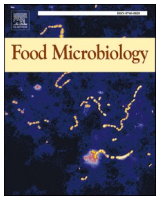
Food Microbiology 107 (2022) 104077

Contents lists available at ScienceDirect

Food Microbiology

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



Insect chitosan as a natural antimicrobial against vegetative cells of *Bacillus cereus* in a cooked rice matrix

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ARTICLE INFO

Keywords:
Insect chitosan
Bacillus cereus
Growth
Cooked rice

ABSTRACT

This study investigates the antimicrobial activity of insect chitosan against vegetative cells of *Bacillus cereus* in a rice matrix. Sample culture solutions were prepared with different concentrations of insect chitosan (150, 180, 220 and 250 μg/mL) and tested at three temperatures (30 °C, 20 °C and 10 °C), which simulate different storage temperature scenarios of precooked rice. The results indicate that insect chitosan has antimicrobial activity that depends on temperature and chitosan concentration. For the assays with chitosan at 10 °C, all concentrations were bactericidal during the study time, reaching a maximum inactivation of 6 log cycles for 250 μg/mL. At 20 °C and at 30 °C a bacteriostatic activity was observed for concentrations of 150 μg/mL and 180 μg/mL. Results also showed that concentrations of 220 μg/mL and 250 μg/mL were bactericidal for all the temperatures tested during the storage time. When rice is cooked and not stored at an appropriate temperature, below 10 °C, the consumer's health is at risk. In these cases, insect chitosan could be a good additional control measure to control *B. cereus* growth and toxin formation in cooked rice.

1. Introduction

Bacillus cereus is present in many foods due to its ubiquitous nature and has become one of the top ten pathogens responsible for many foodborne cases of infection (Rodrigo et al., 2021). In 2018, *B. cereus* was involved in 31 strong evidence outbreaks and 67 weak evidence outbreaks, with a total of 98 reported among EU member states, representing 1.9% of the total outbreaks in the EU, with 1539 human cases accounting for 111 hospitalizations and 1 death (EFSA and ECDC 2019). In general, *B. cereus* toxico-infection episodes have been associated with complex, mixed food products that may include rice as a component (Little et al., 2002); however, other rice-based products such as pasta and noodles are also frequently contaminated and involved in *B. cereus* toxico-infection (Grande et al., 2006). It has also been found in a wide variety of non-cereals including milk and dairy products, meat products, pasteurized liquid eggs, ready-to-eat vegetables, fruits, and spices (Yu et al., 2020). Due to the extensive distribution of strains in the environment, it is practically impossible to obtain raw materials or food that is free of *B. cereus* spores (Ehling-Schulz et al., 2015; Enosi Tuipulotu et al., 2020; Griffiths and Schraft, 2017). This implies that food contamination can occur during any stage of production, including primary production, harvesting or in slaughterhouses, processing, storage, preparation and consumption of food (Enosi Tuipulotu et al., 2020). *Bacillus cereus* strains can vary with respect to their growth and survival characteristics, with growth limits that are not absolute and depend on the strain and environmental factors, such as the composition of the medium, temperature, pH and water activity (Enosi Tuipulotu et al., 2020). *B. cereus* can produce two types of toxi-infections: diarrheal and emetic syndromes. The first one occurs when a high number of *B. cereus* cells are consumed, the microorganism implants and grows in the small intestine producing the enterotoxin, while emetic syndrome occurs when a food containing preformed cerulide toxin is consumed produced during the growth of *B. cereus* (Kramer and Gilbert, 1989).

