Catalogue of predictive models of microbial inactivation and growth as function of processing and storage conditions

[31-08-2023]

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1. INTRODUCTION

1.1. Statement of the problem

Bacillus cereus is an important spore-forming microbe human pathogen (Rodrigo et al. 2021). It is one of the top ten pathogens responsible for many foodborne diseases in humans. In 2021, EFSA reported 679 cases of *B. cereus* food contamination in the European Union.

It is present in many foods due to its ubiquitous nature. *B. cereus* causes two types of food contamination, one of an emetic nature and the other of a diarrheal nature (Griffiths and Schraft, 2017). Diarrheal syndrome is caused by a gastrointestinal disorder due to the ingestion of *B. cereus* spores present in food and at a dose given, an appreciable probability that dells cross the stomach barrier and implanting themselves in the small intestine is possible. Once they germinate in the small intestine they produce enterotoxins that cause disease. On the other hand, emetic syndrome is associated with the production of a cereulide toxin in the food contaminated with spores that germinate and produce the toxin resulting in foodborne poisoning (Rouzeau et al. 2020).

In general, this microorganism is associated with complex food products that may include rice as a component; however, other rice-based products and farinaceous foods such as pasta and noodles are also frequently contaminated and involved in cases of *B. cereus* toxi-infection (Grande et al. 2006). In addition, the ability of *B. cereus* to form spores and biofilms enables its persistence in various ecological niches and food products resulting in its presence in processed foods such as cooked rice (Navaneethan and Effarizah, 2021). Furthermore, it is the bacteria most commonly present in rice and rice-based products (Hwang and Huang. 2019).

Rice is a basic cereal in many diets and is widely consumed by the general population given its ample supply of nutrients and its relatively low cost. This cereal is one of the most important staple crops feeding almost half of the world's population (Wei and Huangm 2019). Starch is the most abundant component of a rice grain constituting about 80% of the dry weight of a brown rice grain and approximately 90% of a milled rice grain (Bao 2019). Rice also provides an important variety of micronutrients including vitamins such as niacin, thiamine, pyridoxine or vitamin E and minerals such as potassium, phosphorus, magnesium and calcium (Base de Datos Española de Composition de Alimentos 2021). These conditions provide a very good substrate for *B. cereus* growth and subsequent toxin production.

One of the main factors contributing to *B. cereus* outbreaks related to cooked rice is poor cooling, or storage at inappropriate temperature (higher than 4ºC or lower than 55ºC) 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 ºC) (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 remaining spores can germinate and grow up to 10^7 or 10^9 CFU/g after 24 hours 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 is the procedure commonly accepted by food safety authorities. 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). Figure 1 shows schematically the statement of the problem.

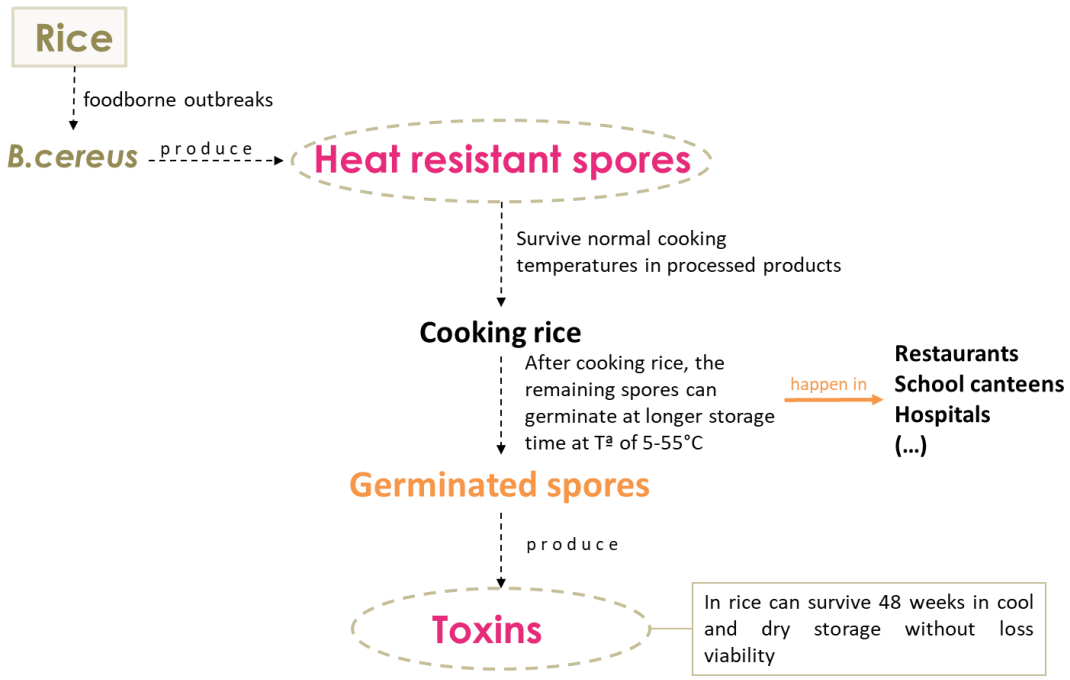

Figure 1. Statement of the problem of B. cereus in rice cooked products.

1.2. Chitosan as natural antimicrobial

Different control measures have been proposed to control *B. cereus* in foods. As an additional strategy, heat treatment can be combined with other control measures (hurdle technology). In this respect, chitosan from different sources (crustacean or fungi) has received attention as antimicrobial. It is a polysaccharide with a well-documented antibacterial activity towards vegetative cells which has already been effectively applied as edible chitosan films (Elsabee 2014) and in food packaging applications (Kumar et al., 2020; Priyadarshi and Rhim. 2020). Existing chitin resources have some natural challenges, including insufficient supplies, seasonal availability, and environmental pollution. As an alternative, insects could be utilized as unconventional but feasible sources of chitin and chitosan. According to Van Huis et al. (2013) rearing insects is a sustainable activity friendlier with the environment than fishing or traditional farming. Besides, as indicated by Mohan et al., (2020), the extraction of chitin and chitosan from insects is more advantageous in terms of extraction methods, chemical consumption, time and yield compared to existing sources. According to previous in vitro studies (Ibañez et al., 2020), insect chitosan could be used as antimicrobial instead of chitosan from other sources. Based on those results it could be also applied as an additional control measure during heat processing and storage of rice thus favouring the destruction of *B. cereus* spores by affecting their heat resistance and subsequent viability. Currently there are no data on the joint effect of insect chitosan and heat on the resistance of *B. cereus* spores since chitosan from other sources is used as a natural antimicrobial in the preservation processes.

1.3. Grape extract as natural antimicrobial

Another natural antimicrobial that can be considered is grape. Grape extract is one of the ingredients to have received attention in recent years. 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, 2010; Yu, Ahmedna, & Goktepe, 2005). In addition to providing compounds

with functional properties, revalorization reduces the environmental impact of eliminating part of the grape, such as seeds and skins, which are generally not used by industry (Goncalves, Lorenzo, & Trindade, 2021). 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). 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.

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; 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 semipermeable 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).

1.4. Microbial mathematical modelling

A model is defined as a simplified description of the reality and describes the relationships between observations of a system (response, e.i., growth or inactivation) and the factors thought to cause the observed response. It can be used for predictive and control purposes, and can be included in an exposure assessment model. In this sense, a mathematical model is a useful tool to simplify the description of a collection of data acquired on a specific response, thus obtaining an inference of reality. Mathematical modelling of the behaviour of microorganisms allows, within the range of experimental conditions, to predict the inactivation or growth of microorganisms as a function of different variables through a model.

In scientific bibliography different inactivation and growth models have been published being the oldest one the Bigelow model for canning processes, but it can be applied to other processing technologies. With the development of non-thermal technologies and mild thermal technologies, it was necessary to seek or develop other models suitable for developing adequate processing or storage conditions and for establishing the shelf life of foodstuffs. Depending on the nature of the model, they can be classified as deterministic or stochastic. The deterministic models are used to calculate the kinetic parameters and provide fixed output values predicting how bacterial proliferation or declination will be under given conditions. On the other hand, in the stochastic models, the input parameters and output data are defined by probability distributions, being results also defined by an uncertainty. Considering the above, and using deterministic models as a baseline, quality and industrial food safety departments need to assess risk based on probability, considering real variations in conditions to obtain a more realistic approximation that can facilitate the decision-making process. To make those decisions, stochastic predictive models, are developed as the starting point in an exposure assessment process that leads us to simulate the final loading results with their associated probability of occurrence.

