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THÈSE

Présentée par

KERNOU Ourdia dite Nouara

Pour l'obtention du grade de

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Thème

Cinétiques de destruction bactérienne par couplage micro-ondes – ultrasons. Etude de cas : *Escherichia coli*, *Enterococcus faecalis*

Soutenue le 04/07/2021 Devant le Jury composé de :

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THESIS

Presented by

KERNOU Ourdia dite Nouara

To obtain the degree of

DOCTOR OF SCIENCE

Sector : Biology
Option : Microbiology

Theme

Kinetics of bacterial destruction by microwaves-ultrasounds coupling. Case study: *Escherichia coli, Enterococcus faecalis*

Supported on: 04/07/2021 In front of the Jury composed by:

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LIST OF ABREVIATION

A	Pre-exponential factor (empirical constant)
a_w	Water activity
CFU	Colony forming unit
CUT	come-up time
D	Decimal reduction time (min.)
DNA	Deoxyribo nucleic acid
Ea	activation energy
EMB	Eosin methylene blue
FDA	Food and Drug Administration
GHz	Giga Hertz
HSP	HeatShockProtein
K	Reaction constant (s - 1)
Log	Decimal logarithm
MHz	Mega Hertz
MSE	Mean square error
MW	Microwave
N	Microbial population
ND:	Not determined
pH	hydrogen potential
RMSE	Root Mean Squared Error
Rpm	Rotations Per Minute
SD	Standard deviation
SEM	scanning electronic microscope
T	Measurements or calculations at different temperatures
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UHF	Ulra High Frequency
US	Ultrasound
V	Volt
W	Watt
Y	Measured (experimental) values

Y	Predicted values
α	Weibull model scaling parameter
Z	Temperature increase to reduce the value of D (°C) by a factor of ten

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GENERAL INTRODUCTION

Microorganisms were the first forms of life on earth, they include bacteria, yeasts, mold, algae and protozoa. These living beings are ubiquitous and colonize all environments. They are essential to man and the environment (Alnnasouri, 2010) ; Nevertheless, there are unwanted pathogenic forms that need to be treated and eliminated. Moreover, several techniques have been developed in order to destroy or inhibit these pathogens. These are methods of heat treatment (pasteurization ...) which is commonly used to inactivate microorganisms in liquidfoods to improve food safety and extend shelf life (Uemura, Kobayashi, & Inoue, 2010), and athermal radiation such as ultraviolet radiation (Rodríguez, Oteiza, Giannuzzi, & Zaritzky, 2017), pulsed electric fiel treatment (Gurtler, Rivera, Zhang, & Geveke, 2010), ozone treatment (Patil, Bourke, Kelly, Frías, & Cullen, 2009), high dynamic pressure (Tahiri, Makhlouf, Paquin, & Fliss, 2006), clarification (Anvarian, Smith, & Overton, 2016), cold atmospheric plasma (Dasan & Boyaci, 2018), or combination of these processes with thermal treatments (Rifna et al., 2019).

However, these treatments some times alter the products on which they are used and present a danger to humans and the environment; therefore, it is essential to look for alternative treatment techniques that destroy the micro-organisms and have no negative consequences for the environment and human health (Levy, 2010).

Among these innovative techniques of microorganisms destruction , we cite the wave methods, namely microwaves and ultrasounds. The latter are physical methods, aimed at replacing chemical treatments, thus offering a better image of preservation of the natural. The effectiveness of these methods in the destruction of microorganisms, has been demonstrated by numerous published studies (Ahmed & Ramaswamy, 2004; Veillet, 2010). Indeed, these studies show that microwaves and ultrasound can induce a significant reduction up to the elimination of bacteria. These techniques could well be used in the treatment of fruit juices, which offer good conditions for the growth not only of spoilage microorganisms, but also of foodborne pathogens such as *Escherichia coli, Listeria monocytogenes*, *Cryptosporidium* and *Salmonella*, and of wastwater dairy effluent which favors the growth of *Cryptosporidium parvum*, *Giardia* sp., *Escherichia coli*, *Clostridium perfringens, Enterococcus faecalis*, *Salmonella* sp. etc. (P. M. Chapman, 2000; P. M. J. E. I. Chapman, 2007; Di, Cameron, Silva, Russell, & Barnett, 2002; Dungan & Leytem, 2013; Ibekwe & Grieve, 2003).

Ultrasound (US) is one of the innovative technologies that can ensure microbial inactivation in fruit juices while preserving their quality and disinfecting wastewater (Blume & Neis, 2004). It is considered to be energy efficient and environmentally friendly with reduced chemical and physical hazards (Mohideen et al., 2015).

The mode of action of ultrasound on microorganisms in liquids involves hydrodynamic effects (intracellular cavitation and microstreaming phenomenon) and formation of radicals that disrupt cell structure (Tiwari, Muthukumarappan, O'Donnell, & Cullen, 2008). However, as a preservation method, the application of ultrasound alone is not effective enough to kill all microorganisms. In addition, a high level of ultrasound power can adversely affect the nutritional and sensory properties of foods (Ferrario, Alzamora, & Guerrero, 2015). Also, it has been reported that ultrasound treatment alone (at room temperature) may be ineffective in inactivating some bacterial species (Gao, Lewis, Ashokkumar, & Hemar, 2014; Kentish & Feng, 2014). In order to achieve significant microbial reduction, high power and long treatment times are required, resulting in enormous energy costs.

The microwave (MW) heating instead of conventional heat exchangers offers certain advantages for heat treatment, such as rapid volumetric heating, lower equipment surface temperature, uniform heating, energy efficiency and possible preservation of food quality (Chandrasekaran, Ramanathan, & Basak, 2013). Many studies have demonstrated the great potential of microwave radiation in the microbial inactivation in various foods, particularly in liquid foods (Benlloch-Tinoco, Martínez-Navarrete, & Rodrigo, 2014). Indeed, High-temperature short-time (HTST) type processes are preferred by the food processing industry to reduce the adverse thermal degradation in food quality while ensuring food safety for liquid food (Varghese, Pandey, Radhakrishna, & Bawa, 2014). In this sense, the advantage of microwave heating is that the process is fast and can significantly reduce the come-up time (CUT) to the desired process temperature (Di Rosa, Bressan, Leone, Falqui, & Chiofalo, 2019). Woo, Rhee, and Park (2000) have investigated the mechanism of microbial cell inactivation by microwave heating, they revealed that in addition to inactivation, the microwave radiation caused severe damage on the surface of *Escherichia coli* cells, and there was no significant change in *Bacillus subtilis* cells (Najdovski, Dragaš, & Kotnik, 1991).

The combination of ultrasound with microwaves can induce synergistic effects in terms of reduction of pathogenes is bacteria present in orange juice and wastewater dairy effluent

In this respect, we have set the following objectives: (i) to compare the two inactivation processes and their combined effect on the inactivation of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 (ii) to fit the Weibull model to describe and compare the inactivation kinetics (iii) to evaluate the combined effects of microwave and ultrasonic treatment.

The work of this thesis will be presented in three parts:

-The first part includes the bibliographicstudy,

-The second part illustrates the experimental part and is in turn subdivided into two parts: I-Inactivation of *E.coli* ATCC 25922 in orange juice beverage.

II-Inactivation of *Enterococcus faecalis* ATCC 29212 in waste water dairy effluent

The third part announces the results obtained and their discussion.

Finally, our manuscript is punctuated by a general conclusion and possible perspectives.

1 LITERATURE REVIEW

1.1 Heat decontamination

1.1.1 Thermal destruction of microorganisms

The temperature sensitivity of microorganisms makes it a key aspect of their development. This factor is indeed very much used to regulate the development of microorganisms (McSwane, Rue, & Linton, 2000)**.** Heat treatment, when possible, allows the destruction of microorganisms present on the food. This requires the application of the effective time-temperature couple.

Heat treatments at low temperatures (of the order of 80ºC to 100ºC) are sufficient to destroy microorganisms in their vegetative form. The product may nevertheless still contain microorganisms in sporulated form, which may give vegetative forms again (Stumbo, 1973).

These treatments continue to be the most preferred method of ensuring food safety and quality. Cheftel and Culioli (1997) distinguish 3 types of heat treatments according to their intensity and objectives:

-*Sterilization*: high temperature heat treatment, above 100ºC, capable of destroy all microbial forms present, including bacterial endospores;

-P*asteurization*: low-temperature heat treatment aimed at the destruction of the heat-sensitive vegetative forms excluding bacterial endospores which, if the pH and aw (water activity) allow them to do so, can germinate and alter the product;

-*Stabilizing pasteurization*: heat treatment of the same type as the previous one. For products with a low pH and/or water activity that does not allow the germination of bacterial endospores.

To express bacterial resistance to heat, two essential notions are defined; the rate of thermal destruction and the rate of decimal reduction. The first refers to the shortest period of time required to kill a bacterial or sporal suspension at a given temperature and under certain conditions. The decimal reduction rate represents the time required to kill 90% of a bacterial population. Using these definitions, it can be seen that temperature-time pairs are important in determining the sensitivity of microorganisms to heat.

1.1.2 Notion of heat resistance

When a suspension of microorganisms is subjected to a heat treatment at constant temperature, an exponential decrease in the number of countable colonies is observed over time. The destruction of the microorganisms can therefore be monitored by representing the logarithm to the decimal of the ratio N/N_0 of the number of surviving microorganisms N to the initial number N_0 as a function of time (Larousse, Martinuzzi, & Tropea, 1991).

The curve obtained is generally linear and the important element for the technologist is the smallest of the slopes of the linear parts, which reflects the maximum heat resistance observed.

The inverse of this slope is noted D: it is the time required for the survival curve of the microorganism, at temperature T, to go through a logarithmic decimal cycle. D is also therefore the processing time for the number of microorganisms to be divided by 10. It is therefore called "decimal reduction time": the larger the number, the better the micro-organism resists heat. The following relationships are established between N_0, N and t (processing time) (Koutchma, Le Bail, & Ramaswamy, 2001).

$$N = N_0 e^{-kt} \quad (1)$$

and

$$D = \frac{\ln 10}{k} = \frac{2.303}{k} \quad (2)$$

K: the constant of the speed of destruction.

To study the influence of temperature on the decimal reduction time D, the logarithm of D as a function of temperature is plotted (log D = f(T)). The points obtained form a straight line called the "TDT phantom curve" (TDT = Thermal Destruction Time) characterized by the inverse of its slope (symbol Z).

$$Z = \frac{\mathrm{T2} - \mathrm{T1}}{\mathrm{Log}\ \frac{\mathrm{D1}}{\mathrm{D2}}} \qquad (3)$$

Z corresponds to the temperature rise necessary to reduce the duration of the standard heat treatment to 1/10 of the time required to obtain the same destruction rate (Figure 1). It is a thermoresistance parameter characteristic of each species of microorganism.(Koutchma et al., 2001).

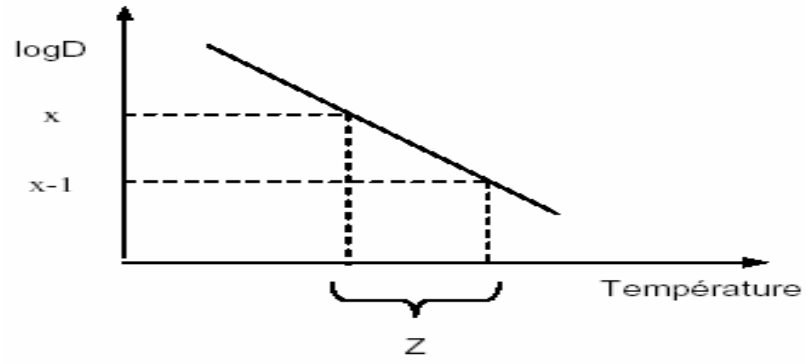

Figure 1. Graphical determination of z-value

These values are less fluctuating than D and are generally of the order of 4 to 7°C for the vegetative forms and of the order of 10°C for the spores. The smaller it is, the more the process of thermal destruction of the microorganisms is influenced by the temperature of the treatment. The behavior of each strain of microorganism during a heat treatment can therefore be characterized by a simple couple (D, z).

Many food scientists still rely on linear inactivation kinetics, although concave and sigmoidal tendencies are frequently observed in pressure treatments.

1.1.3 Factor influencing heat resistance

The survival of microorganisms is greatly influenced by the chemical and physical nature of the environment. It is essential to see the adaptability of microorganisms to different

environmental conditions. It is often accepted that the main factors influencing microbial thermoresistance, apart from temperature, are pH and aw, followed by a multitude of factors whose effects can be more or less marked depending on the species or strain of microorganism. Indeed, according to Jérôme et al (2008), three factors have been identified as the main responsible for variations in thermal resistance

pH

Microorganisms often multiply in a wide range of pH, and the vast majority prefer media with a pH value close to 7, but there are some bacteria that are adapted to acidic or alkaline media. Acidophiles have their growth optimum between pH 1 and 5.5; neutrophils between pH 5.5 and 8.0; alkalophiles between 8.5 and 11.5 (Booth, 1985; Gottschal, Prins, & evolution, 1991).

However, each species grows within a defined pH range and has a pH optimum for growth. The pH influences (Table 1).

In order to survive, microorganisms often have to adapt to changes in the pH of the environment. In bacteria, potassium/proton and sodium/proton antiport systems probably correct small pH variations. Other mechanisms come into play if the pH becomes too acidic. Anellis, Lubas, and Rayman (1954); Corry and Barnes (1968) have shown that the decimal reduction time of bacteria decreases as the pH increases, a low pH increases the sensitivity of microorganisms. If the pH falls below 5.5-6.0, *Salmonella typhimurium* and *E. coli* synthesize a series of novel proteins that characterize their response to acid tolerance

Table 1. pH limits for the growth of some microorganisms

	Minimum	Optimum	Maximum
Bacteria in general	3.2	6.5-7.5	11
Acetic bacteria	4.0	5.4-6.3	9.2
Lactic bacteria	3.2	5.5-6.5	10.5
Pseudomonas	5.6	6.6-7.0	7.0
Entérobacteria	5.6	6.5-7.5	9.0

Staphylococcus	4.2	6.8-7.5	9.3
Clostridium	4.6-5.0		9
Bacillus	5-6	6.8-7.5	9.4-10

Solute and water activity:

Water is essential for the survival and development of all microorganisms.

In food, some of it is called "free", meaning that it is available to microorganisms. The other part is bound to the constituents of the food and cannot be used (Corry & Barnes, 1968).

The water activity (aw) represents the water available (free water) for carrying out metabolic reactions, especially enzymatic reactions. The values of aw are between 0 and 1, the value of aw=1 being reserved for with pure water (Branger et al., 2005).

A low aw decreases the sensitivity of microorganisms. Therefore, a wetter product is easier to sterilize than a partially dehydrated product. Any lowering of aw affects the rate of bacterial growth, most bacteria have an optimum rate around 0.990 to 0.995. At lower aw values growth is slowed down;-so for *Staphylococcus aureus* at a growth rate reduced to 10% of its maximum for an aw of 0.900 (Beuchat, 1983). The decrease in aw leads to a phenomenon of plasmolysis, *Staphylococcus aureus* loses 50% of its intracellular water when aw decreases from 0.995 to 0.950 (Koujima et al., 1978); this phenomenon decreases or stops the growth of microorganisms by hinibition of enzymatic activity. Table 2 shows the water activities (aw) required to support the growth of some microorganisms.

Most often, microorganisms keep the osmotic concentration of their cytoplasm above that of their habitat with compatible solutes, so the plasma membrane is always held firmly against the cell wall (Brown, 1976; Stetter, 1995). The addition of high amounts of sugar, such as sucrose, or salt, such as NaCl, to a medium tends to lower its water activity because these solutes bind to water molecules, reducing the amount of "free" water available (Herbert & Codd, 1986).

Microorganisms do not react in the same way depending on whether salt or sugar is present. Some bacteria, such as halophils, require NaCl to survive. Such microorganisms can tolerate 15% NaCl (MacLeod, 1985).

Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, and Salmonella species will not grow or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not grow below 0.86 and *Aspergillus Niger* will not grow below 0.77. Moreover, even osmophilic yeasts and xerophilic fungi will not proliferate below 0.60 (MacLeod, 1985).

Table 2. Water (aw) required to support the growth of representative microorganisms (Troller, Bernard, & Scott, 1984)

Bacteria	Water activity (aw)	Molds and yeasts	Water activity (aw)
Pseudomonas aeroginosa	0.97	*Nigricans rhyzopus*	0.93
Bacillus cereus	0.95	*Mucor plumbeus*	0.92
Clostridium botulinum, type A	0.95	*Rhodotorula mucilaginosa*	0.92
Escherichia coli	0.95	*Saccharomyces cerevisiae*	0.90
Clostridium perfringens	0.95	*Paecilomyces vartotti*	0.84
Lactobacillus viridescens	0.95	*Penicillium chrysogenum*	0.83
Salmonella spp.	0.95	*Aspergillus fumigates*	0.82
Enterobacter aerogenes	0.94	*Penicilium glabrum*	0.81
Bacillus subtilis	0.90	*Aspergillus flavus*	0.78
Micrococcus lysodekticus	0.93	*Aspergilus niger*	0.77
Staphylococcus aureus	0.86	*Zygosachhromyces rouxii (levure osmophile)*	0.62
Halobacterium halobium	0.75	*Xeromyces bisporus (champignons xérophyles)*	0.61

The minimum water activity for the growth of *E. coli* O157:H7 is 0.953 with an optimum of 0.995 (Nauta & Dufrenne, 1999).

1.2 Predictive microbiology

Predictive microbiology is a tool for predicting by mathematical models the impact of different environmental conditions of temperature, pH, water activity (Membré et al., 2005), organic acid

and inhibitor concentrations (Cárdenas, Giannuzzi, & Zaritzky, 2008) and their possible interactions on the growth, inactivation or survival of microorganisms (Buchanan & Whiting, 1996). Specific models have thus been developed to predict microbial growth on the one hand, and decay or inactivation on the other hand, for pathogenic germs (Shimoni, Labuza, & technology, 2000) or for spoilage germs (K. J. J. o. f. p. Koutsoumanis, 2009).

1.2.1 Modeling of the thermal inactivation of microorganisms

1.2.1.1 Primary modeling

Primary modeling consists of developing mathematical expressions based on theoretical principles, empirical observations, or a combination of both, to predict changes in microbial counts, enzyme activity, or chemical concentrations as a function of treatment time.

Depending on the shape of the predicted kinetic behavior, primary models are classified as linear, concave or sigmoidal.

- First-order kinetic model

First-order kinetics continues to be the model most often used to describe microbial and enzymatic inactivation, although low Estimates can be expected because non-linear trends are often observed experimentally (M. Corradini, Normand, Newcomer, Schaffner, & Peleg, 2009; Wilson et al., 2008). The change in survival rate over time is described by the integrated equation:

$$\frac{N^{(t)}}{N^{(0)}} = \exp(-kt) \qquad (4)$$

With $N_{(0)}$ the initial microbial population, $N_{(t)}$ the surviving microbial population after a treatment duration t and k the inactivation rate. By integrating log10 with respect to the high number of N, the resulting model (Eq. 5) states that the log N/N_0 will result in a decreasing straight line through the origin.

$$\log_{10}\left(\frac{N^{(t)}}{N^{(0)}}\right) = -\frac{t}{D_T} \quad (5)$$

D_T is the so-called decimal reduction time at temperature T, which corresponds to the treatment time required to reduce the surviving population by a factor of ten. It is expressed in minutes. This formulation allows the linearization of log10 ($N_{(t)}/N_{(0)}$) as a function of time and facilitates the identification of D.

Numerous models have been developed as alternatives to kinetic linear inactivation systems (M. van Boekel et al., 2010) Both mechanical and empirical have led to an adequate adjustment to the experimental data, but they are often too specific and/or complex (Mafart, Couvert, Gaillard, & Leguérinel, 2002).

Considering the asymmetric distribution of heat-sensitivity values within a spore population, Fernandez et al (1999) propose a model based on Weibull's statistical distribution function:

$$\frac{N^{(t)}}{N^{(0)}} = \exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right] \quad (6)$$

α and β are the two parameters of the thermoresistance distribution. α is called the scale parameter (characterized by time) and β is the shape parameter. Mafart et al. (2002) have reparameterized this model in a more interesting form whose parameters have a practical meaning for the inactivation of microorganisms:

$$\log_{10}\left(\frac{N^{(t)}}{N^{(0)}}\right) = -\left(\frac{t}{\delta}\right)^{\beta} \quad (7)$$

Where δ is called the time of the first decimal reduction. The shapes of curves are described by the parameter β (Figure 2): when $\beta < 1$ the curve is concave, when $\beta > 1$ the curve is convex and when the curve is log-linear β=1.

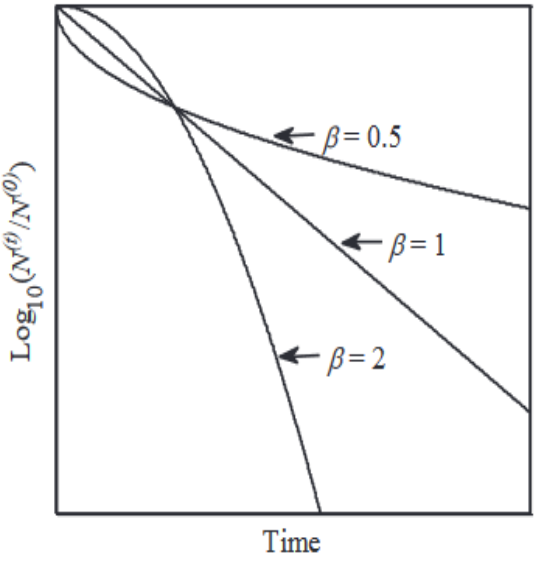

Figure 2. Simulation describing microbial inactivation using the model (Mafart et al., 2002) for the shape parameter $\beta < 1$, $\beta > 1$ and $\beta = 1$

The classical hypothesis that the inactivation of microbial spores follows first-order kinetics has been questioned by several authors (Geeraerd, Herremans, & Van Impe, 2000; M. van Boekel et al., 2010). However, the kinetics remain undoubtedly the most used for the calculation of pasteurization/sterilization scales in industry; it appears as a reference in microbiology text books.

1.2.1.2 Secondary modeling

To extend the application of models, so-called secondary mathematical expressions of models can be developed to estimate the pressure and/or the effect of temperature on the primary parameters predicted by the model.

As in the case of primary models, secondary models can be obtained from theoretical considerations or empirical observations.

Secondary modelling allows the simulation of the evolution of the primary model parameters (lag, μmax or Nmax) as a function of abiotic environmental conditions such as temperature, pH, aw, or biotic conditions such as competition between different microbial species (Drosinos, Mataragas, & Metaxopoulos, 2006) or the physiological state of the inoculum.

Biglew model

The Bigelow model was developed to obtain log-linear estimates of the decimal reduction time as a function of temperature. (Bigelow, 1921; Morales-Blancas, Torres, & Biological Engineering, 2003).

This model takes the parameter D from equation (2) whiche volves with a factor of 10 with respect to the treatment temperature. It is written in the following form :

$$D = D_{ref} 10^{\left(-\frac{T^{(t)} - T_{ref}}{z}\right)} \tag{8}$$

D_{ref} is the decimal reduction time at the reference temperature Tref. z represents the temperature increase to reduce the value of D by a factor of ten.

Arrhenius Model

Describes the effect of temperature on the speed of a chemical reaction. It is also used in microbiology to describe the effect of temperature on the inactivation constant k (s-1).

$$k = A\exp\left(-\frac{E_a}{RT}\right) \tag{9}$$

where A is a frequency factor and Ea is the activation energy of inactivation, which are constant in this model. R is the gas law constant. T is the absolute process temperature. The disadvantage of this model is that An' has no physical meaning, and from a statistical point of view there is a strong correlation between A and Ea which makes it difficult to converge during the procedure of identifying the parameters (M. van Boekel et al., 2010). This is why the reparameterized form is desired (Pritchard & Bacon, 1975; Schwaab & Pinto, 2007), and is written in the form:

$$k = k_{ref} \exp\left[-\frac{E_a}{R}\left(\frac{1}{T^{(t)}} - \frac{1}{T_{ref}}\right)\right] \tag{10}$$

Where k_{ref} is the inactivation rate at the Tref reference temperature. The reference temperature should preferably be chosen as the median of the studied temperature range. $T_{(t)}$ is the treatment temperature at time t.

