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Inhibiting and Inactivating Effect of Chitosan on Heat Resistant Moulds Responsible for Spoilage of Pasteurized Fruit Products

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Abstract

The antimicrobial properties of chitosan, a deacetylated derivative of chitin, were assessed in a synthetic medium and in a blueberry and red grape juice against six heat resistant moulds (HRM) associated with food spoilage, in order to evaluate its use as a potential food preservative of natural origin. In both synthetic medium and natural substrate, the Minimum Inhibitory Concentration (MIC) of chitosan proved to vary in a wide range, regardless to both fungal genus and medium considered. Although *Aspergillus neoglaber, Talaromyces bacillisporus* and *Aspergillus niger* proved most resistant to chitosan (MIC > 5000 mg/L) on both media, a marked difference was detected for other strains tested. On HRM inoculated in MEB, the MIC was equal to 100 mg/L for *Byssochlamys fulva* or *Monascus floridanus*, and to 1000 mg/L for *Talaromyces macrosporus*. On HRM inoculated in blueberry and red grape juice, the MIC was equal to 2500 mg/L for *Byssochlamys fulva* or *Monascus floridanus*, and to 5000 mg/L for *Talaromyces macrosporus*.

If combined with thermal treatment (80°C for 5 min) at a concentration (500 mg/L) that did not prove effective in inhibiting fungal spores, chitosan did not affect spore survival of microorganisms tested, thus allowing the spoilage of tested fruit juice after 5 days at 30°C. Also heat resistance parameters of a selected heat-resistant strain, *T. bacillisporus,* were not significantly affected by supplementation of blueberry and red grape juice with 500 mg/L of chitosan, D values ranging between 47.6 and 71.4 minutes at 82°C; 13.3 and 23.3 minutes at 85°C; 3.6 and 5.9 minutes at 88°C; 0.9 and 1.8 minutes at 91°C (z=5.2°C to 5.5°C). Since chitosan did not prove effective alone or combined with thermal treatment either to inactivate heat resistant microorganisms tested or to modify heat-resistance parameters of a heat resistant mould such as *T. bacillisporus*, its use cannot be considered as an interesting strategy to apply milder thermal treatments and to achieve stability of acid products.

Keywords: Heat resistant moulds (HRM); *Neosartorya*; *Monascus*; *Talaromyces*; Chitosan; Inhibition; Inactivation

Introduction

Fruit juices are susceptible to the growth of yeasts, moulds, lactic acid bacteria and spore-forming microorganisms such as *Alicyclobacilli* and Heat Resistant Mould Spores (HRMS). Bacterial and fungal spores are widely distributed in vineyards, orchards, and fields [1-5], where they can survive for long times due to their so-called "quiescence" and can consequently contaminate raw materials being exposed to soil, before their delivery to a processing plant. Pasteurization treatments are normally sufficient to allow the inactivation of enzymes and microbial vegetative cells, but are ineffective to kill these spores. Consequently, when a spoilage occurs, this may result in great economic losses for food industries involved, especially when spores are present in raw materials at high concentrations [6,7]. The use of synthetic preserving agents as an ingredient have been for a long time one of the most effective ways to counteract spoilage by heat-resistant microorganisms. Nevertheless, food industries are now turning their attention to natural molecules. Among these, chitosan is one of the most interesting ones, since it is a nontoxic biocompatible, bio-functional, and biodegradable deacetylated derivative of chitin, present in cell walls of crustaceans (e.g., shrimp shells) and of some Filamentous Fungi (mainly *Aspergillus niger*) [8,9]. Its main application as a technological aid is in the water treatments [10] or in the wine, beer, and spirits-industry where it is used as a fining or clarifying agent [11] and where it proved to reduce Ochratoxin A [12]. Nevertheless, chitosan is receiving great attention as a potential preservative of natural origin in fruit acid products because of its strong antioxidant, antimicrobial and filmogenic activity [13].

