/mL) of the *Enterococcus faecalis* ATCC 29212 strain was used for the inoculation, which was confirmed to be sterile physiological water by a spectrophotometer (OD600 = 0.08 – 0.13) [2].

2.2 Microwave and ultrasound treatment procedure

Using an ultrasonic probe (SONICS Vibra cell, VCX 130 PB, stepped microtips and probes, No. 630-0422) at 20 kHz (amplitude 60%, 80% and 100%, pulse (3 s continuous, 3 s discontinuous)), 40 mL of sterile physiological water infected with 1.5 10^8 CFU/mL *E. faecalis*, present in a 100 mL beaker were subjected to ultrasonic treatment for 5 and 20 minutes. The sample temperature was held at 30 ± 2 °C by gradually adding ice to the bath containing the beaker.

Forty (40 mL) inoculated sterile physiological water samples were exposed to microwave (Samsung GE614ST, Malaysia) radiation at 300W, 600W, and 900W at varying exposure intervals, ranging from 10 to 70 s. After treatment, the sample was immediately removed from the cavity and refrigerated in an ice bath.

We looked at the impact of ultrasound as a pre- or post-treatment method on the microwave inactivation of *Enterococcus faecalis* ATCC 29212.

The combined microwave and ultrasound treatments (80%) used were as follows: 5 minutes and 20 minutes for pre- or post-ultrasound treatment and 600 W for microwave inactivation. The experimental process was the same as what was previously explained. Triplicates of each experiment were performed.

2.3 Enumeration of *E. faecalis* ATCC 29212

Using sterile physiological water, 1 mL aliquots of the treated and untreated samples were serially diluted and spread-plated on Muller-Hinton medium in triplicate. After 24 hours at 37 °C, surviving bacteria were counted. Every experiment was tripled.

2.4 Statistical methods and inactivation kinetics modeling

Considering the asymmetric distribution of thermo-sensitivity values within a bacterial population, Yildiz et al. [3] proposed a model inspired by the Weibull statistical distribution function. For their part, Mafart et al. [20] parameterized again this model in a more interesting form whose parameters have practical significance for the inactivation of microorganisms: where α is called the time of the first decimal reduction and β which describes the shape of the curves (eq. 1):

$$log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\alpha}\right)^{\beta} \quad \ldots\ldots\ldots\ldots (1)$$

α and β being the two parameters of the thermoresistance distribution. α is called the scale parameter (characterized by time) and β is the shape parameter.

Nonlinear regression and a curve fitting toolbox were used to obtain the parameters of the modified Weibull model (MATLAB 6.5, The Math-Works Inc., Natick, MA, USA). As indications of the calculated parameters' goodness-of-fit, the adjusted coefficients of determination (R^2), confidence intervals (generated with a 95% probability), and root mean square error (RMSE) between all experimental and projected data were used.

Statistical significance ($p < 0.05$) of the effect of ultrasound and microwave treatments was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The survival rates were also statistically compared to control (initial number of microorganisms) using ANOVA followed by Dunnett's post hoc test.

3 Results and discussion

3.1 Modeling and kinetic parameter estimation

As shown in Figures 1 and 2, the experimental survival data of *Enterococcus faecalis* ATCC 29212 were well matched by the modified Weibull model in the current investigation. Additionally, Table 1's R^2 adjusted values (0.896 – 0.992) and RMSE (0.18 on average for all treatments) show that the modified Weibull model predictions adequately reflect the experimental data.