1.2.2 Applications of predictive microbiology

The use of predictive microbiology has become widespread for several foods such as fish (K. J. J. o. f. p. Koutsoumanis, 2009), poultry (Dominguez & Schaffner, 2008), pork (Liu, Yang, & Li, 2006) and industrial applications are now numerous. Predictive microbiology can be used, for example, to determine and validate microbiological life spans based on simulations of the evolution of limiting microorganisms in the food studied (Dalgaard, 1995). It also makes it possible to optimize the formulation of recipes with regard to bacterial hazards and to adapt the manufacturing process for a given food while reducing the number of tests required obtaining the final product. Used in addition to other tools such as growth tests, predictive microbiology allows the extrapolation of results obtained in the laboratory to real food preservation scenarios (K. Koutsoumanis, 2001). In the HACCP approach, predictive microbiology can intervene at different stages of the process. For example, when determining the tolerance limits to be assigned to critical points, modeling can be used to evaluate these limits by associating for each level a contamination expected at the end of the shelf life. Thanks to predictive microbiology, it is also possible to determine in advance the corrective actions to be applied in case of failure, such as a modification of the heat treatment for example. Finally, since predictive microbiology is recognized by the control authorities, it is entirely possible to use the results of simulations as evidence to show an inspector that the product is safe (Thuault and Couvert 2009).

1.3 Microwave heating and biological systems

1.3.1 Microwaves and the electromagnetic spectrum

Many natural and artificial sources emit energy in the form of electromagnetic waves characterized by an electric field and a magnetic field. Depending on their frequency and energy, these waves can be classified as "ionizing radiation" (X-rays and gamma rays) or "non-ionizing radiation" (ultraviolet, visible, infrared, radiofrequency and microwave, low frequency) (Figure 3).

Microwaves (UHF: Ulra High Frequency) are in the frequency range between 300 and 3000 MHz. The microwave technology was born with the conception of the radar around 1930, then widely developed in many fields (industrial, scientific and medical)(Banik, Bandyopadhyay, & Ganguly, 2003; Vela, Wu, & Microbiology, 1979).

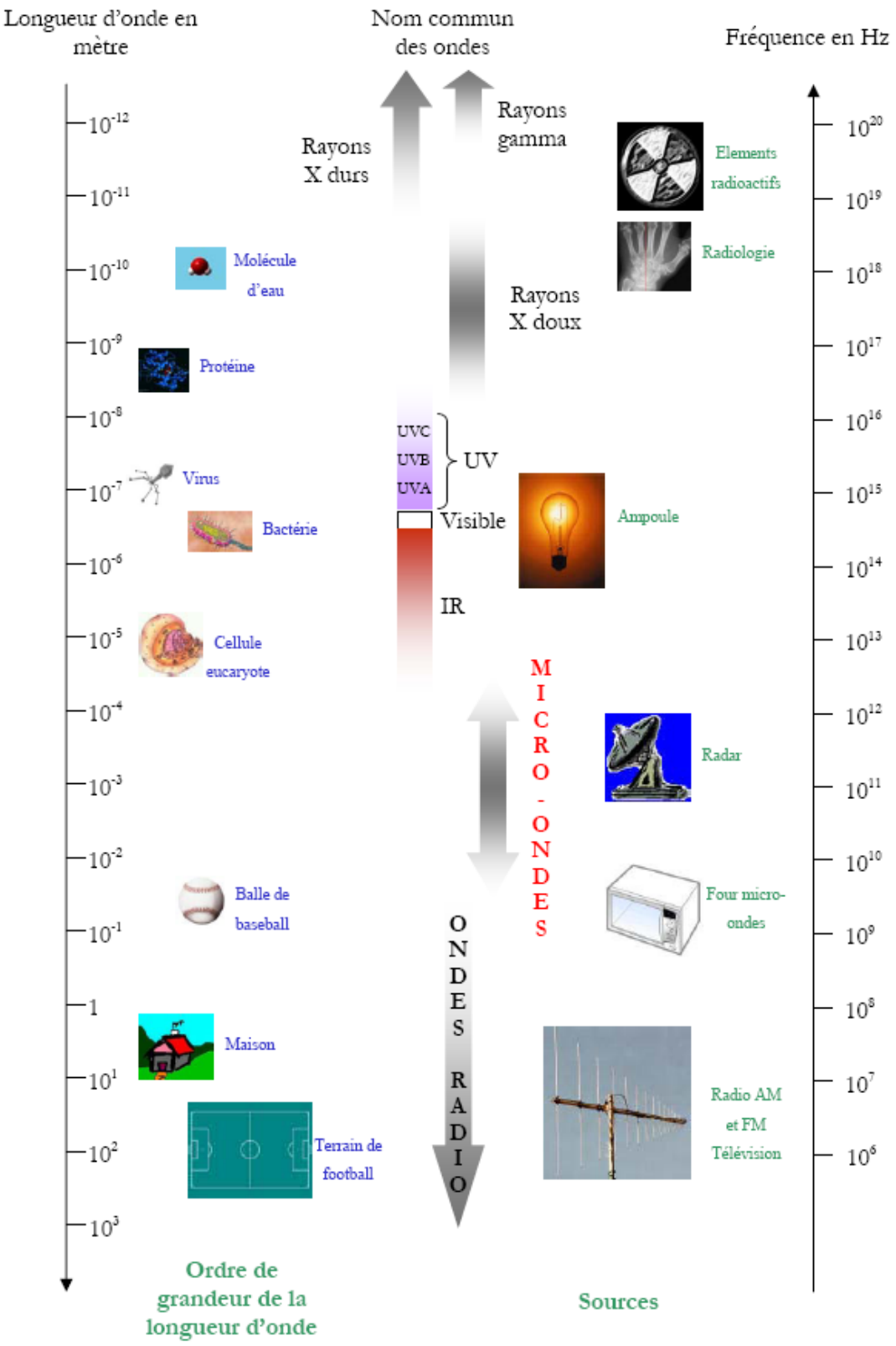

Figure 3. The electromagnetic spectrum and examples of order of magnitude and associated uses (Rougier, 2003)

In the field of food processing, the best-known process using the rapid heating properties of microwaves is the microwave oven. Other processes using microwave heating are developed for the decontamination of dry products and packaged products.

Microwaves have the ability to generate a rapid increase in temperature. The mechanism involved can be explained as follows; when a polar molecule is subjected to a microwave field, it seeks to align itself with the electric field of the wave. This field being alternating (2450 MHz), the molecule will try to rotate at the same frequency in order to remain aligned with the field. This is called coupling between the molecule and the field (Kozempel, Annous, Cook, Scullen, & Whiting, 1998). If the molecule is in a condensed medium (liquid, solid) it will be in interaction with its neighbors and thus will not be able to rotate without hindrance. The friction generated by the oscillation of all these molecules results in a rise in temperature (Figure 4). This phenomenon is also called loss by dielectric relaxation. (Hanna, 2008).

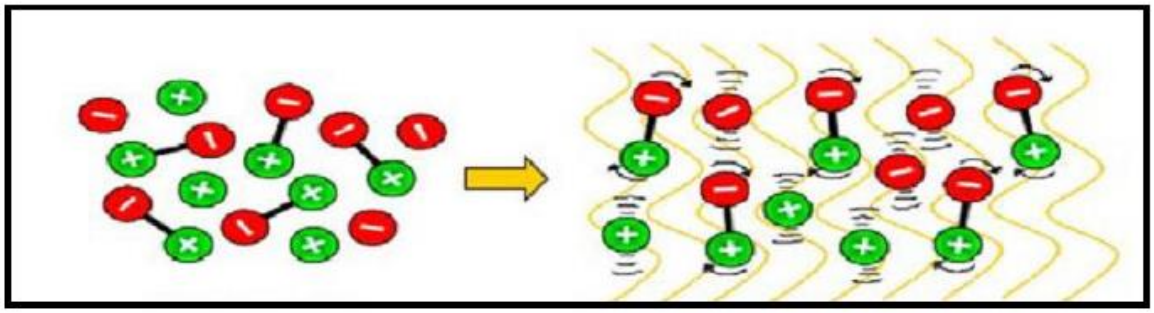

Figure 4. Microwave heating mechanisms (Ştefănoiu, Tănase, Miteluţ, Popa, & Procedia, 2016).

1.3.2 Microwave inactivation mechanisms

Microwaves have been widely studied as one of the alternative energy sources for sterilization or decontamination for many years. In addition, the widespread use of the microwave oven, which operates at a frequency of 2.45 GHz, has contributed to the development of studies on the interactions between microwaves and microorganisms at this frequency (Jeng, Kaczmarek, Woodworth, Balasky, & Microbiology, 1987; Rougier, 2003).

The existence of these effects has led to much controversy. For example, in the area of bacterial decontamination, studies (Atmaca et al., 1996; Salvatorelli et al., 1996) have shown that microwaves, applied under the same temperature conditions as traditional heating, have a

greater bactericidal effect than the latter. Some authors (Dreyfuss & Chipley, 1980; Khalil & Villota, 1988; Tajchakavit, Ramaswamy, & Fustier, 1998) believe that there is a non-thermal bactericidal effect of microwaves, while others (Fujikawa, Ushioda, & Kudo, 1992; Goldblith & Wang, 1967; Vela & Wu, 1979; Welt, Tong, Rossen, & Lund, 1994) refuteits existence. It seems that at present the existence of these non-thermal effects is accepted, but their nature and importance remain to be determined. Two mechanisms are proposed for the inactivation of microorganisms by microwaves:

- The first mechanism indicates that microwaves inactivate microorganisms entirely by heat through mechanisms comparable to other heat-induced processes, such as denaturation of enzymes, proteins, nucleic acids, or other vital components, as well as disruption of cell membranes (Heddleson & Doores, 1994).

- The second mechanism involves non-thermal effects. The possibility of non-thermal effects of microwaves remains a controversial topic (Dholiya, Patel, & Kothari, 2012).Researchers observe that microorganisms irradiated with microwaves are destroyed at temperatures below their Thermal Death Point and show greater damage than cells treated with conventional heating at similar temperatures (Eskicioglu, Terzian, Kennedy, Droste, & Hamoda, 2007). The authors suggested that the athermal effect of microwaves is related to changing protein structures by altering the direction of biochemical reactions (Duhan, Kar, Nain, Patel, & Dash, 2017).

1.3.3 **Action of microwaves on product quality**

The physical state of matter depends on its temperature and chemical reactions are accelerated by heat. Examples include Maillard and caramelization reactions that may occur following the release of juice from animal or plant tissues subjected to heat (Cheftel & Culioli, 1997).

Microwave treatment is a relatively rapid treatment, which allows us to say that the quality of the treated product is not greatly affected. Thanks to the short heating time, the characteristics of cooked and/or pasteurized products are close to those of a fresh product: color, taste, texture, vitamins.

Microwave treatment does not change the nutritional value any more than other cooking methods. As far as proteins are concerned, the same denaturation is observed as in any other heat treatment; there are no abnormalities in the "in vitro" or "in vivo" digestion processes. Fat

or fatty acid compositions are not particularly modified and oxidation phenomena would develop less than in the case of other conventional treatments.

The loss of vitamins (A, E) does not seem to be accentuated by microwave heating and in the case of water-soluble vitamins, there would even be an overall favorable effect on the preservation of these vitamins because the heat treatment is less severe (Finot, 1996).

1.4 Ultrasound treatment

1.4.1 Principle of ultrasound

Ultrasound, in its most basic definition, refers to pressure waves with a frequency of 20 kHz or more (Butz & Tauscher, 2002).

In general, ultrasonic devices use frequencies from 20 kHz to 10 MHz. Higher power ultrasound at lower frequencies (20 to 100 kHz) (Figure 5), which is called "power ultrasound", has the ability to induce cavitation, which has uses the inactivation of microbes (Piyasena, Mohareb, & McKellar, 2003).

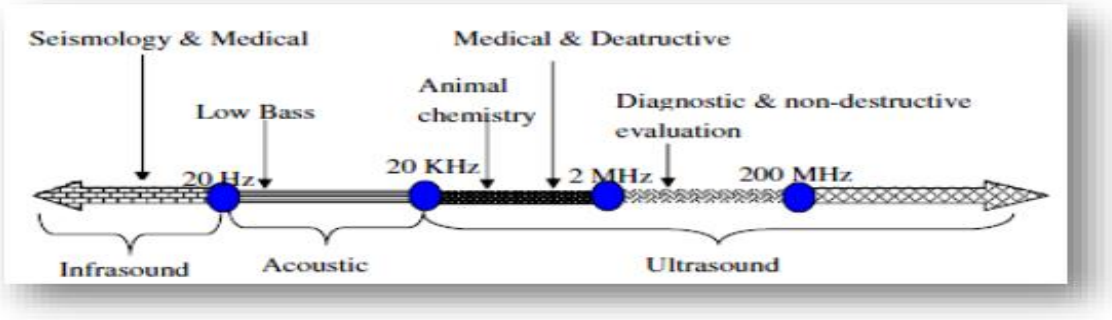

Figure 5. Diagram of the ultrasound ranges (Luque-Garcıa & De Castro, 2003)

However, in the ultrasonic process, cavitation caused by pressure variations created by the ultrasonic waves is responsible for the destruction of bacteria (Figure 6).

the frequency of ultrasonic waves is a crucial parameter, as it determines the size of cavitation bubbles (Giannakis et al., 2015), The literature suggests that the average size of the cavity is proportional to the acoustic power and inversely proportional to the ultrasonic frequency (Brotchie, Grieser, & Ashokkumar, 2009).

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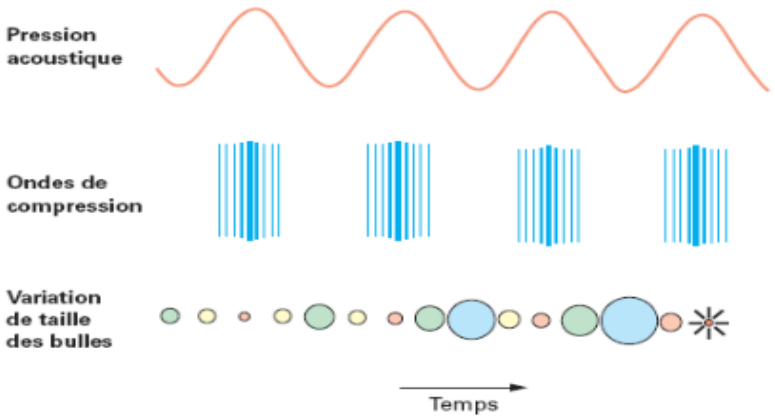


Figure 6. Generation, growth and bursting of bubbles resulting from the cavitation phenomenon (Draye, Estager, Kardos, & Pressure, 2019)

These waves produce strong cavitation in aqueous solution causing shock waves and reactive free radicals (OH, -HO, -O) by the violent collapse of the cavitation bubble (E. Joyce, Phull, Lorimer, & Mason, 2003) These effects contribute to the physical inactivation of microbial structures as well as the degradation of toxic elements (Antoniadis, Poulios, Nikolakaki, & Mantzavinos, 2007; Furuta et al., 2004).

1.4.2 Mechanism of action of ultrasounds

Ultrasound is capable of inactivating bacteria and clumps of de-clumping bacteria through a number of physical, mechanical and chemical effects (Furuta et al., 2004). Ultrasonic irradiation can eliminate surface contamination and biofilms (T. J. Mason & Lorimer, 2002).

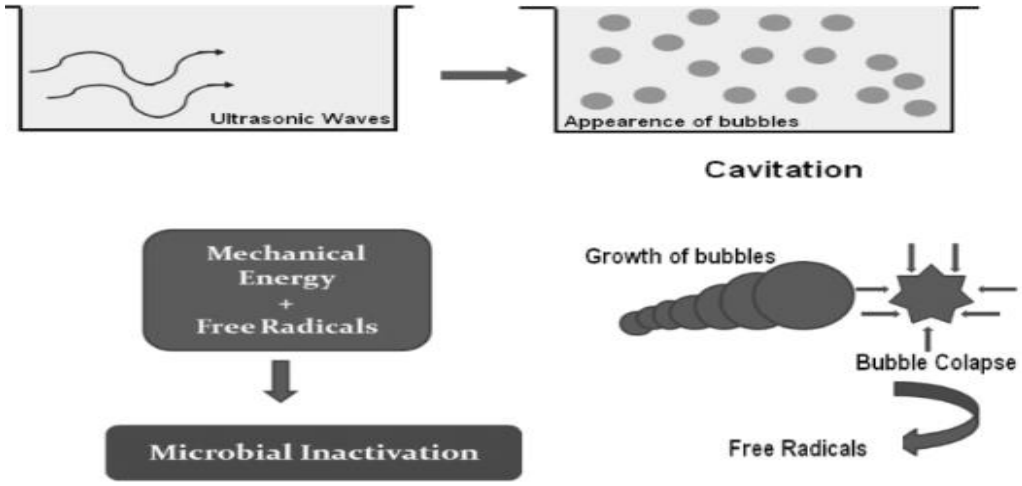


Figure 7. Ultrasonic waves and cavitation phenomenon (de São José et al., 2014)

Cell death is due to the high pressure and temperature caused by bubble collapse and the common forces that destroyed the bacterial cell membrane (Doosti, Kargar, & Sayadi, 2012).

Exposure to high mechanical pressure waves in liquids creates an acoustic current and subsequent acoustic cavitations that cause the formation, growth and implosive collapse of micro and nanobubbles in a liquid. These bubbles have a large specific surface area that increases gas diffusion and generates intense localized heating (about 5000°C) and high pressure (1000 ATM) (E. Joyce et al., 2003; Mahvi, Maleki, REZAEI, & Safari, 2009) The lethal effect of ultrasonication has also been reported for the reduction of *Yersinia enterocolitica*, spores of *Bacillus subtilis, Listeria monocytogenes*, Salmonella spp, *Aeromonas hydrophila, Legionella pneumophila, castellanii acanthamoeba, Saccharomyces cerevisiae, Pseudomonas aeruginosa* and *Staphylococcus aureus* (P. Declerck et al., 2010; Ince, Tezcanli, Belen, & Apikyan, 2001; Kalantar, Maleki, Khosravi, & Mahmodi, 2010; Kwak, Kim, & Rhee, 2011; Lee, Zhou, Liang, Feng, & Martin, 2009)

Some of the effects of ultrasound on the microbial cell are summarized in Table 3.

Table 3. Some effects of ultrasound on the microbial cell (Piyasena et al., 2003)

Ultraound	Effects on bacterials cells
Cavitation	-Thinning of bacterias -Pore formation and disruption of cell membranes.

	-Repture of cell wall structura and release of cytoplasmic content
The destruction by free radicals	-DNA repture and fragmentation

Several factors are involved in the effectiveness of ultrasound in the destruction of microorganisms, namely:

a)Factors intrinsic to microorganisms: It is generally accepted that the sensitivity of microorganisms increases with cell size, that bacilli are more sensitive than cocci, Gram-positive bacteria are more resistant than Gram-negative bacteria, and aerobic bacteria are more resistant than anaerobic bacteria (Sala, Burgos, Condon, Lopez, & Raso, 1995).

*b)Composition of the medium:*Pagán, Manas, Alvarez, and Condon (1999) compared the effects of ultrasound treatment (20 kHz, 200 kPa, 117 μm, 40°C) on Listeria monocytogenes in different media. They find that milk has a protective effect and that an acidic pH increases microbial inactivation. Similarly, the inactivation rate of an ultrasound treatment of *Salmonella typhimurium* in a model medium is 4 log, whereas it is only 2 log in milk, and less than 1 log in egg.

c)Duration of treatment : As with other physical treatments, the number of survivors after ultrasound treatment decreases exponentially with application time.(López-Malo, Guerrero, & Alzamora, 1999; Pagán, Mañas, Palop, & Sala, 1999),

d)Amplitude: The lethal effect of ultrasound treatments increases exponentially with the amplitude of the wave(Pagán, Manas, et al., 1999; Pagán, Mañas, et al., 1999; Palacios, Burgos, Hoz, Sanz, & Ordonez, 1991).

*e)Temperature:*The increase in temperature of a treatment of 30 minutes by ultrasound, from 0 to 40°C, decreases the population of Salmonella typhimurium, by 4 log in model medium, and by 3 log in milk (Wrigley & Llorca, 1992).

f)Pressure:When the ultrasound treatment is performed under the influence of pressure, one speaks then of mano-sonication, for low to moderate temperatures, and of manothermo-sonication, for high temperatures. It should be remembered, however, that the pressures used during this type of treatment (100 to 600 kPa), are much lower than the pressures used during high pressure treatments (100 to 1000 MPa) (Federighi, Vidon, Mescle, & Pilet, 1995).

1.4.3 Action on product quality

Power ultrasound can be applied to induce changes in liquid, solid and gaseous media.

In addition to microbial inactivation, ultrasound has other effects on food, and the purpose of its use is broad, namely; effect on foam formation (Zúñiga, Kulozik, & Aguilera, 2011), degassing/air removal (Laborde, Bouyer, Caltagirone, & Gérard, 1998), and the effect on the formation of foam (Boistier-Marquis, Lagsir-Oulahal, & Callard, 1999; Matsuura, Hirotsune, Nunokawa, Satoh, & Honda, 1994; Zúñiga et al., 2011), Cooking (Pohlman, Dikeman, & Zayas, 1997), Tenderization of Meat (Pohlman, Dikeman, & Kropf, 1997), Brining and Pickling (Hatloe 1995), Drying (Chemat & Khan, 2011; Gallego-Juárez, 1998), Extraction (Chemat, Tomao, & Virot, 2008), , Homogenization/emulsification (Chendke & Fogler, 1975; Mongenot, Charrier, & Chalier, 2000); Cutting (C. Schneider et al., 2009; P. M. Schneider et al., 2008), Cleaning (Chemat & Khan, 2011), Enzyme inactivation (Earnshaw, Appleyard, & Hurst, 1995).

1.5 Orange juice

1.5.1 Orange juice manufacturing processes

1.5.1.1 Manufacturing process of pure orange juice

The orange juice industry involves a large number of operations that can be grouping into three sectors: agricultural production, mining and quarrying industry, and the production and distribution industry. packaging and the chain of storage, transport and marketing of the packaged juice.

1.5.1.1.1 Juice extraction

Before the extraction of the orange juice two primordial stages are carried out namely; The picking (the quality of the orange juice depends largely on the properties of the oranges used (Hendrix & Redd, 1995; Nagy & PE Wardowski, 1990) and the washing and brushing (in order to remove the dust, micro-organisms and other foreign matters).

In most cases, citrus juice extractors mimic the operation carried out with a domestic juicer, with the fruit being cut in half.

1.5.1.1.2 Refining and centrifugation

The orange juice, after extraction, is very pulpy and contains pieces of seeds and other impurities. It then goes through a refining stage. This is the physical separation of some of the pulp and other fibrous material from the juice. The juice can then be centrifuged to refine a fine pulp content of 6-12 %, resulting in a juice with a viscosity that meets consumer expectations (Braddock, 1999).

Finally, prior to heat treatment, the juice is heated to 50ºC in heat exchangers (Braddock, 1999). heat pipes and then subjected to a deaeration process in vacuum tanks. This operation has the advantage for the industrialist to avoid the formation of foam and to avoid oxidation of the product. The juice, once degassed, should not be stored more than one hour before the next step of pasteurization.

1.5.1.1.3 Pasteurization

Pasteurization is the heat treatment that is most used for the preservation of fruit juices. This pasteurization aims to kill microorganisms, and to inactivate enzymes (such as pectin methylesterase (PME) or polyphenoloxidase) that can alter the product or make it unfit for human consumption (Chen, Peng, & Chen, 1995). It is carried out according to a time-temperature schedule that can vary but generally lasts from 30 to 60 seconds. For pure juice, the temperature is quickly raised to 90-96ºC in tubular heat exchangers and then drops in about 30 seconds to a temperature of a few degrees, this is "flash pasteurization" and is the technique used by NCA Ruiba.