Despite its antibacterial activity has been widely investigated with concern to vegetative microorganism [14-16], there are still no literature data about chitosan effectiveness against HRMS. For this reason, the aim of this study was: (i) to assess the inhibiting and inactivating effect of chitosan on heat resistant Fungi, representing a matter of concern for manufacturers of pasteurized fruit products, and to test the synergistic effect of chitosan and thermal treatments on (ii) survival and (iii) heat-resistance parameters of selected strains, to evaluate its possible use as a technological aid during the pasteurization of fruit-based products.

Materials and Methods

Microorganisms

This study was carried out on different microbial strains:

1. *Aspergillus neoglaber* (≡ *Neosartorya glabra*) *SSICA 131014*, isolated from a spoiled plum jam;

2. *Byssochlamys fulva SSICA 18615*, isolated from a spoiled fruit juice;

3. *Monascus floridanus SSICA 26115*, isolated from heat-treated lemon cells;

4. *Talaromyces bacillisporus SSICA 10915*, isolated from heat-treated blueberries;

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5. *Talaromyces macrosporus SSICA 141205*, isolated from heat-treated lemon cells;

In addition to the above-mentioned strains, one *Aspergillus brasiliensis* ATCC 16404 strain were used as reference strains.

HRMS strains were identified according to Guarro et al. [17] for *Monascus* isolate, Samson et al. [18] for *Neosartorya* isolate, Ylmaz et al. [19] for *Talaromyces* isolates, and Samson et al. [20] for *B. fulva*. The observation of the microscopic structures was performed by a Differential Interference Contrast (DIC) Microscope (Eclipse 80i, Nikon, Tokyo, Japan). Apart from *B. fulva* and *T. bacillisporus*, that were easily recognisable by means of their phenotypic characteristics, the identity of all other strains was confirmed by means of molecular techniques. For the *Aspergillus* strain with Neosartorya ascospores, genomic DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) [21] and the partial β-tubulin gene were amplified using the primers N2F (5'-GGCTCTGGCCAGTAAGTTCG-3') and N2R (5'-TTGTCACCGTTGGCCTAGTA-3'), as described by Yaguchi et al. [22]. The resulting amplicons were purified using the QIAquick PCR 163 purification Kit (Qiagen), and sequenced by MACROGEN Europe (Amsterdam, The Netherlands). For all other strains, genomic DNA was extracted using Cetyltrimethyl Ammonium Bromide (CTAB) following the protocol of Graham et al. [21] and the nrDNA ITS1–5.8S–ITS2 were amplified using the universal primers ITS1F/ITS4. PCR amplifications were performed following the parameters by White et al. [23] for the ITS region and carried on the sequencing of the purified samples. All sequences obtained in this study were compared to those available in the GenBank database.

Preparation of fungal suspensions

The control strain (*A. brasiliensis* ATCC 16404) was spread on Malt Extract Agar (MEA; OXOID, Cambridge, UK) and incubated at 25°C for 7 days; it was then collected into a 0.1% (v/v) Tween 80 solution, filtered through sterile glass wool and counted on MEA. The heat-resistant strains were spread on Potato Dextrose Agar (PDA; Oxoid, Cambridge, UK) and incubated at 30°C for 60 days to enhance ascospore production and to increase heat-resistance [24-27]. Ascoma were then collected into a 0.1% (v/v) Tween 80 solution containing sterile glass beads (3 mm diameter). All spore suspensions were shaken for 5 minutes using a mixer (Vortex, Continental Instruments) and filtered through sterile glass wool. The obtained 0.1% (v/v) Tween 80 suspensions were finally heated at 65°C for 10 minutes in a thermostatic bath to kill all fungal conidia, and then stored at -20°C until use. Prior to freezing, spore suspensions were checked to confirm they were all single-cell suspensions and were counted using a Differential Interference Contrast (DIC) Microscope (Eclipse 80i, Nikon, Tokyo, Japan).

