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Effects of ultrasound treatments on wine microorganisms

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ABSTRACT

Ultrasound is one of the most promising non-thermal an emerging technique in food technology. The objective of the present work was to evaluate the effect of different ultrasonic treatments on the most important wine microbiota (*Saccharomyces* and non-*Saccharomyces* yeasts and lactic acid bacteria). Two stages were carried out: the assessment step, where six different ultrasonic treatments (with varying power, time, and pulses) were used on *Saccharomyces cerevisiae*, *Brettanomyces* spp., and *Lactiplantibacillus plantarum*; and the validation step, where two chosen ultrasonic treatments were used on *Zigosaccharomyces bailli, Brettanomyces* spp., *Saccharomyces cerevisiae, Saccharomyces bayanus, Pichia membranifaciens, Schizosaccharomyces pombe*, and *Hanseniaspora osmophila.* The most sensitive microorganism was *Brettanomyces* spp., and the most resistant was *Lactiplantibacillus plantarum*. Ultrasonic treatments had varying effects on vitality (delay of growth or maximum OD reduction) and on viability (reduction of microbial growth).

1. Introduction

In the winemaking process, both bacteria and yeasts play important roles. Understanding their characteristics, as well as the available control technologies, is vital to consistently producing high-quality wine. Some of these microorganisms can cause spoilage during wine production or storage through unwanted growth or through the production of unpleasant textures or odors and flavors, such as bitterness, overly buttery character, and excessive volatile acidity (acetic acid and ethyl acetate) [1,2]. The presence of non-*Saccharomyces* yeasts such as *Zygosaccharomyces bailli* may result in refermentation and CO_2 production in sweet wines or in grape juice concentrate, whereas *Brettanomyces bruxellensis* spoilage often contributes to off-odors and flavors in red wines [3].

In recent years, ultrasound has become one of the most researched and developed techniques in the food field. The procedure has great potential for industrial use, since the equipment required is reliable in practice [4].

The use of ultrasound in food is based on the generation of mechanical waves with a frequency above the threshold of human hearing, i.e., above 16 KHz. These waves travel through the material or over its surface at a speed that is characteristic of both the wave and the material through which it is propagating [5]. The waves are responsible of at least three effects: mechanical shock on biological systems, localized heating, and free radical production [6].

Ultrasounds can be divided into different frequency ranges. Until recently, most applications in food technology involved non-destructive analysis, which was a part of quality assessment. Such applications use high frequency (100 MHz) and low power (<1 W). Low-intensity ultrasounds are most commonly used as an analytical technique to provide information on the physico-chemical properties of foods, such as firmness, ripeness, sugar content, acidity, etc. [6]. In contrast, low frequency / high potency treatments are used to cause alterations in the physic, microbial and chemical characteristics of foods [5].

The effects of ultrasound on liquid systems are mainly related to the phenomenon of cavitation. Ultrasounds are propagated by a series of compression and rarefaction waves induced in the molecules of the medium traversed, forming cavitation bubbles from the gas nuclei inside the liquid. These bubbles, distributed throughout the liquid, grow over a few cycles until they reach a critical size where they become unstable and collapse violently. Their implosion leads to accumulations of energy in the hot spots, generating high temperature and pressure, which in turn produce many new waves [7] that transfer both the heat and mass and the vibration. The waves can reach 570 km/h in the food [8,9].

It is known that the process can affect biological cells. If combined with a heat treatment, ultrasonic waves can accelerate the rate of food

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sterilization. The microbial damage and death is mainly due to the thinning of cell membranes, localized heating, and production of free radicals [4].

On biological systems, mainly on cells, the effect of US relies upon the combination of at least six different mechanisms, generally known as sonoporation [10]. The six mechanisms generally cause the formation of pores on the membranes through phenomena known as jetting, putting and pushing, or the disturbing of microbial homeostasis through the acoustic streaming. As a result, cells tend to release intracellular components, like proteins, nucleic acids (bacteria), polysaccharides (yeasts) [11].

So far, high power ultrasound (HPU), has been used successfully in several areas of winemaking. Its effects are key to the modulation of the microbial load at various stages in musts, wines, and barrels [9]. The kinetics of microbial inactivation are of the highest order, with disinfection varying with potency [11] and frequency of treatment [12]. On the other hand, temperature, duration of treatment, type of microorganism, wave amplitude, volume and composition of the matrix affect the efficacy of ultrasound. High power and long treatment times are responsible for microbial inactivation. Combination with other treatments can improve the efficacy of ultrasound application [13].

Nevertheless, up to now, there have been few attempts to use ultrasound for winemaking, above all to promote the release of pigments, for protein stabilization etc., but to the best of our knowledge there are not records on spoiling or useful microorganisms of wine origin. There are only some data on collection isolates in model system or in other matrices (juices, almond beverage, dairy products). Therefore, the main objective of this study was to evaluate the effects of different ultrasonic (US) treatments on the most important microbiota in winemaking (*Saccharomyces* and non-*Saccharomyces* yeasts and lactic acid bacteria).

2. Materials and methods

2.1. Materials: strains, media, and equipment

Saccharomyces cerevisiae (UCLMS 1), *Brettanomyces* spp. (UCLMNS 1560), *Zgosaccharomyces bailli* (UCLMNS 1098), *Brettanomyces* spp (CECT 11045), *Saccharomyces cerevisiae* (UCLMS 3), *Saccharomyces bayanus* (UCLMS 2), *Pichia membranifaciens* (UCLMNS 1539), *Schizosaccharomyces pombe* (UCLMNS 1579), and *Hanseniaspora osmophila* (UCLMNS 1056) and *Lactiplantibacillus plantarum* (UCLM 43) were used throughout this research.

All microorganisms were chosen as representative wine microbiota since all of them were isolated from wine environments (grapes, mumrsst, wineries, fermentation tanks or distilleries) and can be present in the process. The only exception, acquired from a Spanish type culture collection (CECT), was *Brettanomyces* spp (CECT 11045). All the rest belong to the culture collection of the yeast laboratory of University of Castilla–La Mancha, where they were preserved at − 80 °C with glycerol (15%) as cryoprotective agent. For the study, the yeasts were grown on Peptone and Dextrose Yeast extract (YPD) at 30 °C, and the bacteria were grown on De Man, Rogosa and Sharpe agar (MRS) at 37 °C, both for 24 h.

For US, the equipment used was a Q700 Ultrasonic Processor (Qsonica, Newtown, USA), where the generator transforms the energy of the alternating current into a 20-kHz signal that drives a piezoelectric converter/transducer. The US wave is dissipated in the sample thanks to a probe system.

The amplitude, treatment duration, and presence or absence of pulses were combined through a mixed design of 2/3 levels. The first two conditions (amplitude and time) were set at three levels (20, 50, and 80 %; 4, 6, and 8 min, respectively; coded with −1, 0, and + 1), while the pulses were set at two levels (on/off with a 3-minute pause, coded with + 1 and −1). Different values of power (W) were generated by the combination of the three parameters and the values were divided by the microorganism suspension volumes for calculating the relation power/volume (W/L) **(**Table 1**).** One further combination was added to the design (control), where the samples were not treated.

Growth was assessed through a plate reader (HiPo MPP-96, Biosan, Latvia).