1.5.1.1.4 Transportation

The pure pasteurized juice can be packaged on the production site right after the heat treatment, as is common practice in Spain. It can also be stored for up to 12 months in aseptic tanks equipped with a refrigeration system or transported after manufacture in tankers (refrigerated or not) to the packaging plants.

1.5.1.2 Process for the production of orange juice from concentrate

1.5.1.2.1 Pasteurization after extraction and refining

In order to manufacture the concentrate, the juice is extracted in the sameway as described in paragraphs 1.5.1.1.1 and 1.5.1.1.2.

For pasteurization, it is carried out in flash-pasteurization (95°C/30 s) with a longer temperature drop compared to pure juice, and the juice remains warm until the next stage of concentration (is not completely cooled).

1.5.1.2.2 Concentration and Freezing

The concentration operationconsists of removing about 80% of the water contained in the juice, with as little alteration as possible to the pulp and flavour compounds. The most commonly used process is concentration by the combined effects of heat and vacuum (evaporation) in heatex changers that separate the vapours formed from the concentrated liquid product.

The resulting orange juice concentrates are first rapidly cooled down to 0°C, and then frozen in heat exchangers ; the resultingdoughy mass is cooled to -40°C, thenstored at a temperature not exceeding -18°C.

1.5.1.2.3 Transport of Frozen Concentrate

The frozen concentrate can be transported in bulk in tanker trucks to packaging companies. It can also be dedicated to export, in which case the tankers unload the concentrate in tanks located in trading ports commercials.

The concentrate is then loaded into ships and crosses the oceans, for example from Brazil to Europe. In Europe, the concentrate is transported back to the packaging plant by tanker trucks. The concentrate can also be stored in metal drums containing a plastic pouch of polyethylene.

1.5.1.3 Packaging

1.5.1.3.1 Pure orange juice

After flash-pasteurization, the juice is cooled to 82-85ºC. At this temperature it is introduced into the containers. These are immediately closed, turned over or stirred so that the hot liquid comes into contact with the entire inner surface of the container and sanitizes it.

Cold aseptic filling is another filling technique that consists of cool the juice to room temperature (17-22ºC) after flash-pasteurization and to fill and close the containers under aseptic conditions. The bottles have at previously decontaminated by washing with a hydrogen peroxide solution, or of peracetic acid then rinsed with water.

1.5.1.3.2 Juice from concentrate

The concentrate is transported to another site with non-aseptic trucks. At the conditioner, a new pasteurization is thus essential to eliminate any risk. microbiological. This pasteurization takes place after re-flavouring with an oily phase and an aqueous phase.

The oily phase is reincorporated into the concentrate which is then diluted in the water. with water to return to a Brix degree of 11-12. An aqueous phase is then added, the juice is degassed and then pasteurized for about 30 s at 95ºC in the case of flash-pasteurization. The two conditioning processes used today in the packer after flash-pasteurization are, as for pure juice, either hot filling or flash pasteurization. cold aseptic filling. Thus, the production processes for the fruit juices that come to be described show that the fruit undergoes a first heat treatment to pass from the fruit" stage to the "fruit juice" stage. Then, these juices must undergo other treatments to allow their conservation and packaging and to guarantee their quality.

Microbiological until the moment of consumption. This is particularly the case for juice from concentrate. These multiple treatments will affect the quality of the orange juice, which will then be kept for several months.

The last phase of production, this phase is divided into two categories of operations according to the type of packaging; Tetra pack (Plastic / Cardboard /Aluminum) which are They are used for packaging fruit juice, UHT milk, butter, yoghurt, soup, feta ... (MULTON, BUREAU et al, 1998), or PET, and in this case the material used is a PET bottle with two layers of semi-rigid protection, which has undergone an anti-oxygen and anti-UV treatment with a suitable viscosity and a rigid PE cap.

1.5.2 Major contaminants

Fruit juices contain a wide variety and a large complex mixture of nutrients for health. These nutrients provide good conditions for the growth not only of spoilage microorganisms, but also of foodborne pathogens. Depending on the type of juice, *Escherichia coli*, *Listeria monocytogenes*, Cryptosporidium and Salmonella are considered relevant pathogens for

processes to control the effectiveness of disinfection treatments (Lima Tribst, de Souza Sant'Ana, & de Massaguer, 2009). Fruit juice treatments are based on thermal pasteurization and their inherent acidity. However, concerns about their microbiological safety have been raised due to a number of pathogen-related outbreaks, including *E. coli* O157:H7 (Eblen et al., 2004).

1.5.2.1 The "*E. coli*" contaminant

Escherichia coli, otherwise known as *E. coli*, is a gram-negative bacterium. It is a mesophilic bacterium, a bacillus in the form of elongated rods (Flandrois, 2000). It multiplies at temperatures between 7 °C and 50 °C, the optimal temperature being 37 °C. Some strains grow in acidic foods, up to a pH of 4.4

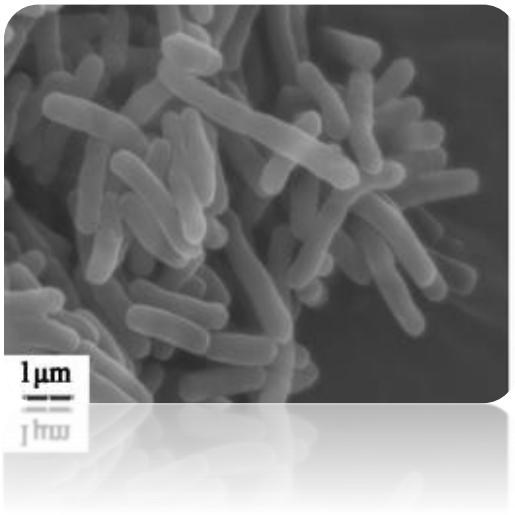


Figure 8. *Escherichia coli* under scanning electron microscope (resolution 1um) (Henderson, 2019)

Commensal strains of *E. coli* are non-pathogenic (except for opportunistic infections); however, there are pathogenic strains of *E. coli* that have specific virulence factors,separated into four groups or "pathovars" depending on the type of infection:

- **Enterohemorrhagic *E. coli* (EHEC or STEC)**

Shigatoxin-producing *Escherichia coli* (*E. coli*) (or STEC, "Shiga-Toxin-producing *E.coli*") is a bacterium commonly found in the digestive tract of humans and warm-blooded animals. Most strains are harmless. However, some, such as shigatoxin-producing *E. coli*, can cause serious foodborne illness. Transmission to humans occurs mainly through the consumption of

contaminated food, such as raw or undercooked ground meat, raw milk, raw vegetables and contaminated sprouted seeds. *E. coli* O157:H7 is the most important serotype for public health (OMS. 2018) . Shiga toxin-producing *E. coli* that is pathogenic to humans is part of *Escherichia coli* 0157:H7.

The code O157:H7 corresponds to the identification of a particular phenotype of surface proteins of the bacterium. Other *Escherichia coli* can give the above pathologies but it is the O157:H7 type that is most often implicated. Most *Escherichia coli* are benign.

- **Enteropathogenic *E. coli* (or EPEC, 'EnteroPathogenic *E. coli*)** are largely responsible for childhood diarrhea in developing countries
- **Enterotoxinogenic *E. coli* (or ETEC "Entero Toxinogenic *E*. coli")** are responsible for traveler's diarrhea ("tourista"), and also the main cause of childhood diarrhea in developing countries.
- **Enteroinvasive *E. coli* (or EIEC "Enteroinvasive *E. coli*")** are responsible for a condition located primarily in the colon. This condition is similar to shigellosis.

1.6 Wastewater Dairy effluent

The milk processing industry is one of the basic industries in the world, which is why the possibilities for treating dairy effluents are attracting more and more attention.

With the rapid industrialization observed in the last century (Nadais, Capela, Arroja, & Hung, 2010) and the growth rate of milk production (about 2.8% per year), dairy processing is generally considered to be the largest source of industrial food waste water, especially in Europe (Britz, Van Schalkwyk, & Hung, 2006).

Milk processing effluents mainly include milk or dairy products lost in technological cycles (spilled milk, spoiled milk, skim milk and pieces of curd); starter cultures used in manufacturing; by-products of processing operations (whey, milk and whey permeates) ; contaminants from washing trucks, tanks, cans, equipment, bottles, and soils; reagents applied in CIP procedures, cooling of milk and dairy products, for sanitary purposes, equipment damage, or operational problems; and various additives introduced into manufacturing (Doble & Kumar, 2005; Schifrin, Ivanov, Mishukov, dairy industry. Moscow, & Industry, 1981; Tawfik, Sobhey, & Badawy, 2008; Watkins & Nash, 2010). The loss of milk to wastewater is

approximately 0.5-2.5% of processed milk, but can be as high as 3-4% (Janczukowicz, Zieliński, & Dębowski, 2008).

Wastewater from dairy products consists of complex components (Borja, Banks, & toxicology, 1995; Demirel, Yenigun, & Onay, 2005). Although milk manufacturing produces waste streams similar to milk and dairy product losses, each process results in an effluent that is unique in volume and composition (Nadais et al., 2010).

1.6.1 Majors contaminants

A wide range of microbial profiles have been identified in food effluents, including *Cryptosporidium parvum*, Giardia sp., *Escherichia coli, Clostridium perfringens*, *Enterococcus faecalis*, Salmonella sp.etc (P. M. Chapman, 2000; Dungan & Leytem, 2013; Ibekwe & Grieve, 2003).

The predominant species have been shown to be *E. faecalis* (39%) and *E. faecium* (29%) in samples from environmental sources (compost, sewage, sediment and pool water), followed by *E. durans*/*E. hirae*, *E.casseliflavus*/*E. gallinarum* and *E. raffinosus*, with different prevalence of species depending on the source (Pinto, Pierotti, Canale, & Reali, 1999).

1.6.1.1 The contaminant « *Enterococcus faecalis* »

Enterococcus are coccoids, Gram+, mesophilic, which develop in a temperature range from 10 to 45 °C, with an optimal temperature of 35 °C. Some species can survive at 60°C for 30 min. They grow under hostile conditions of 6.5 % NaCl, 0.1 % methylene blue milk, 40% bile salt concentration and a pH range of 4.4 to 9.6 (Aguilar Galvez, Dubois Dauphin, Destain, Campos, & Thonart, 2012; Schleifer, Kilpper-Bälz, & Microbiology, 1984). The cells are ovoid and occur as isolated cells, in pairs or as a chain (Schleifer et al., 1984).

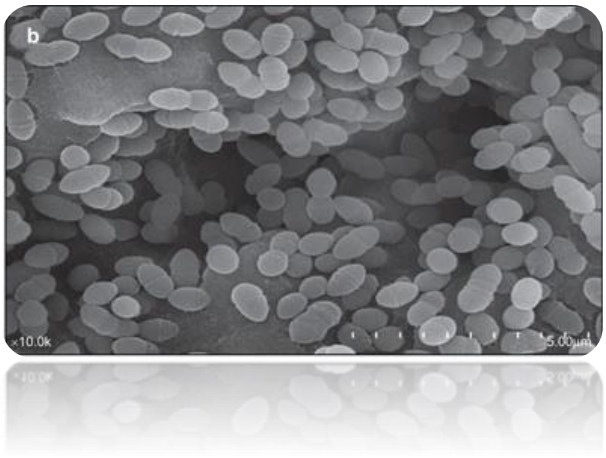


Figure 9. *Enteroccus faecalis* under scanning electron microscope (resolution 5um) (H.-S. Kim et al., 2013)

Enterococcus faecalis are lactic acid bacteria present in cheeses (Giraffa, 2003), meats that are contaminated during slaughter as they belong to the commensal microflora of the gastrointestinal tract of animals (Franz, Holzapfel, & DEKKER-, 2004). And fermented vegetables (Omar et al., 2004)**.**

Enterococci, mainly *Enterococcus faecalis* , and *Enterococcus faecium* , have probiotic potential, producing bacteriocins (substances that eliminate certain microorganisms or inhibit their growth) called enterococins. These enterocins are effective against foodborne pathogens such as *Listeria monocytogenes* (responsible for listeriosis). At the very least, before using *E. faecalis* or *E. faecium*, their presumption of safety must be proven by correctly identifying the factors that may promote the emergence of the opportunistic pathogenic trait, as well as the ability of these species to acquire genes from genetically related species (Ferreira et al., 2007).

Enterococcus faecalis is not a very virulent bacterium compared to Staphylococcus or Pneumococcus. To become pathogenic, she needs to express virulence characteristics associated with adhesion, translocation and disappearance of the immune response. Problems between enterococci and humans are their gastrointestinal origin, their entry into the food chain, their resistance to antibiotics and their possible role in foodborne diseases. Other problems include their ability to exchange genetic material or, for some strains, to produce large quantities of biogenic amines associated with fermentation (Jett, Huycke, & Gilmore, 1994; Omar et al., 2004).

E. faecalis is responsible for 80-90% of Enterococcus infections (Kayser, 2003). Indeed, these are markers of faecal contamination (*Enterococcus faecalis* and *Enterococcus faecium*) also involved in the appearance of nocosomal diseases. The bacteria's high level of natural resistance to antibiotics contributes to its pathogenicity (Ryan & Ray, 2004).

2 MATERIALS AND METHODS

2.1 Inactivatation of *E. coli* ATCC 25922 in orange juice beverage

2.1.1 Search for *E. coli* in the orange juice beverage

E. coli ATCC 25922 was chosen as a model organism because outbreaks involving pathogenic *E. coli* have been observed in unpasteurized or inadequately pasteurized orange juice and is the target organism for juice pasteurization (Mazzotta, 2001).

E. coli ATCC 25922, a substitute for *E. coli* 0157:H7, is ideal because it has similar thermal inactivation kinetics to *E.coli* 0157:H7 (Duffy, Churey, Worobo, & Schaffner, 2000).

Under sterile conditions, 1 ml of the well-stirred ROUIBA orange juice beverage is flooded on a Petri dish containing Eosin methylene blue medium (EMB), which is incubated at 37°C for 48 hours.

According to the "JORA Standards Nº. 35 27-05-1998" no presence of *E. coli* is tolerated in orange juice (the list of microbiological standards is presented in the Table 4).

Table 4. List of microbiological standards according to "JORA Standards No. 35 27-05-1998"

	Total germs		**Coliform**		**Yeast**	**Mould**
	37°C	**22°C**	**Total**	**Fecal**		
Standart (JORA N°35 du 27-05-1998)	20	< 100	Absent	Absent	< 20	10
Incubation temperature (°C)	37	22	37	44	30	30
Incubation time (hour)	48	48	48	48	120	120
Medium of Culture	PCA	PCA	VRBL	VRBL	OGA in depth	OGA on the surface

Reading	Mass lenticular shape	Mass lenticular shape	Gaseous release, Color change (yellow)	Gaseous release, Red ring on the surface	Appearance at the surface	Appearance at the surface

2.1.2 Bacterial strain and culture conditions

Experiments were conducted on *Escherichia coli* ATCC 25922 obtained from Pasteur Institute Collection (Alger, Algeria). The strain was maintained on tryptone soy agar (TSA; Conda, pronadisa, spain) at 4°Cuntil used. Young cultures were prepared by suspending colonies in Tryptone Soy Broth (TSB; Conda, pronadisa, spain) and incubated at 37°C for 18 hours. The bacterial cells were then recovered by centrifugation (4000 x g for 15 min at 4°C) (Cabassi, Falanga, & Romani, 2017).

The inoculation of the *E. coli* ATCC 25922 strain was adjusted to a final concentration of 1.5 x 10^8 colony forming units (CFU/ml) in the orange juice beverage ; as verified by a spectrophotometer (OD_{600}=0.08-0.13) (Pourhajibagher et al., 2016).

2.1.3 Orange juice beverage inoculation

The experiments were performed using commercial orange juice beverage with a pH of 3.27 and soluble solids of 12°Brix. Orange juice beverage was filtered, to remove the pulp, and inoculated with the concentrated suspension of *E. coli* ATCC 29522 previously prepared. Inoculated juice was then adjusted to a final concentration of approximately 1.5 x 10^8 CFU/ml (Cabassi et al., 2017).

2.1.4 Microonde equipement and traitement

Irradiation was carried out in a house hold microwave (samsung GE614ST, MALAYSIA) showed in Figure 10. The microwave device is equipped with a turntable (diameter 280 mm) at a speed of 2.46 revolutions per minute. It emits eight different power ratings of 100, 180, 200, 300, 450, 600, 750 and 900 W at 2450 MHz.

Figure 10. Images of the microwave used during the inactivation experiments.

Microwave irradiation of a volume of 40 ml of orange juice beverage containing a bacterial load of 1.5×10^8 CFU/ml in a 100 ml beaker ; at 300 and 600 W are carried out at 5 s, 10 s, 15 s, 20 s, 25 s, 30 s and 35 s.

The beaker is placed in the centre of the microwave turntable ; and is placed directly into an ice tray after each treatment.

2.1.5 Ultrasound equipement and traitement

The same bacterial load in the same volume described above have been treated in an ultrasonic bath with a frequency of 42 kHz at different times (5, 10, 15, 20, 30, 45 and 60 min). The temperature of the ultrasonic batch was maintained below 20°C by the addition of ice cubes.

Figure 11. Images of the ultrasound used during the inactivation experiments.

2.1.6 Irradiation by microwave/ultrasound ; ultrasound /microwave coupling

In order to carry out treatments by coupling microwave and ultrasound on *E. coli* ATCC 25922 in the orange juice drink; the powers of the microwaves used are: 300 W, 600 W and 900 W, with ultrasound at 10 min; 20 min and 30 min either in post or in pretreatment. All experiments were carried out in triplicate.

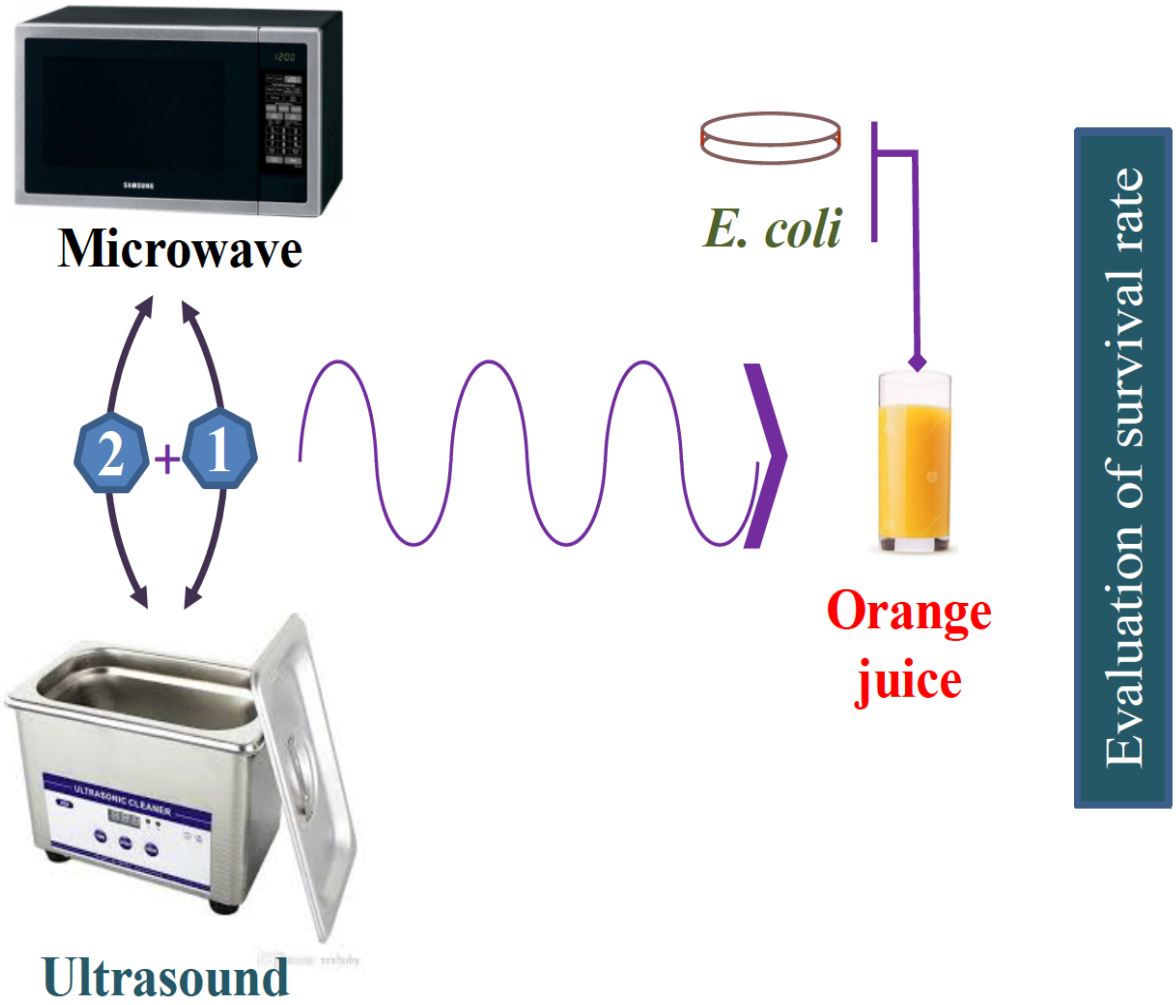


Figure 12. Effect of ultrasound and microwave simultaniously on *E. coli* ATCC 25922 in orange juice beverage

2.1.7 Determination of physico-chemical parameters

2.1.7.1 pH

The pH measurement is based on the potential difference between a glass electrode and a reference electrode immersed in the product. This determination is carried out using a pH meter. The pH meter is calibrated using a buffer solution, a sufficiently large volume V of the formulated drink is taken as a test sample to allow the electrode to be immersed, the pH value is then noted.

Take at least 3 readings for each test.

2.1.7.2 Degree Brix

The refractometer is a measuring device that determines the refractive index of light of a solid or liquid matrix. This index is observed by the deviation of a light beam according to the nature of the medium in which it is propagated. The angle of the beam deflects according to the rate of soluble dry matter (sucrose) in the medium, the higher the concentration of soluble dry matter, the greater the refraction. Soluble solids were measured using a refractometer, refractive index was recorded and converted to °Brix.

The refractive index is read with a hand refractometer (OTAGO)

2.1.7.3 Measurement of acidity

Consists of an acidity titration with a solution of sodium hydroxide (NaOH) in the presence of phenolphthalein as a color indicator (NF V 05-101,1974).

Take 10 ml of juice in a beaker, then add 1ml (1%) phenolphthalein solution. While stirring, pour the 0.1 N sodium hydroxide (NaOH) solution with a burette, until a pink color is obtained

Acidity measurement formula:

$$\text{Acidity} = V \times 0.64 \quad (11)$$

Where :

- V is the volume of NaOH used.

- 0.64 is the coefficient of acidity

2.2 Inactivation of *Enterococcus faecalis* ATCC 29212 in waste water dairy effluent

2.2.1 Preparation of model effluent

Model dairy effluent of pH 6.0 was prepared as reported by Daverey, Pakshirajan, and biotechnology (2011) containing 2 g/l semi-skimmed milk powder (Régilait; France), 0.2% (w/v) milk fat (Ghee; nature foods, Portugal), 0.01% (w/v) sodium hydroxide (Sigma) and sterile distilled water. The milk powder consisted of 31% proteins, 45% carbohydrates, 14% Fat content with saturated fatty acids 9%, 0,92% salt.

2.2.2 Bacterial strain and culture conditions

Experiments were performed with *Enterococcus faecalis* ATCC 29212 collected from the Pasteur Institute (Algiers, Algeria). The strain was maintained on tryptone soy agar (TSA; Conda, pronadisa, spain) at 4°Cuntil use. Young cultures were prepared by suspending colonies in Tryptone Soy Broth (TSB; Conda, pronadisa, spain) and incubated at 37°C for 18 hours. Bacterial cells were then recovered by centrifugation (4000 x g for 15 min at 4°C) (Cabassi et al., 2017; Muñoz-Cuevas et al., 2013).

