LWT - Food Science and Technology 175 (2023) 114481

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Evaluation of the antimicrobial activity of grape extract against *Bacillus cereus* in rice

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ARTICLE INFO

Keywords:
By-products
Natural antimicrobials
Survival
Predictive microbiological modelling

ABSTRACT

The antimicrobial potential of grape extract was assessed in cooked rice against *Bacillus cereus*. Grape extract efficacy was tested at 1, 5 and 10 mL/L, at pH 4.5, 5.5 and 6.5; and at incubation temperatures simulating different storage scenarios, specifically temperature abuse (10 °C), cool chain break (20 °C) and optimal *B. cereus* growth temperature (30 °C). Survival curves for grape extract concentration versus time were obtained. The results indicate that antimicrobial activity of grape extract was dependent on temperature, pH and grape extract concentration. A bactericidal effect of the grape extract was shown at concentration levels $\geq$ 5 mL/L at all temperatures and pHs studied. Inactivation curves of *B. cereus* under grape extract exposure were fitted to a Weibull distribution function for 5–10 mL/L grape extract concentration. Observations showed that the higher the incubation temperature and grape extract concentration, the lower the kinetic rate value. In other words, lower resistance of the microorganism to environmental conditions. The maximum inactivation level was 6 $\log_{10}$ cycles after 24 h of exposure at 10 mL/L of grape extract concentration and pH 4.5. Results indicate that the grape extract could be a good additional control measure for preventing *Bacillus cereus* growth in cooked rice during storage.

1. Introduction

Rice (*Oryza sativa* L.) is a basic cereal, widely consumed by the general population due to its abundant nutrients and relatively low price. It is one of the most important staple crops on the planet and feeds almost half the world population (Wei & Huang, 2019). However, it is also frequently involved in foodborne *B. cereus* outbreaks (Rodrigo, Rosell, & Martinez, 2021). Once cooked, it has a pH close to 7, being an excellent growth medium when the humidity of the substrate reaches adequate water activity values for the growth of the microorganism (Delbrassinne, Botteldoorn, Andjelkovic, Dierick, & Denayer, 2015; Pao, Khalid, & Kalantari, 2006; Rodrigo et al., 2021). *B. cereus* is a gram-positive spore forming bacterium, which is a habitual saprophyte, resistant to high temperatures and low humidity (Hendriksen, Hansen, & Johansen, 2006; Rodrigo et al., 2021). It is a public health issue because produces food toxins and can survive 48 weeks in rice in cool dry storage without loss of viability (Gilbert, Stringer, & Peace, 1974; Sánchez, Correa, & Castañeda, 2016).

One of the main factors contributing to *B. cereus* outbreaks related to cooked rice is poor cooling, or storage at room temperature for a prolonged period, particularly when cooking large quantities in restaurants and/or canteens (Juneja et al., 2019). The main source of this contamination is the presence of heat-resistant spores that survive normal rice cooking temperatures (close to 100 °C) (Gilbert et al., 1974). During standard cooking conditions, 2-3 decimal log reductions in the initial spore load can be achieved, therefore final product risk levels depend largely on the initial concentration of microorganisms and hygienic measures during handling, cooking or processing (Rodrigo et al., 2021). After being cooked, the spores that have survived the process can germinate and grow up to 10^7 or 10^9 CFU/g after 24 h at 26 or 32 °C, respectively (Lake, Hudson, & Cressey, 2004; Yu et al., 2020) and produce a heat-stable (emetic) toxin (Little, Barnes, Mitchell, FSA, & PHLS, 2002). Around 95% of outbreaks of an emetic syndrome are mainly caused by the consumption of cooked or fried rice (Juneja et al., 2019). Controlling the storage temperature (lower than 4 °C or higher than 55 °C) is the procedure commonly accepted by food safety authorities.

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https://doi.org/10.1016/j.lwt.2023.114481
Received 10 October 2022; Received in revised form 23 December 2022; Accepted 15 January 2023
Available online 16 January 2023

Consequently, it is of great interest to have an additional control measure, other than post-cooking storage temperature, in these products, especially if they are not going to be consumed immediately after preparation (Juneja et al., 2019).

Grape (*Vitis vinifera* L.), represents the second-largest crop in the world. Its skin and seeds, are also rich in phenolic compounds, as well as lipids, proteins and polysaccharides (Ferreira, Nunes, Castro, Ferreira, & Coimbra, 2014; Gokturk Baydar, Ozkan, & Yasar, 2007). Phenolic compounds are phytochemicals with functions related to pigmentation, astringency, protection against ultraviolet rays, as well as antioxidant and antimicrobial activity (Oliveira, Brunini, Salandini, & Bazzo, 2003). As antimicrobial agents, these polyphenols can penetrate the semi-permeable cell membrane where they react with the cytoplasm or cellular proteins. Therefore, these polyphenolic compounds with a highly negative charge can be used to prevent the growth of pathogenic bacteria (Arts & Hollman, 1998; Cheng, Bekhit, McConnell, Mros, & Zhao, 2012; Oki et al., 2002; Yadav, Kumar, Kumar, & Mishra, 2015). Grape extract is one of the ingredients that has received attention in recent years due to its natural antimicrobial capacity. One advantage of these extracts is that they are derived from industrial waste products, such as grape juice/wine production (Shi, Yu, Pohorly, & Kakuda, 2003; Yu, Ahmedna & Goktepe, 2005, 2010). In addition to providing compounds with functional properties, revalorization reduces the environmental impact by reusing part of the grape, such as seeds and skins, which are generally not used by industry (Goncalves, Lorenzo, & Trindade, 2021). Identifying the value of these products will result in reducing waste, reusing raw products, and providing an all-natural alternative to synthetic preservatives (Levy et al., 2017), which will improve food-industry sustainability and positively impact on the UN's Sustainable Development Goals. Therefore, the use of this raw material as a natural substitute for synthetic antimicrobial additives represents a positive alternative to prevent foodborne outbreaks (Goncalves et al., 2021; Prado Martin et al., 2012).