Rice is a basic cereal in the diet, widely consumed by the general population due to its ample supply of nutrients and its relatively low price. Generally referred to as Asian grown rice (*Oryza sativa* L.), it is one of the most important staple crops and feeds almost half the world population (Wei and Huang, 2019). This cereal, however, is frequently contaminated by *B. cereus* spores, from cultivation to the later stages of processing and consumption (Kramer and Gilbert, 1989). The primary habitat of emetic strains could be related to roots, tubers, and mycorrhizae of rice, which could explain their generally high prevalence in

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https://doi.org/10.1016/j.fm.2022.104077
Received 22 December 2021; Received in revised form 6 June 2022; Accepted 7 June 2022
Available online 10 June 2022

carbohydrate-rich foods (Navaneethan and Effarizah, 2021). In fact, starch has been shown to promote *B. cereus* growth and emetic toxin production. This would explain why most outbreaks of emetic disease are associated with starch-rich farinaceous foods (Ehling-Schulz et al., 2015). *B. cereus* is able to generate spores that are resistant to the typical rice cooking or pasteurization processes (Fernandez et al., 1999), making it the main opportunistic pathogen found in this substrate (Rodrigo et al., 2021). The main safety issue arises when *B. cereus* 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 and 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.

Chitosan is a polymer of animal origin with a great interest at the moment. It has gained considerable attention in recent decades, due to its biodegradability, biological compatibility, antimicrobial activity, antioxidant and high safety (Jadhav and Diwan, 2018; Avelelas et al., 2019; Aranaz et al., 2021), which is why it is already used in a large number of pharmaceutical, medical and food applications, among others (Rinaudo, 2006; Aranaz et al., 2021). Chitosan is an approved food ingredient in Europe, Japan, Korea, and the United States, and has already been used as a food preservative to prevent spoilage and act as a natural antioxidant (Abd El-Hack et al., 2020). Furthermore, its potential as an antimicrobial has continued to gain ground as a natural food preservative against diverse Gram + and Gram - bacteria (Abd El-Hack et al., 2020; El-Saber Batiha et al., 2021). Insect exoskeleton is also rich in chitin that could be transformed into chitosan. Due to the increase in western countries for insect farming and consumption promoted by FAO recommendations (FAO 2013), chitosan could be easily obtained from insect's by-product. However, the vast majority of studies have been carried out with chitosan from crustaceans while very little information can be found on the properties of chitosan from insects. In this sense, it would be necessary to investigate whether insect chitosan has antimicrobial capacity and whether this, as described by Kumirska et al. (2011) and Kong et al. (2008) for crustacean chitosan is dependent on the environmental conditions of application, such as the temperature and the concentration applied.

Consequently, the main objective of this work is to evaluate the antimicrobial capacity of insect chitosan against vegetative cells of *B. cereus* in a cooked rice substrate, considering different storage temperatures and chitosan concentrations.

2. Material and methods

2.1. Test microorganism

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.

The culture was rehydrated with 0.2 mL of sterile Nutrient Broth (NB) liquid medium (Scharlab Chemie S.A., Barcelona, Spain). After 30 min, 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 min, in a Beckman centrifuge (JLA-16,250 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.

2.2. Insect chitosan

The antimicrobial used for the tests was an insect chitosan 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).

2.3. Rice substrate

For the growth of *B. cereus*, powdered freeze-dried cooked rice was used, with a moisture content of 8.66%. This substrate was prepared in the laboratory.

The lyophilized rice was used as a growth matrix 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.

2.4. Bacillus cereus growth 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 tests also included two *B. cereus* controls (rice substrate without chitosan). 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 (6.25 ± 0.2) and acetic acid concentration that have the chitosan 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 °C; 20 ± 0.5 °C; and 10 ± 0.5 °C, simulating different storage temperatures of precooked rice. Being 30 °C a temperature within the optimal range for the growth of *B. cereus*; 20 °C, a temperature that would represent a cold-chain breakdown in the storage process; and 10 °C as an example of refrigeration temperature abuse.

The antimicrobial effect of chitosan was evaluated by taking sampling points at different incubation times (between 0 and 170 h), 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 °C for 24 h before counting.

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

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

where N is the bacterial concentration (CFU/mL) at time t (h) and N_0 the initial bacterial concentration (CFU/mL) (t_0).