2. OBJECTIVE

In this context, the main objective to accomplish with the deliverable 2.4 was to have a catalogue of mathematical models and seek the more suitable models for each process considered for a rice-based

matrix. This knowledge can pave the way to a better control of *B. cereus* during and after the cooking processes of rice and its derivatives.

Bigelow or Weibull based-models were considered to quantify microbial inactivation whereas Gompertz, and Baranyi and Robert models were considered when microbial growth was found. Those models were used to evaluate and quantify the antimicrobial activity of two natural antimicrobials, grape extract and insect chitosan, against *B. cereus* in a rice matrix, as an additional control measure during the cooking process (thermal treatment) and subsequent storage before consumption. Figure 2 shows schematically the experimental procedure of the study. In a later stage, and based on the deterministic models applied, an exposure assessment to *B. cereus* by the ingestion of cooked rice was performed.

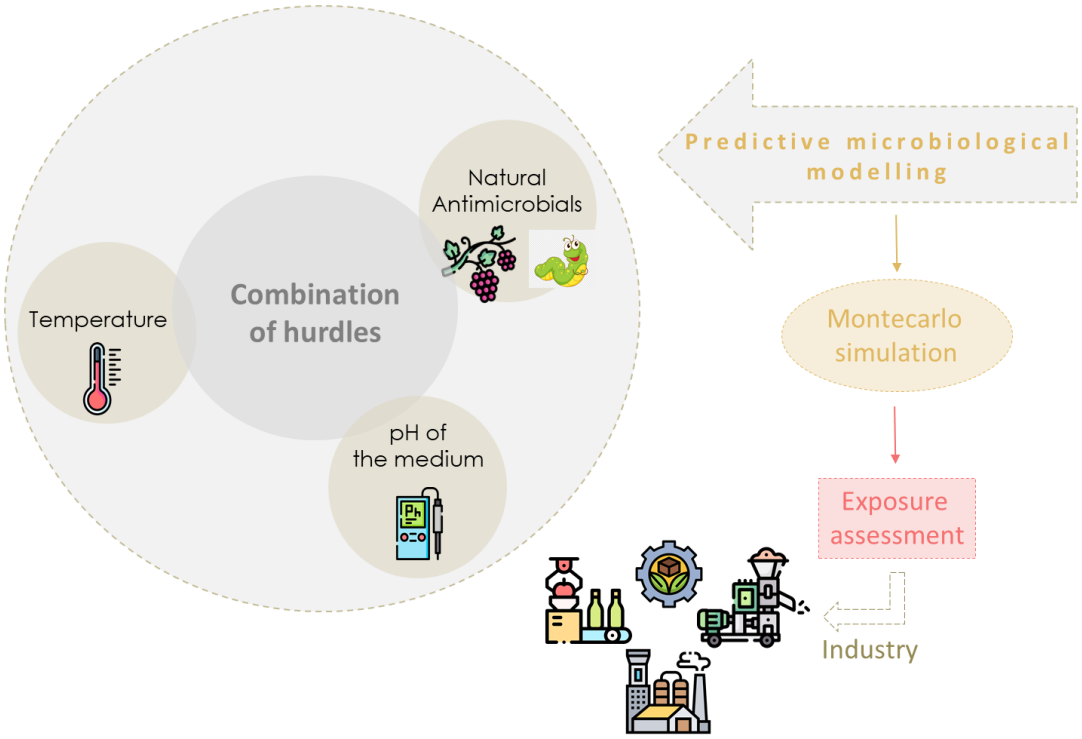

Figure 2. Schematic work plan for the study.

3. MATERIALS AND METHODS

3.1. Microorganism, sporulation and vegetative cells preparation

The tests were carried out with a pure lyophilized culture of *B. cereus* provided by the Spanish Type Culture Collection (CECT 148) that is equivalent to ATCC 13061.

For the thermal inactivation studies, *B. cereus* spores were tested. The strain was reactivated in nutrient broth by shaking for 24 hours at 32 ºC and subsequently 0.5 mL of the *B. cereus* culture was inoculated in 20 Roux flasks (Fisher Scientific SL, Madrid, Spain) with Fortified Nutritive Agar (Scharlab. Barcelona, Spain) and incubated at 30 ºC. When the sporulation level reached approximately 90% the spores were collected. Spore harvesting was performed using a modified metal Digralsky loop (Deltalab, Barcelona, Spain) gently sweeping the agar surface and washing it with double distilled water. The collected solution was centrifuged at 2500 g for 15 minutes at 5ºC in a Beckman centrifuge (JLA-16, 250 mL rotor), the supernatant was removed suspended again in 5mL of double distilled water

and was centrifuged under the same previously described conditions. This process was repeated 4 times. Finally, the spores from the pellet were stored at 4 ºC in distilled water.

For the growth studies, vegetative cells from *B. cereus* were tested. The culture was rehydrated with 0.2 mL of sterile Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). After 30 minutes, the entire suspension was inoculated in an Erlenmeyer flask with 500 mL of NB medium. This was incubated at a temperature of 30 °C in a thermostatic bath with continuous shaking for 14 h, to obtain cells in a stationary growth phase. The cells were centrifuged twice at 5000 revolutions per minute (rpm), 4 ° C and 15 minutes, in a Beckman centrifuge (JLA-16, 250 mL rotor). After decanting the supernatant, the cells were resuspended in 50 mL of NB medium. After the second centrifugation the cells were resuspended in NB and then distributed in 2 mL cryovials, adding 1 mL per cryovial. To each cryovial, 1 mL of 20% glycerol in NB was also added, which acts as a cryoprotectant. The 2 mL samples were immediately frozen and stored at -80 ° C until use. The concentration of B. cereus in the cryovials was determined by plate count having a concentration of 10^8 CFU / mL.

3.2. Rice matrix preparation

The rice solutions from cooked and lyophilized rice supplied for a local company were prepared at a concentration of 2% (w / v). For this, 1 g of lyophilized rice was diluted in 50 mL distilled water in a bottle with a magnet and a screw cap. Before being used, the rice solutions were sterilized in an autoclave.

3.3. Insect chitosan

Insect chitosan was from the *Tenebrio molitor* beetle (MealFood Europe S.L, Salamanca, Spain; reference 6101. Currently TEBRIO, Salamanca, Spain) purity 90-95%, deacetylation degree >85%.

Chitosan stock solutions were prepared at a concentration of 1% (w / v) of insect chitosan, diluted in a 1% (v / v) acetic acid stock solution (Scharlab Chemie S.A., Barcelona, Spain). This organic acid is used as a diluent to solubilize the chitosan. To improve chitosan solubilization, the solutions were left under continuous stirring for 48 h. Subsequently, and before use, the chitosan solutions were filtered with a sterile 0.45 µm membrane filter for sterilization (MF-Millipore® Membrane Filters).

3.4. 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 -80ºC for later use.

3.5. Thermal inactivation studies

For efficient and homogeneous heat treatment, an adaptation of the capillary tube method was used (Fernández, et al., 1999). Specifically, Vitrex capillaries measuring 1.50 x 2.00 x 100 mm sealed at one end were used. The capillaries were filled with the samples chitosan or grape extract solutions using a sterile chamber, fitted with a vacuum pump; once the capillaries were filled, they were sealed at the other end with silicone (Quiadsa, Madrid, Spain). The capillaries were placed on racks and heated in a silicone oil bath with shaking. Ten capillaries were prepared as replicates for each treatment time (0-50 min depending on the temperature) and temperature (90-105ºC). After each heating period, the capillaries were immediately cooled in an ice water bath and immersed in ethanol 70% to avoid external contamination. For sample plating, the capillaries were broken and serial dilutions were made. Samples were plated on starch-enriched nutrient agar and viable counts were based on duplicate counts of the dilutions. Plates were incubated at 30°C for 24 h.