In this context, the main objective of this work was to evaluate the antimicrobial capability of the grape extract against vegetative cells of *B. cereus* (germinated spores) in a rice matrix, using different storage conditions resembling real ones, at different storage temperatures (10, 20 and 30 °C), different grape extract concentrations (1, 5 and 10 mL/L) and different pH values (4.5, 5.5 and 6.5).

2. Materials and methods

2.1. Microbial strains

Pure culture of *B. cereus* (Spanish Type Culture Collection (CECT) 148) were provided by the Spanish Type Culture Collection and, following their procedure, lyophilized samples were rehydrated with 0.2 mL of sterile Nutrient Broth (NB) (Scharlab S.A., Barcelona, Spain). After 30 min, rehydrated cultures were transferred to 500 mL of NB medium, and incubated in a water bath shaker for 14–18 h at 30 °C, to obtain cells in a stationary growth phase. The *B. cereus* cells were centrifuged twice at 5000 revolutions per minute (rpm), 4 °C and 15 min, in a Beckman centrifuge (JLA-16,250 rotor), the supernatant was decanted and resuspended in 50 mL of NB. Process was repeated twice, and then cells were resuspended in 50 mL of NB and distributed in cryovials (1 mL); 1 mL of 200 mL/L glycerol in NB was added as protectant and kept at −80 °C until further use. The final inoculant concentration was determined by plate count and was of 10^8 colony forming units/mL (CFU/mL).

2.2. Rice matrix

Commercial rice acquired in the market was used. Rice was cooked into an electric cooker, and after cooling down cooked rice was freeze-dried. Rice powder had moisture content of 8.66 g water/100 g of product. Three different batches were prepared. The rice matrix was prepared by diluting 20 g of powder rice/L, and it was sterilized in an autoclave.

2.3. Grape extract

Grape color liquid EV-3 is a natural red colorant extracted from red grapes (EEC code: E-163). The grape color extract was provided by Sociedad Española de Colorantes Naturales y Afines SA - SECNA (Chiva, Valencia, Spain). This extract, with a pH of 2.5, was frozen in Eppendorf at −80 °C for later use. 1, 5 and 10 mL/L grape extract concentrations were added to the rice matrix for testing the antimicrobial activity against *B. cereus*.

2.4. Evaluation of antimicrobial activity of grape extract

The grape extract antimicrobial activity was tested at pH 4.5; 5.5 and 6.5, by using a sodium hydroxide solution (1 mL/100 mL) to modify the pH value of samples Microbial growth at these conditions was compared with control samples at the same pHs but without grape extract. In order to carry out the experiment, 10^7 UFC/mL of *B. cereus* was inoculated to each solution of rice. The inoculated media were incubated under shaking at 10 °C, 20 °C and 30 °C for 168 h (10 °C) and 24 h (20 and 30 °C). For each media, samples were taken at different time intervals. Two series of each sample (A and B) were serially diluted in peptone water (0.1 mg/100 mL), plated in duplicate and incubated in nutritive broth agar (NBA) (Scharlab S.A., Barcelona, Spain) at 37 °C for 24 h. After incubation, colonies were counted (CFU/mL). Experimental results are shown as log10 of the survival fraction (log S) calculated as equation (1):

$$Log\ S = Log_{10}\left(\frac{N}{N_0}\right) \tag{1}$$

Where N is the bacterial concentration (CFU/mL) at time t (h) and N_0 initial bacterial concentration (CFU/mL) (t_0). Therefore, positive values indicate microbial growth while negative values show microbial inactivation compared to the initial inoculation value (N_0).

2.5. Mathematical modelling of B. cereus inactivation

For the concentrations of 5 and 10 mL/L grape extract, the survival curves were fitted with the model proposed by (Mafart, Couvert, Gaillard, & Leguerinel, 2002) based on the Weibull distribution function. The Weibull model is a simple non-linear model, which has been successfully used to fit the nonlinear inactivation curves of many microorganisms under different conditions.

$$Log_{10}(N) = Log_{10}(N_0) - \left(\left(\frac{t}{\delta}\right)^p\right) \tag{2}$$

Where N is the population concentration at time t (CFU/mL); N_0 is the initial population concentration (CFU/mL), δ is the first 10-fold reduction (kinetic parameter) and p is the shape parameter. For the case $p < 1$, the equation allows the tailing portion fitting, inward concavity, of the inactivation curve, for $p = 1$ linear survival curves. For $p > 1$, the shoulder portion, outward convexity, can be predicted (Albert & Mafart, 2005; Marugan, van Grieken, Sordo, & Cruz, 2009). Values of p and δ were determined by using GInaFIT add-in for Microsoft® Excel solver function (Version 1.7) (Geeraerd, Valdramidis, & Van Impe, 2006).

