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A case study on the use of ultrasound for the inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in almond milk

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ABSTRACT

The interest towards not-dairy beverages is increasing and almond milk is widespread diffused. The main topic of this paper was a focus on Ultrasound (US) to inhibit *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

The variables of the treatment (power, duration, and pulse) were combined through a randomized design; the use of DoE theory (Design of Experiments) and its outputs (Pareto chart, 3D plots, desirability profiles) pointed out that the effect of the treatment relies upon the total energy distributed into the system on *E. coli* O157:H7, as suggested by the significance of interactions of power, pulse and time, while power was the most important factor for *L. monocytogenes*. A final challenge test was done by using two combinations (H-80% of power, 8 min and pulse at 6 s- for *E. coli* and F-80%; 2 min; 6 s- for *L. monocytogenes*) and storing the samples at 4 °C for 2 weeks. This experiment suggests that the treatment could exert a sub-lethal injury on the pathogens, which, combined with the storage under refrigeration, could contribute to increase the shelf life.

1. Introduction

Almond milk is present in the European market as an alternative nutritive beverage for consumers suffering from lactose intolerance, hypersensitive to cow milk, and looking for plant-based beverages as a substitute to dairy milk [1]. It is promoted as healthy food for its unique nutritional properties, such as the high content of monounsaturated fatty acids. Moreover, the almonds have a balanced composition in protein and fat content and other beneficial plant compounds as fiber, vitamins, antioxidants and minerals [2].

Despite these benefits, little is known on the ability of almonds to support the growth of pathogenic bacteria. The safety is generally assured by thermal treatments, but they cause a loss of the nutritional and organoleptic properties of food products [3], with an adverse effect on sensory and nutritional quality attributes [4]. Non-thermal approaches have been proposed as alternative strategies to avoid these drawbacks, as they are effective at sub-lethal or ambient temperature, leading to minimal or no impact on some key-nutritional parameters of food [5,6].

Among non-thermal technologies, ultrasound processing (US) has received increasing attention because it is reasonably inexpensive and energy saving [7–9].

US are acoustic waves with a frequency from 2 Hz to 10 MHz. This large range can be divided in 3 regions: a) Infrasonic (2–20 Hz); b) Acoustic (20 Hz–20 kHz); c) Ultrasonic (20 kHz–10 MHz). US for food processing have a frequency of 20–100 MHz [10].

The antimicrobial efficacy of US is related to two main mechanisms, namely, cavitation and sonolysis (or sonoporation) [10]. The cavitation is a physical phenomenon of bubble generation; bubbles increases their size and then collapse, producing areas of high temperature (approximately 5500 °C) and pressure (approximately 50 MPa). The high temperatures and pressures created within the bubble are responsible for the generation of hydrogen atoms and hydroxyl radicals [6,11]. The cellular membrane is the first target for the lethal effects of cavitation, with at least 6 different injuries or modes of actions, generally referred to as sonoporation [10]: a) simple cavitation (increase and implosion of bubble size near the membrane); b) push (expansion of bubble may touch and push the membrane); c) pull (bubbles may pull the membrane during the compression/contraction phase, thus leading to membrane disruption); d) jetting (an asymmetric collapse of bubbles can create a funnel); e) streaming (the stream of fluid around the oscillating bubble creates shearing effects); f) translation (high intensity ultrasonic radiation forces may diffuse through the membrane).

Various studies addressing the effect of US alone or combined with other treatments showed microbial inactivation in liquid media, such as

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water, juices, and milk and US exerted promising performances against spoiling dairy microflora and pathogens, such as *Escherichia coli*, *Pseudomonas fluorescens*, *Listeria monocytogenes*, *Listeria innocua*, and yeasts [7,6,9,12]. The lethal effects of US rely on some leading variables [7,13,14,15]:

a) the size of the cells: the larger is a cell the more is sensitive
b) Gram status: some studies reported that Gram negative bacteria were more sensitive, while other authors found that there were not differences between Gram positive and Gram negative bacteria
c) matrix, as some compounds could exert a protective effect on cells;
d) the initial cell number: Gao et al. [16,17] found that the D-value increased by increasing the initial viable count.

The effect of US on yeasts and bacteria include morphological changes, thinning and/or disruption of cell membrane, and possible injuries on DNA [6].