2.2. Design

The experimental design was structured in two steps: the assessment phase and the validation phase. In the assessment phase, *Saccharomyces cerevisiae* (UCLMS 1), *Brettanomyces* spp. (UCLMNS 1560), and *Lactiplantibacillus plantarum* (UCLM 43) were used. Later, in the validation step, the two best combinations (based on results from the first step) and their controls were analyzed to investigate the behavior of *Zigosaccharomyces bailli* (UCLMNS 1098), *Brettanomyces* spp (CECT 11045), *Saccharomyces cerevisiae* (UCLMS 3), *Saccharomyces bayanus* (UCLMS 2), *Pichia membranifaciens* (UCLMNS 1539), *Schizosaccharomyces pombe* (UCLMNS 1579), and *Hanseniaspora osmophila* (UCLMNS 1056).

From the pure cultures obtained, pre-cultivation was carried out in the corresponding broths. Each sample was inoculated with a concentration of 10^6 cfu/mL in a Falcon tube with sterile distilled water. All samples were treated following the design. Each test was carried out in triplicate.

Before each assay, the ultrasonic probe was washed with a 75% alcohol solution. Immediately after each processing, the samples were placed on ice for 5 min.

For better understanding, in Fig. 1 it is showed a graphical for clarifying the experimental setup.

2.3. Influence of ultrasound on viability

An aliquot of 1 mL was taken from each Falcon that had been treated and from the untreated controls. With the help of an automatic spiral seeder (EdyJet-2, IUL instruments), the adequate dilutions were seeded on YPD (yeast) and MRS (bacteria) agar and incubated at 30 and 37 °C for 24/48 h. The grown colonies were counted using an automatic counter (Flash & Go, IUL instruments).

2.4. Influence of ultrasound on vitality

An aliquot (1 mL) from each Falcon, corresponding to each sample subjected to the different treatments and each control, was centrifuged (5 min/4500 rpm). The supernatant was discarded, and the pellet was resuspended in 1 mL of YPD broth (yeasts) or MRS (bacteria).

Then 240 μL of each cell suspension, together with a control (broth without cells), were placed onto 96p microplates and incubated at 30 °C for yeasts and at 37 °C for bacteria for 96 h. The growth curves were monitored doing measurements every 30 min. Before each reading, the samples were agitated for 5 s at 150 rpm.

The parameters were calculated using the model described by Warringer and Blomberg [16]. The most important were the lag phase (λ), generation time (G), and maximum OD (ODmax) reached at the stationary phase. The specific growth rate constant (μmax) was also calculated (μmax = log2/G, where G stands for generation time).

2.5. Statistical analysis

Each experiment was done on at least three independent batches, that is the experiment was done on three independent samples (samples prepared independently from each other). The statistical analysis of the results was performed using the IBM SPSS Statistics for Windows ver. 24 program. An analysis of one factor variance (ANOVA) was performed to determine whether there were significant differences in the results between the different treatments. In addition, the Duncan test was carried out to group values according to these differences.

The lag phase was also analyzed through a multiple regression approach (forward method). The significance of each factor was

Table 1
Amplitude (%), duration (s), pulses, power (W/L) and energy (J) of the different treatments applied.

Treatment	Coded values			Actual values				
	Amplitude	Time	Pulse	Amplitude (%)	Time (s)	Pulse	Power (W/L)	Energy (J)
A	+1	−1	−1	80	4	0	1325	11.14
B	−1	+1	−1	20	8	0	850	12.84
C	−1	−1	+1	20	4	3	600	4.84
D	0	0	−1	50	6	0	1050	11.75
E	0	−1	+1	50	4	3	850	7.28
F	−1	+1	+1	20	6	3	475	6.02
Control	–	–	–	0	0	0	0	0

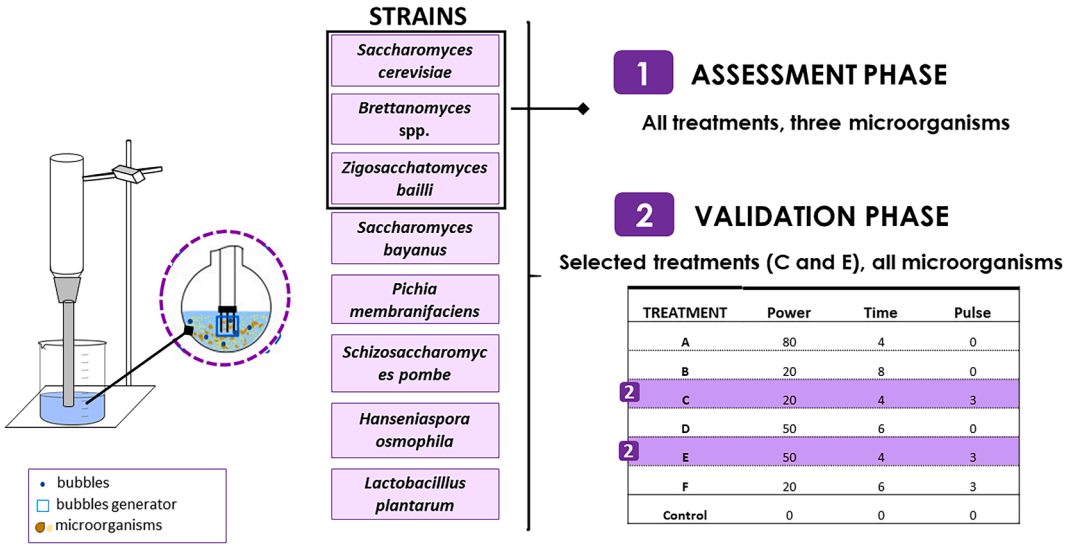


Fig. 1. Graphical abstract of experimental setup used for microorganism treatment.

evaluated with an F-test and 3D plots.

In addition, the lag phase (λ) and maximum OD values were standardized as increase in lag phase and decrease in OD from the control value and used as input values for a multifactorial analysis of variance. The microorganism type and the treatment were used as categorical predictors. The significance of the two individual terms (microorganism and treatment), as well as of their interaction, were evaluated through the Fisher test and the decomposition of the statistical hypothesis (Tukey's test, $p < 0.05$).

The last two analyses (multiple regression and multifactorial analysis of variance) were done by using the software Statistica for Windows, ver. 10.0 (Statsoft, Tulsa, Oklahoma, USA).

3. Results and discussion

3.1. First step: assessment phase

For this step, *S. cerevisiae* (UCLMS 1), *Brettanomyces* spp. (UCLMNS 1560), and *L. plantarum* were submitted to every ultrasonic treatment.

3.1.1. Influence of ultrasound on viability

Counts on YPD and MRS plates were obtained after treatments of the three microorganisms with all combinations of conditions. The loss of viability was calculated in percent, with the untreated controls assigned a value of 0% (data not shown).

Brettanomyces spp. did not resist any of the treatments, and no growth was detected in treated samples. The other two microorganisms responded to the treatment in varying way. *S. cerevisiae* was more sensitive than *L. plantarum*. For both, treatments A, B, and D totally inhibited the growth (100% loss of viability). After combinations C, E, and F, they showed different inhibition grade (26, 74, and 80% loss of viability, respectively). *L. plantarum* showed more resistance, and only treatment A led to 100% inhibition. The rest of the treatments affected the viability to varying extents, with treatment C again being least effective (62, 55, 52, 46, and 5% loss of viability for D, E, F, B, and C, respectively).