The inoculation of the *E. faecalis* ATCC 29212 strain was adjusted to a final concentration of 1.5×10^8 colony forming units (CFU/ml) in the dairy effluent; as verified by a spectrophotometer (OD_{600}=0.08-0.13) (Pourhajibagher et al., 2016)**.**

2.2.3 Microwave equipement and treatement :

The irradiation was performed in a house hold microwave (whirpool talent, MT263, Malysia) with a rotating plate (diameter 280 mm) at a speed of 2.46 rpm. The equipment emits nominal powers of 90, 160, 350, 500, 650, 750 and 850 W at 2450 MHz.

Microwave irradiation of a 40 ml volume of sterilewastwaterdairy effluent containing a bacterialload of 1.5×10^8 CFU/ml in a 100 ml beaker ; at 350 and 650 W is performed at times 10, 20, 30, 40, 50, 60 and 75s.

The beaker is placed in the center of the microwave turntable ; and is placed directly in an icetray after each treatment.

2.2.4 Ultrasounde quipement and traitement

The same bacterial load in the same volume described above were treated in an ultrasonic bath of a frequency of 35 kHz, at the following time: 5, 10, 15, 20, 30, 45 and 60 min. taking into account the temperature which does not exceed 20°C which is controlled by the addition of ice cubes in the ultrasonic bath.

2.2.5 Microwave and ultrasound combination treatments

Microwave and ultrasound coupling treatments on *E. faecalis* ATCC 29212 in the dairy model effluent are performed at microwave powers of 350 W and 650 W with ultrasound at 10 min; 20 min and 30 min either in post or pretreatment. All experiments were performed in triplicate.

Immediately after treatment by ultrasound and microwave, the becker containing 40 ml bacteria suspensions was placed in ice bath for 10 min , the colonies were determined by enumeration.

For microbial numeration, the samples were taken after treatment; this treated samples were serially diluted in sterile physiological water and spread plated onto gelose Eosin methylene blue medium EMB for *E. coli* ATCC 25922 and Muller Hinton for *E. faecalis* ATCC 29212.

Surviving bacterial count was determined after 24h of incubation à 37°C. All experiments were performed in triplicate.

2.2.6 Protein dosages

The amount of proteinreleasedinto the extracellular medium, after exposure or no bacterial cells to microwaves and/or ultrasound was performed using the Bradford method (Bradford, 1976), with the reagent marketed by the company BIO-RAD.

2.2.6.1 Macro method

This technique makes it possible to measure protein quantities between 0.2 and 1mg/ml. The assay consists, in a first step, in adding 100 µL of sample, properly diluted, to 5 ml of Biorad reagent diluted 1/5. The mixture, after homogenization, is then placed in the dark for 5 min and the absorbance is measured at 595 nm (Uvikon 922, Kontron).

Protein concentrations were calculated by linear interpolation from a standard range containing bovine serum albumin (Appendix II).

Standard range :

BSA stock solution at 1mg/ml

Table 5. Calibration range for protein determination by the macro-method

BSA (mg/ml)	0	0.2	0.4	0.6	0.8	1
BSA (µl)	0	20	40	60	80	100
Demineralized water (µl)	100	80	60	40	20	0

2.2.6.2 Micro method

This technique allows to measure protein quantities between 2 and 10µg/ml. The assay consists of adding 800µl of sample, suitably diluted, to 200µl of Biorad reagents. The mixture, after homogenization, is then placed in the dark for 5 min and the optical density is measured at 595 nm.

Standard orange:

BSA stock solution at 10 µg/ml

Table 6. Calibration range for protein determination by the micro-method

BSA (µg/ml)	0	2	4	6	8	10
BSA (µl)	0	160	320	480	640	800
Demineralised water (µl)	800	640	480	320	160	0

2.2.7 Observation under a scanning electron microscope (SEM)

Cells of *E. faecalis* ATCC 29212 (incorporated in dairy wastewater effluent) treated under ultrasound and microwave (the conditions of inactivation: MW (650 W/ 20 s), MW (650 W/ 40 s), MW (650 W/ 60 s), US (30 min), US (30 min)/ MW (650 W/ 20 s), US (30 min)/ MW (650 W/ 40 s), US (30 min)/ MW (650 W/ 60 s) and their controls (untreated cells)) were visualized by scanning electron microscopy (SEM) to assess the impact of the treatments on the morphology and integrity of the bacteria.

Following treatement, dairy wastewater effluent (containing the bacteria) were filtered through 0.45 µm cellulose acetate filters (Sartorius Stedim Biotech, Germany) and *E. faecalis* ATCC 29212 cells retained in the filters (together with the pulp) were fixed and dehydrated. Fixation was performed by immersing the filters in a 2.5% (v/v) glutaraldehyde (Sigma-Aldrich) solution for, at least, 1 hour. Afterwards, glutaraldehyde solution was removed and the filters were washed with deionized water and dehydrated by a graded series of ethanol (30, 50, 70, 80, 90 and 100% (v/v)), by immersing the filters in each solution for 10 min before removing it and substituting by the following. After removing the 100% ethanol solution, a couple of drops of hexamethyldisilazane (HMDS; Sigma-Aldrich, United States) were poured over the filters and immediately evaporated with a stream of nitrogen. Sections of the dried filters were cut and placed on observation stubs, covered with a double sided adhesive carbon tape (NEM tape; Nisshin, Japan), and coated with gold/palladium. Observation was performed in a JEOL JSM-5600LV (JEOL, Japan), with an acceleration voltage of 30 kV and a spot-size of 25.

2.2.8 Incubation of the treated samples and survival estimation

Immediately after treatment by ultrasound and microwave, the becker containing 40 ml bacteria suspensions was placed in ice bath for 10 min , the colonies were determined by enumeration.

For microbial numeration, the samples were taken after treatment; this treated samples were serially diluted in sterile physiological water and spread plated onto gelose Eosin methylene blue medium EMB for *E. coli* ATCC 25922 and Muller Hinton for *E. faecalis* ATCC 29212 (Appendix I).

Surviving bacterial count was determined after 24 h of incubation à 37°C. All experiments were performed in triplicate.

2.2.9 Modeling inactivation kinetics and statistical methods

Several models can be applied, but the Weibull model has been more widely accepted because of its flexibility, simplicity and convenience (M. G. Corradini & Peleg, 2004).

The survival rate at time *t*, denoted *S*, was calculated as the change in the ratio of viable *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 at any time $N^{(t)}$ (CFU/mL) to the initial number of microorganisms $N^{(0)}$ (CFU/mL) as control:

$$S = \frac{N^{(t)}}{N^{(0)}} \tag{12}$$

The decrease in the log-survival rate data of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 obtained in sonication and microwave treatments was described by the modified Weibull model (Mafart et al., 2002).

$$log(S) = -\left(\frac{t}{\alpha}\right)^{\beta} \tag{13}$$

Where α is the time of the first decimal reduction, can be called scale parameter, and β is the so-called shape parameter. In order to compare the time of the first decimal reduction of the obtained inactivation kinetics: equation (13) was fitted for a second time by setting β equal to the mean of the previously estimated β.

Modified Weibull model parameters were estimated using nonlinear regression with a curve fitting toolbox (MATLAB 6.5, The Math-Works Inc., Natick, MA, USA).

2.2.9.1 Estimation of parameters

In order to model the experimental data of the inactivation kinetics, a non-linear regression analysis was performed (Weibull) using Matlab software (version 7, The MathWorks Inc, Natick, MA, USA). The coefficient of determination (R^2) and Root Mean Square Error (RMSE) were used to determine the ability of the tested mathematical models to represent the experimental data.

The parameters of the non-linear models were iteratively adjusted by minimizing the Mean Square Error (MSE) between measured (y) and predicted (ŷ) values using the Nelder-Mead simplex method (Lagarias et al., 1998) using the "fminsearch" function in Matlab software (version 7, The MathWorks Inc, Natick, MA, USA). IEM is called an objective function

$$MSE = \frac{1}{(n-p)} \sum_{i=1}^{n} \left(\overline{Y}^{(t)} - Y^{(t_i)} \right)^2 \qquad (14)$$

With n the number of experimental data, and p the number of model parameters.

The confidence interval for each parameter was determined by the Monte Carlo method (Hessler, 1997) with 2000 to 5000 simulations. The square root of the sum of the deviations (RMSE, Root Mean Square Error) between the experimental data and the predicted data was used as an indicator of the goodness of fit of the model. The correlation between the parameters of each model was evaluated using the Matlab corrcoef function (version 7, The MathWorks Inc, Natick, MA, USA).

The root mean squared error (RMSE) between all experimental and predicted data, adjusted coefficients of determination (R^2 adjusted) and confidence intervals (calculated with 95% of probability) were used as goodness-of-fit indicators for the estimated parameters.

The RMSE measures the average difference between the observed and adjusted (predicted) values. Therefore, lower RMSE values indicate a better fit of a model (Buzrul, Alpas, & Bozoglu, 2005; Buzrul & Alpas, 2004) , and a value of the adjusted correlation coefficient (R^2) close to 1 indicates a good fit. However, some authors have criticized the use of R^2 which is of little importance when the model sleepis non-linear (Davey & therapy, 1993, 1994; Ratkowsky & Giles, 1990)**.**

2.2.10 **Statistical analysis**

Statistical significance ($P < 0.05$) of the effect of the combination 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 DISCUSSIONS

3.1 Inactivation of *E. coli* ATCC 29522 in orange juice beverage

3.1.1 *E. coli* in the orange juice beverage " ROUIBA "

After incubation of the plates inoculated with the orange juice drink "Rouiba" at a temperature of 37°C for 48 hours in the oven, no colony was counted.

3.1.2 Modeling and kinetic parameter estimation

The first-order kinetic model described by the *D* and *z* parameters is widely used to describe the microorganism inactivation kinetics (Benlloch-Tinoco, Martínez-Navarrete, et al., 2014). However, the survival curves of microorganism inactivation kinetics are not often log-linear. Peleg and Cole (1998) have described the survival curves based on a Weibull distribution of microorganism resistance. This model was then modified by Mafart et al. (2002) to give practical significance for its parameters. In the present study, the modified Weibull model was well fitted to the experimental survival data of *E. coli* ATCC 25922, as illustrated in Figures 15, 16 and 17. Furthermore, the R^2_{adjusted} (0.942 – 0.998) and the *RMSE* (0.255 in average for all treatments) values reported in Table 7 indicate that the modified Weibull model predictions describe the experimental data satisfactorily.

3.1.3 Ultrasound effect

Figure 15 shows the inactivation kinetic of *Escherichia coli* ATCC 25922 in an ultrasonic bath at a frequency of 42 kHz.

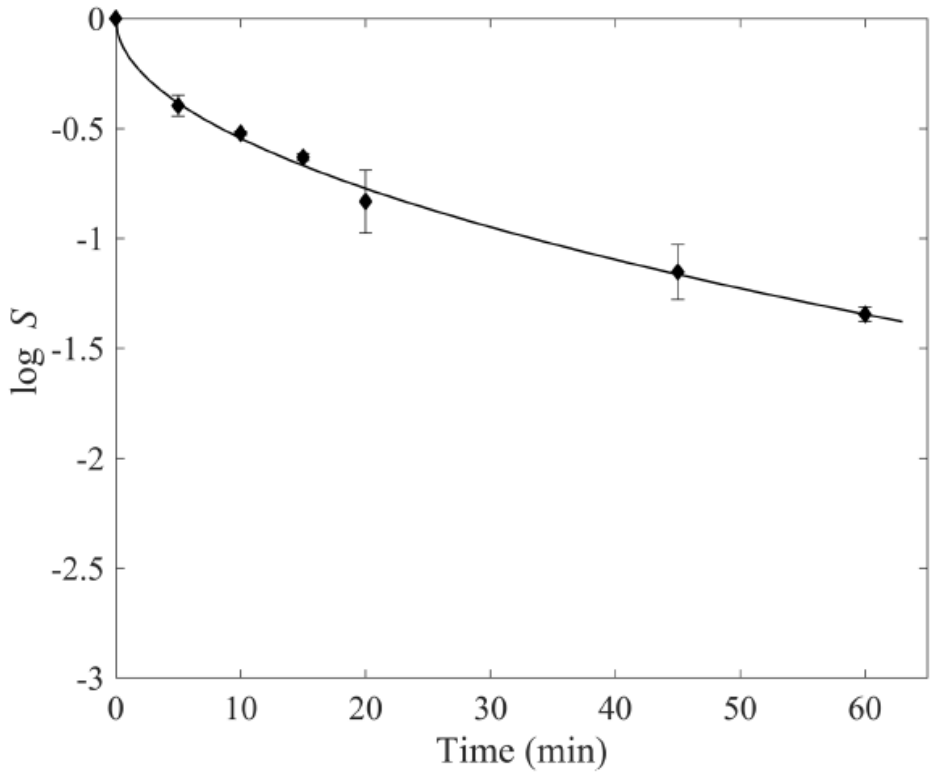


Figure 13. Inactivation kinetic of *E. coli* ATCC 25922 by ultrasonic (42 kHz) treatment. Error bars represent the standard deviation of the mean (n = 3).

The shape parameter β was found as 0.5 ± 0.02 (Table 7), which is clearly seen as an upwardly concavity curve with a more pronounced drag. This curve shape can be explained either by the presence of aggregates or "flocs" in the suspension, or the distribution of resistances within the population i.e. the most sensitive members of the population are inactivated first and the most resistant remain, or due to the physical protection provided by agglutination of the inactivated cells (Peleg & Penchina, 2000).The same shape of the curve ($\beta < 1$) was obtained by Ugarte-Romero, Feng, Martin, Cadwallader, and Robinson (2006) for *E. coli* K12 treated by ultrasounds (20 kHz) at 5 different temperatures in apple cider.

Increasing the duration of the ultrasound treatment results in a reduction in the number of *E. coli* ATCC 25922 in orange juice beverage. However, it can be seen that there is no substantial reduction in the survival rate. Ultrasound treatments applied at a frequency of 42 kHz have a low impact on the inactivation of *E. coli* ATCC 25922. Indeed, the maximum reduction achieved is 1.3 log of the population after 60 min of ultrasound treatment. Char, Mitilinaki, Guerrero, and Alzamora (2010) reported that ultrasound treatment (20 kHz, 95 μm-wave amplitude) in orange juice caused a reduction in *E. coli* ATCC 35218 of up to 2.2 log after 20 min.

Patil et al. (2009) and E. Joyce et al. (2003) concluded that the efficacy of ultrasound treatment was found to be a function not only of environmental conditions and treatment durations, but also a function of frequency. Most studies reported the efficacy of high frequency ultrasound on *E. coli* survival in fruit juices (Yildiz, Pokhrel, Unluturk, & Barbosa-Cánovas, 2019).

In the present study, the choice of using a low-power ultrasonic bath may reduce the viscosity and pectin content of the juice, thus improving the clarity of the juice. In addition, it has been demonstrated that, sonication increases the amount of bioactive compounds in orange juice (Guerrouj, Sánchez-Rubio, Taboada-Rodríguez, Cava-Roda, & Marín-Iniesta, 2016). In contrast, high-power ultrasonic can cause undesirable changes in the rheology of the juice (Chemat & Khan, 2011). The use of ultrasound in food products has proved to be advantageous in many processes. Although low-power ultrasound has been reported to be poorly effective in bacterial inactivation when used alone, there are increasing reports of changes in physicochemical parameters or component structures and the degradation of certain compounds. Studies show that orange juice quality parameters such as color, ascorbic acid reduction, β-carotene content and hydroxyl radical formation can be altered by ultrasonic treatment (Pingret, Fabiano-Tixier, & Chemat, 2013).

Table 7. Estimated scale (α) and shape (β) parameters for the fit of survival rate S (t) at different treatments of *Escherichia coli* ATCC 25922.

Treatments		***α***	***β***	$R^2_{adjusted}$	***RMSE***	$\alpha^{\S}$ **(β = 2.1)**
US treatment only		33.34±2.67	0.5±0.05	0.995	0.034	Nd
MW treatment only						
300 W		32.3±2.8	2.6±1.4	0.942	0.103	32.5±2.6
600 W		15.7±2.3	2.1±0.4	0.991	0.189	16.1±0.5
900 W		15.5±2.8	2.5±0.6	0.982	0.384	13.6±0.8
US pre-treatment						
300 W	US 10min	40.1±3.2	10.0±5.4	0.993	0.010	Nd
	US 20min	32.6±0.6	7.3±1.8	0.991	0.061	Nd
	US 30min	30.6±2.6	7.0±4.6	0.955	0.218	Nd
600 W	US 10min	16.4±7.1	1.9±1.3	0.946	0.384	17.5±1.3
	US 20min	18.4±6.2	2.7±1.5	0.992	0.243	15.6±1.2
	US 30min	9.1±5.0	1.7±0.9	0.969	0.595	13.0±0.4

900 W	US 10min	14.5±2.8	2.4±0.6	0.985	0.388	13.2±0.6
	US 20min	13.1±3.5	2.5±0.9	0.974	0.469	11.6±0.8
	US 30min	9.7±1.0	2.9±0.4	0.998	0.127	7.8±0.6
US post-treatment						
300 W	US 10min	Nd	Nd	Nd	Nd	Nd
	US 20min	36.4±1.0	6.6±2.1	0.979	0.186	Nd
	US 30min	Nd	Nd	Nd	Nd	Nd
600 W	US 10min	20.5±3.4	2.9±1.0	0.981	0.250	17.7±1.5
	US 20min	16.8±4.6	1.8±0.7	0.995	0.129	16.0±1.2
	US 30min	12.6±2.6	2.1±0.5	0.988	0.351	13.0±0.4
900 W	US 10min	16.8±5.5	1.8±1.0	0.968	0.269	18.3±1.5
	US 20min	16.2±3.4	2.0±0.7	0.976	0.267	16.7±0.9
	US 30min	14.3±3.4	2.8±1.0	0.974	0.435	12.0±0.9

Parameter value ± confidence interval (P=0.05).

RMSE, Root Mean Squared Error between experimental and estimated data ($\log S^{(t)}$).

nd, not determined because the fit did not converge.

§ Fitted for a second time by setting β equal to 2.1 (the mean of previously estimated β).

3.1.4 **Microwave effect**

Figure 14 shows the fitting of the modified Weibull model to the experimental survival rate kinetics of *E. coli* ATCC 25922 subjected to microwave treatments at 300, 600 and 900 W, and the corresponding time-temperature profiles.

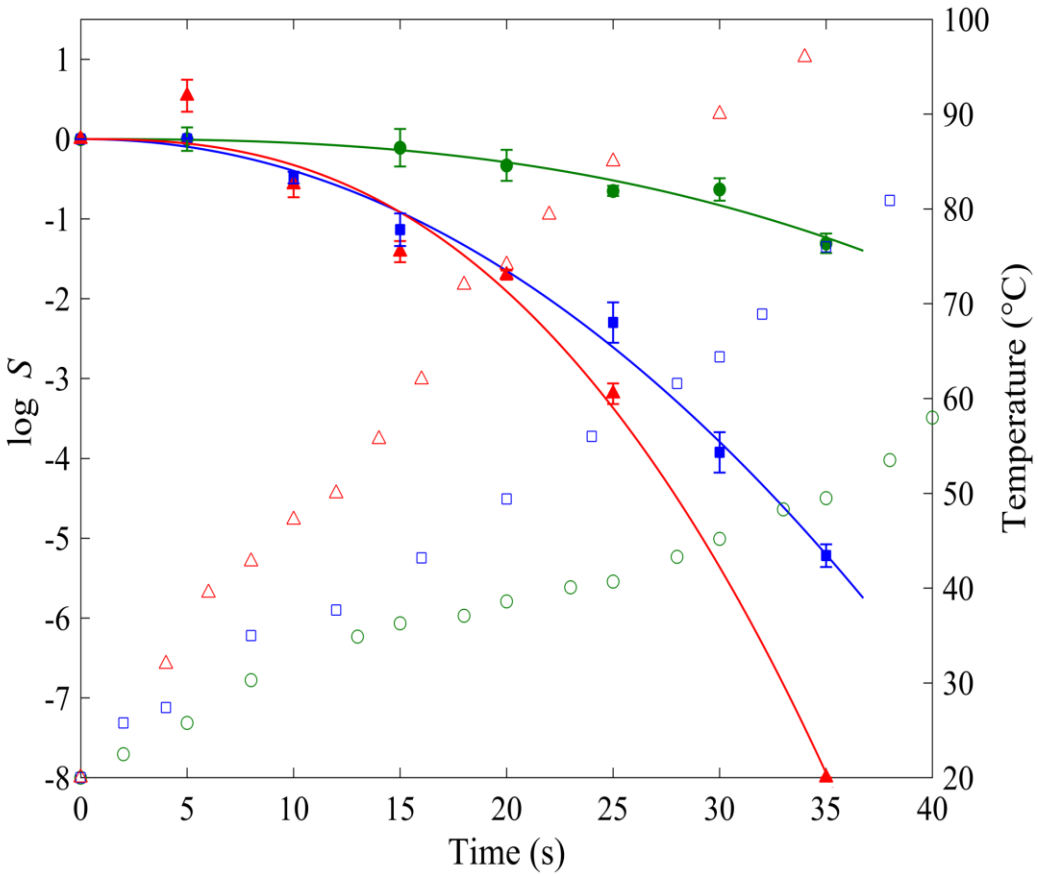


Figure 14. Fitting of the modified Weibull model (solidlines) to the experimental survival rate $S^{(t)}$ (symbols) of *E. coli* ATCC 25922 subjected to microwave treatment (● 300, ■ 600 and▲ 900 W). Time-temperature profiles are represented by symbols (○ 300,□ 600 and△ 900 W). Error bars represent the standard deviation of the mean (n = 3).

Downward concavity (shoulder) of the curves can be interpreted as evidence that accumulated damage caused by a constant exposure to irradiation, and the destruction rate increases with exposure time. The observed reductions of survival rates become statistically significant ($P < 0.05$) only from 20 s for 300 W and 10 s for 600 and 900 W (Table 7). The β values shown in Table 7 do not seem to show a clear trend with changing microwave power. This observation is consistent with the findings of some previous studies (Béchet, Laviale, Arsapin, Bonnefond, & Bernard, 2017; Fernández, López, Bernardo, Condón, & Raso, 2007; Hassani, Mañas, Pagán, & Condón, 2007; M. A. J. S. van Boekel, 2002; Virto, Sanz, Álvarez, Condón, & Raso, 2006; Zhang et al., 2018); they reported that the shape parameter β was independent of heat-inactivation temperature. Given the influence of inactivation conditions on the shape of survival curves and in order to quantify the microwave effect on *E. coli* ATCC 25922, the scaling

parameter α of each survival curve was re-estimated at a fixed β value of 2.1 for each inactivation condition. An increase in microwave power resulted in increased inactivation rate: at 900 W, the estimated scaling parameter α (13.6 ± 0.8 s) was the lowest, while the one at 600 W (16.1 ± 0.5 s) it was about twice as low as that obtained at 300 W (32.5 ± 2.6 s). Total mortality of *E. coli* ATCC 25922 was observed at 900 W after 35 s and achieved a reduction of 5.2 log at 600 W and 1.3 log at 300 W. These reductions were obtained with a linear trend of the time-temperature profiles, reaching temperatures of about 96, 76 and 50°C for 900, 600 and 300 W, respectively.

Table 8. Times from which the observed reductions of survival rates become statistically significant ($P < 0.05$).

	Microwave only	**US pre-treatment**			**US post-treatment**		
		10 min	**20 min**	**30 min**	**10 min**	**20 min**	**30 min**
300 W	25 s	>35 s	30 s	25 s	35 s	35 s	35 s
600 W	10 s	20 s	15 s	15 s	20 s	20 s	15 s
900 W	10 s	15 s	15 s	0 s	20 s	15 s	5 s

Picouet, Landl, Abadias, Castellari, and Viñas (2009) reported that a 1 log reduction of *E. coli* O157: H7 was obtained in apple puree using irradiation at 652 W for 35 s. A 5 log reduction of *Listeria monocytogenes* was obtained during microwave heating of kiwi fruit puree at a time of 75 and 82 s at 900 and 1000 W, respectively (Benlloch-Tinoco, Pina-Pérez, Martínez-Navarrete, & Rodrigo, 2014). Canumir, Celis, de Bruijn, and Vidal (2002) indicated that microwave pasteurization of inoculated apple juice with *Escherichia coli* at 720 and 900 W for 60 and 90 s resulted in a reduction of 2 and 4 log, respectively. Comparing the estimated decimal reduction times, authors have found no significant difference between conventional pasteurization and microwave processing. However, Siguemoto, Gut, Martinez, and Rodrigo (2018) demonstrated that microwave heating is more effective than conventional thermal treatment for *Escherichia coli* O157: H7 and *Listeria monocytogenes* inactivation in apple juice. Furthermore, it has been reported that the exposure effect to microwave irradiation of microorganisms suggests the existence of specific effect (non-thermal) (Shamis, Croft, Taube, Crawford, & Ivanova, 2012). Nevertheless, the topic of non-thermal effects of microwave on microbial inactivation remains controversial in the literature (Kubo et al., 2020).