The only evidence on the use of US in almond milk is the paper by Maghsoudlou et al. [4] reporting the effect of the technologies on particle size, with a beneficial effect on the shelf life. To the best of our knowledge, there are not papers dealing with the use of US as a mean to control microorganisms in almond milk. Thus, the main goal of this study was to evaluate the reduction and inactivation of two foodborne pathogens (*E. coli* O157:H7 and *L. monocytogenes*) in almond milk, by focusing on the weight of the variables of a US treatment (power, duration, and pulse) and then running a challenge test at 4 °C.

2. Materials and methods

2.1. Microorganisms

E. coli O157:H7 and *L. monocytogenes* belong to the Culture Collection of the laboratory of Predictive Microbiology, University of Foggia. Bacteria were stored at 4 °C on Nutrient agar slants (Oxoid, Milan, Italy). Before each assay the strains were grown in Nutrient broth (Oxoid) at 37 °C for 24 h. The cultures were centrifuged at 4000 rpm/4 °C for 10 min; then, the strains were washed with sterile saline solution (9 g/L NaCl) and inoculated into a commercial almond milk.

2.2. Almond milk

A commercial almond milk gluten-wheat-lactose free (Water; Sugar, 3 g; Almond, 20 g; Calcium as tri-calcium phosphate, 120 mg; Sea salt 0.13 g; Stabilisers-Locust bean gum, Gellan gum; Sunflower lecithin; Vitamins (B2, B12, E, D23),14 g) was purchased from a local market in Foggia (Italy).

2.3. Effects of US

E. coli and *L. monocytogenes* were individually inoculated to 5 log CFU/mL in aliquots of 25 mL of almond milk and US-treated with a VC Vibra Cell Ultrasound (US) equipment, model VC 130 (Sonics and Materials Inc., Newtown, CT, USA); the equipment works at 20 kHz (frequency) 130 W (acoustic energy). The probe (5 × 60 mm; diameter × the active component of horn) was put 2–3 cm below the surface of milk.

Power level, the duration of the treatment and pulse were combined through a fractional design 3^{k-p} (Table 1). The number of surviving cells immediately after the treatment was evaluated through spread plating (Nutrient agar, incubated at 37 °C for 24 h). Inoculated and untreated samples were used as controls. The experiments were done on three independent batches.

The reduction of the viable count referred to the control (log CFU/mL) was used as input value for a multiple regression approach; power, time (duration of the treatment) and pulse were used as independent variables. The analysis was done through the software Statistica for Windows (StatSoft, Tulsa, OK, USA). The model was built by using the option "quadratic," for the evaluation of the individual ("Power", "Time" and "Pulse") and interactive effects ("Power * Time", "Power * Pulse" and "Pulse * Time").

Table 1
Combinations of power, duration of US treatment and pulse.

Combinations of the design	Power (%)	Duration of the treatment (min)	Pulse (s)
CONTROL	–	–	–
A	20	2	2
B	20	2	6
C	20	8	2
D	20	8	6
E	80	2	2
F	80	2	6
G	80	8	2
H	80	8	6
I	60	6	4

The significance of the model was evaluated through the adjusted regression coefficients and the mean square residual, whereas the significance of each factor was assessed through the Fisher test ($P < 0.05$).

The individual effect of each factor of the design (power, time, pulse) was also assessed through the desirability approach; the desirability is a function, reading as follows:

$$d = \begin{cases} 0, & y \le y_{min} \\ (y - y_{min})/(y_{max} - y_{min}) & y_{min} \le y \le y_{max} \\ 1, & y \ge y_{max} \end{cases} \quad (1)$$

where y_{min} and y_{max} are the minimum and maximum values of the dependent variable, respectively.

The desirability was included in the range 0–1 (0 for the lowest reduction of the viable count and 1 for the highest one). The desirability profiles were built by setting the variables to the mean values (power to 50%, time to 5 min, and pulse to 4 min) (*a priori* conditions).

2.4. Final validation

Afterward, aliquots of almond milk (25 mL) inoculated with either *E. coli* and *L. monocytogenes* (ca. 5 log CFU/mL) were US-treated with the following combinations: H (80%; 8 min; 6 s) for *E. coli* and F (80%; 2 min; 6 s) for *L. monocytogenes*, and stored at 4 °C for 16 days. Cell viability was evaluated through plate count, immediately after the treatment (time 0) and after 2, 5, 8, 13, and 16 days.