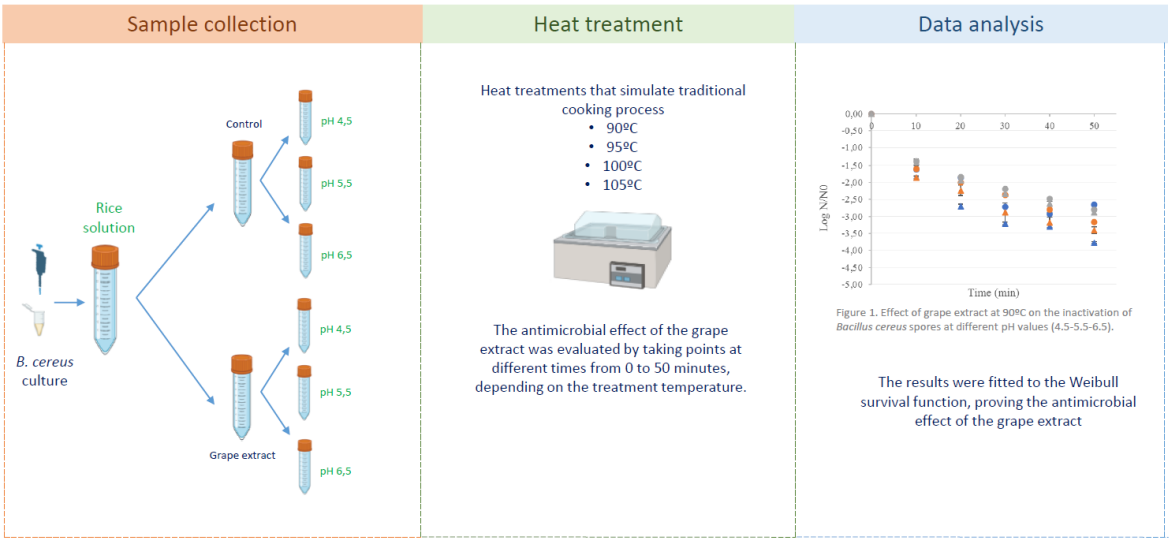

Figure 3. Schematic work plan for thermal treatment studies.

For chitosan studies, two solutions of rice with insect chitosan (150 and 250 µg/mL chitosan) were used. Those concentrations were chosen based on previous studies (Valdez et al. 2022). The pH was adjusted to between 6.8 and 6.9. For the study grape extract study, 0.1% (1 µg/mL) of grape extract was tested with different pHs 4.5, 5.5 and 6.5. In both the chitosan and rice studies, control samples were also tested under the same conditions. The final spore concentration in the rice solutions was 10^8 CFU/mL. Figure 3 shows a schematic work plan for thermal treatment studies.

3.6. Growth studies

For chitosan studies, sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 µg / mL), which were tested at pH 6.25 ± 0.2. The grape extract antimicrobial activity was tested at 1 µg/mL, 5 µg/mL, 10 µg/mL and at pH 4.5; 5.5 and 6.5. The tests also included two *B. cereus* controls (rice substrate without antimicrobial). The first one at the natural pH of the rice substrate (6.85 ± 0.2), and a second one, acidified (acetic acid 0.025% v/v), to reach the same pH and acetic acid concentration that have the samples studied solutions. This second control was considered to evaluate a possible antimicrobial effect of acetic acid under the study conditions.

The different sample solutions were inoculated with the content of a previously thawed and resuscitated (overnight growth in Nutrient Broth, NB, Scharlab Chemie, Barcelona, Spain) vial from the stock, up to a final cell concentration of approximately 10^7 CFU / mL. Subsequently, the solutions were kept in an incubator with continuous shaking at 350 rpm, at temperatures of 30 ± 0.5 ºC; 20 ± 0.5 ° C; and 10 ± 0.5 ºC, simulating different storage temperatures of precooked rice. Being 30 ºC a temperature within the optimal range for the growth of *B. cereus*; 20 ºC, a temperature that would represent a cold-chain breakdown in the storage process; and 10 ºC as an example of refrigeration temperature abuse.

The antimicrobial effect of chitosan or grape extract was evaluated by taking sampling points at different incubation times (between 0 and 170 hours), depending on the storage temperature. The samples at each control time were diluted by serial decimal dilutions in 0.1% (w / v) peptone water, and plated in NB agar culture medium (Scharlab Chemie, Barcelona, Spain). Plates were incubated at 30 ºC for 24 hours before counting. Figure 4 shows a schematic work plan for growth (storage) studies.

The experimental results were shown as $\log_{10}$ of the survival fraction (log S) calculated by (Equation 1)

$$\text{Log}\, S = Log_{10}\left(\frac{N}{N_0}\right) \qquad \text{Equation 1}$$

Where N is the bacterial concentration (CFU / mL) at time t (h) and N_0 the initial bacterial concentration (CFU / mL).

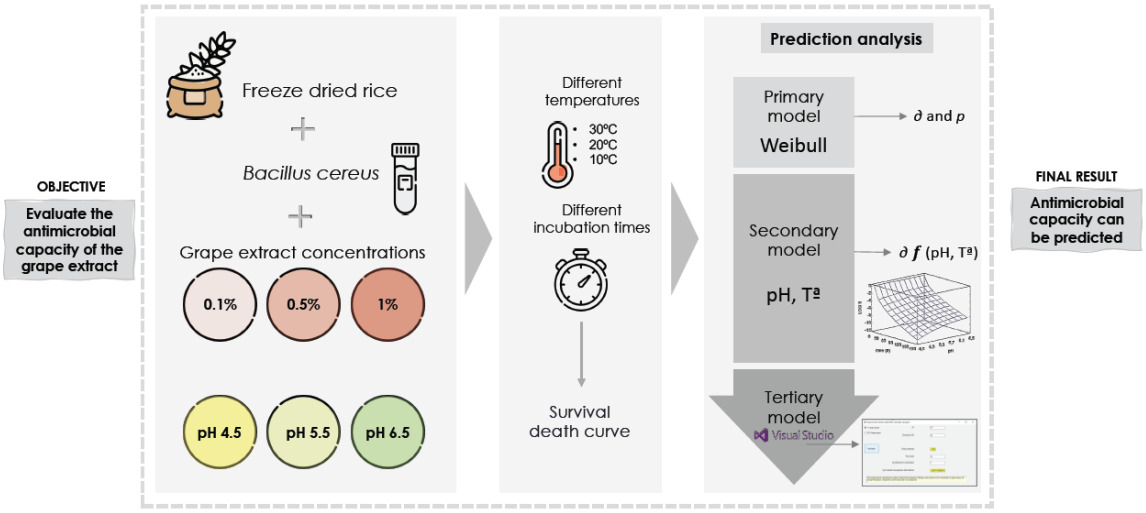

Figure 4. Schematic work plan for growth (storage) studies.

3.7. Mathematical modelling

All mathematical modelling including nonlinear regression were performed using Statgraphics Centurion XVI Software (STATGRAPHICS, Warrenton, VA, USA).

3.7.1. Primary model

Survival or growth data were fitted to primary models linear or non-linear depending on the data obtained.

For linear data, Bigelow model was used with the following equation:

$$Log(S) = -\frac{t}{D} \qquad \text{Equation 2}$$

Where *S* is the survival fraction at time *t*, and *D* the decimal reduction time (min).

For non-linear data, Weibull distribution function (Equation 3) were fitted to experimental data when microbial inactivation was shown:

$$S_t = e^{-(t/a)^b} \qquad \text{Equation 3}$$

Where *a* is the scale parameter, considered as a non-biological kinetic parameter representing the change of the microbial load along the treatment time, and *b* is the shape parameter. The *b* parameter describes the shape of the survival curve so, when $b < 1$ the survival curve is concave, when $b > 1$ the survival curve is convex, and when $b = 1$ the survival curve is a straight line on a log scale (equal to the Bigelow model).

For growth data two models were used:

- The modified Gompertz equation (Equation 4) reads as follows:

$$Log\ (S) = A * e^{\left(e^{\left(\frac{e(1)*K}{A}\right)*(Tlag-t)+1}\right)} \qquad \text{Equation 4}$$

Where Log (*S*) is the log of the survival fraction; *A* asymptote estimation; *K* is the growth rate; *T lag* is the duration of the latency phase; *t* is the storage time.

- Baranyi and Roberts model

$$Log\ y(t) = \log y_0 + \mu_{max} A(t) - log\left(1 + \frac{e^{\mu max A(t)} - 1}{e^{(\log K - h_0)}}\right) \qquad \text{Equation 5}$$

Where y_o is the initial value of contamination, μ_{max} is the maximum growth rate, *A* is the lag phase duration, *K* is the maximum level of microorganisms and h_o specifying the initial physiological state of the microorganism.

