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Use of Frequency Distribution Functions to Establish Safe Conditions in Relation to the Foodborne Pathogen *Bacillus cereus***

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Summary

Minimal processing implementation greatly depends on a detailed knowledge of the effects of preservation factors and their combinations on the spoilage and foodborne pathogenic microorganisms. The effectiveness of mild preservation conditions will become increasingly dependent on a more stochastic approach linking microbial physiological factors with product preservation factors. In this study, the validity of frequency distributions to efficiently describe the inactivation and growth of *Bacillus cereus* in the presence of natural antimicrobials (essential oils) has been studied. For this purpose, vegetative cells were exposed to 0.6 mM of thymol or cymene, obtaining survival curves that were best described by the distribution of Weibull, since a tailing effect was observed. *B. cereus* was also exposed in a growth medium to a low concentration (0.1 mM) of both antimicrobials, separately or combined, and the lag times obtained were fitted to a normal distribution, which allowed a description of dispersion of the start of growth. This allowed a more efficient evaluation of the experimental data to establish safe processing conditions according to accurate parameters and their implementation in risk assessment.

Key words: *Bacillus cereus*, natural antimicrobials, frequency distributions

Introduction

The success and failure of minimal processing depends on a deep knowledge of the effects of individual factors and their combinations on the behaviour of microorganisms. Different preservation factors such as storage temperature, heat, pH and the presence of antimicrobial compounds play a key role in establishing mild combination treatments that can guarantee food safety, while maintaining high standards of organoleptic and quality properties. Consumers look for additive free, fresher and more natural tasting food products (*1,2*) that maintain the same level of microbiological safety. The use of natural antimicrobial systems for preservation of foods could accomplish this increasing demand. Herbs and spices have been known for their antimicrobial activity since antiquity and their safe use has led to their current status as generally recognized as safe (GRAS) food ingredients. Essential oil components, extracted from several types of plants, have been used as flavour-

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ings in the food industry and they represent a highly interesting source of natural antimicrobials for food preservation due to their antimicrobial properties (*3,4*).

Thymol and cymene (a precursor of thymol) are phenolic compounds present in the essential oil fraction of *Origanum* and *Thymus* genera (*4,5*). These compounds are hydrophobic and likely to dissolve in the cytoplasmic membrane, causing modifications of the membrane permeability. Thymol and cymene can be considered as natural preservative agents with an inhibitory effect on bacteria and fungi, including food pathogens (*5,6*).

Bacillus cereus is a facultative anaerobe, spore-forming and Gram-positive rod. Its presence in raw and processed food products has been extensively described (*7,8*). *B. cereus* is associated with two kinds of foodborne illnesses: a diarrhoeal and emetic syndromes, caused by two different types of toxins (*9*). Due to its capability to produce these toxins, *B. cereus* is becoming one of the most important causes of food poisoning in the industrialized world (*10*).

The success of mild processing depends on a deeper knowledge of microbial physiological factors, such as inoculum size, storage conditions, and product preservation factors such as pH or natural antimicrobial compounds. Preservation conditions can influence the outcome of sublethal injury on survival/subsequent outgrowth of bacteria. This could mean that cell injury may not be lethal immediately but can impair the organism's ability to survive. It has been shown that heat stress conditioned the subsequent duration of lag time in individual cells of *Lactobacillus plantarum* (*11*), where distributions of lag times were influenced by the heat treatment to which bacteria were exposed. Many factors such as those mentioned (individual cell response and stress conditions) may influence the ability of bacteria to recover from a preservation treatment, such as heat, and reduce the probability of outgrowth.

It has been pointed out that deterministic models defined for populations may not be valid for individual cells, and therefore stochastic ones should be used (*12*). Stochastic (probabilistic) models can describe the dynamics of individual cells, in order to predict bacterial parameters (lag time, growth, and survival) more accurately (*13*). These models could allow the prediction of shelf life extension or the probability of spoilage, according to more precise calculations of the ability of the surviving microbial population to grow, so that prevention of a food product to become hazardous would be more effective and realistic.