Table 1. Parameters of the Mafart model fitted to the inactivation kinetics of *E. faecalis* by microwave treatment and ultrasound treatment

Microwave treatment	α	β	RMSE	R²
300W	73.0 ± 6.5	1.4 ± 0.4	0.05	0.976
600W	32.2 ± 4.4	1.7 ± 0.4	0.18	0.982
900W	26.0 ± 5.9	2.1 ± 0.5	0.45	0.974
Ultrasound treatment				
60%	23.5 ± 10.1	0.51 ± 0.04	0.17	0.896
80%	11.2 ± 5.5	0.46 ± 0.03	0.16	0.946
100%	11.9 ± 2.0	0.70 ± 0.10	0.07	0.992

3.2 Ultrasound effect

E. faecalis ATCC 29212's inactivation kinetics are shown in Figure 1 using an ultrasonic probe with a frequency of 20 kHz. For amplitudes of 60 %, 80 %, and 100%, the shape parameter was determined to be 0.51 ± 0.04, 0.46 ± 0.03, and 0.70 ± 0.10 for 60 %, 80 % and 100% amplitude respectively (Table 1). This is visibly a more intense drag and an upward concavity curve.

The distribution of resistances throughout the population, wherein the more resistant members of the population persist while the most sensitive members are inactivated first, or the physical protection offered by the agglutination of the inactivated cells may all be used to explain this curve shape [21].

The quantity of *E. faecalis* ATCC 29212 in sterile physiological water declines as the ultrasonic treatment time increases (Figure 1). However, it is clear that the survival rate was not significantly decreased. Inactivation of *E. faecalis* ATCC 29212 is not significantly affected by ultrasound treatments at a frequency of 20 kHz at 60 %, 80 %, and 100 %. After 30 minutes of ultrasound treatment, the population is reduced by a maximum of 1.9 log. The observed reductions in survival rates only become statistically significant ($p < 0.05$) after 5 min, 5 min, and 1 min of treatment at US 60 %, 80 %, and 100 %, respectively, compared to the control.

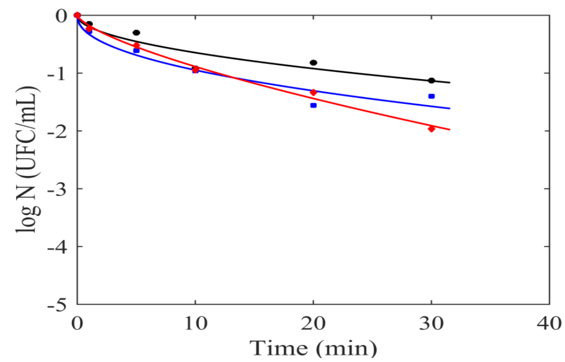

Figure 1. Kinetics of inactivation of *Enterococcus faecalis* ATCC 29212 by ultrasound. Comparison of experimental values (amplitudes: ● 60%, ■ 80% et ◆ 100%) and those simulated using the Weibull model (—)

According to Gholami et al. [22], at 20 kHz, a 4-log decrease in *E. coli* and *E. faecalis* ATCC 29212 cells found in drinking water was achieved at 9 min.

According to research carried out by Amabilis-Sosa et al. [23] on the impact of ultrasound on bacterial inactivation wastewater, *B. subtilis*' bacterial density decreased by 3.16 Log after 15 minutes of sonication (20 kHz, 35 % amplitude, and 600 W/L). After 30 minutes, there was no detectable *B. subtilis* CFU/mL in municipal wastewater, and after 45 minutes, total and fecal coliforms had decreased by more than 6.45 log.

3.3 Microwave effect

The experimental survival rate kinetics of *E. faecalis* ATCC 29212 treated with microwaves at 300, 600, and 900 W are fitted using the modified Weibull model in Figure 2. It is possible to interpret the downward concavity (shoulder) of the curves as proof that cumulative damage resulted from continuous irradiation, and the destruction rate rises with exposure duration.

Weibull estimates the scaling parameter (α) and shape parameter (β) in order to measure the resistance of *E. faecalis* ATCC 29212 while taking into account the impact of growing circumstances on the form of survival curves. According to the model's regression findings, it was discovered that the parameter typically exhibited a declining trend and that its value tended to rise with rising power levels.

The inactivation rate decreased as microwave power increased. Table 1 shows that at 900 W, the estimated scale parameter was lowest (α =26.0), and at 300 W, it was greatest (α =73.0).