3.7.2. Secondary model

In those cases where it has been possible, a secondary model was derived that related the logarithm of a of the primary model with the independent variables, temperature and pH.

3.7.3. Exposure assessment models

Exposure assessment modular models for thermal and growth studies in the presence of chitosan/grape extract will be developed based on Monte Carlo simulation. For the first module (thermal studies) the final number of microorganisms will be calculated for a given temperature and cooking time. As the input data, the Bigelow model or Weibull model will be used according to the inactivation curves, to obtain the inactivation kinetic parameters. The other input data will be the initial number of *B. cereus* contaminating a portion of food.

Next, the second module (growth studies) will be added. The input data will be the final number of microorganisms after the cooking process (first module) and the growth kinetic constants obtained through the Baranyi and Roberts or Gompertz growth models. Figure 5 shows a schematic flow of the methods followed for the exposure assessment study.

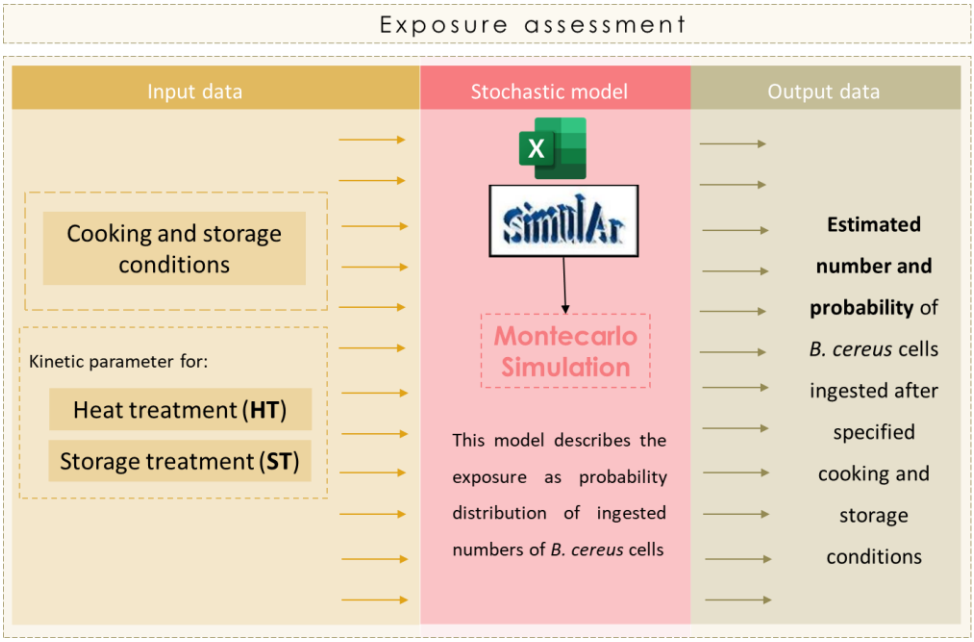

Figure 5. Schematic flow of the methods followed for the exposure assessment study.

Monte Carlo simulation will be performed by using software SimulAr (https://www.simularsoft.com.ar/), which is a Monte Carlo simulation software developed in Argentina and designed for the analysis and evaluation of businesses and decision making that involves risk. SimulAr is a program designed as a complement to Microsoft Excel (Add-in) and is characterized by its simplicity and flexibility, allowing the user to manage within a familiar environment, and it is free. As indicated before, SimulAr focuses on the method called Monte Carlo Simulation to carry out a risk analysis. It consists of assigning frequency distributions to the variables of the model that have risk and, subsequently, generating random numbers according to those distributions "simulating" the behavior that they are considered to have in the future. In this way it is possible to give more realism to the model, obtaining more reliable results when making a decision. This Excel add-in works on Excel 2010 32 or 64 bits.

For Excel 2016 the Add-In Argo can be also used (https://boozallen.github.io/argo/). Argo is a Dynamic Simulation model that serves as investigative tool, allowing decision makers to explore scenarios and measure the effectiveness of potential decision strategies. This free version is the first step in releasing Argo as an Open Source platform for spreadsheet-based risk analysis and decision support.

4. RESULTS

4.1. Thermal inactivation studies

The present work has studied the antimicrobial effect of chitosan and grape extract against *Bacillus cereus* spores when a rice solution, was subjected to four temperatures (90, 95, 100, 105ºC), which include those simulating traditional cooking heat treatment.

The logarithm of the survival fraction was plotted as a function of treatment time for each temperature and chitosan or grape extract concentration in figures 6 and 7, respectively.

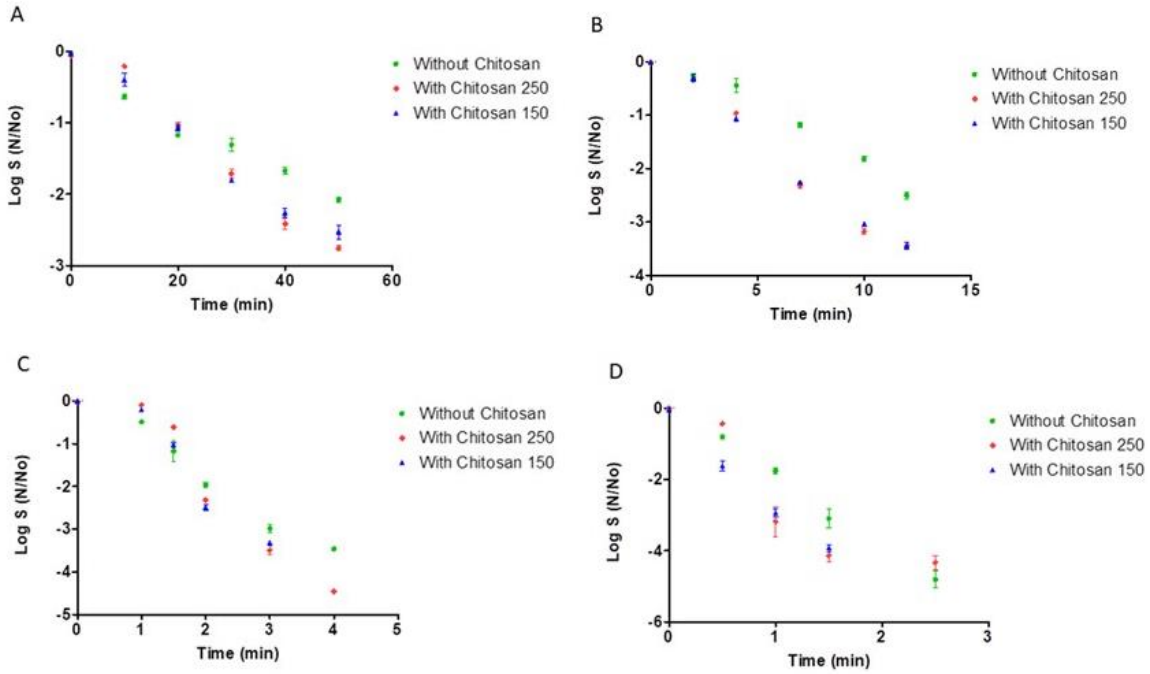

Figure 6: Effect of chitosan (control, 150 µg/mL and 250 µg/mL) at 90ºC (A), 95ºC (B), 100ºC (C) 105ºC (D) on the inactivation of B. cereus spores.