3.1.5 Combination effect

The ultrasound pre-treatment can increase the efficiency of the microwave inactivation of *E. coli* ATCC 25922 in orange juice beverage. A microwave treatment at 600 W for 30 s combined with an ultrasound pre-treatment for 20 and 30 min provided respectively a reduction of 4.1 and 8.0 log, while only 2.7 and 6.3 log was achieved when the sonication is carried out in post-treatment. The same observation for 900 W, where it can be seen that the microwave treatment during 30 s followed by ultrasound at 20 min results in a destruction of 4.0 log, while a population reduction of 8.0 log is achieved when the ultrasound used in pre-treatment. These results demonstrate that ultrasound pre-treatment can reduce the microwave treatment time.

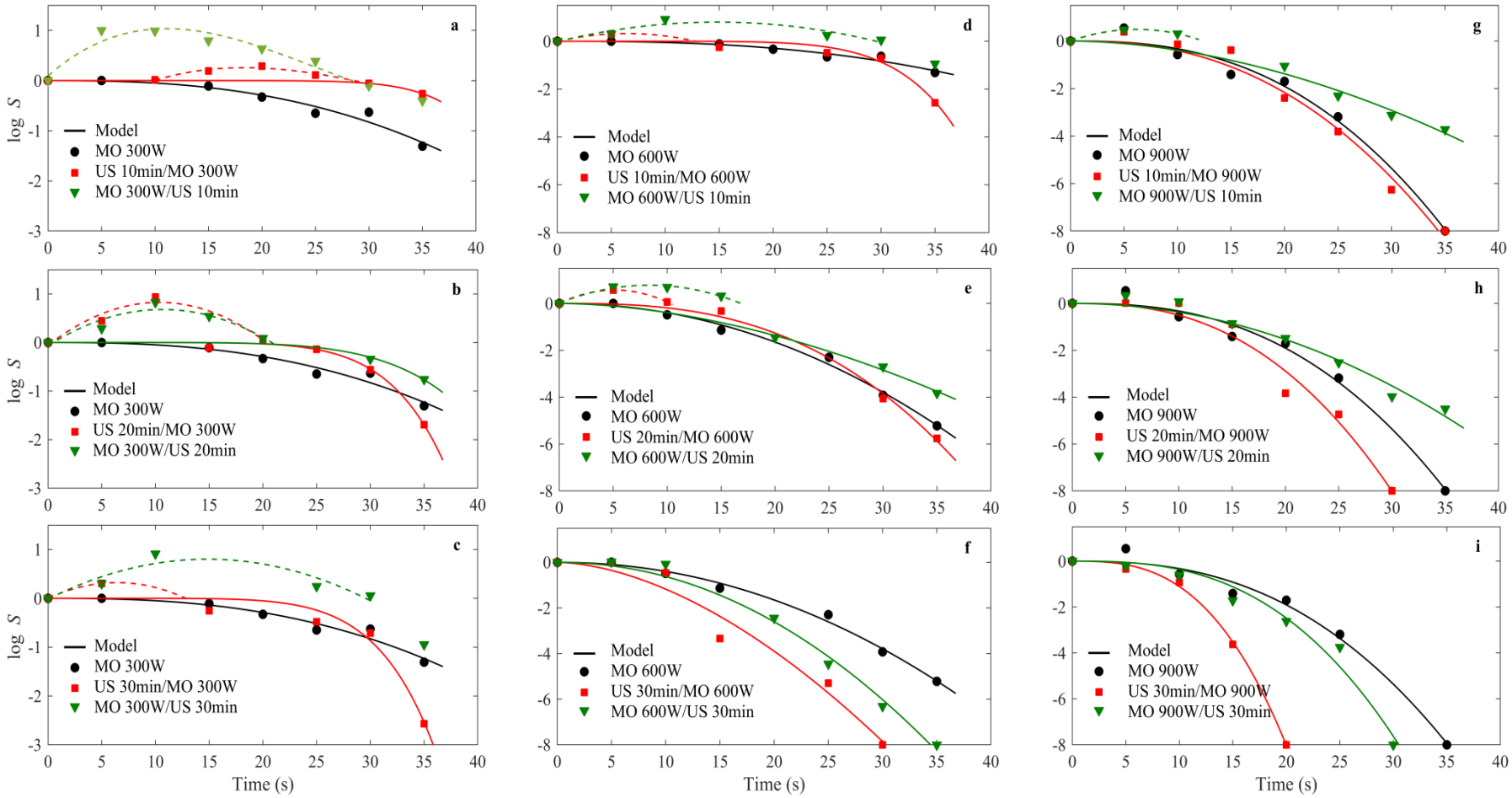


Figure 15. Fitting of the modified Weibull model (solidlines) to the experimental survival rate $S^{(t)}$ (symbols) of *E. coli* ATCC 25922 subjected to combined microwave (300, 600 and 900 W) and ultrasonic (42 kHz) treatments. The dashedlines are curves added as a visual guide to highlight an increase in survival rate. Error bars represent the standard deviation of the mean (n = 3)

Figure 15 shows the fitting of the modified Weibull model to the experimental survival rates *S* of *E. coli* ATCC 25922 under combined microwave (300, 600 and 900 W) and ultrasound (42 kHz) treatments.

During the first seconds of inactivation, it can be seen that in Figure 15 (a, c, d, e and g) there is a small increase (< 1 log) in the survival rate (highlighted by curves with dashed lines), indicating declumping effect that occurred as a result of the sonication treatment. This effect is more pronounced ($P < 0.05$) for the microwave inactivation kinetics at a power of 300 W with sonication as post-treatment, (Figure 15 a, b and c). Moreover, it is associated with longer shoulder periods of the survival curves obtained by combination treatments. For example, the observed reduction rates become statistically significant ($P < 0.05$) from 15 s at 900 W combined with a short ultrasound treatment (10 min), while it is 10s for 900 W without combination. However, this time is reduced to 0 s when the ultrasound treatment time is extended to 30 min. This declumping phenomenon was observed by E. Joyce et al. (2003) for *Bacillus subtilis* which was sonicated at low frequency by a probe (20 kHz) and an ultrasonic bath (38 kHz) without temperature control. It was also reported that sonication at low powers (40 kHz) resulted in a declumping effect on cyanobacteria over the first 2 min (E. M. Joyce, Wu, & Mason, 2010; Wu, Joyce, & Mason, 2012). This effect is reflected by the fact that ultrasound can disaggregate bacterial clumps producing more individual cells. The colony forming unit (CFU) enumeration reflects the viability of cells after treatment, although it is important to note that CFU can be a single cell or a group of cells. Then, if ultrasound triggers the cells, more CFUs will be formed (T. Mason, Duckhouse, Joyce, & Lorimer, 2003).

ANOVA results revealed that there was no significant difference ($P > 0.05$) between the different treatments shown in Figure 15 (d and e). It also revealed that there was no significant effect of short ultrasound pre-treatment (10 min) combined with 900 W treatment (Figure 15 g). As shown in Figure 15 (g and h), the microwave treatment alone at 900 W was more effective when combined with short ultrasound treatments (10 and 20 min) because the latter produces a declumping effect, thus increasing the microbial load. However, the inactivation effect of ultrasound during post-treatment is more pronounced with a treatment time of 30 min (Figure 15 f and i). An overlap of the kinetic inactivation curves is observed in Figure 15 (a, b and c). This suggests that the major effect of low power microwave and the ultrasound combination on the inactivation rate (*S*) is attributed to the dispersion of bacterial clumps with little inactivation effect. In other words, the declumping effect induced by sonication masks the

actual inactivation rate. Indeed, the expected effect of microwave and ultrasound combination did not occur at 300 W.

Table 8 shows that values of the schape parameter β are greater than 1and do not show any particular trend for microwave and ultrasound combination treatments. Nevertheless, high values were observed for combinations at 300 W. By setting β to 2.1, it can be seen that values of α decrease with increase in the ultrasound treatment time at the same irradiation power. The estimated α for combinations using ultrasound as pre-treatment are lower than those estimated for post-treatments, which, in turn, are lower than those estimated for microwave or ultrasound alone. This provides further evidence of the effectiveness of ultrasound as a pre-treatment to improve the inactivation of *E. coli* ATCC 25922 by microwave processing.

Wang, Zhao, Liao, and Hu (2010) found that treatment of three different strains of *Alicyclobacillus* by ultrasonic waves followed by microwave (400 W and 900 W) was significantly better than the treatment of microwave followed by ultrasonic waves, and this combination had a markedly better effect than separate microwave processing. The effectiveness of ultrasound as a pre-treatment was also observed for thermal inactivation at high temperatures (70, 80, 85, 90 and 95°C) of *Alicyclobacillus acidoterrestris* spores in apple juice (Tremarin, Canbaz, Brandão, & Silva, 2019). Char et al. (2010) also reported the greater efficacy of simultaneous ultrasonic and UV-C treatment of orange and apple juices against *E. coli* ATCC 35218 compared to individual treatments. They found that a combination of high intensity ultrasound followed by UV-C was more effective than combination UV-C followed by high intensity ultrasound treatment.

Shorter processing times in microwave coupled ultrasound treatment processes may allow for lighter impacts on the overall juice quality. However, studies on the impact of heat treatment on the overall Juice quality are necessary for practical optimization of process conditions (Tremarin et al., 2019).

3.1.6 **Physicochemical analysis**

The choice of the points for which the physicochemical parameters are measured depends on the number of log reductions of *E. coli* ATCC 25922 after US post-treatment of the orange juice beverage (reaching a 5 log reduction recommended by FDA and a total reduction).

Table 9. Values of pH, Brix, acid titrable and tempertures after treatement by US/ MW.

	Control±SD	(US (20min)/ MW(600)) ±SD	(US (30min)/ MW (600)) ±SD	(US (30min)/ MW (600)) ±SD	(US (10min)/ MW (900)) ±SD	(US (10min)/ MW (900)) ±SD	(US (20min)/ MW (900)) ±SD	(US (20min)/ MW (900)) ±SD	(US (30min) /MW (900)) ±SD
Time (s)	0	35	25	30	30	25	25	30	20
Final temperature (°C)	20 ± 2	76	56 - 61.6	64.4	90	85	85	90	74.1
pH	3.27 ±0.03	3.28 ±0.03	3,29 ± 0,02	3.28 ± 0.02	3.25 ± 0.03	3.28 ± 0.02	3.27 ± 0.02	3.285 ± 0.06	3.28 ± 0.014
° Brix	12 ± 0	11.85 ±0.05	11,85 ± 0,05	11.95 ±0.05	12 ± 0	11.9 ± 0	12.0 ± 0	11.95 ± 0.05	12.0 ± 0.05
Acidité	2.17 ± 0	2.11 ± 0.09	2.08±0.04	2.11±0.09	2.14±0.04	2.2 ± 0.04	2.16±0.02	2.12 ± 0.06	2.11 ± 0.09
Inactivation of *E. coli* ATCC25922	0	-5.75 log	-5.29 log	-8 log	-6.25 log	-8 log	-4.73 log	-8 log	-8 log

SD : Standard deviation

Ultrasound/microwave processing was found to have no significant ($P > 0.05$) effects on pH, titratable acidity or °Brix, irrespective of time treatement of ultrasound / treatment time of power of microwave (Table 9).

Pérez-Grijalba et al. (2018) concluded that no significant effects on these pH, Brix and titratable acid were observed on the wall juice drink treated with microwave and ultrasound simultaneously.

The influence of ultrasound and microwave on the pH of wall juice resulted in an increase of 2.5 and 3.5 % respectively, While, the combination of microwaves/ultrasound generated only an increase of 1.9 % (Pérez-Grijalba et al., 2018).

3.2 Partial conclusions

This present study evaluated the effectiveness of microwaves, ultrasounds and their combination to inactivate the cells of *Escherichia coli* ATCC 25922 in the orange juice beverage. Variations in the time-course of survival rate were well represented by a modified Weibull model. Ultrasonic bath treatment (42 kHz) has no substantial effect on the survival rate reduction of *E. coli*, as only 1.3 log reduction was obtained for 60 min of sonication. However, sonication can increase the efficiency of microwave inactivation. The combination treatment of an ultrasound bath followed by a microwave gives better results than using ultrasonic bath as a post-treatment: a reduction of 8.0 log was obtained by a combination of ultrasound treatment (20 min) followed by a microwave (900 W/30 s). In food processing, it is recommended to develop HTST (HighTemperature-ShortTime) processes to eliminate pathogenic bacteria in liquid foods while preserving quality. The combination of microwave and ultrasound processes allows to obtain a significant reduction in microbial load with short times while saving energy compared to the conventional processes. This work is useful to predict and optimize pasteurization processes for fruit juice beverages, particularly orange juice beverages, where the presence of acid and high-temperature resistant strains such as *E. coli* ATCC 25922 is of concern.

The combination of ultrasounds with microwaves can induce synergistic effects in terms of quality preservation in the case of orange juice and efficiency in microbial inactivation, as well as energy saving

The combined effect of microwave-ultrasound on inoculated orange juice beverage was assessed. Weibull model was used to describe the inactivation kinetics. Sonication can increase the efficiency of microwave inactivation, FDA standard of 5 $\log_{10}$ has been achieved under microwave and microwave-ultrasound conditions and reduction of 8 log was achieved by US (20 min) followed by MW (900 W/30 s).

3.3 Inactivation *E. faecalis* ATCC 29212 in wastewater dairy effluent

3.3.1 Ultrasound effect

The destruction of *E. faecalis* ATCC 29212 by ultrasonic bath at a frequency of 35 kHz is shown in Figure 16.

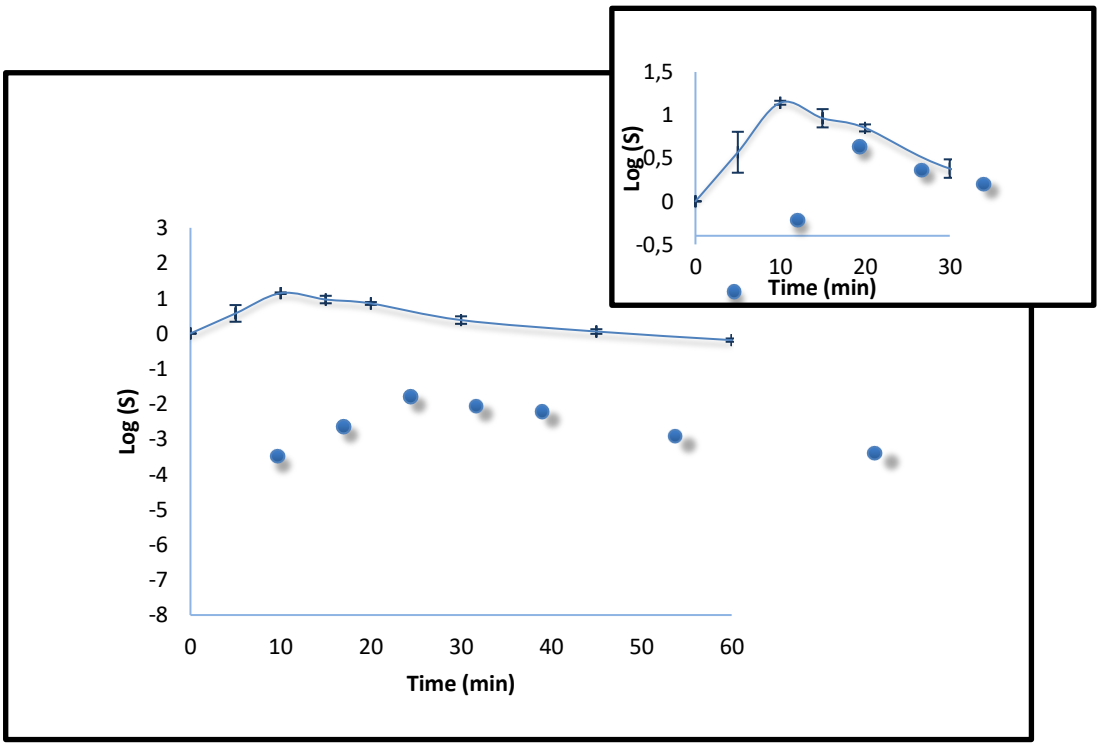


Figure 16. Inactivation of *E. faecalis ATCC* 29212 by ultrasonic bath at a frequency of 35 kHz

The results indicate that the significant effect on the viability of the bacterium, that is due to the declumping effect (increased load; log $N^{(t)}$> log $N^{(0)}$). This frequency produces an increase of 1.14 log within the first 10 minutes, followed by a steady decrease, but the value remains higher than the initial concentration even after 60 minutes of sonication, which could also indicate that the declumping effect at this frequency (produces a higher number of CFUs) masks the actual deactivation. CFU measurement represents cell viability after sonication, although it is important to note that a CFU may be a single cell or a group of cells. Therefore, if US separates the cells, more CFUs will form .

The thicker cell walls present in Gram-positive and coccus-shaped bacteria were also found to be resistant to ultrasonic treatment. It is thought that the ineffectiveness of ultrasonic sterilization may be attributed to distinct physical and biological properties of *E. faecalis* (Drakopoulou, Terzakis, Fountoulakis, Mantzavinos, & Manios, 2009).

Obioma (2015) revealed that *Enterococcus faecalis* (Gram-positive bacterium with a resistant peptidoglycan cell wall) present in drinking water showed a declumping effect after 2 minutes of sonication at 20 kHz probe. This phenomenon was also observed by E. Joyce et al. (2003) on *Bacillus subtilis* at a frequency of 20 kHz for the probe and 38 kHz in ~~a~~ the bath.

Cell death is due to high pressure and temperature caused by bubble collapse and shared forces that have destroyed the bacterial cell membrane (Doosti et al., 2012).

Exposure of high mechanical pressure waves to liquids creates acoustic current and subsequent acoustic cavitation that causes the formation, growth and implosive collapse of micro and nanobubbles in the liquid. These bubbles have a large specific surface area that increases gas diffusion and generates intense localized heating (about 5000°C) and high pressure (1000 ATM) (Mahvi et al., 2009).

Ultrasonic cavitation affects the inner membrane (cytoplasmic membrane) of the bacteria and the lipoprotein layer is disrupted, torn and shredded (Broekman, Pohlmann, Beardwood, & de Meulenaer, 2010). The thickness of the cell membrane mainly affected the effectiveness of themicrobial elimination Butz and Tauscher (2002) that is why *Enterococcus faecalis* has been resistant to ultrasonic waves since it is Gram positive and its cell wall is thicker than Gram negative.

A 4 logarithmic reduction in *E. coli* and *E. faecalis* cells present in drinking water was obtained at 9 minutes as reported by Gholami, Mirzaei, Mohammadi, Zarghampour, and Afshari (2014) and no declumping effect was observed at 20 kHz US.

The lethal effect of US is reported by several authors on several bacteria sach as: *Microcystis aeruginosa*, *Circinalisanabaena* and *Chlorella sp.* (Rajasekhar, Fan, Nguyen, & Roddick, 2012), *Legionella pneumophila* (P. J. E. m. Declerck, 2010), *Pseudomonas aeruginosa, Staphylococcus aureus* (Kalantar et al., 2010*)*, and *Escherichia coli* (Kwak et al., 2011; Lee et al., 2009) while some papers have described the application of US disinfection technologies (Gómez-López, Bayo, García-Cascales, & Angosto, 2009; Toor & Mohseni, 2007)**.**

3.3.2 Modeling and kinetic parameter estimation

In all cases tested in the present study, the modified Weibull model was very reasonable and was well fitted to the experimental survival data of *E. faecalis* ATCC 29212, as illustrated in Figure 17. The fit quality was determined by both R^2 and *RMSE* values, which ranged from 0.927 to 0.995 and 0.040 to 0.842, respectively (Table 11); this does not mean that other models are not applicable; in fact, other models can also work well. However, the purpose of this paper was not to compare models, but to study the dependence of Weibull parameters on power and exposure time to MW and US.

Statistical significance ($P < 0.05$) of the effect of the combination 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.

3.3.3 Microwave effect

Figure 17 shows experimental and simulated survival rate $S^{(t)}$ of *Enterococcus faecalis* ATCC 29212 subjected to combined microwave-ultrasonic treatments

Our results show that in the first 10 seconds at a power of 650 W, there was an increase in the microbial load compared to the initial load which reached of 8.99 ± 0.26 Log, this can be explained by the declumping effect and the reduction starts from 10 s and reaches the total destruction after 75s. On the other hand, the power of 350 W produces an immediate increase in CFU with respect to the first second which reaches 8.57 ± 0.26 log (followed by a steady decrease, but the level remains unchanged above the initial concentration, even after 20 seconds of MW irradiation, so that only the declumping effect can justify this phenomenon. After 75 s the destruction does not exceed 3 log, and thus, *E. faecalis* ATCC 29212 was more resistant to lower power than to higher one.

These results suggest that the major effect of high-powered MW in the first few seconds is the decommissioning of bacterial agglomerates with little deactivation. It could may also indicate that the downgrading effect at low powers hides the actual deactivation. In order to model the obtained results, the inactivation was taken into consideration from 20th second of MW treatment

Considering the influence of growth conditions on the shape of survival curves and in order to quantify the resistance of *E. faecalis* ATCC 29212, the scale parameter (α) of each survival curve was re-estimated with a fixed value β (β = 2.6) for each growth condition.

Based on the regression results for the Weibull model, it was found that the α parameter generally showed a downward trend and that the β parameter tended to increase in value with power.

When the survival curves of these two powers in Figure 23 were compared, it is obvious that the curve with the lowest β value hadmore pronounced drag, while α value of the MW at 350 W is was much higher than the one at 650 W power.

An increase in MW power resulted in a decrease in the inactivation rate: at 650 W, the estimated scale parameter α (32.2 ± 1.7) was the lowest, while at 350 W (50.0 ± 4.2) as shown in Table 11.

Hollywood, Varabioff, and Mitchell (1991) reported that a reduction from 3 to 4 log after 7 to 11 min has been obtained from ground beef with a power of 650 Wand suggests that the effect of the MW on this strain has a thermal effect and no other factor affecting the inactivation of this strain. *Enterococcus faecalis* at a charge of 10^{11} CFU/ml in a dextrose broth at a power of 650 W after 5 min of treatment.Survivors are still present, whereas a dosage of 10 min at 650 W of MW was sufficient to destroy all of them,but have been destroyed at a dose of 650 W after 10min of MW procedure (Najdovski et al., 1991).

Fang, Kang, Hong, and Wu (2012) reported that the mortality of *Aspergillus parasiticus* spores increased with increasing temperature. The mortality rate is 99% at 70°C with MW heating.

Although the effect of bacterial inactivation by MW is much more pronounced in a liquid medium than in a solid medium and as observed by other authors, microbial inactivation was faster for an increase in MW power (Benlloch-Tinoco, Martínez-Navarrete, et al., 2014).