2.5. Statistical analysis

The experimental results were processed in the Microsoft Excel 365 program and the statistical analysis of the experimental data was carried out using the SPSS Statistics V27.0.1.0 program. Outliers were identified and removed prior to data analysis. The statistical significance of the

data was determined by an analysis of variance (ANOVA) (p-value <0.05) and inter-group differences were determined by Tukey's *post hoc* test, which identifies homogeneous subsets of means that do not differ from each other.

3. Results

The present work has studied the effect of different concentrations of insect chitosan on vegetative cells of *Bacillus cereus*, stored at three temperatures, comparing the results with those of growth in a control at pH 6.8 and an acid control at pH 6.25.

Fig. 1 (A, B and C) shows the behavior of the microorganism in the control without acidification (pH 6.8) and in the control acidified with acetic acid (pH 6.25) at the three storage temperatures. Results show that 10 °C is a limiting temperature for the growth of the microorganism used in this study (Fig. 1C). After 200 h of storage, the microorganism concentration decreased with respect to the initial concentration in both control media, acid control and control without acidification. However, at 20 °C, which is a temperature, considered as a cold-chain breakdown, the microorganism exponentially grows after a short lagphase (Fig. 1B). No significant differences were observed in the final *B. cereus* concentration (30 h of storage) between acidified and non-acidified control. At 30 °C, which is the optimal growth temperature for this microorganism, no lag phase was identify and the stationary phase was reached at around 10 h of storage. The final concentration of *B. cereus* cells at 24 h was significantly higher in the acidified medium than in the non-acidified one, probably due to the fact that the cells in the acid control have some stress resistance mechanism active that makes the chitosan less effective.

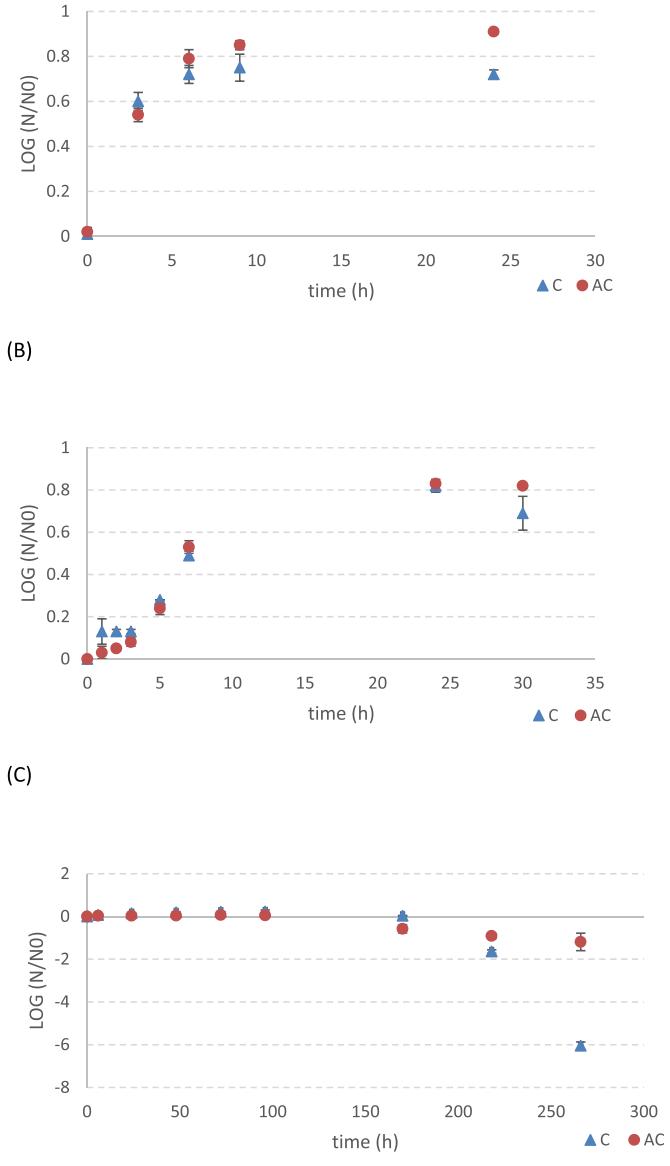

Fig. 1. *B. cereus* behavior (growth/inactivation) in control samples (C) and acid control samples (AC) at 30 °C (A), 20 °C (B) and 10 °C (C).