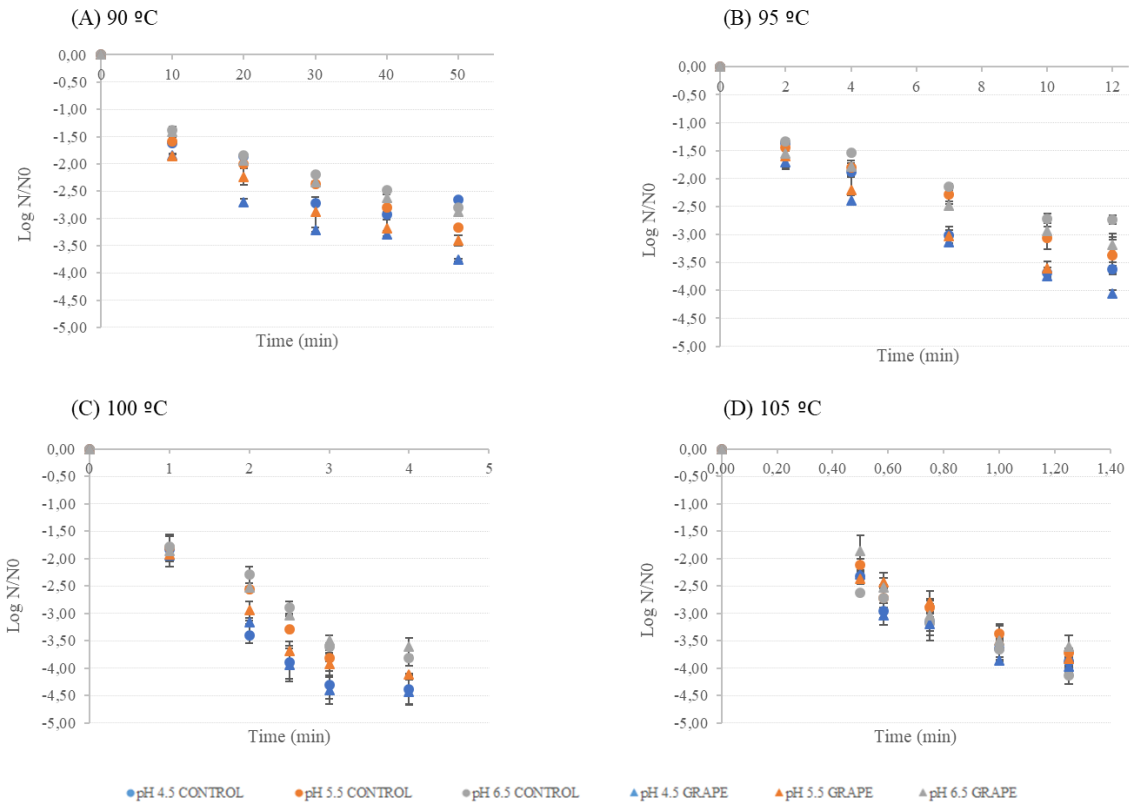

Figure 7. Effect of grape extract at 90ºC (A), 95ºC (B), 100ºC (C) 105ºC (D) on the inactivation of B. cereus spores at different pH values (4.5-5.5-6.5).

The logarithm of the survival fraction was plotted as a function of treatment time for each temperature, chitosan concentration (figure 6) and pH studied in the case of grape extract (figure 7). Both figures show that, as time and temperature increase, the value of the decimal logarithmic reduction increases. Regarding the effect of chitosan, for all temperatures studied *B. cereus* spore's inactivation increases with the addition of chitosan, while in the case of grape extract the effect appears only for 90 and 95ºC, it seems that for higher temperatures, the thermal effect is higher than the one of grape extract.

The experimental data were fitted primary models, Bigelow (equation 2) and Weibull distribution function (equation 3) for chitosan and grape extract respectively (Equation 1) by using a non-linear regression (Statgraphics Centurion 18 version 18.1.13 by Statgraphics Technologies, Inc.), in order to obtain parameters that can be used to compare the effect of pH, temperature and antimicrobial concentration, on the *B. cereus* spore's survival.

Table 1: Estimation of thermal resistance parameters by a nonlinear regression in the different substrates

Temperature (ºC)	**Estimated D_T value (min)**		
	Control	**Chitosan 150 µg/mL**	**Chitosan 250 µg/mL**
90	18.90	15.47	14.17
95	5.87	4.27	4.83
100	1.82	1.18	1.64
105	0.56	0.32	0.56

Table 2: Estimated z values (ºC) in different substrates

Substrate	Estimated z value (ºC)	Standard Error Asymptotic
control	9.84	0.30
chitosan 150 µg/mL	8.95	0.20
chitosan 250 µg/mL	10.70	0.32

Table 1 shows the estimation of the parameters that define the heat resistance of *B. cereus* spore's D_T for each of the chitosan concentration and temperatures studied. Table 2 shows the z value for each of the studied substrate. The value of the parameter D_T estimated by the model is clearly higher in the substrate without chitosan (control) than in the substrate containing chitosan which is related with the lower spore inactivation as previously shown by the survival curves. Regarding the value of the parameter, D_T estimated by the model when chitosan is present little difference was found between the two chitosan concentrations. It seems that the effect of chitosan on the inactivation of *B. cereus* spores does not depend on the concentration of chitosan between 150 and 250 µg/mL during heating.

Table 3. Weibull survival function parameters obtained by fitting experimental data to equation 1.

T ºC	pH	CONTROL				GRAPE			
		a	b	R^2_{adj}	MSE	*a*	b	R^2_{adj}	MSE
90	**4.5**	2.62 ± 0.08 $^{*\ a.\ A}$	0.36 ± 0.01	0.945	0.171	1.87 ± 0.17 $^{*\ a.\ A}$	0.40 ± 0.01	0.991	0.086
	5.5	4.02 ± 0.34 $^{*\ b.\ A}$	0.40 ± 0.02	0.995	0.051	2.43 ± 0.35 $^{*\ a.\ A}$	0.40 ± 0.01	0.995	0.058
	6.5	4.96 ± 0.16 $^{c.\ A}$	0.44 ± 0.01	0.999	0.016	4.38 ± 0.23 $^{b.\ A}$	0.44 ± 0.01	1.000	0.011
95	**4.5**	1.14 ± 0.10 $^{*\ a.\ B}$	0.57 ± 0.01	0.980	0.136	0.66 ± 0.11 $^{*a.\ B}$	0.48 ± 0.02	1.000	0.009
	5.5	1.24 ± 0.01 $^{*\ a.\ B}$	0.52 ± 0.05	0.987	0.091	0.58 ± 0.15 $^{*a.\ B}$	0.42 ± 0.05	0.972	0.149
	6.5	1.30 ± 0.02 $^{*\ a.\ B}$	0.46 ± 0.01	0.986	0.083	0.89 ± 0.01 $^{*a.\ B}$	0.44 ± 0.03	0.993	0.054
100	**4.5**	0.26 ± 0.11 $^{a.\ C}$	0.56 ± 0.06	0.965	0.234	0.25 ± 0.02 $^{a.\ C}$	0.56 ± 0.03	0.968	0.214
	5.5	0.34 ± 0.08 $^{a.\ C}$	0.53 ± 0.01	0.974	0.161	0.25 ± 0.01 $^{a.\ B}$	0.53 ± 0.01	0.979	0.161
	6.5	0.43 ± 0.18 $^{a.\ C}$	0.61 ± 0.13	0.974	0.142	0.28 ± 0.02 $^{a.\ C}$	0.50 ± 0.02	0.986	0.103
105	**4.5**	0.07 ± 0.01 $^{a.\ C}$	0.48 ± 0.01	0.986	0.108	0.08 ± 0.01 $^{a.\ C}$	0.51 ± 0.02	0.977	0.149
	5.5	0.10 ± 0.02 $^{a.\ C}$	0.53 ± 0.04	0.987	0.085	0.12 ± 0.05 $^{a.\ B}$	0.57 ± 0.12	0.996	0.060
	6.5	0.08 ± 0.01 $^{a.\ D}$	0.51 ± 0.01	0.999	0.030	0.06 ± 0.01 $^{a.\ C}$	0.46 ± 0.09	0.997	0.052

T, Temperature; a, scale parameter; b, shape parameter; R2adj, r2 adjusted; MSE, mean square error. The asterisk indicates significant differences between the values per column. Lower case letters compare values per row between pH at a specific temperature; while upper case letters compare values per row but between temperatures at a specific pH. Different letters indicate significant differences (p <0.05).

Data modelling to Weibull distribution function for grape extract is shown in table 3. The fitting parameters, R^2_{adj} and MSE, were used to assess about the fitting accuracy. The Weibull distribution has two parameters, a (scale parameter) and b (shape parameter). Parameter *a*, represents a measure of resistance to the treatment, it decreases when the treatment temperature increases and at acidic pH values; moreover, it can be seen that when grape extract was present, the value of *a* was lower in all treatments, except at 100 and 105ºC, showing that at that temperature range, no combined action was observed between grape extract and heat, so it appears that the inactivation was mostly due to temperature effect.