Data were modelled through the lag-exponential equation modified by van Gerwen & Zwietering [18] and by Baty & Delignette-Muller [19], cast in the following form:

$$y = \begin{cases} y_0 & t \le \lambda \\ y_{max} - \log\{(1 + 10^{y_{max} - y_0} - 1) * \exp[-\mu_{max}(t - \lambda)]\} & t > \lambda \end{cases} \quad (2)$$

where y and t are the dependent and the independent variables, respectively (cell number-log CFU/mL and time-day); λ is the lag phase (day); μ_{max} is the maximal growth rate ((log CFU/mL)/day); y_0 and y_{max} are the inoculum and the population in the stationary phase (log CFU/mL), respectively.

When the populations did not show the lag phase, the lag-

exponential model was used as follows [20]:

$$y = y_{max} - -\log\{1 + (10^{y\,max - y0} - 1) * \exp(-\mu_{max} t)\} \quad (3)$$

The estimation of the fitting parameters of the growth curve (y_0, y_{max}, μ_{max}, and λ) was done through the option advanced models/non linear estimation of the software Statistica for Windows. The lag-exponential model was set as the custom function and the loss of variability measured through the least squares approach. The basic assumptions of the fitting were as follows: a) at least 200 interactions for the estimation of the fitting parameters; b) asymptotic standard errors; c) set ε (random error) to 10^{-8}; c) start values of b_0 set to the observed values immediately after the inoculum to increase the accuracy of fitting.

The accuracy and the goodness of fitting was evaluated through the regression coefficient and the mean square residual.

3. Results

3.1. Effect of US on Escherichia coli

The first step of this research was aimed at assessing the lethality of sonication on the viability of the two pathogens; therefore, the analyses were done immediately after the treatment in order to assess the weight of the different factors on the antimicrobial effect towards the two pathogens. The results were analysed through the DoE approach (Design of Experiments) and gave three main outputs: a) the Pareto chart of standardized effects; b) the 3D plots; c) the desirability profiles. When setting a challenge test there are some critical factors that can affect the result; one the most important variable is the inoculum level [21]. For a stability experiment an inoculum of 2–3 log CFU/mL is used; on the other hand, higher inoculum levels are used to test the lethality of a treatment (at least 6 log CFU/mL) [21]. In this study, we chose a compromise and set the inoculum level to 5 log CFU/mL, as we performed both an evaluation of the lethality (first step) and a stability test (second step).

Fig. 1 shows the Pareto Chart for *E. coli* O157:H7; the significance of individual, quadratic and interactive terms is represented by bars: if a factor is significant, it is longer that the cut-off point (the vertical line). Moreover, the longer is a bar the more it is significant.

The model was significant with a multiple regression coefficient of 0.859 and a mean square residual of 0.046. The most significant terms were power (as linear factor) and the interaction "power by time", followed, in descending order, by the interactive term "power by

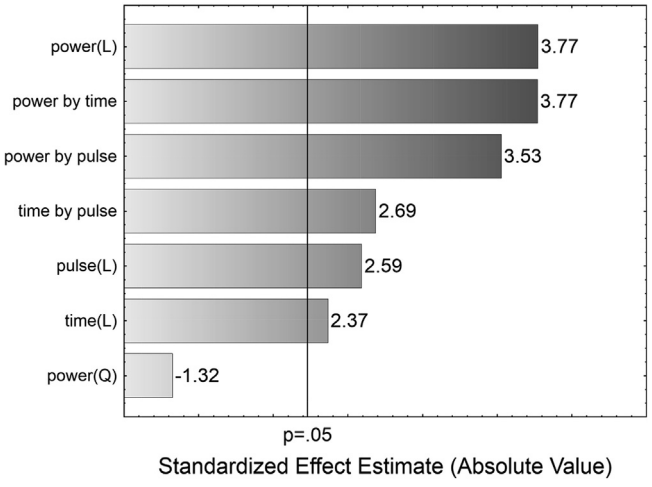

Fig. 1. Pareto charts of individual and interactive effects of power, duration of the treatment (time) and pulse on the reduction of the viable count of *E. coli* O157:H7. The vertical line represents the significance cut-off ($P < 0.05$). L, linear term; Q, quadratic effect.