Chemicals

A powdered chitosan of fungal origin (degree of acetylation: 0-30 mol%) was used. A stock solution was prepared by dissolving the powdered chitosan in a 2.5% acetic acid solution (w/v) (Carlo Erba, Cornaredo, Italy), in order to obtain increasing dilutions (from 2 mg/L to 5000 mg/L) that were used for inhibition and inactivation tests.

Inhibition and inactivation tests

Tests on HRMS were carried out on Malt Extract Broth (MEB; Oxoid, Cambridge, UK) and on an organic 55% blueberry and 45% red grape juice (optical refractometric residue 12.5°Bx; pH 3.50) supplemented with increasing chitosan concentrations (from 50 mg/L to 5000 mg/L). All cultural media were also prepared by adding acetic acid at the same concentrations used for dissolving the chitosan, as a control.

Each medium was transferred into Falcon® Multiwell Flat-Bottom Plates (Corning Incorporated, NY, USA), for a total of 3.9 ml/well. Each well was inoculated with 0.1 ml of the spore or conidial suspensions previously prepared, each at a time, to reach a final concentration of about 3.0 Log CFU/ml. Plates were then incubated at 25°C (vegetative fungal cells) and 30°C (HRMS) in static conditions up to a maximum of 30 days. A visual inspection was carried out daily to evaluate the fate of the strains inoculated. If growth did not occur within the due period, inhibition or inactivation of the targeted strain was checked by means of a cultural test. For HRM, the content of each well where no fungal mycelium had been observed was filtered, plated on MEA and incubated at 25°C for seven days.

Each test was carried out in triplicate and the experiments were repeated twice.

Synergic effect of heat treatment and chitosan on fungal spores

An organic 55% blueberry and 45% red grape juice (optical refractometric residue 12.5°Bx; pH 3.50) supplemented with 500 mg/L of chitosan was used. This concentration was selected, since in the first part of the work it proved not to be fungistatic for any of the strains tested in the above-mentioned medium. All heat-resistant strains were tested, except for *M. floridanus*, whose heat-resistance proved sensibly lower than that of other HRMS, their spores being inactivated at 75°C for few minutes (data not shown).

Thermal death tubes (TDT; 8 mm external diameter, 6 mm internal diameter and 1 mm wall thickness) containing 0.1 ml of spore suspension and 9.9 ml of heating medium were used, the final concentration of spores in TDT tubes being 3.0 Log CFU/ml. TDT tubes not exposed to heat shock were used as controls. The medium was pre-heated accounting to come-up time calculated, rapidly inoculated with the diluted spore suspensions (for their preparation, see section 2.2), each at a time, and treated at 85°C for 5 minutes in a water bath (FA90, Falc, Treviglio, Italy) to mimic the temperature/time conditions usually applied at an industrial level for fruit juices. After the heat treatment, tubes were removed from the water bath, cooled for a short time in water at 4°C. Appropriate decimal dilutions were plated on PDA one hour after the treatment and after 5, 10, and 15 days at 30°C (if no visible growth was observed prior than the sampling time). Colonies were counted after incubation at 30°C up to 14 days.

Thermal resistance studies on *Talaromyces bacillisporus*

Tests were carried out on *Talaromyces bacillisporus SSICA10915* in an organic 55% blueberry and 45% red grape juice (optical refractometric index 12.5°Bx; pH 3.50) supplemented or not with 500 mg/L chitosan. The blueberry and grape juice was assessed for the presence of inhibiting substances, in order to evaluate their suitability as a matrix for ascospores heating, according with method proposed by Scaramuzza and Berni [28]. The spore suspension previously obtained (see Preparation of fungal suspensions section) was diluted 1:10 with the heating medium considered, each at a time. Polythene bags (68 mm × 78 mm) containing about 2.0 ml of the diluted suspension were sealed with no air present and plunged into a stirring water bath (FA90, Falc, Treviglio, Italy) equipped with a Platinum-sensor probe (Delta HOM, Padua, Italy) for continuous temperature check. Analyses were carried out from 75°C to 91°C and from 0.5 to 240 minutes. After the heat treatment, bags were removed from the water bath, cooled for a short time in water at 4°C and opened under sterile conditions. Appropriate

decimal dilutions were then plated on acidified PDA (0.6 ml 10% tartaric acid) and colonies were counted after incubation at 30°C up to 14 days.