A secondary model was built by means of a forward stepwise multiple regression, relating the logarithm of the scale factor of the Weibull model (δ) with temperature and pH. This is intended to limit the number of terms of the secondary model. Likewise, a global model is built in order to improve the value of the coefficients of the secondary model. A tertiary model is developed using the coefficients deduced by the global model in C# (Microsoft Visual Studio Community 2022 (64 bits) Version 17.0.2).

2.6. Statistical analysis

The fit of the models to the polynomials (secondary model) was performed with STATGRAPHICS Centurion XVIII (STATGRAPHICS, Warrenton, VA), including an ANOVA analysis to test significant differences in estimated model parameters depending on pH and temperature in the same grape extract concentration. The goodness of fit of the model was assessed using adjusted the regression coefficient (adjusted-R^2) and root mean square error (RMSE). To assess predictions made by the model, the Accuracy Factor parameter (*Af*) (Ross, 1996) was used:

$$Af = 10^{\left(\sum\left(\frac{\log\left(\frac{predicted}{observed}\right)}{n}\right)\right)} \quad (3)$$

The predicted/observed ratio refers to the relationship between the survival fraction predicted by de model and the one obtained experimentally and n is the number of observations used to make the calculations.

3. Results and discussion

3.1. Effect of grape extract concentration, pH and incubation temperature on B. cereus growth

In the present work, the effect of different grape extract concentrations on vegetative cells of *B. cereus*, stored at three temperatures and three pH values, has been studied and compared with those obtained in controls. Vegetative cells were used because during storage spores germinate to vegetative cells, producing toxins during growth, so this is the bacterial stage that should be controlled.

Fig. 1 represents the behavior of *B. cereus* qualitatively and schematically under all study conditions. In the control (CA), *B. cereus* grew at all pH values at a temperature of 30 and 20 °C, while the temperature of 10 °C had a bacteriostatic effect with long lag phases, independently of pH, and only in the case of pH 6.5 some significant growth could be observed after 96 h of incubation time. Therefore, neither the pH nor the temperature above 10 °C proved sufficient barriers to prevent *B. cereus* growth. This finding justifies the need to introduce an antimicrobial to prevent microbial growth in the event of cold chain breach or temperature abuse.

By contrast, at grape concentrations of 5 and 10 mL/L grape extract acted as a bactericide for all conditions considered in the study, independently of pH or temperature. From a food safety point of view, it is worth pointing out that 1 mL/L grape extract concentration is the scenario in which temperature and pH are more critical. This is because depending on temperature and pH, grape extract concentration will act as a bactericidal, bacteriostatic or have no effect on *B. cereus* growth.

Fig. 2 shows the behavior of *B. cereus* for the lowest grape extract concentration assayed: 1 mL/L. Positive log values (N/N_0) indicate microbial growth, whereas negative values indicate microbial inactivation compared to the initial inoculation (N_0). At 10 °C and all pH values, the final concentration of *B. cereus* (after 168 h of storage) was lower, or slightly lower (for pH 6.5) than the initial concentration of the microorganism, showing a bactericidal (pH 4.5 and 5.5) or bacteriostatic effect (for pH 6.5). For the same grape extract concentration but at a temperature of 20 °C, a lag phase was observed for all pH values studied. At pH 4.5 and 5.5 the grape extract acted as a bacteriostatic, since *B. cereus* did not grow, while at pH 6.5 the lag phase lasted approximately 6 h, which was longer than the result with acidic control, thus the addition of grape extract delayed microorganism growth. Finally, at 30 °C there was no lag phase for any of the pH values studied. Therefore, at 1 mL/L an increase in storage temperature implies a decrease in lag phase duration.

3.2. Effect of grape extract concentration, pH and incubation temperature on B. cereus inactivation

As mentioned above, under the pH and temperature conditions studied, grape extract exerted a bactericidal effect for concentrations equal to or greater than 5 mL/L. Those conditions were selected to quantitatively characterize the bactericidal effect of grape extract. The results showed that the grape extract can be bacteriostatic or bactericidal depending on concentration, storage temperature and pH of the medium. The antimicrobial capacity of grape extract has been attributed to its polyphenol content (Fontana, Antoniolli, & Bottini, 2013; Oliveira et al., 2013; Yadav et al., 2015). Various authors have reported Total Polyphenol Content in grape extract between 128.22 and 215.93 mg Gallic Acid Equivalents/Dry Weight (mg GAE/DW) (Ky & Teissedre, 2015); 36.6–88.7 mg GAE/DW (Ky, Lorrain, Kolbas, Crozier, & Teissedre, 2014); 212–279 mg GAE/DW (Brezoiu et al., 2019); 24.5–60.1 mg GAE/DW (Bosso et al., 2020); 33.2–37.5 mg GAE/DW (Guaita & Bosso, 2019). The quantity of polyphenols depends on the origin (grape seeds or skin), as well as the extraction solvent used for polyphenol quantification and the analysis method; therefore values could differ, accordingly (Balaban, Koc, Sar, & Akbas, 2021). Many classes of negatively