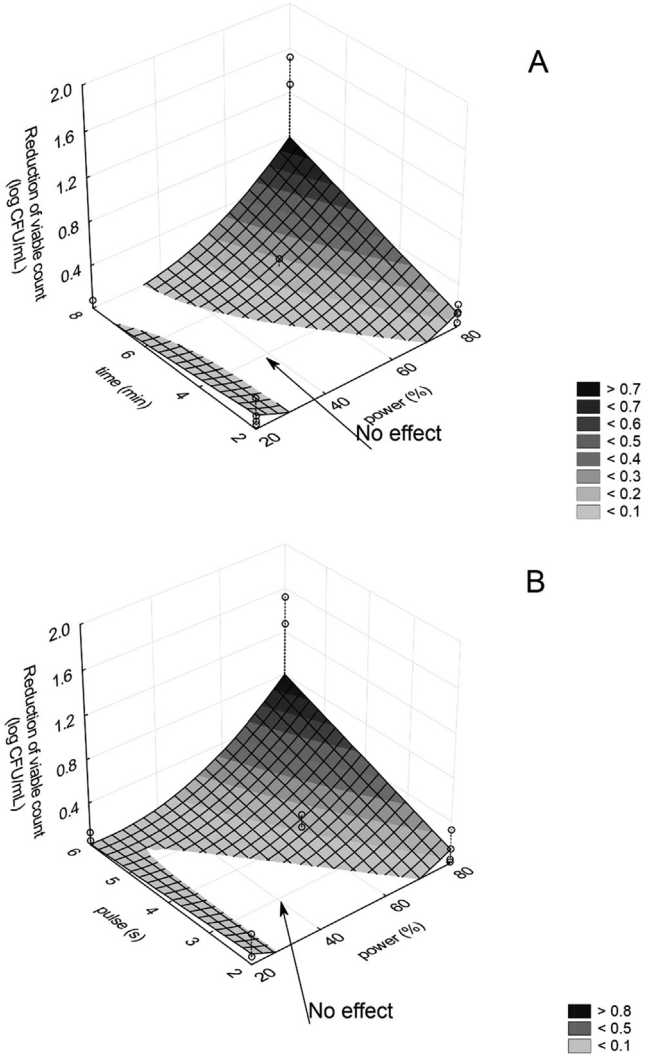

Fig. 2. 3D plots for the interactions "power by time" (A) and "power by pulse" (B) on the reduction of viable count of *E. coli* O157:H7 after US-processing.

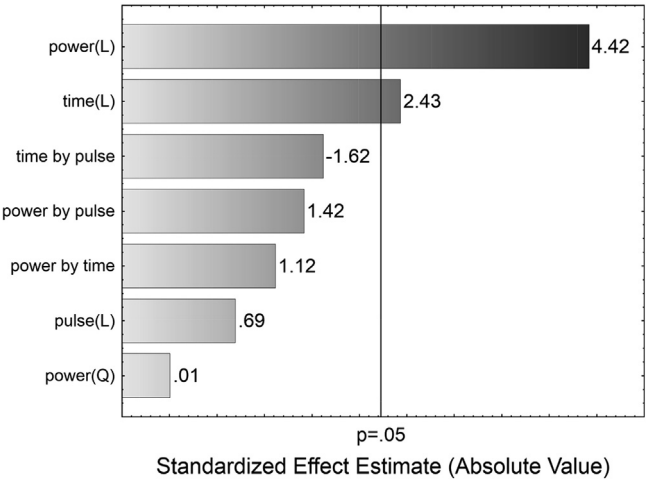

Fig. 3. Pareto charts of individual and interactive effects of power, duration of the treatment (time) and pulse on the reduction of the viable count of *L. monocytogenes*. The vertical line represents the significance cut-off ($P < 0.05$). L, linear term; Q, quadratic effect.