3.1.2. Influence of ultrasound on vitality

The results and the first part of the statistical analysis (ANOVA and Duncan test) are shown in Table 2 A and B, for *S. cerevisiae* and *L. plantarum*, respectively. The data for *Brettanomyces* spp. are not shown since it experienced complete inhibition with all treatment combinations (lag phase > 96 h).

The ultrasound treatments affected *S. cerevisiae* more than *L. plantarum*. Combinations A, B, and D did not allow *S. cerevisiae* to develop (Table 2A). The other treatments affected it in different ways. All treatments prolonged the λ, and two groups were established apart from the one consisting of the controls (treatment C in one, and E and F in the other, with longer time). μmax was very close to the control for combinations C and F, although showed significant differences, and it was significantly affected by treatment E, which led to very slow growth. The same trend was observed for generation time (G) and maximum OD, with combination E leading to the highest G and lowest ODmax values.

For *L. plantarum* (Table 2B), only treatment A was able induce complete inhibition. λ was also prolonged in all cases except with combination C, establishing different statistical groups. Except with treatment A, there were no differences in μmax and G, and ODmax was significantly lower only for treatments B and E.

In the Supplementary Material section, Fig. 1 shows the growth curves after treatment for both microorganisms (1A for *S. cerevisiae* and

Table 2
Kinetic parameters of *Saccharomyces cerevisiae* (UCLMS 1) (A) and *Lactiplantibacillus plantarum* (B) with the different treatments and untreated controls. Results from the first step (assessment).

A				
Treatment	λ (h)	μmax (h^{-1})	G (h)	ODmax
A	$> 96^{a}$	–	$> 96^{a}$	0.14 ± 0.00^{a}
B	$> 96^{a}$	–	$> 96^{a}$	0.14 ± 0.01^{a}
C	12.5 ± 0.00^{c}	0.16 ± 0.01^{c}	1.88 ± 0.07^{b}	1.41 ± 0.05^{c}
D	$> 96^{a}$	–	$> 96^{a}$	0.15 ± 0.00^{a}
E	19.00 ± 0.00^{b}	0.05 ± 0.01^{a}	6.45 ± 1.15^{b}	0.32 ± 0.02^{b}
F	18.67 ± 0.58^{b}	0.15 ± 0.01^{c}	1.96 ± 0.08^{b}	1.51 ± 0.07^{d}
Control	2.00 ± 0.00^{d}	0.14 ± 0.00^{b}	2.15 ± 0.07^{b}	1.44 ± 0.03^{c}
B				
Treatment	λ(h)	μmax (h^{-1})	G (h)	ODmax
A	$> 96^{a}$	–	$> 96^{a}$	0.18 ± 0.01^{a}
B	20.33 ± 0.58^{c}	0.13 ± 0.01	2.30 ± 0.01^{b}	1.09 ± 0.05^{b}
C	11.33 ± 0.29^{e}	0.13 ± 0.01	2.32 ± 0.09^{b}	1.85 ± 0.03^{c}
D	23.67 ± 0.29^{b}	0.14 ± 0.01	2.20 ± 0.09^{b}	1.83 ± 0.05^{c}
E	19.17 ± 0.29^{d}	0.13 ± 0.01	2.31 ± 0.07^{b}	1.10 ± 0.04^{b}
F	20.33 ± 0.58^{c}	0.13 ± 0.01	2.41 ± 0.26^{b}	1.87 ± 0.04^{c}
Control	11.33 ± 0.29^{e}	0.13 ± 0.00	2.35 ± 0.07^{b}	1.87 ± 0.01^{c}

Different letters in the columns indicate significant differences for each treatment (ANOVA and Duncan test, $p < 0.05$).
- indicates "not detected."

1B for *L. plantarum*) in response to the behavior described above.

To better understand the respond to the treatments, a multiple regression analysis of the lag phase of *S. cerevisiae* and *L. plantarum* was carried out. Power, time (duration of the treatment), and pulse were used as independent variables, as single or interactive terms. This statistical analysis is useful for quantitative purposes because it allows the lag phase to be predicted not only for the combinations of power/time/pulse used in the design, but for all possible treatments.

The first output of a multiple regression is a polynomial equation, describing the mathematical effect of the variables on the lag phase.

The equation for *S. cerevisiae* is as follows:

$$\lambda_{SC} = 17.45 + 0.28 \times [\text{power}][\text{time}] - 0.42 \times [\text{power}] \times [\text{pulse}]$$

Thus, the lag phase was mathematically affected by the positive interaction of power/time (the lag phase increased with increasing power and increasing treatment time). On the other hand, there was a negative interaction with power/pulse, which means that the lag phase decreased when either the power or pulse increased.

The second output of the multiple regression approach was a 3D-plot, which shows the changes in the dependent variable (lag phase) as a function of two independent variables. Fig. 2 presents the 3D plot for the power/time interaction. As expected, the model predicts a maximum lag phase value (>140 h) at 80% power with an 8-minute treatment. The figure also shows that for a 2-minute treatment, even at high power, the effect is not significant (low or no lag phase predicted).

Other authors have also observed the sensitivity of *S. cerevisiae* when it is treated with ultrasonic. For example, Liu et al. [17] showed that higher ultrasonic power, longer irradiation time, and lower pulse duty ratio may facilitate its inactivation.

The same approach was used for the lag phase of *L. plantarum*; the equation is as follows:

$$\lambda_{LP} = 11.49 - 2.59*[\text{power}] + 0.04*[\text{power}]^2 + 9.83*[\text{time}] - 0.54*[\text{time}]^2 + 1.47*[\text{pulse}]$$

In this case, the presence of linear and quadratic terms for two variables (power and time) suggests a non-linear trend with quadratic kinetics and a threshold breakpoint; for example, an increase of the lag phase as a function of time up to a critical breakpoint, after which a further increase of time does not influence the lag phase.

The 3D plot for the lag phase of *L. plantarum* is showed in Fig. 3. The results show that from a quantitative point of view, power is more significant than time, and time could act as a reinforcing variable: at 80 % amplitude for 2 min, the model predicts a lag phase of 280 h, while for 80 % during 8 min, the predicted lag phase is 350 h. From a mathematical point of view, this suggest that for *L. plantarum* the main quantitative factor was power, while the duration of the treatment (time) acted only as a strengthening element. Nevertheless, in yeasts, the increase of lag phase at the maximum values of power and time, highlights a strong quantitative effect of interaction. It is different since in