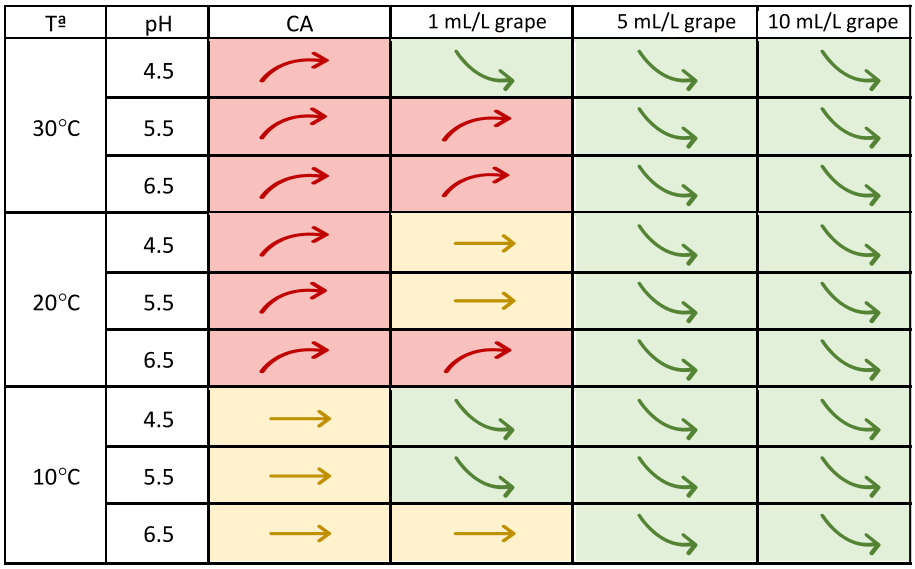

Fig. 1. Schematic representation of *B. cereus* growth/inhibition depending on temperature (10 °C, 20 °C and 30 °C), pH (4.5, 5.5 and 6.5) and grape extract concentration (0 mL/L (CA), 1 mL/L, 5 mL/L and 10 mL/L). Colors code: Red represents unsafe conditions, where the microorganism grows; yellow, bacteriostatic conditions; and green are microbiologically safe conditions, where the antimicrobial acts as a bactericide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