The results for *B. cereus* growth as a function of the storage temperature and the concentration of insect chitosan can be seen in Fig. 2 (A, B, C and D). 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.

On analyzing the results by temperature (Fig. 2) we observe that at 10 °C 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, acidic and non-acidified media (Fig. 1), we can see 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 °C and 20 °C, 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.

Table 1 shows the survival of *B. cereus* after 24 h of incubation for the controls © and Acid Control (AC) and the studies with insect chitosan (ICH). The 24 h incubation time was considered a good control point for comparison, since it is the moment when all controls (C and AC) reach the stationary phase.

According to the table, the statistical analysis only showed significant differences (p-value <0.05) after 24 h of incubation between the C and AC samples for the study at a temperature of 30 °C, where the acid control (AC) grew above the non-acidified control (C), although these differences are not significant in terms of growth. According to these results, the addition of acetic acid at a concentration of 0.025% does not seem to adversely affect microbial growth under these conditions.

Regarding the chitosan exposure studies, the statistical analysis showed significant differences between the different ICH concentrations and between the ICH with the two controls (C and AC), for the different temperatures studied. With the exception of exposures at concentrations of 220 μg/mL and 250 μg/mL at a temperature of 10 °C, where there were no significant differences. The effect of the concentration of insect chitosan was significant in terms of the capacity that the recovery culture had after 24 h of incubation.

Comparison of the results presented in rows (Table 1) indicates that temperature also exerted an important effect on trends in *B. cereus* counts. In this respect, statistically significant differences were recorded depending on temperature for all controls and exposures to chitosan, with the exception of AC at 30 °C and 20 °C, where there were no differences.