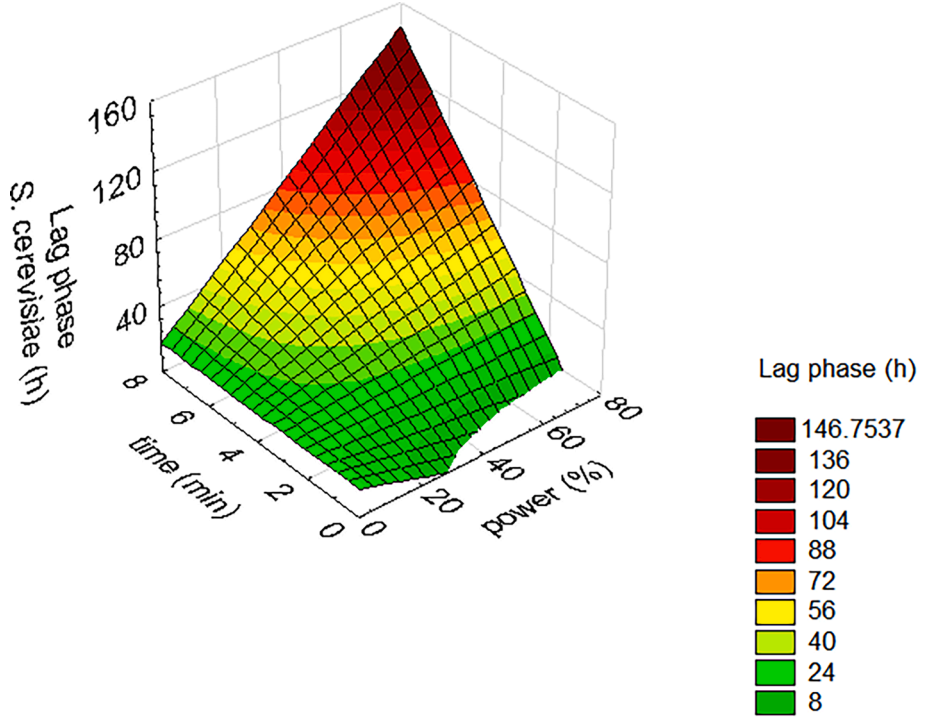


Fig. 2. 3D plot for the power/treatment time interaction on the lag phase of *Saccharomyces cerevisiae* (UCLMS 1). Results from the first step (assessment).

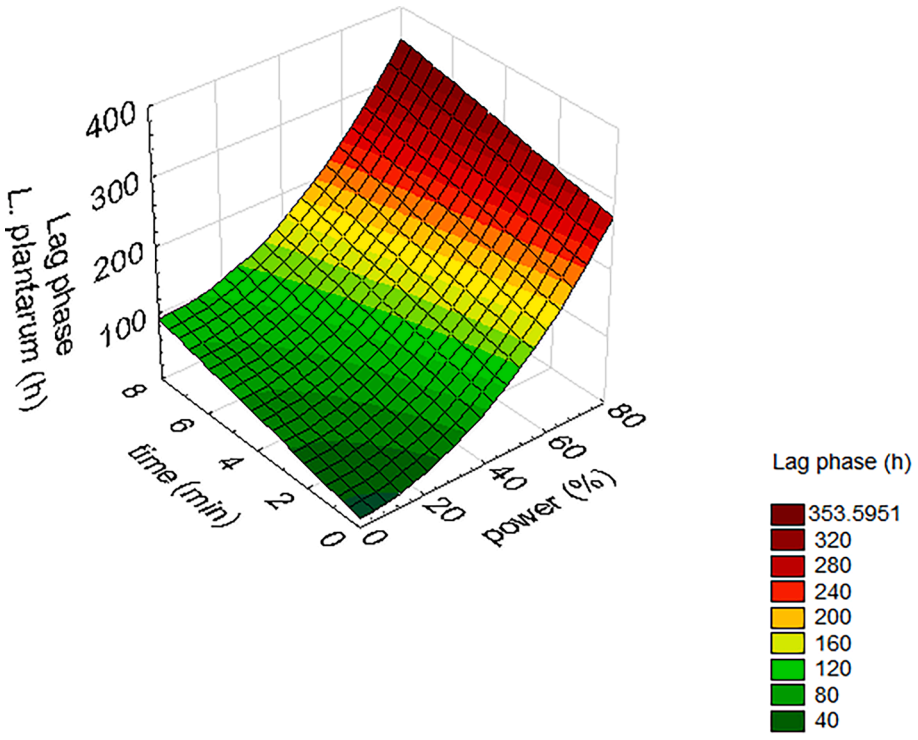


Fig. 3. 3D plot for the interaction power/duration of the treatment on the lag phase of *Lactiplantibacillus plantarum*. Results of first step (assessment).

the bacteria it was related to individual terms of factors.

The effect of ultrasound depends on several factors, including the total acoustic energy, as suggested by the existence of some interactive terms in the best fit equations (power*pulse and power*duration). This result is in agreement with some literature reports [13–15], although the statistical weight of the different variables is not always consistent. In a different matrix, Bevilacqua et al. [19] report that the effect of ultrasound on yeasts was mainly influenced by the power and the duration of the treatment, and that the influence of pulses was not very significant.

Wu et al. [20] suggested a two-step model for the effect of ultrasound on yeasts. The first target of ultrasound is the cell wall, which responds to the stress through the release of polysaccharides. Then, if the ultrasonic power is sufficient to destroy/disaggregate the wall, the second target is cell membrane; the sign of this effect is the release of proteins.

A similar model could be also used for bacteria, although the capsule could also play a role [21,22]. A comprehensive review of the mode of action of ultrasound on cells was presented by Ojha et al. [14]; these authors suggested a unifying mechanism, called sonoporation: ultrasound causes the formation of pores or mechanical injuries on cells. If these phenomena are reduced or controlled, the effect could be simply a delay of growth, as found in some combinations of the design of this research. This delay in growth manifests through the prolonged lag phase, which may be due to the time needed to repair injuries and to restore metabolism, as also found for some bacteria (*Listeria monocytogenes* and *Escherichia coli*) [23]. On the other hand, uncontrolled formation of pores and an efflux of nutrients from cells could lead to irreversible injuries, which could in turn lead to the reduction of maximum OD in some combinations of the design, or to death, as observed for other microorganisms [24].

The microorganisms evaluated present some structural differences that could help to explain their behavior, especially among yeasts and bacteria. The treatment sensibility coincides with the size of cells: the smaller they are, the better they resist the treatments (*L. plantarum* < *Saccharomyces cerevisiae* = *Brettanomyces* spp). Their sizes vary between 0.5 and 1.2 × 1.0–10 μm for bacteria and 1.0–9.0 × 2.0–20 μm for yeasts, being the non *Saccharomyces* smaller than *Sacchoramyces*. Another different characteristic is the wall cell. Although it is present in yeasts and bacteria, its composition is different: in yeasts are formed basically by beta glucans, mannoprotein and chitine, meanwhile in Gram +, it is the peptidoglycan the major component [25].

Another key-point of the higher resistance of *L. plantarum* on cell wall is on the mode of action postulated on bacteria and yeasts. As stated above in bacteria, capsule could play a role, as it could act as a sponge and partially adsorb the mechanical energy of US and then release it in the external medium or to the inner layers. Even if capsule is missing, this mechanism could be also done by cell wall [21,22]. In addition, US create a localized increase of heating and lactobacilli [6], at least for medium temperature, are more resistant than *S. cerevisiae*. Probably the combination of the role of the outer layers (capsule and cell wall) and of the higher heat resistance is responsible of the increased resistance to US.

Finally, a multifactorial analysis of variance was carried out to compare the effect on different microorganisms. The lag phase (λ) and maximum OD (ODmax) were standardized to compare microorganisms with different kinetics and trends. The input values were the prolongation of λ (h) and the reduction of ODmax. The first output of the statistical analysis is the table of standardized effects, which indicates the statistical weight of the two factors and their interaction. The most significant factor was the species of microorganism (F, 477,603), followed by the single effect of the treatment (F, 324,082) and by the treatment/microorganism interaction (F, 110,453).