The aim of the present study was to evaluate the potential use of frequency distributions to describe efficiently inactivation and growth data of microorganisms exposed to preservation treatments, proving their validity to establish more accurately safe processing conditions.

Materials and Methods

Bacterial strain and growth conditions

B. cereus INRA-AVZ421 was kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France). It was isolated from refrigerated processed foods of extended durability (REPFED) based on vegetables. Cells were grown at 30 °C in brain-heart infusion broth (BHI) (Scharlab Chemie, Barcelona, Spain) supplemented with 0.5 % glucose (Scharlab Chemie) up to early stationary phase and maintained at –80 °C in 30 % glycerol as a cryoprotectant.

Chemicals

Thymol (Sigma Aldrich Chemie, Steinheim, Germany) and cymene (Aldrich Chemical Company, Milwakee, USA) stock solutions (0.5 M) were prepared in 95 % ethanol and stored at 4 °C.

Inactivation of Bacillus cereus by thymol and cymene in HEPES buffer, pH=7

The effect of thymol and cymene on the viable counts of vegetative cells in mid-exponential growth phase of *B. cereus* INRA-AVZ421, cultured at 30 °C, was studied. Vegetative cells of *B. cereus* in mid-exponential growth, with an absorbance at 660 nm (A_{660nm}) of 0.4 measured in a spectrophotometer (ZUZI, Madrid, Spain), were harvested by centrifugation, washed twice and diluted in a 50 mM HEPES buffer at pH=7, until an A_{660nm}=0.1 was reached. Cell suspensions were exposed to thymol alone (0.6 mM) and cymene alone (0.6 mM). Samples were taken at different time intervals during exposure, and diluted immediately (10^2 to 10^5 fold) in peptone physiological salt solution (peptone 10 g/L and NaCl 8.5 g/L). Appropriate dilutions were plated on BHI agar and incubated for 24 h at 30 °C.

Effect of thymol and cymene on the growth of Bacillus cereus in BHI broth

The effect of thymol and cymene on the growth of vegetative cells, in lag phase (A_{660nm}=0.001) of *B. cereus* INRA-AVZ421 at 20 and 30 °C in BHI broth, was studied. Cell cultures were exposed to a concentration of thymol (0.1 mM) alone, or cymene (0.1 mM) alone or both combined (0.1 mM of each antimicrobial). The inoculum level was $5 \cdot 10^2$–10^3 CFU/mL. Samples were taken at different time intervals during exposure, and their A_{660nm} was immediately measured using a spectrophotometer (ZUZI 4110RS, Auxilab, Beriaín, Spain). At least 8 replicate experiments per condition were performed. Growth curves were obtained by plotting A_{660nm} against exposure time. Although the measurement of the absorbance of a culture is only an estimation of its growth, it helps to compare the growth parameters of the same culture grown in different conditions.

Analysis of experimental data

The data were obtained from the survival curves of *B. cereus* cells after exposure to the natural compounds by plotting the log of the survivors against treatment time. The interpretation of the resulting parameters and goodness-of-fit test using Weibull model were evaluated. If the inactivation of microorganisms by the exposure of antimicrobials follows a Weibull distribution, the survival function will be (*14*):

$$S_t = 10^{-(t/\alpha)^n} \qquad /1/$$

where S_t is the fraction of survivors achieved by the process, t is time, α is the scale parameter that could be considered as a kinetic rate parameter, and n is the shape parameter, which describes the shape of the curve. The n values lower than 1 indicate the presence of tailing, whereas n values close to 1 indicate first order inactivation kinetics.

Growth curves were fitted using the function of Baranyi *et al.* (*15*) to estimate the main growth parameters, *i.e.* specific growth rate and lag time. Only growth curves with at least 10 data points were used for modelling, as suggested by the authors. The parameters derived from the growth experiments (lag phase) were used to test different distributions with the Best Fit tool of @Risk 4.0, Palisade Europe, London. An ANOVA analysis was performed to establish significant differences between the experimental conditions tested (MATLAB®).