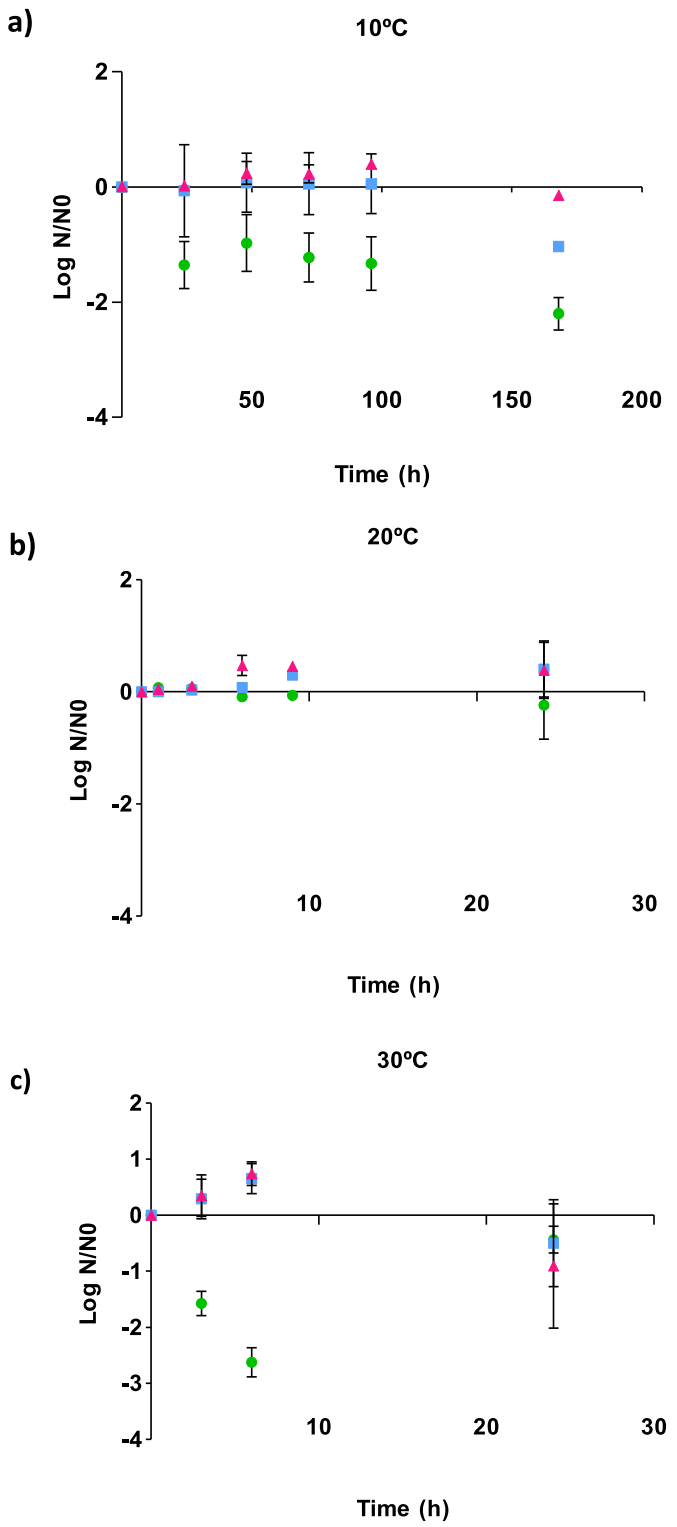

Fig. 2. Growth/inhibition of *B. cereus* with 1 mL/L grape extract concentration depending on pH (4.5 (●), 5.5 (■) and 6.5 (▲)) and temperature (10 °C (a), 20 °C (b) and 30 °C (c)).

charged polyphenols have been identified in grapes, such as phenolic acids (benzoic and hydroxycinnamic acids), stilbene derivatives (resveratrol), flavan-3-ols (catechin, epicatechin), flavanols (kaempferol, quercetin, myricetin), anthocyanins, etc. (Oliveira et al., 2013; Yadav et al., 2015). Gram-positive bacteria were more sensitive to grape extract than Gram-negative bacteria. These differences could be explained by the presence of the lipopolysaccharide cell wall in Gram-negative bacteria, which can limit the penetration of polyphenols (Gerardi, Pinto, Baruzzi, & Giovinazzo, 2021). This potential is greater in grape-skin extract because phenolic acids are present in undissociated forms (Yadav et al., 2015). In this frame, Katalinić et al. (2010) studied the antimicrobial activity of 14 phenolic extracts, both from white grape and red grape (seven and seven), utilizing a broth microdilution test with Gram-negative (*E. coli* O157: H7, *Salmonella Infantis*, *C. coli*) and Gram-positive (*S. aureus*, *B. cereus*) bacteria. Antimicrobial activity was confirmed against all gram-negative and gram-positive bacteria for all extracts. Differences in phenolic efficiency of white and red grape cultivars have been observed for different test organisms but no significant differences were found in the susceptibility of Gram-positive and Gram-negative bacteria (Katalinić et al., 2010). In the present work, results shown that grape extract exerted an antimicrobial activity against *B. cereus*, in accordance with Katalinić et al. (2010). Furthermore, as occurs in the present study with grape extract concentrations, in a study of the antimicrobial effect of pomegranate peel extracts at different dilution ratios against *B. subtilis, B. cereus*, and *E. faecalis* strains, Balaban et al. (2021) observed that the antibacterial activity of the extracts decreased when the extract dilution increased. In addition, in most cases, all the extracts and their dilutions were found to be more effective against *B. cereus* growth.

In this study, a 24 h period was considered a good control point for comparison between different pH values and temperatures since it is the time taken for control samples to reach the stationary phase at 20 °C. This temperature is important as it is considered as a cold chain breach. Fig. 3 shows that the bactericidal effect of the grape extract against *B. cereus* varied depending on the incubation temperature, the grape extract concentration and the pH of the medium. Considering the effect of pH, the greatest bactericidal effect occurred at pH 4.5, which differed with statistical significance to effects at pH 5.5 and 6.5 at 10 and 20 °C, and reaching up to 6 logarithmic reductions at 30 °C. Similar effect occurs in the study of Mau, Chen, and Hsieh (2001) where studied the antimicrobial effect of mixed different natural extracts (Chinese achieve + cinnamon + corni fructus (1:1:1)) in other food matrices (orange juice, pork and milk) against 15 microbiological strains, at different pH (original, 4.5, 5.5 and 6.5) and storage temperature (4 and 25 °C). Higher inhibitory effect was obtained at more acidic pH values and in their study it was independent by the storage temperature. Regarding temperature effects in the present work, the greatest inactivation occurred at 30 °C (Fig. 3), regardless of pH values and the grape extract concentration studied. This may be because these are optimal conditions for microorganism growth and thus defense mechanism against stress are not activated (Rodrigo, Ruiz, Barbosa-Canovas, Martinez, & Rodrigo, 2003). For pH 4.5, there were no significant differences between the inactivation levels reached at 10 or 20 °C, while for pH 5.5 and 6.5, (less stressful conditions) a greater inactivation was achieved at 20 °C. Likewise, Periago and Moezelaar (2001), observed that nisin on its own, showed a small effect at 8 °C against *B. cereus* strain, needing to be combined with lower pH, carvacrol, or both of them for maximum bactericidal action. The combined effect of nisin and carvacrol was significant at pH 6.3 and 7 and was significantly greater at 30 °C than at 8 °C. A similar effect can be observed in the present study since the combination of different factors enhances the effect of the natural antimicrobial.

Regarding the effect of grape extract concentration on inactivation, in general, there were no significant differences between the values reached with 5 or 10 mL/L. Only at a pH of 5.5 and a temperature of 30 °C did the grape concentration produce a significant increase in inactivation (Fig. 3).