A table of standardized effects is a useful tool; however, it does not show trends or quantitative details. These outputs can be found in the figures of the decomposition of the statistical hypothesis, which show the quantitative effect of each factor. Before discussing the practical implications of each figure, a short overview on the meaning of decomposition of the statistical hypothesis. These pictures do not show actual values, at least for the effect of the individual term of each predictor; they are a mathematical extrapolation of the mathematical effect of each independent variable on the output of the process (prolongation of the lag phase) and the bars, as well, as an extrapolation for the variability within the whole data set and they are generated through the

propagation of error.

Fig. 4**A** shows the individual effect of each treatment variable; this is a statistical index, which shows the effect of a factor over time and mathematically excludes the influence of the other variables. As expected, each treatment exerted a different effect on the target strains.

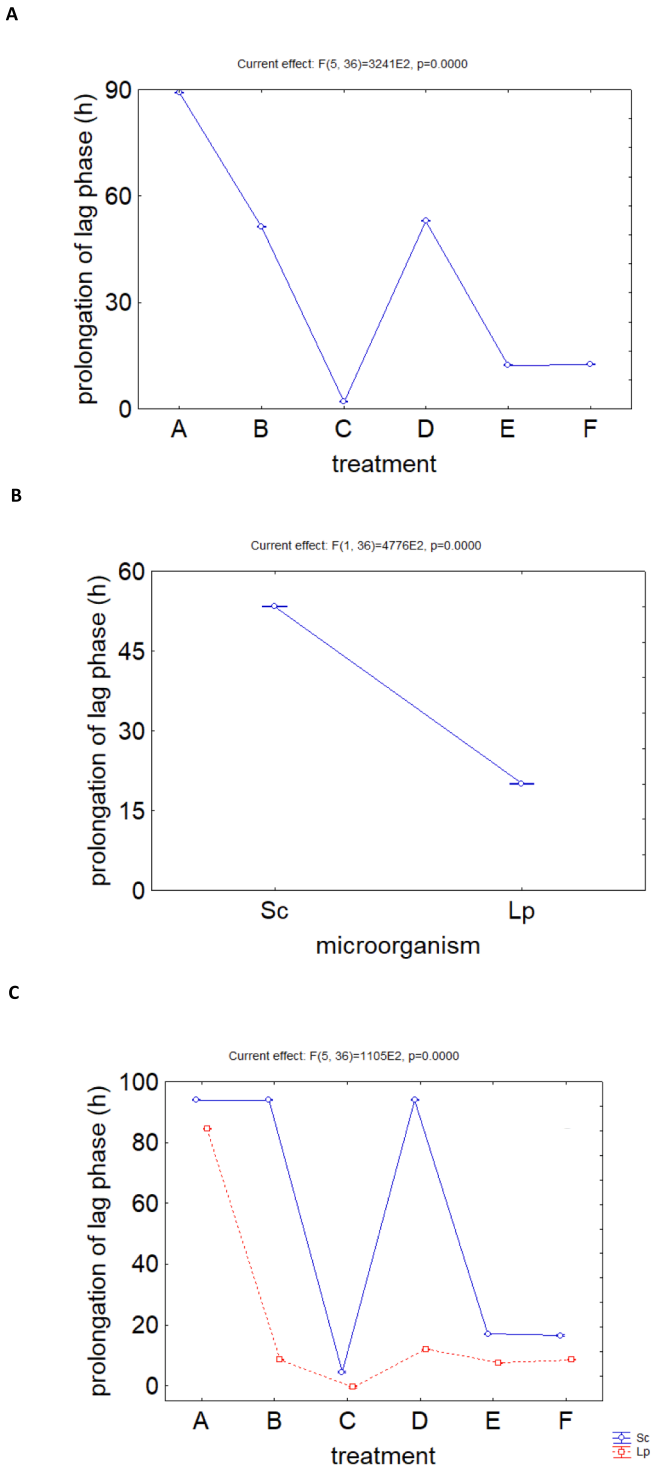


Fig. 4. Decomposition of the statistical hypothesis for the effects of microorganism species (A), treatment (B), and treatment/microorganism interaction (C) on the lag phase of *Saccharomyces cerevisiae* (UCLMS1) (Sc) and *Lactiplantibacillus plantarum* (Lp). Bars denote 95% confidence intervals. Written above each figure are the degrees of freedom for ANOVA (in parentheses) and the F-test. Results of the first step (assessment).

The treatment that caused the longest delay in the lag phase was A, followed by treatments B and D, and finally by C and E.

The effect of the microorganism variable can be seen in Fig. 4**B**; *S. cerevisiae* was generally more sensitive than *L. plantarum*, with a longer delay in the lag phase.

The last output is the decomposition for the treatment/microorganism interaction (Fig. 4**C**). This figure shows the effective trends and the effect of the different combinations on both microorganisms. Only treatment A was able to strongly delay both *S. cerevisiae* and *L. plantarum*, while treatments B and D were more effective on the yeast (complete inhibition) and less effective on *L. plantarum* (lag phase prolonged by about 10 h).

The same approach was used for maximum OD, modeled as a reduction in OD compared to the controls (untreated microorganisms); the most significant term was the kind of treatment (F, 2100.44), followed by the microorganism species (F, 1339.14) and by the treatment/microorganism interaction (F, 809.54).

The decomposition of the statistical hypothesis for the treatment (Fig. 5**A**) shows that the strongest effect was observed with treatment A, followed by treatments E, B, and D, while the effect of treatments C and F was not significant (no reduction in OD). In terms of the lag phase, the most sensitive microorganism was S. *cerevisiae* (Fig. 5**B**). The treatment/microorganism interaction (Fig. 5**C**) shows the actual trend and the different behavior of the two microorganisms in response to some treatments. Both *S. cerevisiae* and *L. plantarum* were completely inhibited by treatment A, while they were not affected by C and F.

With treatments B and D, the effect was species-dependent. The yeast experienced complete inhibition, while the bacterial strain was not influenced by ultrasound. The species dependence is a key factor for ultrasound [6]; generally, the larger cells, the more sensitive they are. Therefore, it is not surprising that at high levels of energy, *S. cerevisiae* appears more sensitive than *L. plantarum*.

Concerning the effect of the combinations on both OD and lag phase, the effect of US probably relies on the total energy released in the system, which is responsible of both the mechanical action on cells and of the localized heating and free radical production. The energy of a US treatment is a function of three parameters: the amplitude, the duration of the treatment and pulse [18], but some preliminary experiments done on other yeasts showed that the most important variable for the total energy are the amplitude and the duration of the treatment, while pulse could only play a strengthening effect [19]. The combination A, B and D showed the highest levels of energy (around 11–12 J), compared to the other combinations and this is the reason why they were generally more effective; however, the difference in the combination B and D for *L. plantarum* and *S. cerevisiae* also suggest that despite the total energy the structure of cells has an important role, being more resistant the smallest ones. It could be due to the ratio surface/volume is higher in bacteria than yeasts, which implies a faster metabolism that help cells to repair their damage structures in a more efficient way.

Finally, the differences in *L. plantarum* amongst the combination A vs B/D, which were characterized by similar level of energy, suggest that apart from the total energy the mechanical effect of US (here represented by amplitude) should be also considered when a US optimization is carried out. The combination A, in fact, was characterized by the highest amplitude (power).