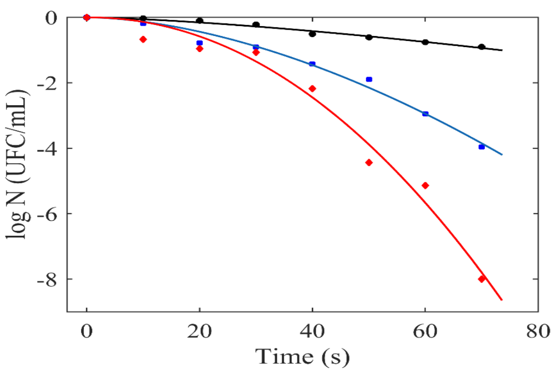

Figure 2. Kinetics of inactivation of *Enterococcus faecalis* ATCC 29212 by microwave. Comparison of experimental values (● 300W, ■ 600W et ◆ 900W) and those simulated using the Weibull model (—)

The inactivation rate increased as microwave power rose: at 900 W, the estimated scaling parameter was the lowest α (26.0 ± 5.9s) and at 600 W, it was approximately twice as low (32.2 ± 4.4 s) as that found for 300 W (73.0 ± 6.5). After 70 seconds, total *E. faecalis* ATCC 29212 mortality was seen at 900 W, with reductions of 3.96 log at 600 W and 0.90 log at 300 W.

The observed reductions of survival rates become statistically significant ($p < 0.05$) only from 30 s, 20 s and 10 s of treatment at 300 W, 600 W and 900 W respectively. ANOVA results revealed that there was no significant difference ($p > 0.05$) between 600 W and 900 W treatments. At least, a significant difference was obtained between treatments at 300W and treatments at 600 W and 900 W.

Benjamin et al. [24] studied the effect of microwaves on the inactivation of *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli* present in 100 mL of sterile physiological water. At a power of 130 W, a declumping effect was observed after two minutes for the three species, then a regression of the number of bacteria was observed, and a total inactivation was obtained after 5 min of treatment for *Enterococcus faecalis* ATCC 29212 and 4 min for *Staphylococcus aureus* and *E. coli.*

3.4 Combined effects of MW and US

The effect of MW at 600 W (30 s and 50 s) and US at 80% amplitude (5 min and 20 min) combination treatments, in inactivation of *E. faecalis*, was studied and the results are displayed in Figure 3. Combined microwave (600W – 30 s) and ultrasound 5 min treatment is less important than micro-wave treatment alone (Figure 3 a-b). MW treatment at 600W for 30 s plus US treatment for 5 min achieved 0.64 log reduction with no significant difference ($p > 0.05$) observed, compared to US treatment at 20 min which reached a reduction of 0.59 log.

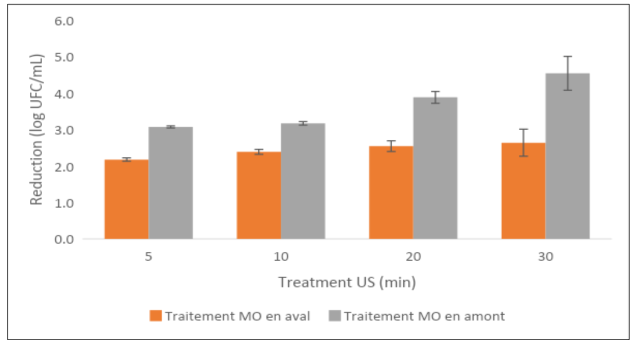

Figure 3. Comparison of two *Enterococcus faecalis* ATCC 29212 inactivation treatment couplings by microwave (MW) and ultrasound (US): (a) US post-treatment; (b) US pre-treatment

The *E. faecalis* ATCC 29212 bacteria in sterile physiological water may be inactivated more effectively by microwaves if they are subjected to a US pretreatment beforehand. The combination of microwave therapy at 600W for 50 s and ultrasound pre-treatment for 20 min resulted in a decrease of 4.34 log, which was much greater than the reduction of just 2.95 log achieved by microwave treatment alone (Figure 3b).