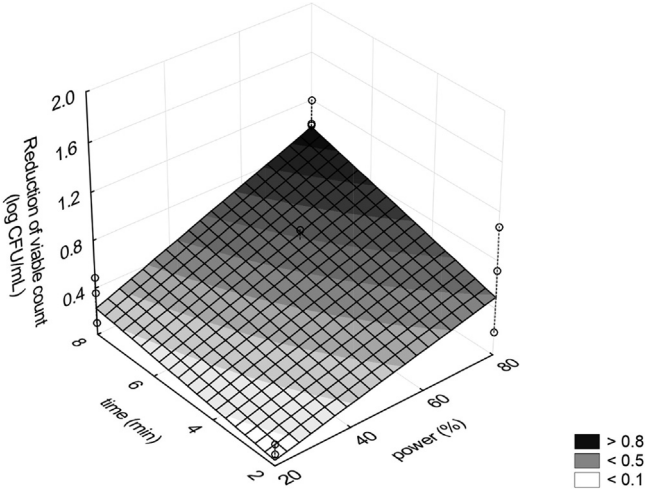

Fig. 4. 3D plots for the interaction "power by time" on the reduction of viable count of *L. monocytogenes* after US-processing.

pulse", "time by pulse" and by the linear terms of pulse and time.

Pareto chart is a qualitative output, because it pinpoints if a factor is significant or not, but it cannot give any insight on the quantitative trends. This output can be easily found in the 3D-plots. Fig. 2 shows the interaction "power by time" and "power by pulse". As one could infer from Fig. 2A, the effect of US-treatment was maximum at the highest levels of power (80%) and time (8 min) with a predicted reduction of the viable count of 1.0 log CFU/mL. The same output was found for the interaction "power by pulse", with a reduction of *E. coli* O157:H7 of 0.8 log CFU/mL for the combination power/pulse of 80%/6s (Fig. 2B).

3.2. Effect of US on Listeria monocytogenes

The same approach was used to model the results from *L. monocytogenes*. The model was significant, and the multiple regression coefficient was 0.764; however, the weight of factors was different as the reduction of the viable count was only affected by power and time as individual terms (Fig. 3). The effect recovered on 3D-plots were similar to those found for *E. coli* O157:H7, that is the effectiveness of the treatment increased by increasing the power and the duration of the treatment and was maximum (reduction of the viable count by 1 log CFU/mL) when the power was set to 80% for 8 min (Fig. 4).

3.3. Optimization of the treatment

3D-plots are probably the most important outputs of the Design of the Experiments. However, they suffer a drawback: they are focused on two-way interactions (for example in this research power by time for *L. monocytogenes* and power by time or power by pulse for *E. coli* O157:H7) and cannot be used to analyse the individual effect of each factor excluding the other variables. This output can be easily recovered through the prediction and desirability approach. The desirability is a dimensionless parameter ranging from 0 to 1 and is the output to the question: is the achieved result good or bad? The reply is: 0 for the worst result and 1 for the best one. Moreover, a desirability profile is often completed by a prediction profile, which shows the predicted values of the dependent variable as a function of the values of the factors of the design [22].

The profiles for *E. coli* O157:H7 are in Fig. 5; as expected by increasing the power the effect of the treatment increased and achieved the most desired value (desirability of 0.35, corresponding to a reduction of the viable count of 0.5 log CFU/mL) at the highest power. An