Modelling the thermal death rate curve

Since ascospores usually survive in a dormant state that can only be broken with a sub-lethal thermal treatment called "activation" [29], all experiments carried out on HRMS are usually characterized by a linear activation phase and then, after a maximum number of activated ascospores was reached, by a shoulder preceding an accelerating death rate. In this paper, the activation and inactivation phases were presented in Figure 2, but just the latter phase has been considered for the determination of the thermal parameter, D_t.

Statistical analysis

Since analyses were carried out in duplicate for each time/temperature combination, thermal reduction data were presented as mean values ± standard deviation (SD). SPSS® Version 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The linear regression function enabled the determination of the D values, defined as the time required bringing a1-log reduction in the population of a given microorganism at a given temperature, and of the z values, defined as the degree of temperature required to bring a 10-fold reduction in each D value.

Results and Discussion

Inhibition and inactivation tests

HRMS

Test in MEB: Results of the test carried out in MEB supplemented with 50-5000 mg/L of chitosan are reported in Table 1.

With regard to the effect of chitosan on germination of the fungal strains investigated, their lag phase was not significantly affected by chitosan increase if concentrations up to 1250 mg/L were considered, compared to controls that always germinated within less than 4 days. A marked difference was observed just at the highest concentrations investigated (2500 and 5000 mg/L) for *A. neoglaber* and *A. brasiliensis*, their lag-phase being respectively equal to 12 and 14 days (our unpublished data).

With regard to the effect of chitosan on growth of the fungal strains investigated, a progressive increment in the number of days needed for mycelium formation was observed if increasing concentrations of chitosan were considered. Compared to their corresponding controls, less resistant strains showed a delay in their growth that was equal to 1 day (*B. fulva*) and 4 days (*M. floridanus, T. macrosporus*) at the last concentration where growth occurred. *T. bacillisporus* retarded its growth from 4 to 7 days at concentrations between 250 mg/L and 5000 mg/L, while a delay ranging from 7 to 8 days (*A. neoglaber*) or from 15 to 16 days (*A. brasiliensis*) at concentrations higher than 1250 mg/L was observed for most resistant strains.

Tests in blueberry and red grape juice: Results of the tests carried out in blueberry and grape juice supplemented with 250 mg/L to 5000 mg/L of chitosan are reported in Table 2. With regard to the effect of chitosan on germination of the fungal strains investigated, their lag–phase was not affected by chitosan increase at any of the concentration tested, compared to controls. A significant difference was observed just for *A. neoglaber* at the highest concentration investigated (5000 mg/L) where spores germinated at 18 days, that means 10 days after those inoculated in juice supplemented with acetic acid and used as a control (our unpublished data). With regard to the effect of chitosan on growth of the fungal strains investigated, a progressive increment in the number of days needed for mycelium formation was observed for most of the combination tested, if increasing concentrations of chitosan were considered. Such increase was proportional to the concentration of chitosan added, according to what observed by Gomez-Rivas et al. [30]. Nevertheless, a delay in the formation of fungal mycelium was not observed for any of the fungal isolates tested, compared to their corresponding controls. As Tables 1 and 2 shows the effect of chitosan proved to be species-specific and depended on the medium considered. This means that Minimum Inhibitory Concentration (MIC) varied in a wide range, regardless to both fungal genus and medium considered. Although *A. neoglaber, T. bacillisporus* and *A. niger* proved most resistant to chitosan, their MIC being higher than 5000 mg/L on