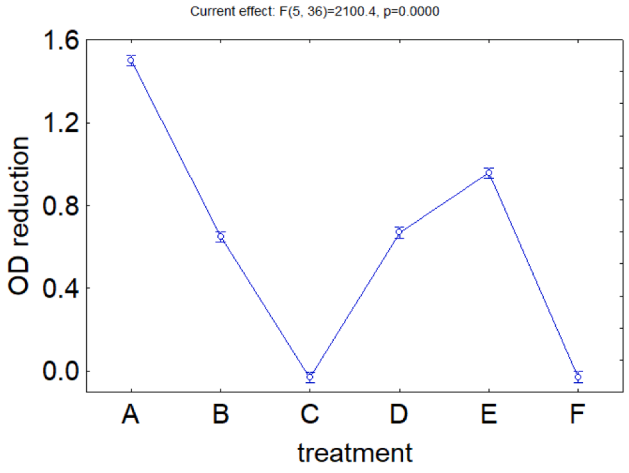
3.2. Second step: Validation

Two treatments, C and E, were chosen for the validation step due to their contrasting inhibition effects observed before, with E being very effective and C being very ineffective.

3.2.1. Influence of ultrasound on viability

The trend of loss of viability was the same as that observed in the first part: the growth was less inhibited with treatment C than with treatment E for all microorganisms. *P. membranaefaciens* was the most sensitive (23

A



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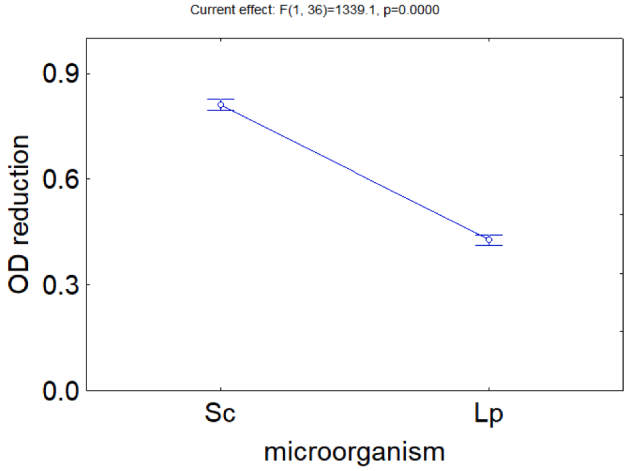

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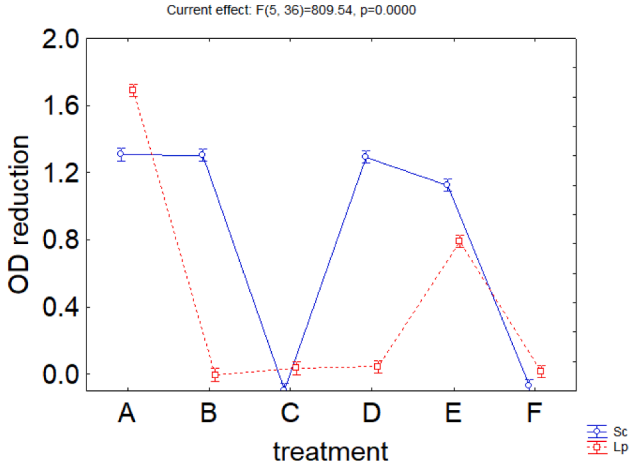

Fig. 5. Decomposition of the statistical hypothesis for the effects of microorganism species (A), treatment (B), and the treatment/microorganism interaction (C) on OD reduction in *Saccharomyces cerevisiae* (UCLMS1) (Sc) and *Lactiplantibacillus plantarum* (Lp). Bars denote 95% confidence intervals. Written above each figure are the degrees of freedom for ANOVA (in parentheses) and the F-test. Results of the first step (assessment).

and 100% loss of viability with C and E, respectively), followed by *H. uvarum* (15 and 100% loss of viability). *Bretanomycess* spp. and *S. bayanus* showed similar intermediate values (around 21 % for C and 53% for E). *S. cerevisiae* (UCLM 3) also behaved similarly, although it was more resistant to treatment C (only 8% loss). The yeasts with the best viability were *Z. bailli* for treatment C and *S. pombe* for E (5 and 51% loss, respectively).

The variability of results can be due to the structural differences, that make some of them to better resist the treatment, or metabolic ones that allow them to growth, even when they are damage [25].

Results also showed that the behavior depends not only on the species, but also on the yeast strain and growth conditions, since *Bretanomycess* spp. and *S. cerevisiae* used in this part were different clones from the used in the first part, and their loss of viability was different as well. This has been observed for many biotechnological characteristics of yeasts related to different issues, including food safety [26], antimicrobial activities [27], probiotic properties [28,29], antioxidant and aroma development [30], and bioremediation and biocontrol characteristics [31].

This behavior might be explained based on the different proteomic profile found among the microorganisms. Many studies justify the different behavior not only among different genera but also at strain level. García-Bejar et al [32], observed that the response of two strains from the same specie (FR19 and ECF61-Diutinia rugosa), revealed slight differences in the proteome alteration in response to stress conditions. Similar results were found in other works related to different strains of *S. cerevisiae* also under stress conditions [33] or related to the cell wall proteomic profile of fungi [34].

These results can conduct new research where are studied the changes on the proteomic profiles after ultrasonic treatments and their comparation with the ones produced under other stress conditions.

3.2.2. *Influence of ultrasound on vitality*

The results for the kinetics parameters after treatment are shown in Table 3. In all cases, λ significantly increased with respect to each control, and there were only two cases with no growth (*P. membranaefaciens* and *H. uvarum*, both with treatment E). For the rest of variables, there were few significant differences, and usually only two groups were formed, except for *Z. bailii* for μmax and to *P. membranaefaciens* for ODmax, where the three values (from C, E, and control) were different.

Table 3
Kinetic parameters of all microorganisms with treatments C and E and no treatment (control). Results of the second step (validation).

Treatment	λ (h)	μmax (h-1)	G (h)	ODmax
Zygosaccharomyces bailii				
C	16.38 ± 0.14^{b}	0.06 ± 0.00^{b}	4.76 ± 0.16^{a}	1.45 ± 0.02^{b}
E	39.38 ± 0.25^{c}	0.04 ± 0.01^{a}	9.54 ± 2.24^{b}	0.45 ± 0.12^{a}
Control	3.44 ± 0.13^{a}	0.07 ± 0.00^{c}	4.06 ± 0.12^{a}	$1,49 \pm 0.01^{b}$
Brettanomyces spp.				
C	13.50 ± 0.13^{b}	0.13 ± 0.01^{a}	2.38 ± 0.11^{b}	1.40 ± 0.06
E	13.31 ± 0.13^{b}	0.13 ± 0.02^{a}	2.36 ± 0.34^{b}	1.49 ± 0.07
Control	0.50 ± 0.20^{a}	0.17 ± 0.00^{b}	1.82 ± 0.02^{a}	1.46 ± 0.01
Saccharomyces cerevisiae				
C	16.06 ± 0.13^{c}	0.02 ± 0.00^{a}	10.90 ± 0.35^{b}	1.53 ± 0.05
E	15.31 ± 0.13^{b}	0.03 ± 0.00^{b}	9.58 ± 0.24^{a}	1.49 ± 0.04
Control	0.50 ± 0.00^{a}	0.03 ± 0.00^{b}	8.75 ± 0.54^{a}	1.52 ± 0.02
Saccharomyces bayanus				
C	8.06 ± 0.12^{b}	0.12 ± 0.01	2.61 ± 0.08	1.43 ± 0.05^{a}
E	10.44 ± 0.13^{c}	0.12 ± 0.02	2.19 ± 0.24	1.37 ± 0.04^{a}
Control	0.69 ± 0.13^{a}	0.13 ± 0.00	2.26 ± 0.07	1.49 ± 0.03^{b}
Pichia membranaefaciens				
C	16.42 ± 0.14^{b}	0.09 ± 0.01	3.34 ± 0.21^{b}	1.01 ± 0.00^{b}
E	$> 96^{c}$	–	$> 96^{a}$	0.17 ± 0.00^{a}
Control	5.33 ± 0.29^{a}	0.09 ± 0.01	3.33 ± 0.39^{b}	1.48 ± 0.02^{c}
Schizosaccharomyces pombe				
C	17.31 ± 0.24^{b}	0.08 ± 0.01	3.59 ± 0.12^{a}	1.44 ± 0.03^{a}
E	21.26 ± 0.21^{c}	0.08 ± 0.01	4.03 ± 0.54^{b}	1.40 ± 0.04^{a}
Control	3.25 ± 0.20^{a}	0.09 ± 0.01	3.36 ± 0.09^{a}	1.52 ± 0.04^{b}
Hanseniaspora osmophyla				
C	10.06 ± 0.13^{b}	0.15 ± 0.00	1.98 ± 0.03^{b}	1.50 ± 0.02^{b}
E	$> 96^{c}$	–	$> 96^{a}$	0.18 ± 0.00^{a}
Control	1.19 ± 0.13^{a}	0.15 ± 0.00	1.95 ± 0.02^{b}	1.52 ± 0.02^{b}