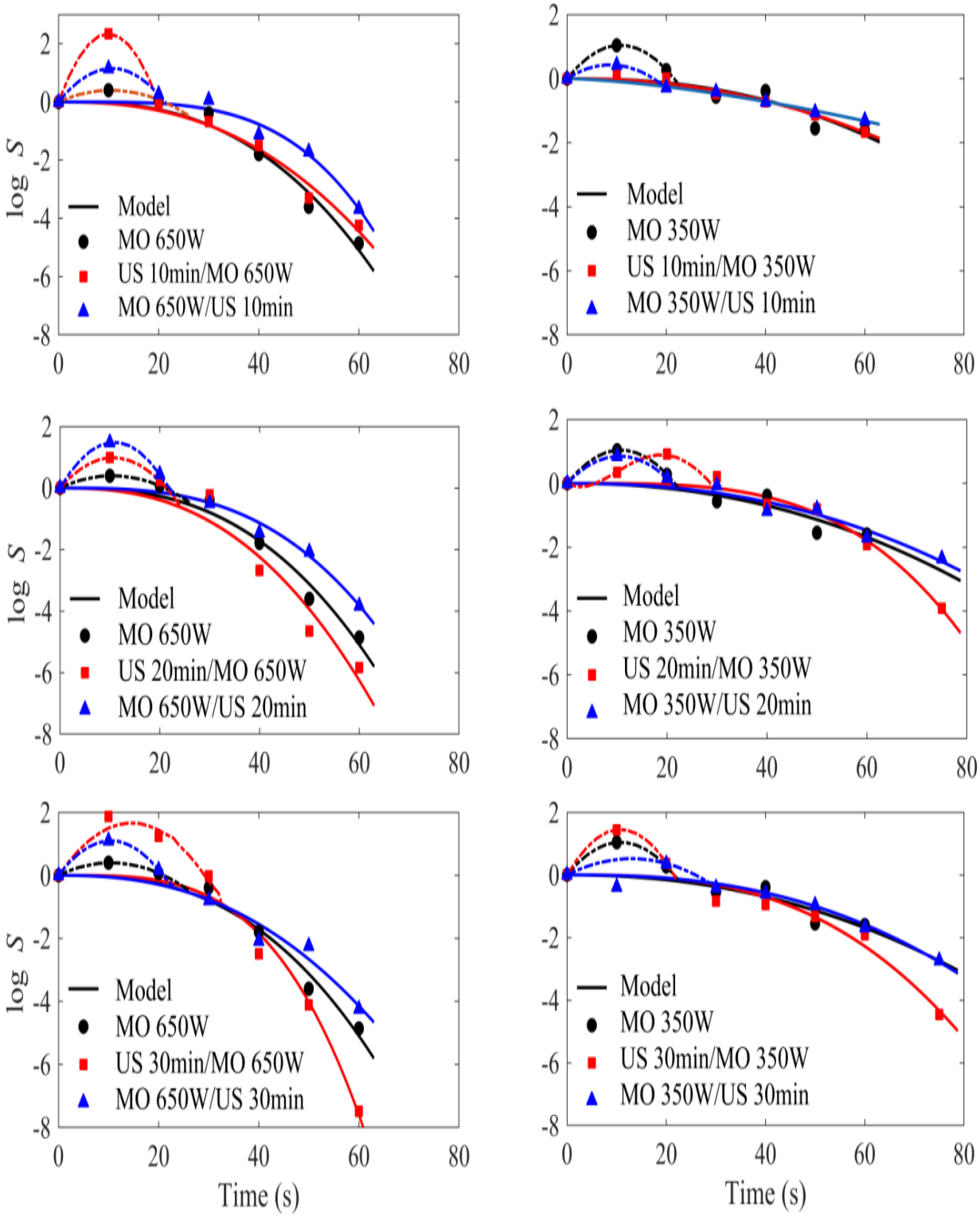


Figure 17. Experimental and simulated survival rate S (t) of *Enterococcus faecalis* ATCC 29212 subjected to combined microwave-ultrasonic treatments. Error bars represents standard deviation, n = 3.

3.3.4 Combination effect

It cannot be completely excluded that the inherent inhomogeneity of the dairy wastewater effluent medium caused by milk fat may pose experimental problems in terms of regulating the concentration of this fat in the medium and the agglutination of microorganisms.

The concentration of this fat affects the inactivation of the microorganism, as it has a protective effect on bacterial cells, preventing interactions between bacteria and reactive species (MW and US) (Patange et al., 2018).

Variations in inactivation of *E. faecalis* ATCC 29212 per MW and US were highly dependent on US exposure time/power and MW exposure time as well as on the process chronology (either the US in pre-treatment or in post-treatment).

No significant effect of ultrasound in pre- or post-treatments combined with microwaves at both 350 and 650 W power. Nevertheless, there was a significant difference after comparing survival rates to the initial number of microorganisms (control), after performing an ANOVA followed by Dunnett's post-hoc test; the results are summarized in the following Table:

Table 10. Times from which the observed reductions of survivalof *E. faecalis* ATCC 29212 rates become statistically significant ($P< 0.05$).

	Microwave only	**US pre-treatment**			**US post-treatment**		
		10 min	**20 min**	**30 min**	**10 min**	**20 min**	**30 min**
350 W	> 60 s	> 60 s	> 60s	60 s	> 60 s	> 60 s	> 60
650 W	50 s	40 s	40 s	40 s	50 s	40 s	40 s

In all treatments; β is greater than 1, which determines the value of its concavity parameter downward, which means that the remaining cells become more and more sensitive to heat. In other words, it indicates that cumulative damage is occurring, making it increasingly difficult for cells to survive.

The values of α decreased from 45.1 ± 2.2 to 43.1 ± 2.8 s for US (10 min)/ MW (350 W) to US (30 min)/ MW (350 W), and from 57.3±6 s to 50.5±2.8 s for MW (350 W)/ US (10 min) to MW (350 W)/ US (30 min) respectively (Table 11).

Also α decrease at a power of 650 W , i.e. 33.6 ± 1.6, 29.6 ± 2.4, 28.3 ± 3 for US (10min)/ MW (650 W), US (20 min)/ MW (650 W), US (30 min)/MW (650 W) respectively, this can be explained by the fact that the more the bacteria are exposed to higher power, the higher the power has a stronger bacterial inactivation effect.

These results suggest that the combination of microwaves and US may be useful in improving the inactivation of *Enterococcus faecalis*.

US pretreatment can increase the efficiency of MW inactivation of *E. faecalis* ATCC 29212 in DE. Microwave treatment at 350 W for 75 s combined with US pre-treatment for 20 and 30 min resulted in a reduction of 3.8 and 4.3 log respectively, and only 2.2 and 3. 6 logs when sonication was performed as post-treatment. The same observation for 650 W, where the MW treatment for 60 seconds followed by US at 30 min resulted in ~~a~~ 5.0 log destruction, while the total population reduction was achieved when US was used as pre-treatment.

Table 11. Estimated scale (α) and shape (β) parameters for the fit of survival rate $S^{(t)}$ at different treatments of *E. faecalis* ATCC 29212

Treatments		***α***	***β***	***R²***	***RMSE***	***α* (*β* = 2.6)**
MO only						
350 W	-	47.2 ± 9.7	2.2 ± 1.2	0.927	0.314	50.0 ± 4.2
650 W	-	32.7 ± 7.3	2.7 ± 1.1	0.974	0.375	32.2 ± 1.7
US pre-treatment						
350 W	US 10min	47.2 ± 3.4	2.2 ± 0.7	0.981	0.101	45.1 ± 2.2
	US 20min	50.8 ± 6.0	3.5 ± 1.2	0.975	0.263	45.7 ± 3.6
	US 30min	45.1 ± 8.5	2.9 ± 1.2	0.953	0.377	43.1 ± 2.8
650 W	US 10min	32.8 ± 6.5	2.5 ± 0.9	0.977	0.301	33.6 ± 1.6
	US 20min	29.1 ± 11.9	2.5 ± 1.6	0.941	0.708	29.6 ± 2.4
	US 30min	33.6 ± 11.6	3.5 ± 2.3	0.947	0.842	28.3 ± 3.0
US post-treatment						

350 W	US 10min	49.9 ± 2.0	1.5 ± 0.2	0.995	0.040	57.3 ± 6.0
	US 20min	50.6 ± 7.9	2.3 ± 1.1	0.942	0.249	52.4 ± 4.0
	US 30min	50.0 ± 6.4	2.5 ± 0.9	0.965	0.217	50.5 ± 2.8
650 W	US 10min	42.8 ± 6.2	3.9 ± 1.8	0.971	0.291	37.8 ± 3.7
	US 20min	38.4 ± 7.8	3.0 ± 1.5	0.962	0.344	36.3 ± 2.6
	US 30min	33.4 ± 9.4	2.4 ± 1.3	0.951	0.413	34.5 ± 2.5

Parameter value ± confidence interval (P=0.05).

RMSE, Root Mean Squared Error between experimental and estimated data (log $S^{(t)}$).

nd, not determined because the fit did not converge.

§ Fitted for a second time by setting β equal to 2.1 (the mean of previously estimated β).

However, combined MW/ US treatment is less important than MW treatment alone at a power of 650 W; MW (650 W)/ US 10 min, MW (650 W)/ US 20 min, MW (650 W)/US 30 min, α= 37.8 ± 3.7s, α=36.3±2.6s, and α=34.5±2.5s, respectively. Based on the obtained data from this study, under the same experimental conditions, the US pre-treatment application had a better effect on the inactivation of *E. faecalis* ATCC 29212 than the MW treatment followed by the US, these results are consistent with the work of Wang et al. (2010).

Since *E. faecalis* ATCC 29212 has shown a high resistance to MW treatments and since ultrasound treatments weaken the bacterial wall and contribute to the extraction of intracellular compounds, it could be presumed that the heat generated by MW in the liquid and the fragility of the bacterial wall thus accelerate the inactivation and death mechanisms of microbial cells.

The obtained results show that a significant reduction in the bacterial population was obtained with a reduced treatment time.

3.3.5 **Protein assays**

Figure18 show a differents proteins concentrations at different processing in dairy wastewater effluent containing *E. faecalis* ATCC 29212.

The release of intracellular proteins was measured, in order to study cell membrane damage caused by MW and US irradiation.

Microwave heating at 650 W/20 s did not result in a large difference, in the amount of protein released from the cells (443.30 ± 0.02 µg/ml), compared to the control (437.6 ± 0.02 µg/ml). However, when the treatment time was increased to 40s, substantial differences in the amount of released protein were observed with a value of 454.8±0.01µg/ml (Table 12). The protein content increased, these results indicate that most of the microwave heated cells may be ghost cells (will be confirmed with SEM). These results indicate that most of the heated cells were ghost cells from which intracellular material was released into the cell suspension (S. Y. Kim, Jo, Kim, Bai, & Park, 2008). Indeed, in response to temperature increases, chemical or physical stresses, prokaryotic cells synthesize specific proteins involved in cell protection. These stress proteins include a large family called HSPs. (for Heat Shock Protein). Some HSPs are constitutively expressed in cells under normal culture conditions. The induction of high expression of these proteins occurs when cells are subjected to stress. These proteins are considered to be "molecular chaperones": they are thought to be involved in protein folding(Cleary, Cao, Liu, Egle, & Shelton, 1997).

A low level of protein leakage was observed when *E. faecalis* ATCC 29212cells were irradiated for 60 seconds, a time sufficient for a 3.31 log reduction in the number of viable cells.

On the one hand, some authors propose mechanisms to explain the existence of a stress response under non-thermal conditions. Depending on the power of the microwaves, the effect could be different. At low power, microwave fields could alter the proper folding of some proteins but not enough to induce a stress response. Misfolded proteins are therefore not protected by the HSP system (Laurence, French, Lindner, & Mckenzie, 2000). On the other hand, according to the principle of the Bradford method, used in this study for the determination of proteins, which is the binding (complexation) of Coomassie Blue G-250 with basic amino acids (arginine, histidine and lysine) and hydrophobic residues of amino acids present in the protein(s). The combination of these two explanations leads us to suggest a hypothesis for which there would be a decrease of the proteins in the medium at 650 W for 60 s; indeed after microwave treatment and refolding of the proteins as explained above, the basic amino acids will not be accessible "hidden" in the three-dimensional structure of the proteins with respect to Coomassie blue, which decreases the rate of the basic amino acid-Coomassie blue couple, consequently, absorbance will be reduced. Nevertheless, according to Woo et al. (2000) who studied the

leakage of proteins from Bacillus subtilis treated by microwave at 20, 40 and 60°C, they found similar results to ours and according to them the reason of this decrease is still unknown.

Studies of interaction between microwaves and the cell membrane have been carried out on liposomes, models of biological membranes. Studies have shown an increase in permeability of unilamellar liposomes under microwave exposure at 2.45 GHz at 37°C (Saalman et al., 1991). However, these two studies disagree since Saalman and his collaborators conclude that there is a non-thermal effect of microwaves on liposome permeability, whereas Berquist and his team show that this increase in permeability is due to the thermal effects of microwaves. Another study on the influence of microwaves on the release of enzyme contained in liposomes shows that there would be a change in permeability induced by microwave exposure and that this change in permeability would be due to the formation of pores (Orlando, Mossa, & D'lnzeo, 1994).

Table 12. Quantity of proteins released in the dairy wastewater effluent medium by Bradford

(Protein) µg.ml^{-1}		**SD**
Control	437 .6	0.02
S1: MW (650 W/ 20 s)	443.3	0.02
S2: MW (650 W/ 40 s)	454.8	0.01
S3: MW (650 W/ 60 s)	441.0	0.01
S4: US (30 min)	441.0	0.01
S5:US (30 min)/ MW (650 W/ 20 s)	452.9	0.01
S6: US (30 min)/ MW (650 W/ 40 s)	463.8	0.01
S7: US (30 min)/ MW (650 W/ 60 s)	475.7	0.01

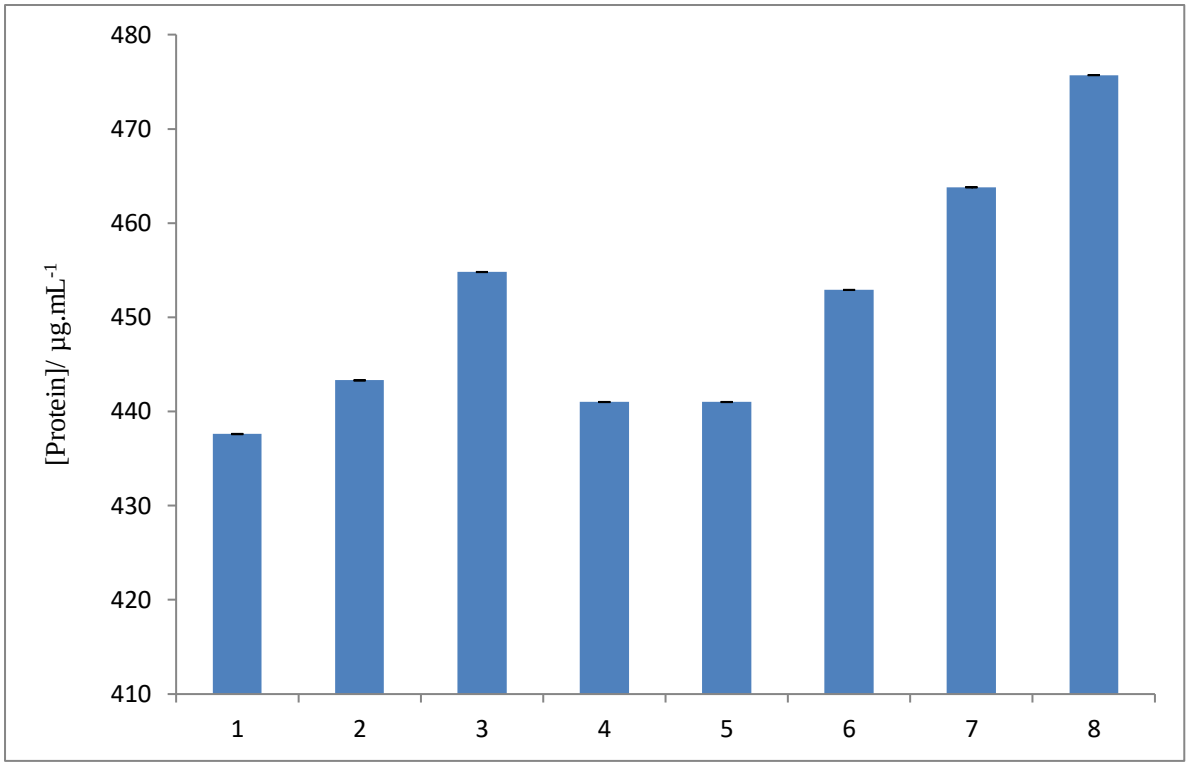
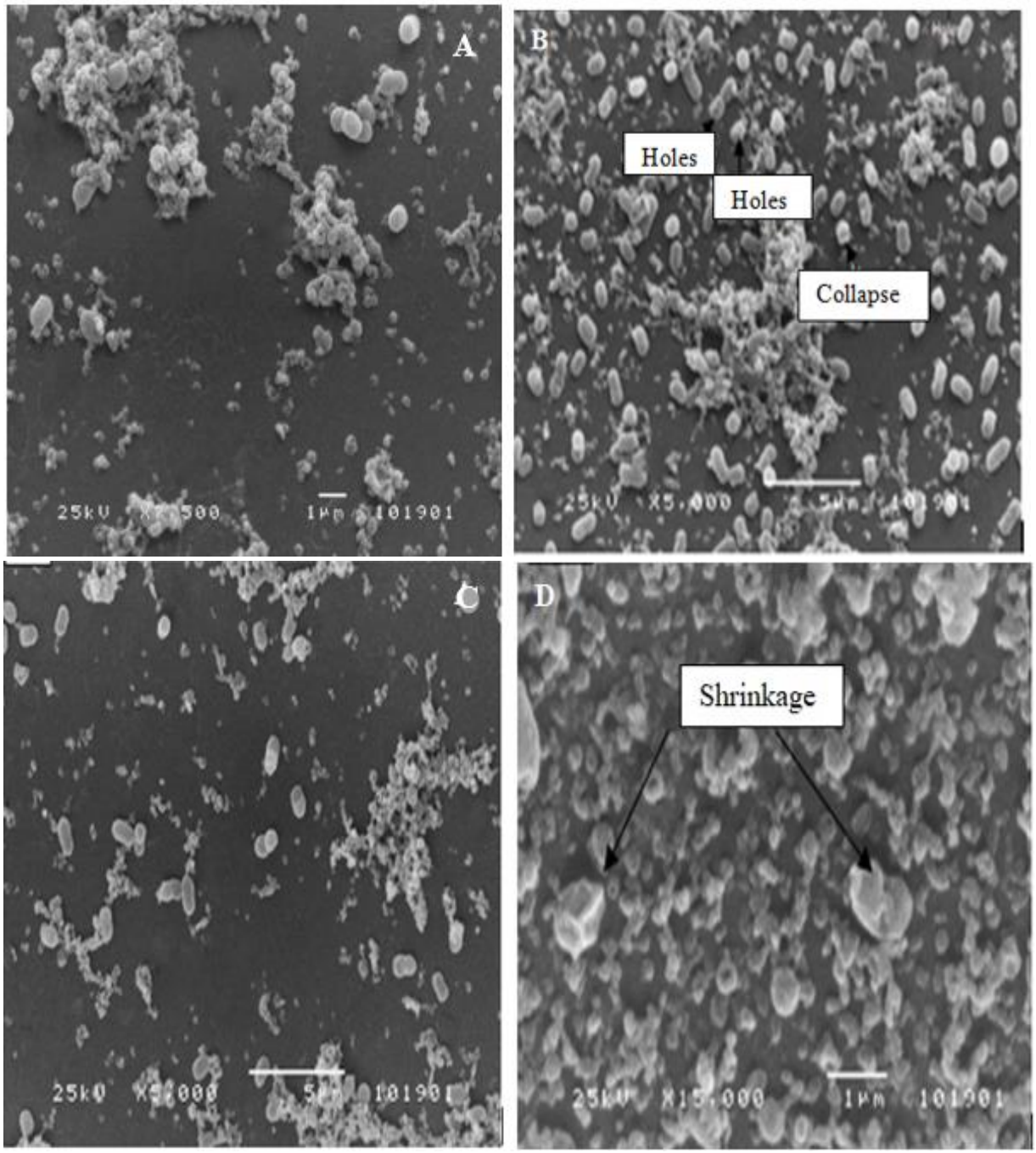


Figure 18. Different protein concentrations at different treatments in dairy effluents containing *E. faecalis* ATCC 29212 ; **1** : control; **2**: MW (650 W/ 20 s) ; **3**: MW (650 W/ 40 s) ; **4**: MW (650 W/ 60 s) ; **5**: US (30 min) ; **6**: US (30 min)/ MW (650 W/ 20 s) ; **7**: US (30 min)/ MW (650 W/ 40 s) ; **8**: US (30 min)/ MW (650 W/ 60 s).

By using slightly less intense ultrasound (35kHz), it is possible to increase the permeability of the membrane to macromolecules. In this case, the membrane is not broken, only slightly damaged (Peuker, 2006), hence the explanation for the low amount of proteins released in the medium.

The combined effect of ultrasound and microwaves leads to an increase in the amount of proteins released into the medium; ultrasound weakens the membrane and microwaves complement the effect of the ultrasound by reacting on the membrane.

3.3.6 **Scanning Electron Microscope (SEM)**



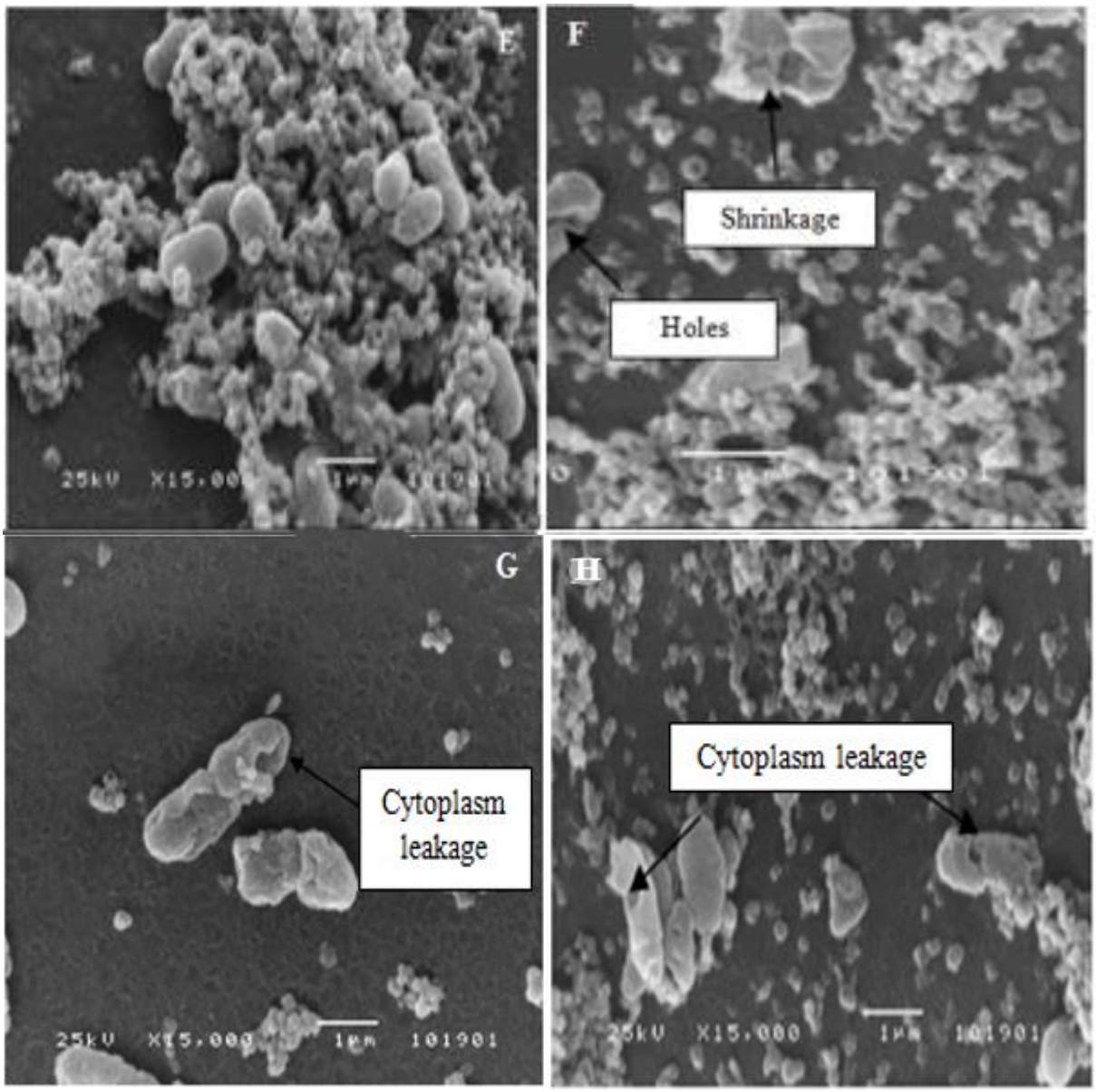


Figure 19. *E. faecalis* ATCC 29212 images obtained by SEM in wastwater dairy effluent after treatment, A : Control ; B : treatement US (30 min) ; C : MW 650 W (20 s) ; D : MW 650 W (40 s) ; E : MW 650 W (60 s) ; F : US (30 min) /MW 650 W (20 s) ; G : US (30 min) / MW 650 W (40 s) ; H : US (30 min) /MW 650 W (60 s).