A comparative study between the inactivation of *E. faecalis* ATCC 29212 by microwave and ultrasound coupling has been shown in Figure 4.

For ultrasonic treatment times at 5 min, 10 min, 20 min and 30 min coupled to microwave at 600 W for 50 s, it was found that the US pretreatment is more efficient than its post-treatment; a reduction of 2.18 log, 2.39 log, 2.55 log and 2.64 log for the US coupling in post-treatment, while it was 3.07 log, 3.17 log, 3.88 log and 4.54 log for the US coupling in pre-treatment at are consistent with many studies [9-12].

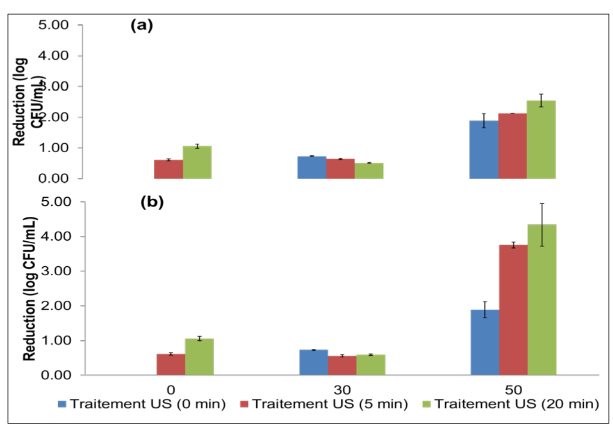

Figure 4. Comparison of the inactivation kinetics of *Enterococcus faecalis* ATCC 29212 by US treatment coupled with MW treatment at 600W for 50 s

Indeed, the combination of ultrasound with microwaves can induce synergistic effects in terms of microbial inactivation, as well as energy savings.

The combination of ultrasound with other techniques has been studied by several researchers; Blume and Neis [25] examined the scientific and economic potential of using US as a pre-treatment step in combination with UV to optimize wastewater disinfection. After 30 s of UV treatment (14 W with 3 W emitted at 254 nm) followed by 5 s of ultrasound treatment at 50 or 310 W/L, the microbial reduction levels were 3.3 log and 3.7 log units, respectively. US/ozone synergistically eliminated enterococci.

Wang et al. [26] and Chen et al. [27] found a value of 1.11 log for enterococcus in wastewater following ultrasonic waves at 200 W for 30 min and ozone at 4.2 mg O3/L. This is still low compared to our findings.

4. Conclusion

The purpose of this research was to determine whether or not microwaves, ultrasounds, or a combination of the two may successfully destroy *E. faecalis* ATCC 29212 cells that are suspended in sterile physiological water. A modified version of the Weibull model was successful in accurately representing the temporal dynamics of the survival rate. Only a 1.9 log decrease was achieved after 30 minutes of sonication using an ultrasonic probe operating at 20 kHz. This indicates that the therapy does not induce a significant impact on the survival rate reduction of *E. faecalis* ATCC 29212.

The treatment times ultrasound for 20 min and 600W of microwaves for 50 s were enough to destroy (4.34 log) heat-resistant bacteria in sterile physiological water, but microwave or ultrasound processing alone showed more resistance to killing the bacteria.

However, to overcome this issue, either the microwave treatment alone was extended for a longer period of time in line with the level of power, or the microwave and ultrasound treatments were combined, and especially the ultrasound was used as a pre-treatment. This was successful in removing the bacterial load that was present in sterile physiological water. Both of these solutions were implemented aiming to address this issue.

Limitations of the study.

The main limitation of the current study consists of the use of large volumes for industrial application. A larger device to meet the needs of the industry must be developed and remains a working prospect.

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Author Contribution: O.N.K. Conception, data collection, funding acquisition, research, first draft writing, and writing that includes revision and editing. A.B. Writing—review and editing; conception; formal analysis; technique; validation. K.B. Research; writing; peer review; and editing. G.K.B. Research; writing; peer review; and editing. L.B.M. Research; writing; peer review; and editing. K.M.: Director.

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