Following the same methodology, a multifactorial ANOVA was used to model the results. As explained above, data were first standardized as increase in the lag phase or decrease in OD.

For the increase in the lag phase, the most significant term was the individual treatment effect (F, 43.14), followed by the microorganism species (F, 18.09) and by the treatment/microorganism interaction (F, 9.20).

Fig. 6**A** displays least two different classes of susceptibility. The first one includes the most sensitive microorganisms (*P. membranifaciens* and *H. osmophila*), and the second, the less sensitive strains (*Z. bailii*, *Brettanomyces* spp, *S. cerevisiae*, *S. bayanus*, *Sc. pombe*).

Concerning the treatment, E was more effective than C, and caused a greater mean increase in the lag phase (39 vs. 13 h) (Fig. 6**B**).

Finally, the figure related to the interaction (data not shown) shows how the treatment affected each strain. It also shows the effective trends, and suggests that treatment E was more effective on the two strains included in the sensitive group (*Pichia* and *Hanseniaspora*), in which is consistent with the results from the other parts.

The statistical analysis on the reduction of OD shows that the most significant term was again the treatment (F, 2475.63); however, the decomposition of the statistical hypothesis suggests a different grouping, with at least 3 classes of sensitivity. P. *membranifaciens* and *H. omsophila* were the most sensitive yeasts (OD reduced by 0.8–1.0), while *Brettanomyces* spp., *Sc. pombe*, and *S. bayanus* were the least affected, because they did not experience a significant reduction in OD after ultrasonic treatment. There was also an intermediate class, composed of *S. cerevisiae* and *Z. bailii* (OD reduction of ca. 0.5) (Fig. 7**A**). This grouping can also be explained by the strength of the effect of treatment E on some yeasts (Fig. 7**B**).

In the validation step, both variables, increase of lag phase and maximum DO, in the two treatments, show that the bacteria is in the most resistant group. This indicates, one again that the smaller the cells are, the less sensitive they are, probably due to the high relation surface/volume.

It is also clear that the two variables can show different behavior for the same treatment/strain. This happens with *S. cerevisiae* (UCLM3): its lag phase does not increase, but its ODmax decreases. In the rest of cases, the trend is the same. The strain-dependent behavior is observed again. As stated before, it has been reported in relation to many other aspects of yeast metabolism; however, this is the first time that this behavior has been observed following ultrasonic treatment in wine.

4. Conclusions

Ultrasound is an effective treatment for microbial quality and safety. It has been used with a wide variety of foods and microorganisms, but to the best of our knowledge, few data are available on its use with yeasts and bacteria from wine. This paper is the first structured overview on the effects of ultrasound on wine microorganisms, confirming some previous literature reports and adding new evidence.

The results corroborate the greater resistance of bacteria (at least *L. plantarum*) compared to that of yeasts, and the importance of the total energy for the antimicrobial effect, suggested by the existence of the interactive terms in the best fit equations.

On the other hand, the data also suggest that ultrasound could have a dual effect on wine yeasts, depending on the duration of the treatment and the power. Ultrasound could have either a reversible effect on vitality (prolongation of the lag phase, followed by a recovery of growth) or an irreversible effect on viability (reduction of the maximum OD value and plate count.

In addition, the experiments show that the behavior of microorganisms depends not only on the species but also on strains. For