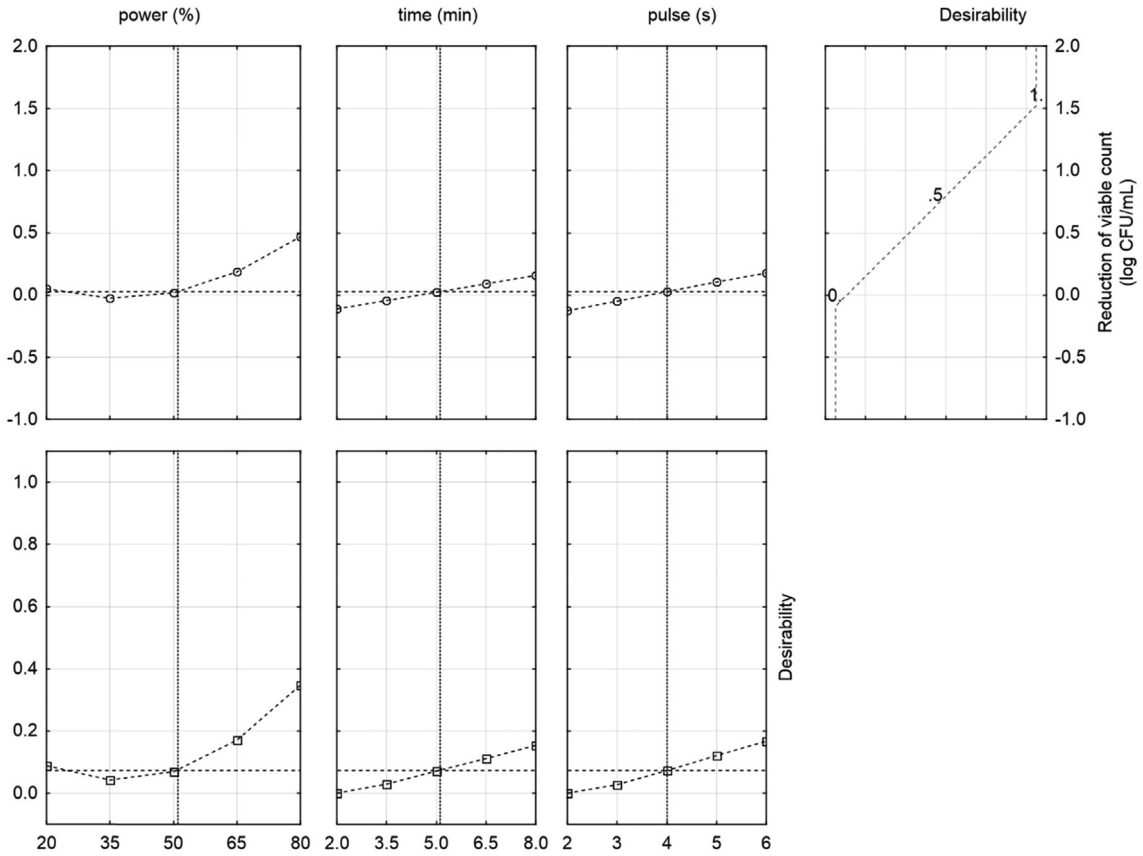

Fig. 5. Prediction (up) and desirability profiles (down) for the individual effects of power, time and pulse on the reduction of the viable count of *E. coli* O157:H7. The dotted vertical lines represent the *a priori* conditions for the evaluation of desirability (power to 50%, time to 5 min, and pulse to 4 min).