3.3. Kinetics of *B. cereus* inactivation by grape extract

In order to complete the evaluation of the grape extract, quantify its natural antimicrobial activity in cooked rice products, and make

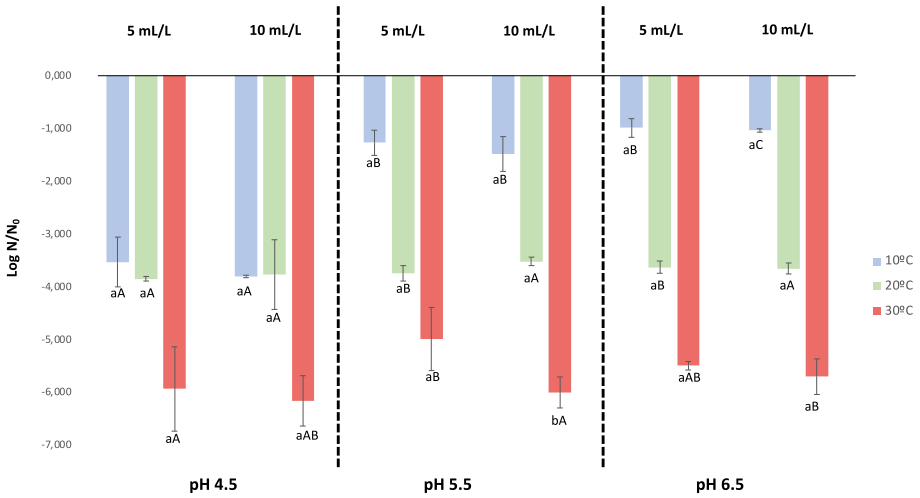

Fig. 3. *B. cereus* inactivation levels at 24 h storage time under exposure to concentrations of 5 and 10 mL/L of grape extract, at incubation temperatures of 10 °C, 20 °C and 30 °C, and pH 4.5, 5.5 and 6.5. Letters on the bars indicate significant differences ($P \leq 0.05$) between concentrations of 5 and 10 mL/L for the same pH and temperature (lowercase letters) and between pH (4.5–5.5 - 6.5) for the same temperature and concentration (capital letters).

predictions for different scenarios, the experimental results were adjusted for concentrations of 5 and 10 mL/L of grape extract (conditions in which the expected result was achieved) to the Weibull distribution function, using the GInaFIT excel add-in (Version 1.7). Each individual experimental survival curve obtained was fitted separately and the estimated parameters (scale parameter δ and shape parameter p) were derived for each pH, temperature, and grape extract concentration (Table 1). The scale parameter δ is the first 10-fold reduction, related to the microorganism's resistance to the inactivation treatment, as the higher the treatment intensity, the lower the resistance. In general, as shown in Table 1, increasing extract concentrations and temperatures was associated with a δ parameter reduction although it was not always statistically significant. Additionally, there was a significant effect of pH (4.5 versus 6.5) for resistance to microorganism death for any concentration and temperature, except 20 °C – 10 mL/L.

A secondary model was developed by a forward stepwise multiple regression to define the dependence of log δ with temperature and pH for fixed concentrations of grape extract (5 or 10 mL/L), equations 3 and 4. The p value is less than 0.05, thus there is a significant relationship between the dependent variable (log δ) and independent variables at the 95% of confidence level. Kwon, Rhee, and Yoon (2020) obtained shape p values lower than 1 at all temperatures studied, indicating rapid decrease of the microorganisms, which agrees with our results as shown in Table 1.

$$Log\ \delta = -2.28 + 0.418 * \mathrm{pH} \pm 0.0175 * \mathrm{pH} * \mathrm{T} + 0.204 * \mathrm{T} - 0.0040 * T^2 \quad R^2 = 97\% \tag{3}$$

$$Log\ \delta = -8.11 + 3.10 * pH - 0.248 * pH^2 - 0.0104 * pH * T \quad R^2 = 94\% \tag{4}$$

To improve the value of coefficients of the secondary model (polynomial model) obtained, a global model was built using all data points (*Log S*) and fitting them to equations 5 and 6, using one-step nonlinear regression (Statgraphics Centurion XVIII). The estimated coefficients and the confidence intervals obtained are shown in Table 2.

$$Log\ S = -\left[\left(\frac{t}{10^{(0.780+0.165*pH-0.00095*pH*T-0.0746*T+0.00077*T^2)}}\right)^p\right] \tag{5}$$

$$Log\ S = -\left[\left(\frac{t}{10^{(-7.29+2.84*pH-0.231*pH^2-0.0101*pH*T)}}\right)^p\right] \tag{6}$$

The performance of the global inactivation model was validated by the Accuracy factor (*Af*). *Af* is a measure of how close the predicted value is to the experimental value (the further from 1, the more inaccurate) (Oscar, 2005). In the present study, *Af* for 5 and 10 mL/L of grape concentration were 1.26 and 1.15, respectively, which indicates an error rate of 26% and 15% for these predictions. Therefore, the models developed can accurately predict *B. cereus* inactivation in a rice matrix, at different pH values and in a temperature range between 10 and 30 °C, which is considered critical from the food safety point of view. Simillar to this study, Rodrigo, Barbosa-Canovas, Martinez, and Rodrigo (2003) analyzed *Escherichia coli* inactivation by pulsed electric fields and the experimental data were fitted to the Weibull distribution function using a one-step non lineal regression and scale and shape parameters were deduced. A secondary model for the scale parameter was also built. To improve the precision of the coefficients of the secondary model, a global model using all experimental data was constructed similar to equations (5) and (6) of the present study, with very good results. A problem that could arise in applying the kinetic parameter obtained by a secondary model to estimate the residual microbiological load is related with the shape parameter in the primary models. Couvert, Gaillard, Savy, Mafart, and Leguerinel (2005) solved this problem using a single p value, estimated from the entire data set. Despite a slight loss of fit goodness, this modification improved the robustness of the model, a fact that also occurred in the present work, in which a single p value has been used within the data set of each concentration of grape extract. This method was also suggested by other authors (Corradini, Normand, & Peleg, 2008; Mafart et al., 2002).