Morphological changes of *Enterococcus faecalis* ATCC 29212 induced by ultrasound and MW were observed using SEM (Figure 19).

As shown in the SEM images of *Enterococcus faecalis* ATCC 29212, the cells not treated by microwaveshad a coccoid shape (control Figure 19-A) with intact cellwalls and membranes smooth. while the cells of *Enterococcus faecalis* ATCC 29212 exposed to 650 W microwaves (Figures 19 C-D-E), they were deformed. The effect of the microwave is manifested in the first

second by the shrinking of the wall, the cellshad a dehydrated appearance ; A reduction in the size of the bacterial cell and a transformed and amorphous structure were observed. This phenomenonis due to oxidative damage that altered the permeability of the cell membrane, causing a leakage of intracellular potassium and unbalancing the osmotic pressure, this was explained by Megha et al. (2015).

For *Enterococcus faecalis* ATCC 29212 exposed to 650 W microwaves for 20, 40 and 60 s, little or no deformation of the bacterial cells observedafter a 20 s treatment under 650 W, this can be explained by the limited time of exposure of the bacteria (Figure 19-C); however, the level of proteins released in the medium was remarkable, soit is assumed that the membrane is deformed, so that the intracellular components can escape; the observation of the intact membrane as well as the high level of proteins at 20 s can be explained by the factthat the cell membrane has regainedits initial shape; the effect of microwaves is there fore temporary at this time. This was demonstrated by Shamis et al. (2011) who observed the effect of microwaves on *E.coli* under SEM just after treatment and after an additional 10 minutes after treatment, they foundthat the cell morphology returned to a state identical to that of the untreated controls.

The cells were more or less damaged. For MW (650 W/ 60 s) (Figure 19-E); there is shrinkage of the cells and perforation, however, thiswas not sufficiently altered compared to the other treatments, indeed, it is noted that the sample was taken near fat and it protects the cells from the treatment.

In addition, the longer the duration of exposure to microwaves, the greater the deformation of the cell shape and the rupture of the membrane.

The cell membrane is damaged. The results obtained in this study provided evidence that *E. faecalis* ATCC 29212 bacterial cells in dairy wastewater effluent are lysed during exposure to a time of 60 s MW irradiation at 650 W. This suggests that cell membrane damage is a crucial mechanism of bacterial inactivation by MW irradiation. Similar results were reported by Woo et al. (2000).

Numerous studies have been conducted on the effect of microwaves on the membrane of microorganisms. Thus, a change in cell morphology has been observed by electronmicroscopy on bacterial suspensions irradiated at 2.45 GHz (Rosaspina, Anzanel, & Salvatorelli, 1993; Rosaspina, Salvatorelli, & Anzanel, 1994; Rosaspina, Salvatorelli, Anzanel, & Bovolenta, 1994; Salvatorelli, Marchetti, Betti, Rosaspina, & Finzi, 1996). Different bacteria subjected to

microwave irradiation show membrane "breaks". The same bacteria subjected to the same temperature (about 100°C) under traditional heating do not show membrane alterations. Shin and Yun (1997) have shown that microwaves, applied at 50°C, induce damage to the membrane of *Lactobacillus plantarum*, associated with increased permeability.

After treatment with ultrasound alone for 30 minutes, In some cells of *E. faecalis* ATCC 29212, invaginations were present and the membrane or cell wall appeared to be ruptured in some places. The partial rupture (collapse) of the cell walls was observed, which resulted in the flow of some cell contents (such as proteins according to the previous results). In addition, groups of *E. faecalis* ATCC 29212 were dispersed and observed as single cells (Figure 19-B). In a study using transmission electron microscopy, Cameron, McMaster, and Britz (2008) also reported similar results showing extensive damage to bacterial cells and in wardfolding and vesicle formation in *E. coli* after sonication. cell death involving cell membranes has generally been attributed to either pore formation or mechanical disruption of the cell wall or membrane. Pore formation couldalso have led to mechanical disruption of the cells. Small pores are created in the bacterial membrane during physical/chemical treatments. These pores are small enough to prevent the passage of molecules responsible for osmotic equilibrium but they allow the entry of water molecules into the bacterial cells, which leads to an increase in cell volume. The entry of water into the cells leads to an increase in turgidity, pressure and there fore cell rupture leading to cell death (Tsong, 1989).

In this study, SEM images of ultrasonically treated samples of *E. faecalis* ATCC 29212 cells did not show a significant increase in cell volume indicating that pore formation may not be involved in the destruction of this bacterium by US.

On the contrary, the presence of invaginations and discontinuities in the membrane show that the membrane was physically damaged by US. This would have resulted in a loss of cytosol and DNA from the cells causing their death. The cell membrane controls metabolic activities by maintaining an osmotic balance between the cell and its environment. Any damage to the cell membrane could result in an alteration of this balance leading to cell death (Pelczar & Filipowicz, 1998). SEM images suggest that the possible mechanism of inactivation of these bacteria by US is physical damage to the cell wall and membrane of the bacteria.

From the images obtained at the SEM, it became clear that the ultrasound combined with the microwave had a significant impact on the biological structure of *E. faecalis* ATCC 29212. The

deagglomeration effect of the ultrasound increased the probability of individual cells coming completely into contact with the acoustic wave. The chemical bonds between the molecular components of the cell membranes werebroken by the ultrasound, so the membrane fragility caused by the ultrasound favoured the bactericidal effect of the *E. faecalis* microwave (Nan et al., 2010; Piyasena et al., 2003).

SEM observation of samples treated by coupling showed that the coupling effect has a more destructive effect on wall structure and cell shape than microwave or ultrasound treatment alone. That is, there is a synergistic effect between microwave and ultrasound (Figures 19 F-G-H).

3.4 Partial conclusions

The study demonstrated the proof-of-principle of food wastewater safe treatment effluents using US (in pre-treatment) coupled with microwave for decontamination, with useful efficiency over short treatment periods. The coupling effectiveness varies with experimental parameters, i.e. the US exposure time and the power of the microwave. US-MW coupling treatement has proved to be a promising technology in which main indicator micro-organisms in dairy wastewater effluentes are reduced and fully inactivated.

US 30min/ MW 650 W at 60 s was sufficient to effectively eliminate heat-resistant bacteria in dairy production effluents, while greater resistance to inactivation was presented to MW-only or US-only processing. . This problem was therefore solved either by extending the combination of US and MW and more specifically by carrying out a pre-treatment by US, which allowed to effectively eliminate the total bacterial load present in the dairy effluent.

4 GENERAL DISCUSSION

Statistics reveal that more than 55 billion liters of orange juice are drunk every day in the world. It is the most appreciated and consumed drink, at least it can be the vector for pathogens such as *E. coli* O157:H7 (Martinez-Gonzales et al., 2003). This bacterium is the cause of various infections ranging from simple diarrhea to serious manifestations such as hemorrhagic colitis (March & Ratnam, 1986).

The global consumer market requires that foods be high in bioactive compounds to improve human health. Indeed, the use of heat treatment is nowadays one of the main means of decontaminating food products and improvement efforts are focused on minimizing changes in the organoleptic properties of products.

Because of these questions, the objective of this research was to compare the combined effect of a new thermal (microwave) and non-thermal (ultrasound) treatment.

HTST type processes are preferred by the food industry to reduce harmful thermal degradation of food quality while ensuring the food safety of liquid foods (Varghese et al., 2014). Microwave heatingis fast and can significantly reduce the time to reach the desired process temperature. The hydrodynamic action of ultrasound on microorganisms is a great potential to improve the efficiency of microbial decontamination by microwave treatment. This study contributes to the design and control of an effective treatment combining ultrasound and microwaves to improve the pasteurization processes of orange juice beverage.

To this end, the beverage cavitation (ultrasound) at low frequency (a nutritional interest for orange juice beverage because a high frequency ultrasound treatment of the juice brings modifications on viscosity and pectin content) and by microwave thus their coupling is carried out, this study is pioneering.

As explained in the materials and methods section, the use of *E. coli* O157:H7 at the laboratory level is very dangerous, there for *E. coli* ATCC 25922, which is a substitute for *E. coli* 0157: H7, is ideal because it has a thermal inactivation kinetics similar to that of *E. coli* 0157 :H7.

Not all the survival curves of the inactivation kinetics of microorganisms are linear (log-linear), for this reason we opted for the modeling of kinetics and the application of the Weibull model. Through to the parameters of this model, we were able to have a physiological and biological

explanation, and it also allowed us to predict the evolution of bacterial inactivation, and to understand what happens to *E. coli* ATCC 25922 in orange juice beverage and *E. faecalis* ATCC 29212 in wastewater dairy effluent after sonication and microwave treatment.

In fact, after treatment of the orange juice beverage with ultrasound at 42 kHz containing *E. coli* ATCC 25922 at a load of 1.5 x 10^8 CFU/mL, it was noticed that there is no dramatic effect on the inactivation of the bacteria. After 60 min of treatment, a reduction of 1.3 log was observed, which is very low compared to the time used and the structure of the bacterial wall which is Gram negative (Gram negative which has the thin membrane compared to gram positive) supposed to be influenced by the acoustic cavitations of the ultrasound (Petit, Ritz, & Federighi, 2002).

After microwave treatment of *E. coli* ATCC 25922 in the juice at different powers (300, 600 and 900 W) from 0 s to 35 s. the shape parameter $\beta > 1$ (2.6 ± 1.4, 2.1 ± 0.4 and 2.5 ± 0. 6 for 300, 600 and 900 W, respectively), i.e. the concavity of the curves is downward (shoulder) , this means that exposure to microwave irradiation causes accumulated damage and the inactivation rate increases with the exposure time (Peleg & Penchina, 2000).The observed reductions in survival rates only become statistically significant *($P < 0.05$*) after 20, 10 and 10 s of treatment at 300, 600 and 900 W respectively.

In order to quantify the effect of microwaves on *E. coli* ATCC 25922 and to compare the scale parameter α which corresponds to the time of the first decimal reduction. The shape parameter β is re-estimated to a fixed value of 2.1 for each inactivation condition.

An increase in microwave power resulted in an increase in the inactivation rate: at 900 W, the estimated scale parameter α (13.6 ± 0.8 s) was the lowest, while at 600 W (16.1 ± 0.5 s), it was about half as low as that obtained for 300 W (32.5 ± 2.6 s). Total mortality of *E. coli* ATCC 25922 was observed at 900 W after 35 s and achieved a reduction of 5.2 log at 600 W and 1.3 log at 300 W. These reductions were obtained with a linear trend in time-temperature profiles, reaching temperatures of about 96, 76 and 50°C for 900, 600 and 300 W respectively.

After coupling ultrasound and microwave, the ultrasound post-treatment kinetics only become interesting after a 600 W microwave treatment followed by ultrasound at 30min.

Indeed, statisticalan analysis shows that the effect of ultrasound post-treatment with microwave at 300 W is significant, this is due to the declumping effect (increased load; $\log N^{(t)} > \log N^{(0)}$)

(Figure 3 a, b and c). This phenomenon is observed even with 10 min and 20 min of ultrasound post-processing at a power of 600 W and 10min ultrasound post-processing at a power of 900w but it remains insignificant. This effect called declumping produces a higher number of CFU's, and it masks the effective deactivation. The CFU measurement reflects the viability of the cells after sonication, although it is important to note that a CFU can be a single cell or a group of cells. Logically, therefore, if the US triggers the cells, more CFU swill be formed. And no significant effect of US as pre-treatment has been found (Figure 3 a, b and c).

At 600 W microwave, no significant effect of short US pre- or post-treatment (10 and 20 min) (Figure 3 d and e). But Significant effect of US pre- or post-treatment (30 min) was observed (Figure 3 f). At 900 W of microwave treatment, we observed significant difference between the different treatments (Figure 3 g, h and i), except the US pre-treatment with a short time (10 min).

After this coupling; the kinetics reveal important results, especially when it comes to a 30 min ultrasound pre-treatment with MW at 600 and 900 W power. A total inactivation (8 log) of *E. coli* ATCC 25922 is obtained at US 30 min /MW 600 W (30 s), while at MW 600 W (30 s) only 4 log reduction was achieved (representing half inactivation compared to the coupling).

Equally important was the spectacular inactivation after pre-treatment by ultrasound followed by microwave at 900 W for 20 s where total destruction is recorded while inactivation that does not even reach 2 log was recorded after treatment by microwave alone at 900 W for 20 s. It is known that orange juice beverage, like other high acidity products, requires a heat treatment of 80 to 95°C for 15 to 30 seconds. What is also of interest in this study is the temperature obtained at the end of each treatment; the temperature noted after 900 W for 20 s is 74.1°C to reach total destruction attreatment ; betteryet, to achieve a 5 log destruction, recommended by the FDA, by ultrasound 30 min/ microwave 600 W, the necessary treatment time is from 15 s which corresponds to a temperature of less than 62°C.

The quality of this coupling is there fore not evaluated only after measurement of the physicochemical parameters pH, °Brix and titratable acidity.

No significant differences are observed in these parameters after treatment with :

- US (20 min)/ MW(600 W) 35s which correspond to a reduction of 5.75 log ;
- US (30 min) /MW (600 W) 25 s which correspond to a reduction of 5.29 log ;

- US (30 min) /MW (600 W) 30 s which correspond to a reduction of 8 log ;
- US (10 min)/ MW (900 W) 30 s which correspond to a reduction of 6.25 log ;
- US (10 min)/MW (900 W) 25 s correspond to a reduction of 8 log ;
- US (20 min)/MW (900 W) 30 s which correspond to a reduction of 4.73 log (close to 5 log) ;
- US (20 min) /MW (900 W) 35 s which correspond to a reduction of 8 log,
- US (30 min) /MW (900 W) 20 s which correspond to a reduction of 8 log

In fact, the choice of measurements of these parameters at these points, is that the reduction of the number of bacteria reached 5 log recommended by the FDA and reached a total destruction.

Indeed, we are currently working on the effect of the combination MW and US pre-treatment on these quality parameters of the orange juice beverage, and we intend to evaluate the effect of US-MW treatments on the kinetic growth parameters (growth rate and latency time) of *E. coli* survival after storage at 4°C. This aspect is necessary and should be explored to support the practical application of the results of this study.

Another axis of the study is to invest in the use of these techniques in a non-consumable matrix, which is why we used waswater dairy effluent. Although, we had chosen to explore this combination on the inactivation of a microorganism that has the highest thermoresistance of the bacterial vegetative forms; which is a *Enterococcus faecalis* Gram positive.

After inactivation by ultrasound (35 kHz) of *E. faecalis* ATCC 29212 in the dairy wastewater effluent, a significant effect ($P<0.05$) due to the declumping effect was obtained during the first 30min, wich push us suggest that there is a bacterial inactivation effect but concealed by the declumping effect. Indeed, the sonication treatment dissociates aggregated cells into chains or diplococcoci (Figure 20).

A significant difference ($P<0.05$) was observed between microbial inactivation at 10 and 30 min using ultrasound, it is related to the declumping effect.

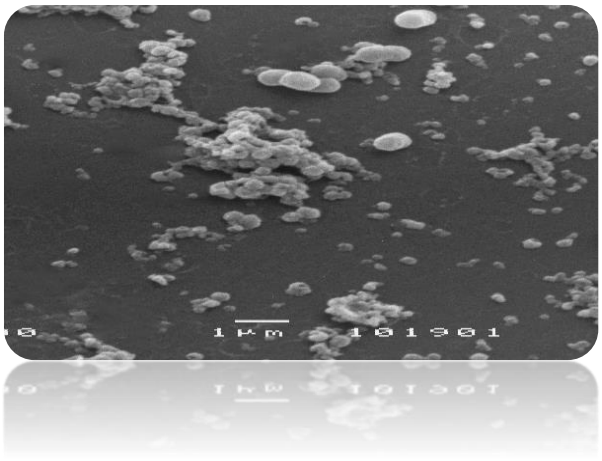


Figure 20. Chain grouping under scanning electron microscopy of a strain of *Enterococcus faecalis* ATCC 29212

The results show that the first few seconds (10 s – 20 s) under microwave at either 650 W or 350 W power, there was an increase in the microbial load compared to the initial one. The power at 350 W produced an immediate increase in CFU which reached 8.57 ± 0.26 log followed by a steady decrease, but this was insignificant (not significant). The level of bacterial load remains unchanged above the initial concentration even after 20 s of MW irradiation, this can be explained by the declumping effect. After 60 s, inactivation does not exceed 2 log.

At 650 W, the significant inactivation effect starts from 50 s, but the destruction was only 4.6 log after 60 s, and thus *E. faecalis* ATCC 29212 was more resistant to a lower power than to a higher one.

After coupling ultrasound in pre or post treatment with microwave, we didn't observed any dramatic effect on the bacteria even after 60 s at 350 or 650 W. Indeed, inactivation with microwave alone at 350 W is better than the coupling with ultrasound either in post or pre-treatment at 10 min. Inactivation with US in pre-treatment (20 and 30 min) starts to become significant ($P < 0.05$) from 75s in the microwave.

A slight difference between 650 W alone and the coupling with ultrasound 20 min in pre-treatment but it remains insignificant. The 30 min ultrasonic coupling followed by microwave at 60 s gives a total destruction, these results are better than US in post-treatment or microwave alone.

In all the tested cases, the fit of the Weibull model was very reasonable with $0.99>R^2>0.92$, this does not mean that other models are not applicable; in fact, they could also work well. However, the purpose of this work was not to compare models, but to study the dependence of Weibull parameters on power and exposure time to microwave and ultrasound.

Contrary to our expectations, the values of α are not clear in relation to the treatment that *E. faecalis* ATCC 29212 underwent, for example the ultrasound pre-treatment with the microwave at 650 W, the values of α are 32. 8 ± 6.5 s, 29.1 ± 11.9 s and 33.6 ± 11.6 s for US at 10 min, 20 min and 30 min respectively ; Also when it comes to the ultrasound post-treatment at 350 W, or α = 50.0 ± 6.4 s at 30min and α=49.9 ± 2.0 s at 20 min. Indeed, after setting the value of β=2.6 for all alpha values, the results were clear and quite logical. The values of α became 33.6 ± 1.6 s, 29.6 ± 2.4 s and 28.3 ± 3.0 for pre-treatment at US 10 min, 20 min and 30min with 650 W microwave respectively.

Combined MW/ US processing is less important than MW processing alone at 650 W; MW (650 W)/ US 10 min, MW (650 W)/ US 20 min, MW (650 W)/ US 30 min, the values from α are 37.8 ± 3.7 s, 36.3 ± 2.6 s, and 34.5 ± 2.5 s, respectively. Based on the data obtained in thisstudy, the application of a pre-treatment in the US had a better effect on the inactivation of *E. faecalis* ATCC 29212 than the MW treatment followed by the US.

In order to have more information on the mechanisms of action of microwaves and ultrasounds on the bacterial cell, its observation under electron microscope scanning and dosage of proteins in the treated or untreated medium have been carried out.

The proteins released by *E. faecalis* ATCC 259212 in the extracellular medium after treatment with ultrasound at 30 min, microwave at 650 W for 20 s, 40 s and 60 s, as well as the coupling in pretreatment by ultrasound at 30 min; were measured.

The results show that the applied microwaves have an effect on the quantity of proteins released in the extracellular medium at 650 W.

It seems that an exposure for 20 s and 40 s allows a higher protein leakage than an exposure for 60 s. After combining the principle of bradford assay, which is the reaction of coomassie blue G 250 with basic aminoacids (arginine, histidine, lysine), and the principle of non-thermal action mechanisms of microwaves on protein folding, it is suggested that basic aminoacids are not accessible to react with coomassie blue during protein folding.

Indeed, even if the cells subjected to ultrasound 30 min with microwaves at 20s and 40s are permeabilized with an increase of 3.5 % and 6 % of proteins released in the external environment compared to the control, we were not able to demonstrate significant cell mortality, we even had a declumping effect at 20 s in the microwave and a reduction of 3.5 log at 40s thisis due to a non-bactericidal effect. On the other hand, a 9% increase in protein was obtained at 60 s of post-treatment in the microwave, which corresponds to the total mortality of *E. faecalis* ATCC 29212. Shamis et al. (2011) observed the non-bactericidal effect on *E. coli* ATCC 25922 after short-term microwave treatment, more over they deduced that this MW effect seems to be temporary, because after an additional period of 10 min, the morphology of the cell seems to return to a state identical to that of the untreated controls.

Nevertheless; if we correlate these results with those obtained in terms of membrane integrity, the quantity of proteins released in the extracellular medium is proportional to the state of the cells permeabilized for bacterial suspensions exposed to ultrasound and microwaves.

In fact, the time of protein determination and the observation under scanning electron microscope of *E. faeaclis* in the wastewater dairy effluent, is not random and that's because we made our calculations basing on the fact that the selected points gave a general overview of all the observed phenomena, which are the declumping effect, the inactivation effect, the effect of the microwave alone and ultrasound alone and the coupling of both technics effect, that which gives a synergistic effect.

The membrane seems to be the seat of the most interesting phenomena in the presence of an electromagnetic field. The same applies to ultrasonic cavitation.

Observations under SEM of *Enterococcus faecalis* ATCC 29212 before treatment show the morphology, appearance and grouping mode of the latter, which is diplococcal or chain-like with a very smooth intact membrane. The cells of *Enterococcus faecalis* ATCC 29212, exposed to 650 W microwaves at different times ranging from 20 s to 60 s, were deformed. Wall shrinkage, a dehydrated appearance ; a reduction in the size of the bacterial cells and a transformed and amorphous structure were observed. Megha, et al. (2015) suggest that an alteration in membrane permeability due to oxidative damage has occurred.

Indeed, many hypotheses concerning the non-thermal effect of microwaves on the cell membrane were emitted. A change in ion concentration on either side of the membrane and the reorientation of large molecules, such as membrane proteins, could be a hypothesis on the non-

thermal mechanism of microwaves (Barnes and Hu, 1977). Moreover, the dielectric behaviour of biological macromolecules (proteins, lipids, carbohydrates, nucleica cids) is a function of the amount of water molecule in close contact withthem (bound water). The water bound to the membrane could there fore be responsible for the effects of microwaves.on the cell membrane (Liu and Cleary, 1995). Water may there fore be needed to induce and potentiate specific microwave effects (Vela and Wu, 1979).

The bacterial cells inside fat milk are protected from microwaves.

SEM observation of *E.faecalis* ATCC 25922 sample's treated by coupling effect showed that it effect has a more destructive effect on wall structure and cell shape than microwave or ultrasound treatment seperately.

A synergistic effect is to be noted because the declumping effect of the ultrasound increases the probability that the individual cells come completely into contact with the acoustic wave. The chemical bonds between the molecular components of the cell membranes were broken by the ultrasound, sothat the brittleness of the membranes caused by the ultrasound favored the bactericidal effect of the microwaves of *E. faecalis* (Nan et al., 2010; Piyasena et al., 2003).

5 GENERAL CONCLUSION

On the one hand, orange juice is one of the most consumed juices in the world. Beyond its sweet and refreshing taste, orange juice is a mine of antioxidants, vitamins, minerals and a host of other excellent health nutrients. Nevertheless, it remains a reserve for a possible contamination by *Escherichia coli* "STEC". The search for microbiological stabilization processes of fruit juices that cause a minimum damage to the organoleptic characteristics, are considered an alternative to the classic thermal pasteurization. The inactivation kinetics of *E. coli* ATCC 25922 were studied by ultrasound and microwave.