The comparison of the results (Table 3), corroborate the effect that pH and temperature have on the thermo-resistance of *B. cereus* spores. At pH 6.5 there were significant differences at all temperatures (90, 95, 100, 105ºC), when the pH value is very close to neutrality, showing the effect of temperature on *B. cereus* spores alone. Meanwhile, at pH 4.5 or 5.5, there were significant differences only between the temperatures 90, 95 and 100°C; in this case *B. cereus* spore resistance decreased due to the effect of more acidic pH values, and therefore the decimal reduction was higher. Therefore, we can state that *B. cereus* spore development can be inhibited by combining factors such as pH, a natural antimicrobial and a mild heat treatment.

Subsequently, for grape extract, a secondary model has been developed that relates the logarithm of the scale parameter "a" with the independent variables of pH and temperature, both for the control samples (Equation 6) and for the samples with grape extract (Equation 7). To do so, a forward stepwise multiple regression was carried out and the most influential terms of the polynomial were selected.

$$Log(a) = 4.7702 + 0.076338*pH - 0.000567674*T2 \quad \text{(Equation 6)}$$

$$Log(a) = 9.34103 - 0.0993655*T \quad \text{(Equation 7)}$$

Since the p-value in the ANOVA table for both equations is less than 0.05, there is a statistically significant relationship between the variables with a confidence level of 95.0%. With this procedure, the terms that should enter in the polynomial were delimited considering statistical evidence.

Subsequently, a global model (Fernández, et al., 2002) was built in order to improve the estimation of the secondary model coefficients according to equation 7 for the control without grape, and equation 8 for the samples with grape. The shape parameter follows a normal distribution (0.48 ± 0.06) for grape extract and a normal distribution (0.52 ± 0.07) for the control.

$$Log\ S = -\left(\left(\frac{t}{10^{(a+b*pH+c*T^2)}}\right)^{0.52}\right) \quad \text{(Equation 8)}$$

$$Log\ S = -\left(\left(\frac{t}{10^{(a+b*T)}}\right)^{0.48}\right) \quad \text{(Equation 9)}$$

Where S is the survival fraction and t is the time.

The results of fitting these equations using a one-step nonlinear regression can be seen in table 4.

Table 4. Coefficients of the polynomial obtained by fitting the global model (equation 8 and equation 9) to the total experimental data.

Control			Grape extract		
Coefficient	**Estimated**	**Standard error**	**Coefficient**	**Estimated**	**Standard error**
a	5.40	0.14	a	9.68	0.63
b	0.051	0.016	b	-0.1030	0.0054
c	-0.000617	0.000011			

The global model has been validated by calculating the Accuracy Factor (A_F) of the experimental data with respect to that predicted by the model, according to Ross (1996).

The A_F for the global model for the control sample was 1.003, indicating that the percentage error is 0.3% for the predictions; while in the case of grape samples, the A_F for the global model was 1.028 indicating a percentage error of 2.8%. According to the A_F factor, the global model can be used to predict the remaining *Bacillus cereus* spores in rice solution, after being subjected to a preservation process, for both the control and samples containing grape extract, in which the pH and temperature are the independent environmental variables.

4.2. Growth studies

As stated in the previous studies, *B. cereus* spores are able to resist to cooking treatments. The main safety issue arises when those resistant spores, once activated by heat, germinate spontaneously in the cooked rice and grow producing the toxin if the cooked rice is stored at an inappropriate temperature, between 5 ° C to 50 ° C (Griffiths and Schraft, 2017). Consequently, having an additional control measure other than temperature alone is highly recommendable in these types of products, especially if they are not being consumed immediately after preparation. In this respect, natural antimicrobials can play an essential role.

4.2.1. Growth studies in the presence of insect chitosan

The present work has studied the effect of different concentrations of insect chitosan (150, 180, 220 and 250 µg/mL) and grape extract (1, 5 and 10 µL/mL) on vegetative cells of *Bacillus cereus*, stored at

three temperatures (10, 20 and 30ºC), comparing the results with those of a control without antimicrobial.

Figure 8 shows the results for *B. cereus* growth as a function of the storage temperature and the concentration of insect chitosan. The most outstanding result is the effect of the presence of chitosan on the bacterial counts, that induces a decrease in *B. cereus* concentration compared to the initial one at all storage temperatures and concentrations considered in this study.

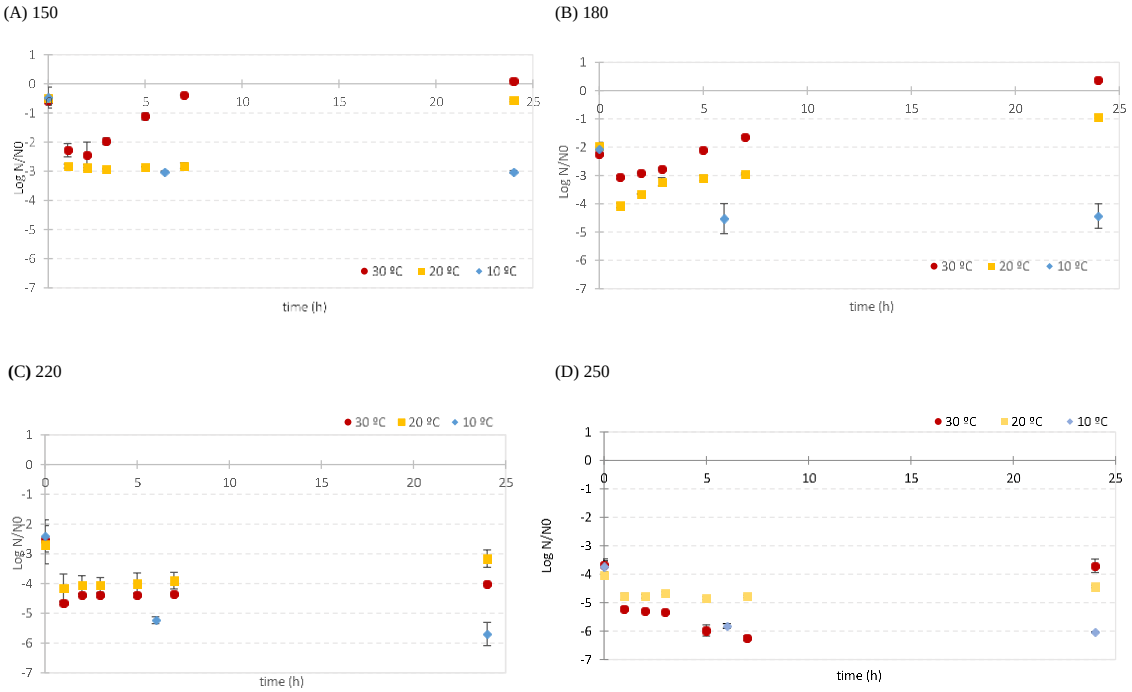

Figure 8: Effect of insect chitosan concentration 150 µg/mL (A), 180 µg/mL (B), 220 µg/mL (C) and 250 µg/mL (D) on the inactivation of Bacillus cereus at 10, 20 and 30ºC.

On analysing the results by temperature, it can be observed that at 10 ºC there is a decrease in the concentration of *B. cereus* as the storage time advances, for all the chitosan concentrations in the study. This decrease in *B. cereus* counts was greater as the concentration of chitosan in the medium increased. If we compare these results with those obtained in the controls (not shown) it can be concluded that the presence of chitosan exerted an additive effect to the temperature in controlling microorganism growth. Thus, a bactericidal effect for chitosan was observed. At temperatures of 30 ºC and 20 ºC, we observed that the cultures treated with the lowest concentrations of chitosan, 150 µg / mL and 180 µg / mL, have a greater recovery capacity during the storage period as compared to the initial inoculation value (N_0). In the cultures treated with chitosan concentrations of 220 µg / mL and 250 µg / mL, the antimicrobial effect of chitosan was higher and, consequently, the recovery capacity of the *B. cereus* cells was reduced, without achieving the levels observed at the lower insect chitosan concentrations during the storage period. Therefore, at these concentrations, chitosan exerted a marked bactericidal effect.

ComBase (ComBase Team, 2019), an internet platform that collects different growth and inactivation models for selected microorganisms included *B. cereus* was used to fit growth curves. The chosen growth model on ComBase was the Baranyi and Roberts model (equation 5). Table 5 shows kinetic parameters obtained for the different chitosan concentrations.