Fig. 4 illustrates the response surface of the global model with the relationship between the influence of pH and incubation time on *B. cereus* inhibition for concentrations 5 mL/L (a) and 10 mL/L (b) of grape extract. The figure reveals that inactivation increased with treatment time and pH decreased. In addition, for the 10 mL/L concentration, a slight decrease in inactivation was observed for pH 5.5.

Considering the global model obtained (equations (5) and (6)), a tertiary model was constructed using Microsoft Visual Studio C^{++}. This developed software can be implemented in industry to help in the decision making regarding pH and temperature at each concentration level with reference to the initial microbial load. It will allow an industrial business operator to adjust the storage conditions (time and temperature) to achieve the microbiological stability of the food based on its initial contamination. Besides, it can be chosen between two concentrations of grape extract, according to the food matrix properties. At the

Table 1
Weibull parameters (δ and p) and model fit (Adjusted R^2 and RMSE) for *B. cereus* inactivation under exposure to 5 and 10 mL/L grape extract concentration at 10 °C, 20 °C and 30 °C.

Grape extract concentration	Temperature	pH	Weibull parameters		Accuracy fit	
			δ	*p*	R^2_{adj}	RSME
5 mL/L	10 °C	4.5	3.07 ± 0.868 ^{aA}	0.34 ± 0.005	0.916	0.691
		5.5	5.68 ± 0.949 ^{bA}	0.42 ± 0.018	0.965	0.347
		6.5	6.95 ± 0.689 ^{bA}	0.40 ± 0.043	0.946	0.458
	20 °C	4.5	*2.256 ± 0.510 ^{aB}	0.572 ± 0.052	0.971	0.294
		5.5	4.224 ± 0.691 ^{bB}	0.840 ± 0.059	0.972	0.308
		6.5	5.649 ± 1.150 ^{cA}	0.905 ± 0.135	0.993	0.134
	30 °C	4.5	0.655 ± 0.283 ^{aC}	0.500 ± 0.061	0.948	0.707
		5.5	0.499 ± 0.119 ^{abC}	0.439 ± 0.051	0.887	1.032
		6.5	0.296 ± 0.168 ^{bB}	0.283 ± 0.043	0.765	1.148
10 mL/L	10 °C	4.5	2.51 ± 0.422 ^{aA}	0.26 ± 0.006	0.837	0.985
		5.5	6.72 ± 0.368 ^{bA}	0.45 ± 0.012	0.95	0.457
		6.5	7.05 ± 0.200 ^{bA}	0.46 ± 0.006	0.929	0.524
	20 °C	4.5	*0.526 ± 0.129 ^{aB}	0.359 ± 0.049	0.927	0.523
		5.5	3.209 ± 0.219 ^{bB}	0.583 ± 0.015	0.998	0.07
		6.5	2.160 ± 0.252 ^{cB}	0.559 ± 0.024	0.98	0.246
	30 °C	4.5	0.363 ± 0.282 ^{aB}	0.420 ± 0.054	0.977	0.46
		5.5	0.394 ± 0.059 ^{aC}	0.462 ± 0.025	0.983	0.442
		6.5	0.322 ± 0.134 ^{aC}	0.389 ± 0.042	0.969	0.62

Values followed by different letters within the same pH (small letters) and within the same temperature (capital letters) are significantly different ($p \leq 0.05$). Values with (*) indicate significant differences ($p \leq 0.05$) for the same pH and temperature between concentrations.

Table 2
Coefficient estimates obtained by fitting the global model (equations (5)–(5) mL/L grape extract concentration and equation 6–10 mL/L) to the total experimental data.

5 mL/L grape extract			10 mL/L grape extract		
Parameter	Estimated	Standard error	Parameter	Estimated	Standard error
a	0.7797	0.4372	a	−7.2862	1.4601
b	0.1648	0.0752	b	2.8449	0.5529
c	−0.0009	0.0032	c	−0.2308	0.0390
d	−0.0746	0.0310	d	−0.0101	0.0001
e	0.0007	0.0006			