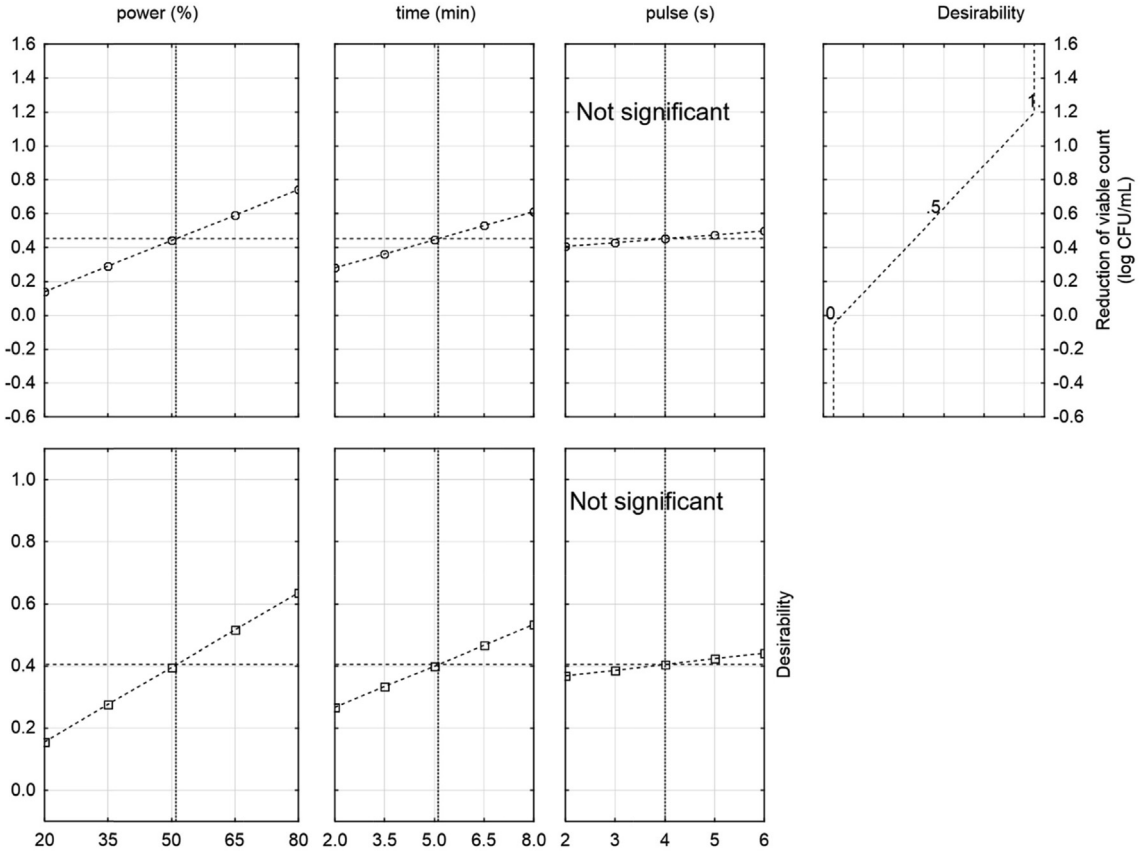

Fig. 6. Prediction (up) and desirability profiles (down) for the individual effects of power, time and pulse on the reduction of the viable count of *L. monocytogenes*. The dotted vertical lines represent the *a priori* conditions for the evaluation of desirability (power to 50%, time to 5 min, and pulse to 4 min).

Table 2
Fitting parameters (mean values ± standard error) of the lag-exponential equation for the evolution of *E. coli* O157:H7 and *L. monocytogenes* in US-treated almond milk. H: power, 80%; time, 6 min; pulse, 6 s. F: power, 80%; time, 2 min; pulse, 6 s.

	y_0	y_{max}	μ_{max}	λ	R
E. coli O157:H7					
Control	5.12 ± 0.22	8.56 ± 0.16	1.19 ± 0.07	–	0.973
Combination H	3.81 ± 0.33*	8.41 ± 0.88	0.79 ± 0.04*	–	0.848
L. monocytogenes					
Control	5.64 ± 0.24	8.36 ± 0.14	1.62 ± 0.12	–	0.943
Combination F	4.86 ± 0.40	7.73 ± 0.35	1.68 ± 0.22	4.91 ± 0.19	0.945

The symbol "*" denotes a significant difference between the control and US-treated sample (t-student test, $P < 0.05$).
y_0, inoculum (log CFU/ml); y_{max}, population in the stationary phase (log CFU/mL); λ, lag phase (day); μ_{max}, maximal growth rate ((log CFU/mL)/day); R, regression coefficient.

important result was that the correlation power/effect was not strictly linear, as the effectiveness of the treatment increased only at power levels > 50%.

On the other hand, the outputs found for the time and the pulse were unexpected, as these parameters, as individual terms, slightly affected or did not influence the effectiveness of the treatment, as the desirability increased for both of them from 0 to 0.17 (reduction of the viable count by 0.19 log CFU/mL)

The individual terms of power and time more significantly affected *L. monocytogenes* (Fig. 6), as suggested by both the prediction and the desirability profiles. The correlation power/effect was strictly linear, and the reduction of the viable count increased up to 0.8–1.0 log CFU/mL at a power level of 80%; similar results were found for the time.

3.4. Challenge test

In the last step of this research a preliminary validation was done on two selected combinations of the design; the choice relies upon the reduction of the viable count in the preliminary step (combination H for *E. coli* because it resulted in the highest effect of the treatment) or the increase of the temperature for *L. monocytogenes* (exit temperature after the treatment < 40 °C; combination F). The results were analysed by the lag-exponential model (Table 2). US treatment (combination H) significantly reduced *E. coli* O157.H7 level from 5.12 to 3.81 log CFU/mL and lowered the growth rate (μ_{max}) (from 1.19 to 0.79 (log CFU/mL)/day). The pathogen never experienced the lag phase. On the other hand, US acted on the shape of the growth curve of *L. monocytogenes*; the lag phase, in fact, was missing in the control sample, but it was 4.91 days in the sample F.