4. Discussion

Crustacean chitosan exhibits antibacterial activity on vegetative

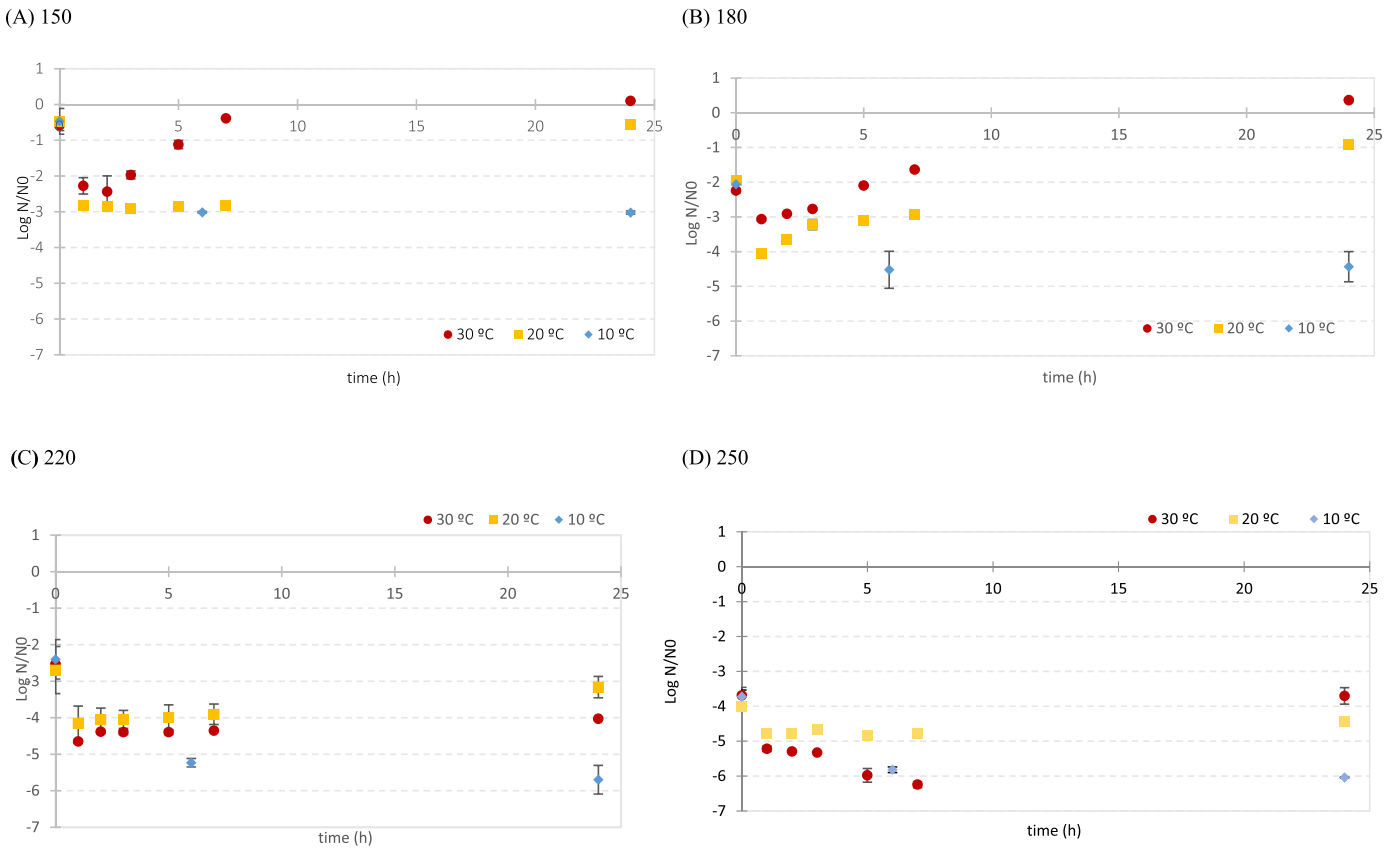

Fig. 2. Effect of insect chitosan concentration 150 μg/mL (A), 180 μg/mL (B), 220 μg/mL (C) and 250 μg/mL (D) on the inactivation of *Bacillus cereus* at 10, 20 and 30 °C.

Table 1
Survival of *B. cereus* after 24 h of incubation, represented as the mean of Log N/N0 (CFU/mL) ± SD (C: Control; AC: Acid Control; ICH: Assay with insect chitosan at concentrations of 150, 180, 220 and 250 μg/mL).

Substrate	Log N/N_0 (CFU/mL)		
	30 °C	20 °C	10 °C
	Time = 24 h	Time = 24 h	Time = 24 h
C	0.72 ± 0.02 (a, A)	0.82 ± 0.03 (a, B)	0.15 ± 0.01 (a, C)
AC	0.91 ± 0.01 (b, A)	0.91 ± 0.02 (a, A)	0.07 ± 0.01 (a, B)
ICH 150	0.1 ± 0.01 (c, A)	−0.57 ± 0.04 (b, B)	−3.02 ± 0.6 (b, C)
ICH 180	0.36 ± 0.04 (d, A)	−0.92 ± 0.06 (c, B)	−4.43 ± 0.62 (c, C)
ICH 220	−4.03 ± 0.03 (e, A)	−3.16 ± 0.29 (d, B)	−5.7 ± 0.39 (d, C)
ICH 250	−3.7 ± 0.24 (f, A)	−4.43 ± 0.04 (e, B)	−6.04 ± 0.01 (d, C)

Values with the same lowercase letter do not differ significantly by column, and values with the same uppercase letter do not differ significantly by row. Different letters indicate significant differences (p-value <0.05). Positive values indicate growth and negative values mean microbial inactivation with respect to the initial inoculation value (N0).