Results and Discussion

Analysis of B. cereus vegetative cells inactivation data

Survival curves obtained with *B. cereus* INRA-AVZ421 showed a downward concavity (Fig. 1). Therefore, interpretation of such data would be limited and difficult to extrapolate to real processing conditions with traditional linear regressions. Table 1 shows the parameters of the Weibull distribution using goodness-of-fit test to determine viable counts for *B. cereus* INRA-AVZ421 exposed to thymol or cymene, compared to the traditional first order kinetics. Any prediction (combination of concentration and time of exposure) using D values would be unrealistic and could lead to false dangerous processes, since high mean square errors were obtained, particularly when exposed to thymol (Table 1). Application of Eq. /1/ (the survival function derived from Weibull distribution) generated a good fit of the experimental data (Fig. 1).

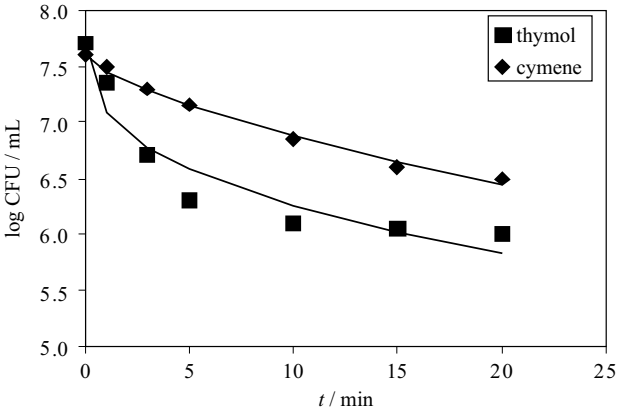

Fig. 1. Survival curves for *B. cereus* INRA-AVZ421 vegetative cells exposed to thymol or cymene with experimental points fitted by Eq. /1/

Table 1. Kinetic parameters fitted to inactivation curves obtained after exposure of *B. cereus* INRA-AVZ421 to 0.6 mM thymol or cymene using Weibull distribution or first order kinetics

Kinetic parameters		*c*(thymol)= 0.6 mM	*c*(cymene)= 0.6 mM
Weibull	α/min	3.67	16.23
	n	0.369	0.682
	MSE	0.029	0.001
First order	a	–0.0748	–0.0559
	b	–0.522	–0.0971
	D value/min	13.4	17.9
	MSE	0.123	0.006

The shape parameter, n, was lower than 1 in both cases, indicating that exposure to these antimicrobials induces tailing in *B. cereus* INRA-AVZ421 vegetative cells, as can be seen in Fig. 1. A similar observation was made for *Listeria monocytogenes* exposed to essential oils (*16*). The n value was lower for thymol than for cymene (Table 1), indicating that the presence of a downward concavity survival curve was much more evident in the case of thymol. The scale parameter, α, indicates how quickly microorganisms are inactivated (the higher the value, the lower the inactivation at the beginning of the exposure). Therefore, a higher »α« value for cymene (16.23) than for thymol (3.67) indicated that cymene had a lower bactericidal effect than thymol at the same concentration for short exposure time. These observations are in agreement with the published data, which show that thymol has a stronger antimicrobial effect than cymene (*17*).

Analysis of growth data of B. cereus exposed to thymol and cymene

When *B. cereus* cells were grown in the presence of low levels (0.1 mM) of natural antimicrobials thymol or cymene, no significant differences were found when compared to the controls at incubation temperatures of 20 or 30 °C (Table 2). These results indicated that the inhibitory capacity of both compounds was limited in an optimal growth medium, even when the growth temperature was not optimal (20 °C). As it was expected, the duration of the lag phase was longer at 20 °C than at 30 °C (30.17 h versus 6.25 h in the presence of thymol, for example), but also the standard deviations were

Table 2. Average lag phase of *B. cereus* INRA-AVZ421 grown in BHI broth in the presence of thymol, cymene or both combined at the concentrations indicated, at an incubation temperature of 20 or 30 °C