4. Discussion

In a preliminary step, the variables of a US treatment were combined through the Design of the Experiments, to point out the leading variable for the antimicrobial effect in almond milk. The results of 3D plots, along with the desirability approach on *E. coli*, showed the significance of individual terms, but above all of interactions, thus confirming that for a US-treatment the effect is generally ruled by the total energy into the system, which is a function of power, time and pulse [23–25]. The weight of interactions was not found on *L. monocytogenes*; for this microorganism, in fact, the effect was ruled by the power and the duration of the treatment. This different role of the variable could be partly attributed to a difference in the external layers. As reported by Gao et al. [16,17], the effect of US relies upon the net power and the shear forces, but the capsule and to a lesser extent all external layers can reduce or enhance its effect by dampening the mechanical action of cavitation, thus affecting the way US acts on cells.

Moreover, it is a matter of debate if the Gram status could influence the effectiveness of sonication. Some authors reported that Gram positive microorganisms are more resistant than Gram negative ones, because of their thicker cell wall which provides a protection against ultrasound effects [26]. However, other studies reported no significant differences between these groups of bacteria [9]. The results for the first step suggested that, although there were some differences in the mode of action of US (interactive effects on *E. coli* and individual variables in *L. monocytogenes*), the reduction of the viable count of both pathogens was included in a defined range (1–1.3 log CFU/mL), mainly when higher power (80%) and longer times (6 and 8 min) were used.

It is possible that US disperses microbial clumps, disrupts cells and modifies cellular activity from the outside to the inside of the structures [27]. These effects result from the combined physical and chemical mechanisms that occur during the collapse of cavitation bubbles, the formation of free radicals (e.g., OH–), and the generation of hydrogen peroxide [6]. In addition, during ultrasound treatment, microorganisms are also subjected to mild temperatures caused by waves, which increase the weakening of the bacteria membrane and lysis [28,29]. Finally, the lethal effect of US can be also related to the sonoporation, as postulated by Ojha et al. [10].

The results of the last step (use of selected combinations, and storage of almond milk under refrigeration) suggest that, apart from the reduction of the viable count, US could determine a transient effect or sub-lethal injury on both pathogens with a delay of growth, which could be the results of sonoporation, streaming, impairment and partial destabilization of the membrane [8]. Sub-lethal effects refer to a stage before cell death, where reversible damage occurs, and the cell can recover if the effect ceases under appropriate physical parameters [30]. Certain ultrasound processing conditions seem to be selective in terms of exclusively destabilizing the outer membrane without severely affecting the cytoplasmic membrane or causing slight effect on it [31]. The practical implication of this transient effect could be strong and significant, as it could result in a delay of bacterial growth and an increase of the shelf-life.

This transient effect can be differently highlighted for the pathogens: *L. monocytogenes* experienced a prolongation of the lag phase, while in *E. coli* there was a reduction of the growth rate. This difference in the fitting parameters could be the result of a different effect on cells. The prolongation of the lag phase was the sign of a stronger sub-lethal injury on *L. monocytogenes*, as there was an impairment of growth and the microorganism required a time to adapt to the environment and probably restore the metabolism. Once restored the metabolism, the microorganisms fully restored the enzymatic pool and thus the difference in the growth rate was not significant.

On the other hand, in *E. coli* the effect was lower as the microorganism did not experience a lag phase; however, the targets of US were not completely restored as the growth rate was significantly lower.

This paper represents the first contribution on the use of US as an alternative approach to reduce the level of pathogens in almond milk; the effect of the treatment relies upon the total energy distributed into the system on *E. coli* O157:H7, as suggested by the significance of interactive terms of power, pulse and time, while power was the most important factor for *L. monocytogenes*. The final validation and challenge test also suggest that the treatment could exert a sub-lethal injury on the pathogens, as evidenced by the lag phase in *L. monocytogenes* or the reduction of the growth rate for *E. coli* O157:H7, which, combined with the storage under refrigeration, could contribute to increase the shelf life.

A possible drawback could be related to the extent of reduction, which points out that at present the treatment could be used only for foods at reduced shelf life (traditional Italian fresh almond milk prepared and stored at 4 °C for few days), thus further efforts are required to design and optimize the treatment at industrial level.

Declaration of interest

None.

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