Table 5. Growth parameter as a function of chitosan concentration at a storage temperature of 20 ºC deduced by Baranyi and Roberts model (Equation 5)

Chitosan concentration (µL/mL)	Growth rate (h)	Lag phase (h)
0	0.087	0.40
150	0.17	6.80
250	-0.16	5.00

4.2.2. Growth studies in the presence of grape extract

As for grape extract studies figure 9 shows the behavior of *B. cereus* for the lowest grape extract concentration assayed: 1 µL/mL. Positive log values (N/N_0) indicate microbial growth, whereas negative values indicate microbial inactivation compared to the initial inoculation (N_0). At 10ºC and all pH values, the final concentration of *B. cereus* (after 168 hours 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 20ºC, 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 hours, which was longer than the result with acidic control, thus the addition of grape extract delayed microorganism growth. Finally, at 30ºC 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.

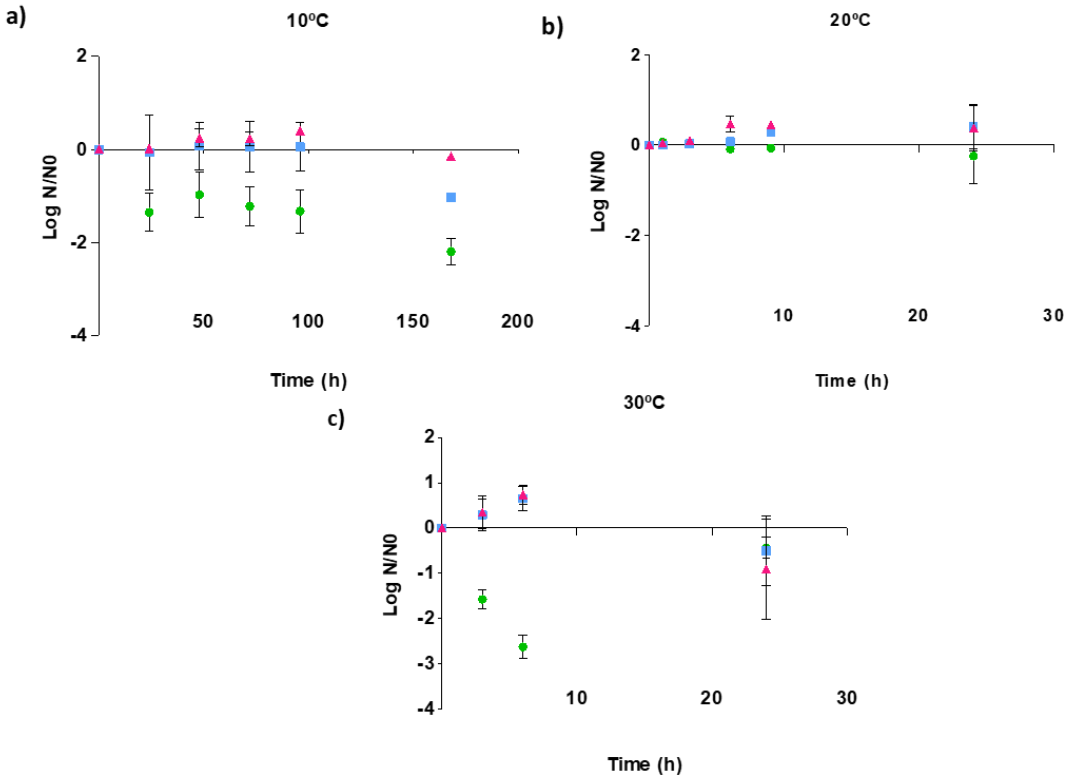

Figure 9 Growth/inhibition of B. cereus with 1 µL/mL grape extract concentration depending on pH (4.5 (●), 5.5 (■) and 6.5 (▲)) and temperature (10ºC (a), 20ºC (b) and 30ºC (c)).

As mentioned above, under the pH and temperature conditions studied, grape extract exerted a bactericidal effect for concentrations equal to or greater than 5 µL/mL. Those conditions were selected to quantitatively characterize the bactericidal effect of grape extract. A 24 hours 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. Figure 10 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.

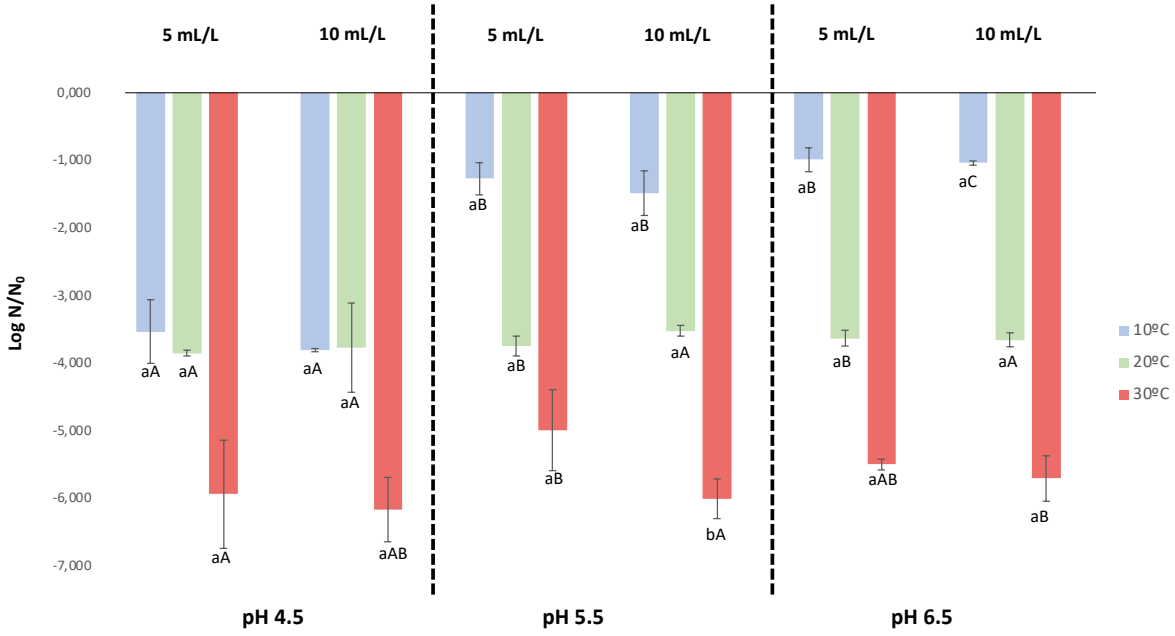

Figure 10. B. cereus inactivation levels at 24 hours storage time under exposure to concentrations of 5 and 10 µL/mL of grape extract, at incubation temperatures of 10ºC, 20ºC and 30ºC, and pH 4.5, 5.5 and 6.5. Letters on the bars indicate significant differences (P ≤ 0.05) between concentrations of 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).

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 20ºC, and reaching up to 6 logarithmic reductions at 30ºC. Regarding temperature effects, the greatest inactivation occurred at 30ºC (Fig. 10), 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 20ºC, while for pH 5.5 and 6.5, (less stressful conditions) a greater inactivation was achieved at 20ºC. Regarding the effect of grape extract concentration on inactivation, in general, there were no significant differences between the values reached with 5 or 10 µL/mL.

Where growth was detected, experimental data were fitted to mathematical models. Data of 1 µL/mL were adjusted to Gompertz equation (Equation 4). Growth rates ranged between 0.06 and 0.13 (CFU/mL *h) and lag phase duration between 0.13 and 0.05 h.

Data for 5 and 10 µL/mL of grape extract were fitted to the Weibull distribution function. Each individual experimental survival curve obtained was fitted separately and the estimated parameters were derived for each pH, temperature, and grape extract concentration (Table 6).

Table 6. 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 10ºC, 20ºC and 30ºC.

Grape extract concentration	T (ºC)	pH	Weibull parameters		Accuracy fit	
			a	*b*	R^2_{adj}	RSME
5 µL/mL	**10**	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	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	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 µL/mL	**10**	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	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	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 ≤ 0.05). Values with (*) indicate significant differences (p ≤ 0.05) for the same pH and temperature between concentrations.