$\frac{T}{°C}$	Antimicrobial	$\frac{t(\text{lag phase})}{h}$	Standard deviation
30	Control	5.83	3.12
	0.1 mM thy*	6.25	1.57
	0.1 mM cym*	6.68	3.29
	0.1 thy + 0.1 cym	36.64	2.55
20	Control	22.55	8.47
	0.1 mM thy	30.17	10.81
	0.1 mM cym	20.55	12.56
	0.1 thy + 0.1 cym	83.21	31.84

*thy: thymol; cym: cymene

much bigger, indicating that in suboptimal conditions, the variability to start growth was far greater (standard deviations in the range of 8.47–12.56 at 20 °C and 1.57–3.29 at 30 °C) (Table 2). This pointed out that, when *B. cereus* was exposed to non optimal conditions, the distribution of the lag times changed.

Exposure of *B. cereus* to the same levels of thymol and cymene combined led to an extension of the lag phase at 30 °C, although the standard deviation was similar to the control, indicating that the effect of the antimicrobials was a delay of the lag phase, with a similar dispersion to the other conditions tested after incubation at 30 °C (Table 2). However, when the microorganism was incubated at 20 °C, the combination of both compounds led to a significant extension of the lag phase (with an average of 83.21 h) and a remarkable increase of the standard deviation (up to 31.84), indicating a great dispersion of the period before the onset of growth (Table 2). The growth rates did not show significant differences in any of the conditions tested (data not shown). Similar behaviour was observed in *Lactobacillus plantarum* when exposed to a sublethal heat treatment (*11*).

Modelling the distribution of the lag times

Figs. 2 and 3 show the results of the distribution of lag of the growth curves obtained at 30 and 20 °C in control experiments (no antimicrobials added) and experiments with either thymol (0.1 mM) or cymene (0.1 mM), since no significant differences were observed between them. The distributions (obtained from 21 observations at 30 °C and from 25 observations at 20 °C) presented a similar pattern, although at 20 °C it seemed to have a wider dispersion. Using the Best fit tool of @Risk, Palisade, different distribution functions were studied and the parameters and statistics describing each distribution were analyzed. Normal distribution gave an adequate description of both sets of data. This distribution had been previously used to describe microbial behaviour in Monte Carlo simulations (*18*). The parameters describing the data are shown in Table 3. From the information derived, it could be concluded that in less than 5 % of the cases, *B. cereus* would start growth before 10.4 h at 20 °C or 2.7 h at 30 °C. With a probability of 95 %, growth would start within 42.05 h at 20 °C or 10.3 h at 30 °C (Table 3). The dispersion of lag times obtained at 20 °C for *B. cereus* exposed to a combination of thymol and cymene would be greater than any of those presented in Table 3 (average 83.21, standard deviation 31.84, Table 2). In this case and with the limitation of the low number of repetitions available (N=8), the 5 % probability of growth would be 40 h, whereas the 95 % probability would be 143.8 h, indicating a much bigger margin of growth possibilities. It has been observed that *L. plantarum* exposed to heat also showed a change in their distribution of lag times (*11*).

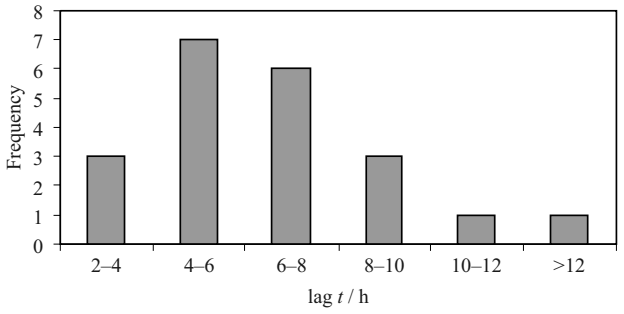

Fig. 2. Distributions of lag times of *B. cereus* INRA-AVZ421 grown at 30 °C, including controls and cells exposed to 0.1 mM thymol or cymene