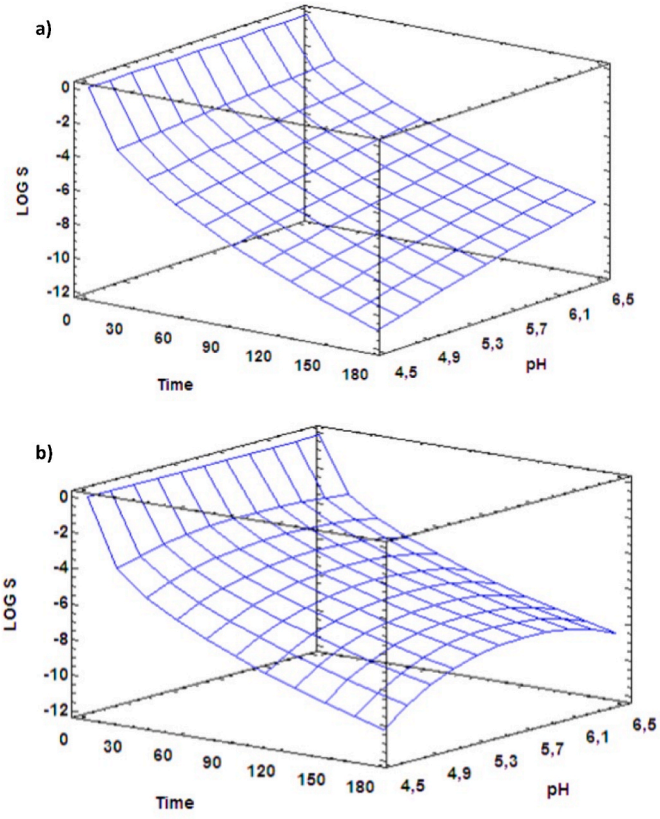

Fig. 4. Three-dimensional relationship between the influence of pH and incubation time at a constant temperature of 20 °C on *B. cereus* inactivation for **a)** 5 mL/L and **b)** at 10 mL/L concentration of grape extract.

same time, for specific pH and storage temperature conditions it can also be adjusted the maximum incubation time that the food matrix can be stored without causing any food safety issue (final microbial concentration remains below a specific value).he software will. Fig. 5 shows a screenshot of the software.

4. Conclusions

The present study proposes a quantitative approach to evaluate the antimicrobial potential of a natural coloring agent coming from an industrial by-product, namely grape extract, against *B. cereus*. Grape extract, at a concentration of 5 mL/L, has proven value as a way to control the bacterial load in a potential cold chain breach. In addition, this food ingredient, used in combination with other preservation treatments such as lowering the pH of the medium and the temperature, can attain maximum levels of *B. cereus* inactivation (6 log reductions) at grape extract concentrations of 5–10 mL/L. Furthermore, software developed from the global model can help in selecting the combination of grape extract concentration, incubation time and temperature, as well as the pH of the medium in order to attain the desired log reduction of *B. cereus* loads. Although the study indeed has some limitations that are indicated by the limits of the parameters that have been studied, temperatures between 10 and 30 °C, pH between 4.5 and 6.5 and grape extract concentrations 1, 5 and10 mL/L.

The results reported in this manuscript provide evidence for the positive effects of adding food ingredients such as grape extract, which have bacteriostatic or bactericidal properties. Further investigation is needed to evaluate the sensory acceptability of such products to consumers.

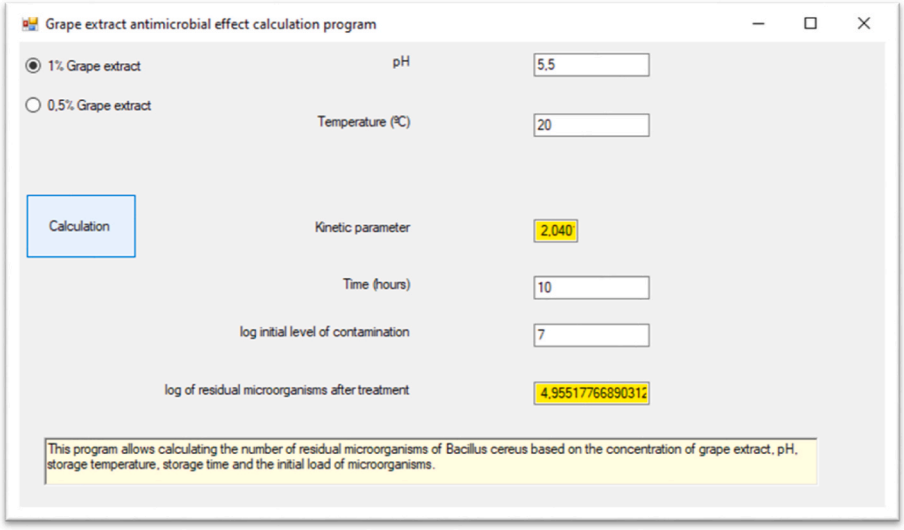

Fig. 5. Screenshot of the software developed (tertiary model) to calculate the microorganism concentration after grape extract treatment combined with pH and temperature.Fig. 1.

CRediT authorship contribution statement

Eva Grau-Fuentes: Investigation, Writing – original draft. **María Úbeda-Manzanaro:** Investigation. **Antonio Martínez:** Data curation, Writing – review & editing. **Raquel Garzón:** Formal analysis. **Cristina M. Rosell:** Funding acquisition, Writing – review & editing. **Dolores Rodrigo:** Supervision, Conceptualization, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgments

We want to thank TRACE-RICE project, Reference Number AMD-1934-1 and grant PID2020-116318RB-C31, funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe", for supporting this Research.

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