On the other hand, water plays an essential role in the dairy food industry with large volumes of wastewater from various treatment units, this water is rich in organic matter, therefore, these wastewaters may contain a multiplicity of contaminants including *E. faecalis.* Several methods of treating this effluent are being considered, however; the use of ultrasounds and microwaves for this purpose is a first. Due to the significant presence of *E .faecalis* ATCC 29212 in the wastewater dairy effluent, decontamination of the latter remains paramount. Inactivation kinetics of *E. faecalis* ATCC 29212 in dairy wastewater effelent by the effect of uncontrolled acoustic cavitation (ultrasound) at a low frequency and by microwaves otheir coupling is achieved, this study is pioneering.

In this research, the comparative study was under taken between ultrasonic and microwave inactivation methods for *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 in orange juice beverage and wastewater dairy effluent, respectively, modelling of kinetics by Weibull, physico-chemical parameters namely pH, acidity and sucrose (° Brix) content for orange juice and protein determinations for waste water dairy effluent and observation under scanning electron microscope (SEM) of *E. faecalis* ATCC 29212 are carried out.

The kinetic results allowed us to deduce that the inactivation process of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 by ultrasoundis not significant and that the behavior of the two species differs with respect to cavitation. Indeed, the most sensitive members of the *E. coli* ATCC 25922 bacterial population are inactivated first and the most resistant ones remain; and a declumping effect followed by inactivation for *E. faecalis* ATCC 29212.

Microwave inactivation of the two species shows that the efficiency of inactivation depend sproportionally on the time and power parameters. A total destruction is obtained at 900 W for

35 s with an α value of 13.6 ± 0.8 s, however no total destruction for *E. faecalis* ATCC 29212 at 650 W. This situation was overcome by simply coupling ultrasound and microwave treatment times which allowed to effectively eliminate both bacterial strains of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212.

The results of the inactivation kinetics by ultrasound and microwave coupling treatment allowed us to deduce that the inactivation process of *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 takes place in two steps: a declumping step at low microwave power followed by an inactivation step.

Indeed, the kinetics of inactivation by a post-treatmentis ultrasound are much more performant compared to the kinetics of inactivation by an ultrasound pre-treatment.

The effectiveness of the treatment varies according to the Gram of the bacteria as well as its thermoresistance.

After measurements of the physico-chemical parameters of the treated orange juice beverage, no significant difference was observed, revealing the quality of the treatment by ultrasound-microwave coupling.

The protein dosage and the microscopic scanning observation shows us that the effect of ultrasound and microwave is indeed the bacterial membrane of *E. faeacalis*, and their combination gives a synergistic effect.

Microwave heating is fast and can significantly reduce the time to reach the desired process temperature. The hydrodynamic action of ultrasound on microorganisms has great potential to improve the efficiency of microbial decontamination by microwave treatment. This study contributes to the design and control of an effective combined ultrasonic and microwave treatment to improve orange juice beverage pasteurization or dairy effluent decontamination processes.

Ultrasound-microwave treatment has proven to be a promising technology for the reduction and complete inactivation of key indicator pathogenic microorganisms in orange juice beverage and in a model of dairy wastewater effluent. However, it is recommended that a wider variety of biological test species be used for conclusive results to advance this technology which can be safely deployed.

In order to complete the study, other elements missing from our work would be necessary to carry the mout:

- The effect of the treatment on the quality parameters of the juice (color, ascorbic acid content, total phenolic content, antioxidant activity and certain sensory attributes) must be studied so that the synergistic effect can be better evaluated.
- Optimization of inactivation processes (MW; US)
- An increase in microwave power would be necessary in order to obtain a short time and high temperature for better inactivation of *E. faecalis* ATCC 29212.
- Studying HSP protein: The interest in studying these proteins during exposure to microwave fields lies in the fact that it is known that electromagnetic fields induce a response to stress (Weisbrot et al., 1993). Moreover, some of them are produced only when there is a rise in temperature and seem interesting to locate the existence of hot spots within a system exposed to microwaves.
- Modeling of *E.coli* ATCC 25912 growth kinetics after US/MW treatment in orange juice beverage.

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APPENDIX

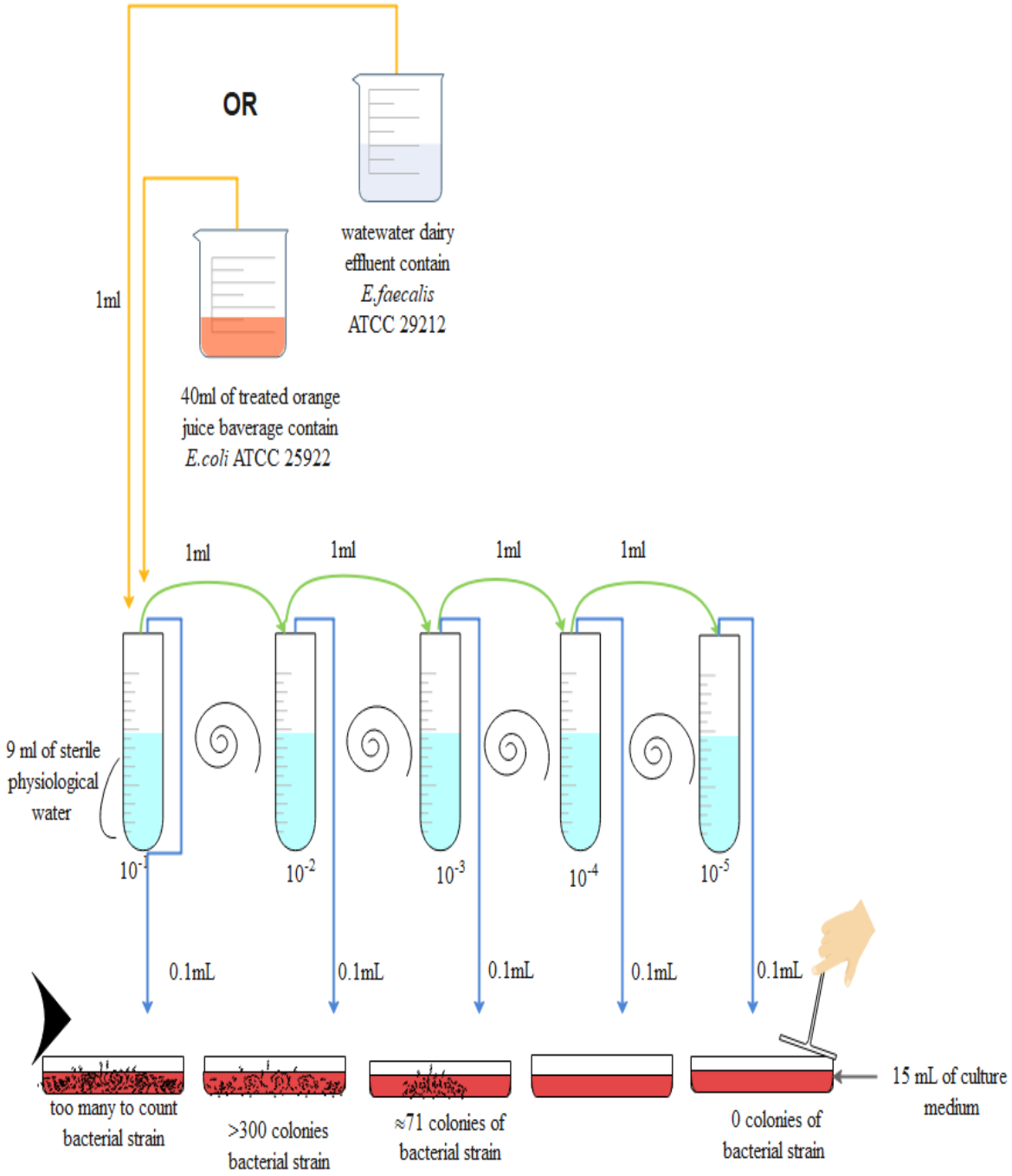

Figure 1. Method of enumeration of survival cells

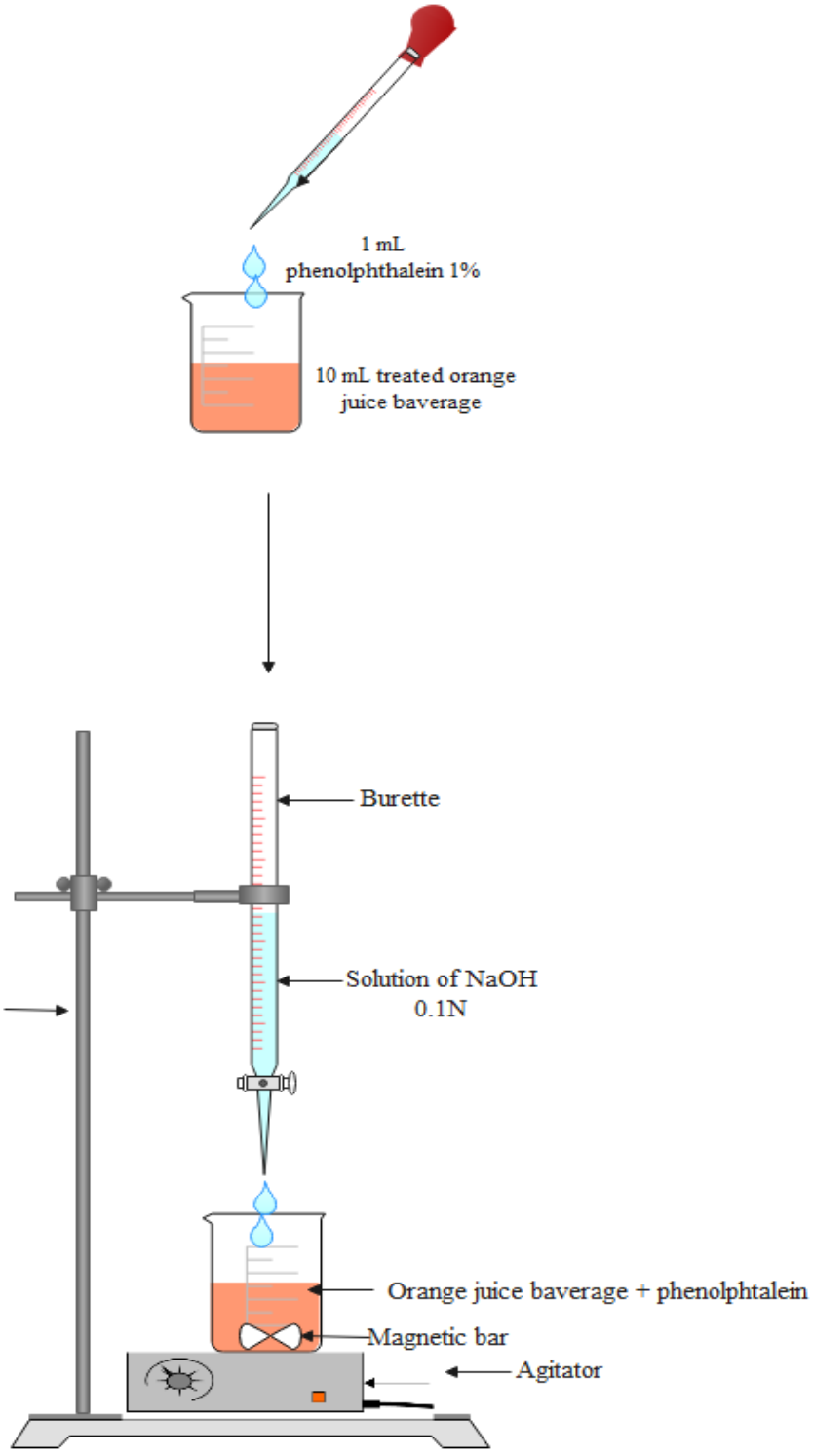

Figure 2. Method of Measuring acidity

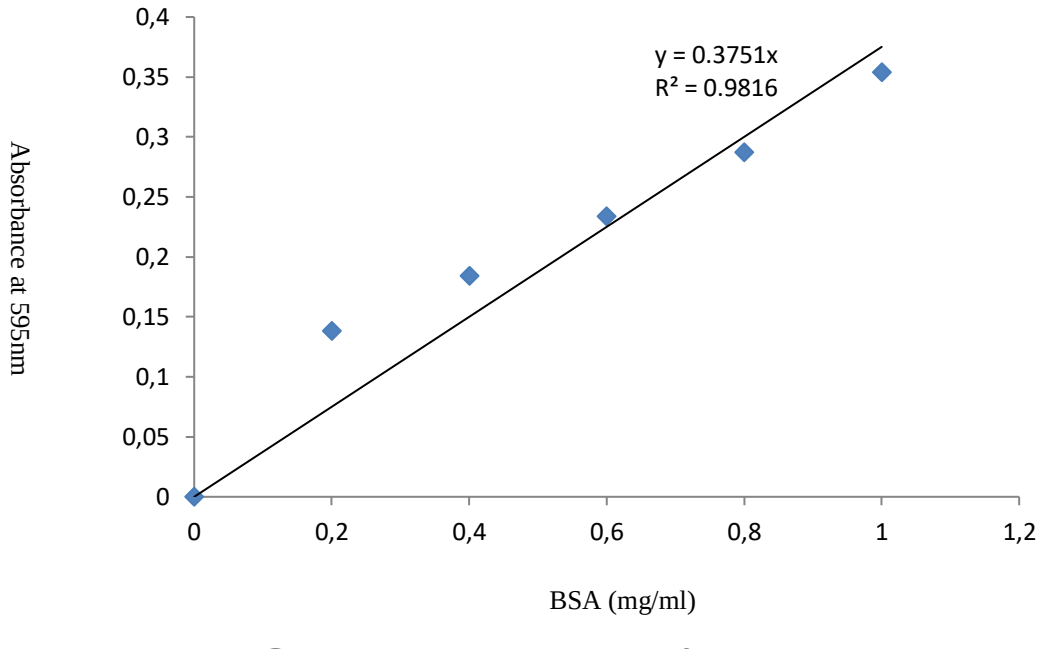

Figure 1. Protein determination by the macro method.

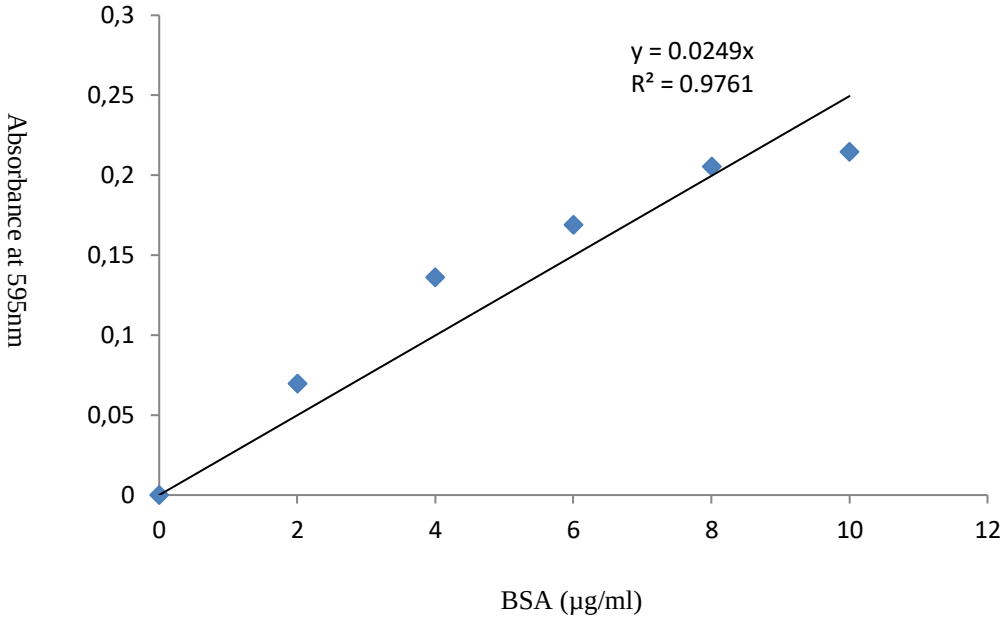

Figure 2. Protein determination by the micro method.

Composition of the cultures media

- **EMB medium composition**

peptone (any)	10 g
lactose	5 g
sucrose	5 g
potassium hydrogen phosphate	2 g
eosin Y	400 mg
methylene blue	65 mg
agar	13,5 g
distilled water	1000ml

Adjust the pH to 7,2 ± 0,2 at 25°C

- **TSA medium composition**

Tryptone	15 g
Soy papain peptone	5 g
Sodium chloride	5 g
agar	15 g
distilled water	1000ml

Adjust the pH to pH 7.3 ± 0.2

- **TSB medium composition**

Glucose (dextrose)	2.5 g
Bipotassium hydrogen phosphate	2.5 g
Sodium chloride	5 g

distilled water	1000ml

Adjust the pH to pH 7.3 ± 0.2

Résumé

La survie d'*Escherichia coli* ATCC 25922 et d*'Enterococcus faecalis* ATCC 29212 traitées par microondes et/ou ultrasons repspectivement dans une boisson du jus d'orange et dans des effluents d'eaux usées de laiterie a été évaluée ; le modèle de Weibull a été ajusté aux courbes de survie pour décrire les cinétiques d'inactivations et comparer les effets des différents traitements combinés micro-ondes-ultrasons. Le traitement par ultrasons (42 kHz) n'a pas d'effet significatif sur la réduction du taux de survie (1,3 log pour 60 min de sonication) d'*E. coli*, mais la réduction est significative lorsqu'il s'agit *d'E. faecalis*, traité par ultrasons (35kHz), due à l'effet declumping. Cependant, la sonication peut augmenter l'efficacité de l'inactivation des micro-ondes. L'utilisation des ultrasons sur *E. coli* ATCC 25922 en prétraitement a été plus efficace que celle en post-traitement, c'est-à-dire qu'une réduction de 8 log a été obtenue par une combinaison d'ultrasons (20 min) suivie d'une micro-onde (900 W/30 s), tandis qu'une réduction de 4,0 log a été obtenue lorsque ces deux processus ont été inversés. Le paramètre d'échelle α estimé à partir de la cinétique d'inactivation des micro-ondes combinée à un prétraitement par ultrasons s'est avéré inférieur à ceux obtenus avec un post-traitement, qui, à leur tour, sont inférieurs à ceux estimés pour les micro-ondes ou les ultrasons seuls. Les résultats ont montré que le pH, Brix et acidité titrisable est non significatif ($P < 0,05$).

En effet, les même résultats sont observés avec *E. faecalis* ATCC 29212 utilisant des ultrasons en pré-traitement qui été plus efficace que son post-traitement. Une réduction totale a été obtenue par une combinaison d'ultrasons (30 min) suivie d'une micro-onde (650 W/60 s) avec $\alpha=28.3 \pm 3.0$s qui est inférieur à ceux obtenus avec un post-traitement. Le dosage des protéines et l'observation microscopique à balayage (SEM) révèlent que l'effet des ultrasons et des micro-ondes se manifeste par des dommages graves sur la morphologie des cellules, trous sur la membrane cellulaire, l'apparition d'effondrements et la perméabilité de la membrane *d'E. faecalis*.

Mots clés : *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, microwave, ultrasons, boisson de jus d'orange, effluents d'eaux usées de laiterie, modelisation cinétiques d'inactivation.

Abstract

Survival of *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 in orange juice beverage and dairy wastewater effluent treated with microwaves and/or ultrasound was evaluated ; the Weibull model wasfitted to the survivalcurves to describe the inactivation kinetics; and the effect of combined microwave-ultrasound treatments was evaluated. Ultrasound treatment (42 kHz) had no significant effect on the reduction of survival rate (1.3 log for 60 min of sonication) of *E. coli*, but the reduction was significant when *E. faecalis* wastreated with ultrasound (35kHz), due to the declumping effect. However, sonication can increase the efficiency of microwave inactivation. The use of ultrasoundon *E.coli* ATCC 25922in pretreatment was more effective than in posttreatment, i.e., an 8 log reduction was achieved by a combination of ultrasound (20 min) followed by microwave (900 W/30 s), where as a 4.0 log reduction was achieved when these two processes were reversed. The scaling parameter α estimated from microwave inactivation kinetics combined with ultrasonic pretreatment was found to be lower than those obtained with posttreatment, which, in turn, are lower than those estimated for microwave or ultrasonic alone. The results showed that the pH, Brix and titratable acidityis not significant ($P < 0.05$).

Indeed, the samere sults are observed with *E. faecalis* ATCC 29212 using ultrasound as a pre-treatment which was more effective than its post-treatment. A total reduction was obtained by a combination of ultrasound (30 min) followed by microwave (650 W/60 s) with $\alpha=28.3 \pm 3.0$s which is lower than those obtained with post treatment. Protein assay and scanning microscopic observation (SEM) reveal that the effect of ultrasound and microwave is manifested by severe damage to cell morphology, holes in the cell membrane, appearance of collapses and permeability of the *E. faecalis* membrane.

Keywords: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, microwave, ultrasound, orange juice beverage, dairy waste water effluent, inactivation kinetic modeling.

الملخص

تم تقييم بقاء *Escherichia coli* ATCC 25922 و *Enterococcus faecalis* ATCC 29212 المعالجين بالميكروويف و / أو الموجات فوق الصوتية على التوالي في مشروب عصير البرتقال وفي مياه الصرف الصحي لمنتجات الألبان ؛ تم تركيب نموذج Weibull على منحنيات البقاء على قيد الحياة لوصف حركية تعطيل النشاط ومقارنة تأثيرات العلاجات المختلفة بالموجات فوق الصوتية بالميكروويف. العلاج بالموجات فوق الصوتية (42 كيلو هرتز) ليس له تأثير كبير على تقليل معدل البقاء على قيد الحياة (-1.3 لوغاريتم لكل 60 دقيقة من صوتنة) للإشريكية القولونية ، لكن الانخفاض مهم عندما يتعلق الأمر بـ *E. faecalis* ، التي يتم علاجها بالموجات فوق الصوتية (35 كيلو هرتز) ، بسبب تأثير التفريغ. ومع ذلك ، يمكن أن يزيد الصوتنة من كفاءة تعطيل الميكروويف. استخدام الموجات فوق الصوتية على كانت الإشريكية القولونية ATCC 25922 في المعالجة المسبقة أكثر كفاءة من المعالجة اللاحقة للموجات فوق الصوتية ، أي تم الحصول على تخفيض 8 لوغاريتمات عن طريق مزيج من الموجات فوق الصوتية (20 دقيقة) تليها موجة ميكروفون (900 واط / 30 ثانية) ، مع تقليل 4.0 لوغاريتم تم الحصول عليها عندما تم عكس هاتين العمليتين. تم العثور على معلمة المقياس α المقدرة من حركية تعطيل الميكروويف جنبًا إلى جنب مع المعالجة بالموجات فوق الصوتية أقل من تلك التي تم الحصول عليها مع المعالجة اللاحقة ، والتي بدورها أقل من تلك المقدرة للميكروويف أو الموجات فوق الصوتية فقط. وأظهرت النتائج أن الرقم الهيدروجيني ، نسبة السكر والحموضة القابلة للمعايرة ليس كبيرا ($P <0.05$) .

في الواقع ، لوحظت نفس النتائج مع بكتريا *E. faecalis* ATCC 29212 باستخدام الموجات فوق الصوتية كعلاج مسبق والذي كان أكثر فعالية من معالجته اللاحقة. تم الحصول على تخفيض كلي عن طريق مزيج من الموجات فوق الصوتية (30 دقيقة) متبوعًا بميكروويف (650 واط / 60 ثانية) مع $\alpha = 28.3 \pm 3.0$ ثانية وهو أقل من تلك التي تم الحصول عليها مع المعالجة اللاحقة. يكشف فحص البروتين والمراقبة المجهرية أن تأثير الموجات فوق الصوتية والميكروويف يتجلى في الأضرار الجسيمة التي لحقت بمورفولوجيا الخلايا ، والثقوب في غشاء الخلية ، وظهور الانهيارات ونفاذية غشاء *E. faecalis*

الكلمات الرئيسية: *Escherichia coli* ATCC 25922 , *Enterococcus faecalis* ATCC 29212 ، ميكروويف ، موجات فوق صوتية ، مشروب عصير برتقال ، مخلفات سائلة لمياه الألبان ، نمذجة حركية التعطيل