cells, including *Bacillus cereus* cells (Gerasimenko et al., 2004; No et al., 2002; Park et al., 2004; Tsai et al., 2002). Nevertheless, its antimicrobial activity varies as a function of its physicochemical characteristics and depends on the type of microorganism. In this sense, the present work has investigated the effect of different concentrations of insect chitosan on vegetative cells of *B. cereus* stored at different temperatures. The results have shown that insect chitosan can exert bactericidal or bacteriostatic effects depending on the concentration and the storage temperature, being bactericidal for 220 and 250 μg/mL at all temperatures tested and for 180 and 150 μg/mL at 10 °C. while at those lower concentrations (180 and 150) and 20 and 30 °C it was bacteriostatic (growth inhibition but not death of microorganisms). Mellegård et al. (2011) studied the ability of chitosan to inhibit B. cereus spore outgrowth and multiplication. They used six different chitosans with defined macromolecular properties. Results of their studies indicated that growth was inhibited by chitosan, but germination was not. Chitosan action was concentration-dependent and also closely related to the molecular weight and fraction of acetylation of the biopolymer. According to these findings, chitosan concentration may play an important role in its antimicrobial capacity, as also observed in the present study, in which the greatest antimicrobial effect of all concentration tested was observed when *B. cereus* vegetative cells were in contact with 250 μg/mL of insect chitosan. Fernandes et al. (2009) studied the antimicrobial effect of chitosans with different molecular weight on vegetative cells of *B. cereus*; however, they did not consider different storage temperatures or diverse concentrations of chitosan. These authors used atomic force microscopy imaging to reveal how chitosans with different molecular weight behave differently against *B. cereus* cells in terms of their antimicrobial effect. Low molecular weight chitosan caused more visible damage in *B. cereus* vegetative cells than high molecular weight chitosan; this was most probably due to cell penetration.

Insect chitosan as an antimicrobial has also been studied for *E coli*, *Salmonella* and *Listeria monocytogenes* cells and compared with crustacean chitosan (Ibañez-Peinado et al., 2020). The authors found a very rapid effect of both chitosans during the first hours of storage. This effect was maintained for 24 h, after which an adaptation of the microbial cells took place, evidenced by an increase in their concentration until reaching the initial levels of inoculation and even exceeding them in some cases. They recorded differences in activity between insect or crustacean chitosan that depended on the microorganism, the initial inoculum concentration and pH of the media, probably due to its different origin, which would affect its physicochemical properties. In this study something similar occurred with *B. cereus* vegetative cells, with a rapid decrease in their concentration followed by a more or less

stable period in terms of the number of cells until 24 h, after which these was an increase in their concentration. The population dynamics of *B. cereus* depended on the incubation temperature and the chitosan concentration. The increase at 24 h was greater at lower chitosan concentrations (150–180 μg/mL) than at higher concentrations (220 and 250 μg/mL) while at 10 °C the bactericidal effect was observed throughout the incubation time.

Regarding the antimicrobial effects of insect chitosan, these results will provide new opportunities for the use of natural antimicrobials, fostering the reuse of waste from the livestock and food industry and reducing impact on the environment. We should consider that the rearing of insects in mini-farms has a lower impact on the environment than the use of conventional livestock (FAO 2013), with lower greenhouse gas production, water and food consumption, and land use. It should also be pointed out that overexploitation of the seas will lead to a shortage of crustacean skeletons from which to extract chitin in order to produce chitosan.

5. Conclusions

The use of insect chitosan as an antimicrobial against *B. cereus* may be useful as an additional control measure in ready-to-eat dishes based on precooked rice and its derivatives. Its use together with an adequate storage temperature can reduce the microbial load of *B. cereus* to levels well below the infective dose; therefore, it is applicable to improved food safety of this type of food.

A concentration of insect chitosan of 150 μg/mL and a storage temperature of 10 °C may be ample to guarantee the microbiological stability of precooked rice and its derivatives.

Declaration of competing interest

None.

Acknowledgements

We want to thank TRACE-RICE project, Reference Number AMD-1934-1 and grant PID 2020-116318RB-C31 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe", the support for this Research.

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