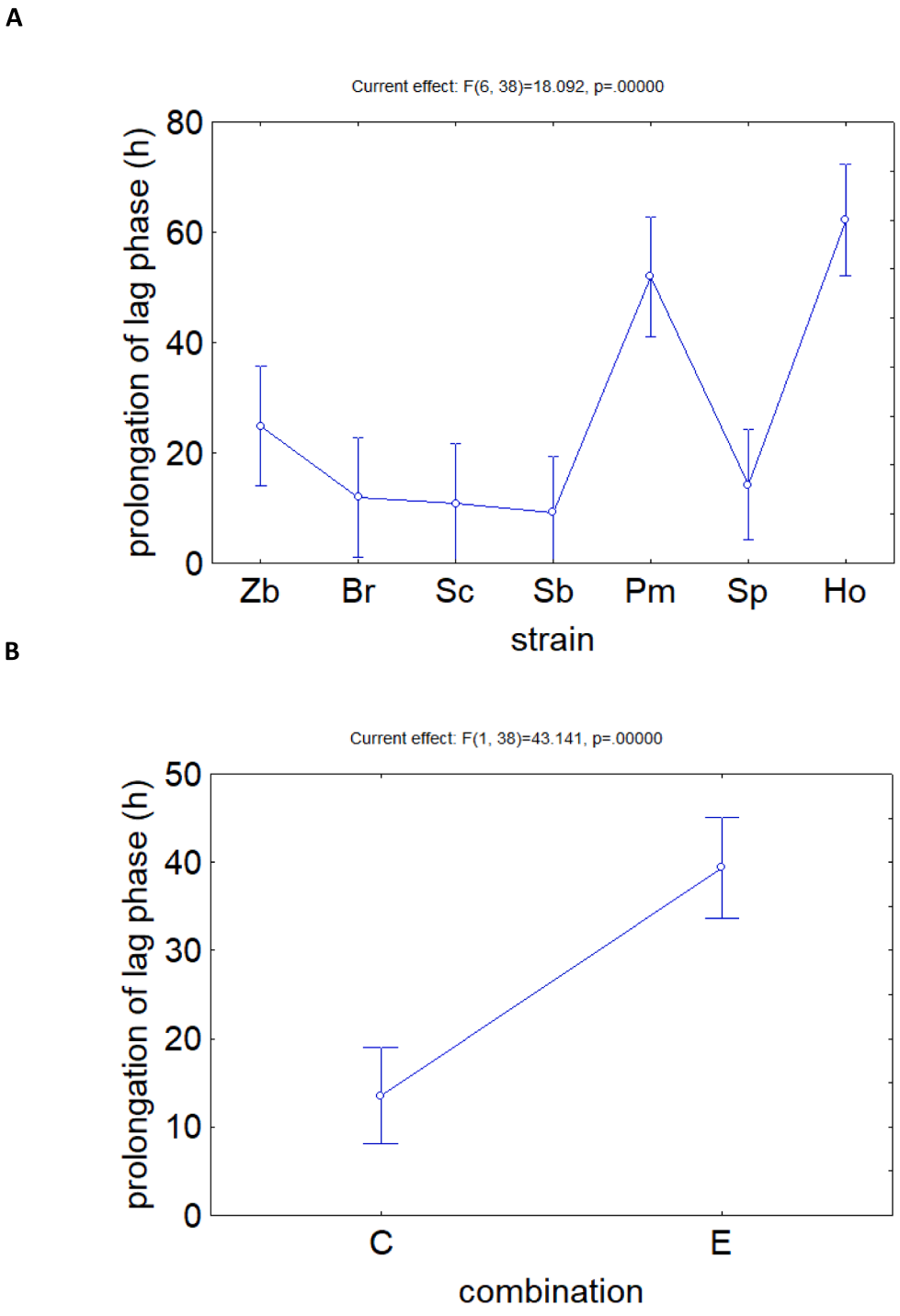

Fig. 6. Decomposition of the statistical hypothesis for the effects of the microorganism (A) and treatment (B) on the prolongation of the lag phase. Bars denote 95% confidence intervals. Written above each figure are the degrees of freedom for ANOVA (in parentheses) and the F-test. Results of the second step (validation).

S. cerevisiae and *Brettanomyces* spp., different clones of the same species were affected differently (one of the *Brettanomyces* was the most sensitive strain from all the yeasts studied, and did not resist any of the ultrasonic treatments).

Finally, two treatments of intermediate effect chosen for use on yeasts revealed the existence of at least two classes of susceptibility: low susceptibility (the less sensitive *Brettanomyces* spp., *Sc. pombe*, and *S. bayanus*) and high susceptibility (P. *membranifaciens* and *H. omsophila*), as well as a possible intermediate class. However, further experiments with multiple strains from each species are required to confirm this result.

In conclusion, this paper shows that ultrasound is suitable for use with wine microorganisms, but in vivo validation is required to design effective treatments and to investigate the effect of other factors on microbial resistance (sugar, ethanol, pH, phenolic compounds).

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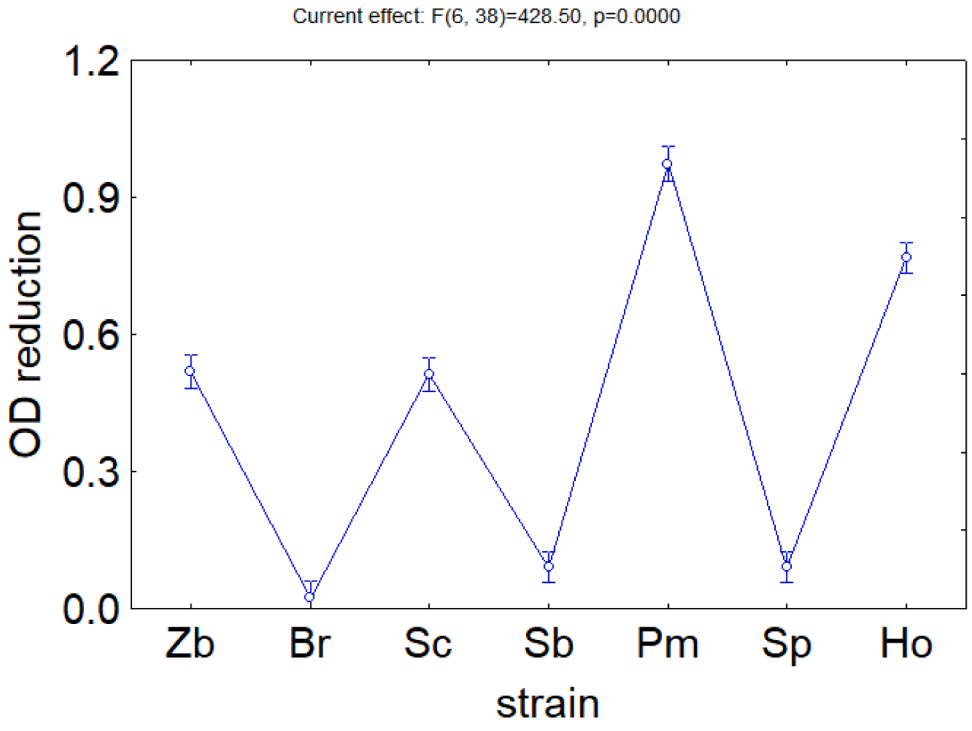

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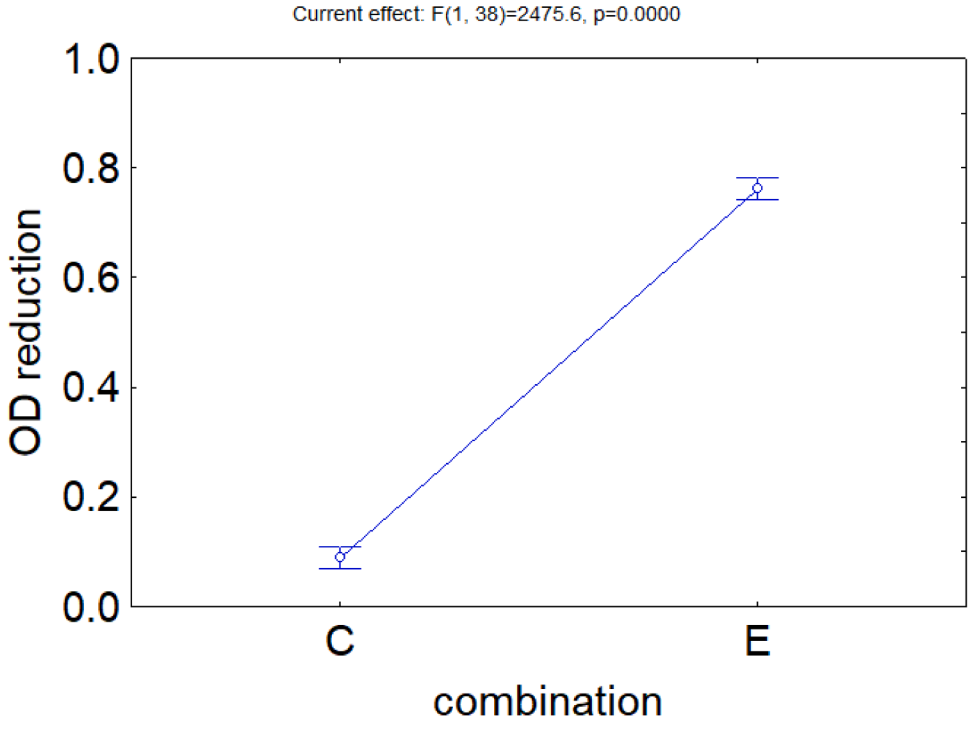

Fig. 7. Decomposition of the statistical hypothesis for the effects of the microorganism (A) and treatment (B). Bars denote 95% confidence intervals. Written above each figure are the degrees of freedom for ANOVA (in parentheses) and the F-test. Results of the second step (validation).

(Spain Government).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2021.105775.

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