A secondary model was developed by a forward stepwise multiple regression to define the dependence of log *a* with temperature and pH for fixed concentrations of grape extract (5 or 10 µL/mL), equation 10 and 11. 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

$$Log\ a = -2.28 + 0.418 * \mathrm{pH} \pm 0.0175 * \mathrm{pH} * \mathrm{T} + 0.204 * \mathrm{T} - 0.0040 * T^2$$ R^2=97% (Equation 10)

$$Log\ a = -8.11 + 3.10 * pH - 0.248 * pH^2 - 0.0104 * pH * T$$ R^2=94% (Equation 11)

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 10 and 11, using one-step nonlinear regression (Statgraphics Centurion XVIII). The estimated coefficients and the confidence intervals obtained are shown in Table 7.

Table 7. Coefficient estimates obtained by fitting the global model (equation 9 to 5 µL/mL grape extract concentration and equation 10 to 10 µL/mL) to the total experimental data.

5 µL/mL grape extract			10 µL/mL 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			

The performance of the global inactivation model was validated by the Accuracy factor (*Af).* In the present study, *Af* for 5 and 10 µL/mL 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 30ºC, which is considered critical from the food safety point of view.

Considering the global model obtained (equations 10 and 11), 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 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). Figure 11 shows a screenshot of the software.

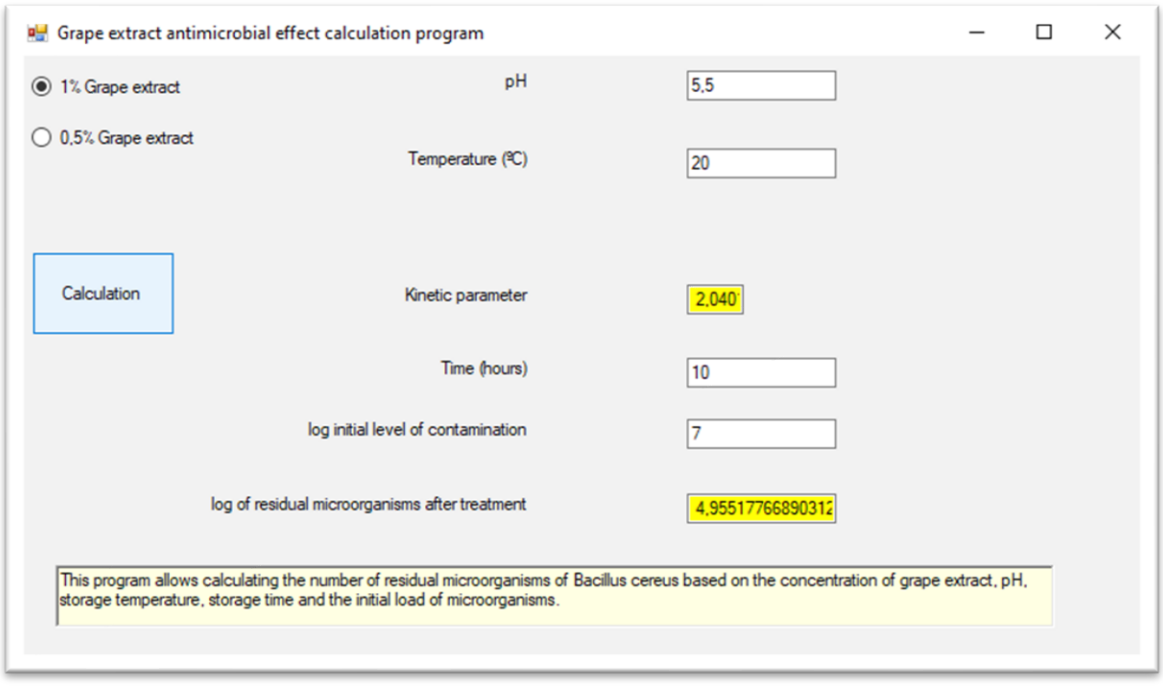

Figure 11. Screenshot of the software developed (tertiary model) to calculate the microorganism concentration after grape extract treatment combined with pH and temperature.

4.3. Exposure assessment models

An evaluation of the exposure to *B. cereus* in cooked rice during its cooking and subsequent storage in the presence (and absence) of natural antimicrobials grape extract and chitosan has been carried out. For this purpose, mathematical predictive models that define the inactivation (cooking process) and growth (storage process) obtained in epigraphs 4.1 and 4.2, respectively, have been integrated into Monte Carlo simulations, obtaining as a result the final number of *B. cereus* in stochastic terms at the end of storage at given conditions.

In this document, the applicability of the models will be explained as an example for the grape extract. Two specific scenarios, presence (scenario I) or absence (scenario II) of grape extract were considered (Table 8).

Table 8. Conditions (cooking and storage) for the two specific scenarios used to explain the application of the exposure assessment model.

	Scenario I	Scenario II
Grape Extract (µL/mL)	0.1	0
Cooking temperature (ºC)	90	
pH	6.5	
Cooking time (min)	20	
Storage temperature (ºC)	20	
Storage time (h)	20	

In these scenarios, a low cooking temperature (90 °C), at a pH close to neutrality (6.5), for the most usual rice cooking time (20 min) where chosen as cooking conditions, and a subsequent storage under cold chain break for several hours (20 °C for 20h) were considered as storage conditions.

Table 9. Output results obtained after running the Monte Carlo simulation for the two scenarios used in the present study

	Scenario I	Scenario II
Iterations Nº	1000	
Minimum	-1.670	0.010
Average	80068.070	283896.356
Maximum	18633109.812	66041662.322
Median	2259.967	7931.553
Standard deviation	684712.587	2427323.796
Kurtosis	552.740	552.351
Skewness	21.599	21.591
Coef. of variation	8.552	8.550
Percentile 5%	21.53	81.51
Percentile 24%	354.08	1266.38
Percentile 25%	380.12	1380.0
Percentile 50%	2259.97	7931.55
Percentile 75%	13824.04	48726.42
Percentile 95%	186319.66	662255.11
Less than infective dose	**71.50%**	**53.90%**

Table 9 shows the Monte Carlo simulation output values for scenario I and II. A total of 1000 iterations, or 1000 random assignments of final *B. cereus* load values, were performed. These values were between -1.670 and 18633109.812 cfu/g for scenario I and 0.010 and 66041662.322 cfu/ g for scenario II. The infective dose for *B. cereus* consumption is considered to be of 10^4 cfu/g (Hazards (BIOHAZ), 2016). Therefore, regarding the probability that the *B. cereus* load is lower than the infective dose (10^4 cfu/ g) the results obtained for scenario I are 71.50% and 53.90% for scenario II. These probability data show that assuming the conditions of the scenario I (1 µL/mL grape extract), out of 100 rice rations cooked and stored, 71 of them will have a final *B. cereus* value ≤ 10^4, whereas in the case of scenario II only 53 rations will have a *B. cereus* load lower than the infective dose. Within all the output values provided by the Monte Carlo simulation, it is worth noting that the addition of grape extract reduces the probability of risk of ingestion of a rice ration with a *B. cereus* load above the infective dose.

This exposure assessment allows associating the probability of occurrence of a given final concentration of *B. cereus* after the combination of different hurdles such as use of antimicrobials (grape extract or chitosan), pH, and storage temperature, to improve the microbiological safety of rice. Therefore, the exposure assessment is a very useful tool for the industry, as it can contribute predictively to decision-making and propose the necessary control measures to reduce the risk associated with B. cereus.

5. Conclusions

Hurdle technology is getting very popular as a food preservation process. Results displayed in this study show that the combined use of mild cooking temperatures (90-95ºC) with natural antimicrobials (chitosan or grape extract) could be considered as a hurdle preservation process for acidic, ready-to-eat rice products. The advantage of combining heat with natural antimicrobials is an improvement of the safety against *B. cereus* spores present on rice.

Bigelow model and Weibull distribution function, as inactivation models, and Gompertz and Baranyi and Roberts, as growth models, were suitable to fit experimental cooking and storage data. They were applied depending on the process condition.

Industrial exploitation strategy: besides the results exposed before, the specific items described below could be offered to the industry:

- A user-friendly software has been developed to be used in industry, allowing calculating the residual number of microorganisms under specific pH, temperature and time as processing conditions for two grape extract concentrations.
- Exposure assessment models are available either for heat/chitosan and heat/grape extract processes that will allow industry make decisions in the process conditions, giving an estimation of the probability of finding a number of residual microorganisms as a function of the heat/antimicrobial combination and other environmental factors as storage temperature.

Arranging meetings with business operators, in line with the incorporation of the models derived for ready-to-eat rice products, should be considered to explain the context, applicability and how to use them.

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