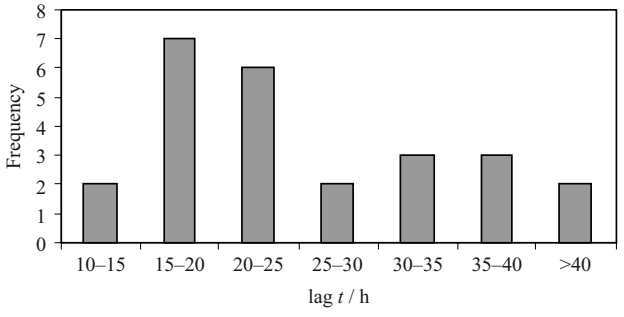

Fig. 3. Distributions of lag times of *B. cereus* INRA-AVZ421 grown at 20 °C, including controls and cells exposed to 0.1 mM thymol or cymene

Table 3. Parameters of a normal distribution for lag phase data obtained at 20 and 30 °C in *B. cereus* INRA-AVZ421 grown in BHI broth with or without the addition of 0.1 mM thymol or cymene

Parameters	T=20 °C	T=30 °C
t(average lag phase)/h	26.23	6.51
Standard deviation	9.62	2.31
t(lag for 5 % probability)/h	10.41	2.71
t(lag for 95 % probability)/h	42.05	10.32
t(90 % probability interval)/h	10.41–42.05	2.71–10.32

Gamma distribution was also fitted to the experimental data, giving a good description of the results. The α and β parameters obtained were 7.73 and 3.39 for the lag phase data measured at 20 °C, whereas for the data at 30 °C they were 9.12 and 0.71, respectively. In this case, for less than 5 % of the cases growth would start before 12.87 h at 20 °C or 3.42 h at 30 °C, which is similar but not identical to predictions of the normal distribution. Gamma distribution has been successfully applied to describe biological behaviour such as bacterial growth or inactivation (*19*).

From these data, a more general and valid information about the potential risk of growth of *B. cereus* in optimal conditions can be derived, and it could be extrapolated to food substrates (with the precaution of performing a limited number of tests in the real substrate). A more realistic shelf life could be established when *B. cereus* is the target microorganism if information on the level of contamination and/or survival and the storage conditions was available.

Conclusion

The use of frequency distributions can be very useful for establishing safe conditions in relation to foodborne pathogens and mild preservation techniques. For inactivation data that do not follow a linear relationship, distributions such as Weibull can efficiently describe experimental data to draw conclusions for appropriate treatment conditions. In relation to growth data, when sufficient data points are available, it is feasible to describe experimental results using frequency distributions (such as a normal distribution) that would give a more complete information in terms of the risk of growth of pathogens, such as *Bacillus cereus*, presented in this study. In this way, data can be implemented efficiently for risk assessment of food products.

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Učestalost patogene bakterije *Bacillus cereus* pri određivanju zdravstvene ispravnosti hrane

Sažetak

Minimalni opseg prerade uvelike ovisi o detaljnom poznavanju utjecaja zaštitnih faktora i njihovih kombinacija na kvarenje hrane patogenim mikroorganizmima. Učinkovitost blagih zaštitnih mjera jako će ovisiti o stohastičkom pristupu povezivanja čimbenika mikrobne fiziologije s faktorima zaštite proizvoda. U radu je istraživana pojava raširenosti bakterija kako bi se uspješno opisala inaktivacija i rast *Bacillus cereus* u prisutnosti prirod-

nih esencijalnih ulja s antimikrobnim djelovanjem. Zbog toga su vegetativne stanice izložene utjecaju 0,6 mM timola ili cimena, a dobivene krivulje preživljavanja najbolje su objašnjene Weibullovim modelom raspodjele jer su uočeni zaostaci preživjelih stanica. *B. cereus* je također bio izložen malim koncentracijama (0,1 mM) timola i cimena u podlozi ili njihove smjese, a dobivena lag faza odgovarala je normalnoj raspodjeli, što je omogućilo njezino opisivanje na početku rasta. Zbog toga su se eksperimentalni podaci mogli uspješnije procijeniti i utvrditi sigurni uvjeti prerade u skladu s točnim parametrima i njihovom implementacijom u procjeni rizika.