Fungal strains	Chitosan concentration (mg/L)								
	50	100	200	250	500	1000	1250	2500	5000
A. neoglaber SSICA 131014	+++ (4)	+++ (4)	+++ (4)	+++ (7)	+++ (7)	+++ (7)	++- (14)	+++ (21)	+ - - (21)
B. fulva SSICA 18615	+++ (5)	- - - [a]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]
M. floridanus SSICA 26115	+++ (19)	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]	- - - [b]
T.bacillisporus SSICA10915	+++ (4)	+++ (4)	+++ (4)	+++ (7)	+++ (11)	+++ (11)	+++ (11)	+++ (14)	++- (29)
T.macrosporus SSICA141205	+++ (4)	+++ (4)	+++ (4)	+++ (7)	+++ (11)	- - - [b]	- - - [b]	- - - [b]	- - - [b]
A. brasiliensis ATCC 16404	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (20)	+++ (22)	+ - - (22)

Note: Values in parentheses indicate the number of days that each strain needed to grow, forming visible mycelium. Superscript letters in brackets indicate if inhibition (a) or inactivation (b) was detected for those combinations where no mycelium was observed after 30 days.

Table 1: Effect of chitosan at increasing concentrations on HRMS spores and *Aspergillus* conidia inoculated in MEB.

Fungal strains	Chitosan concentration (mg/L)					
	250	500	1000	1250	2500	5000
A. neoglaber SSICA 131014	+ + + (8)	+ + - (8)	+ + + (8)	+ + + (7)	+ + + (12)	+ - - (29)
B. fulva SSICA 18615	+ + + (4)	+ + + (4)	+ + + (4)	+ + + (4)	- - - [a]	- - - [a]
M. floridanus SSICA 26115	+ + + (11)	+ + + (11)	+ + + (11)	+ + + (7)	- - - [b]	- - - [b]
T. bacillisporus SSICA 10915	+ + + (4)	+ + + (4)	+ + + (4)	+ + + (5)	+ + + (11)	+ + + (30)
T. macrosporus SSICA 141205	+ + + (4)	+ + + (4)	+ + + (8)	+ + + (4)	- - - [a]	- - - [a]
A. brasiliensis ATCC 16404	+ + + (4)	+ + + (4)	+ + + (4)	+ + + (4)	+ + + (5)	+ - - (29)

Note: Values in parentheses indicate the number of days that each strain needed to grow, forming visible mycelium. Superscript letters in brackets indicate if inhibition (a) or inactivation (b) was detected for those combinations where no mycelium was observed after 30 days.

Table 2: Effect of chitosan at increasing concentrations on HRMS spores and *Aspergillus* conidia inoculated in blueberry and grape juice.

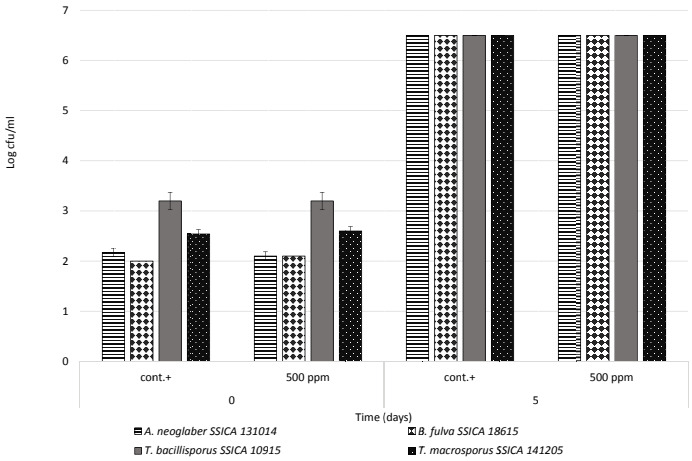

Figure 1: Combined effect of heat-treatment and chitosan on survival and growth of fungal ascospores (Log CFU/ml). Vertical error bars indicate standard deviation for mean values.

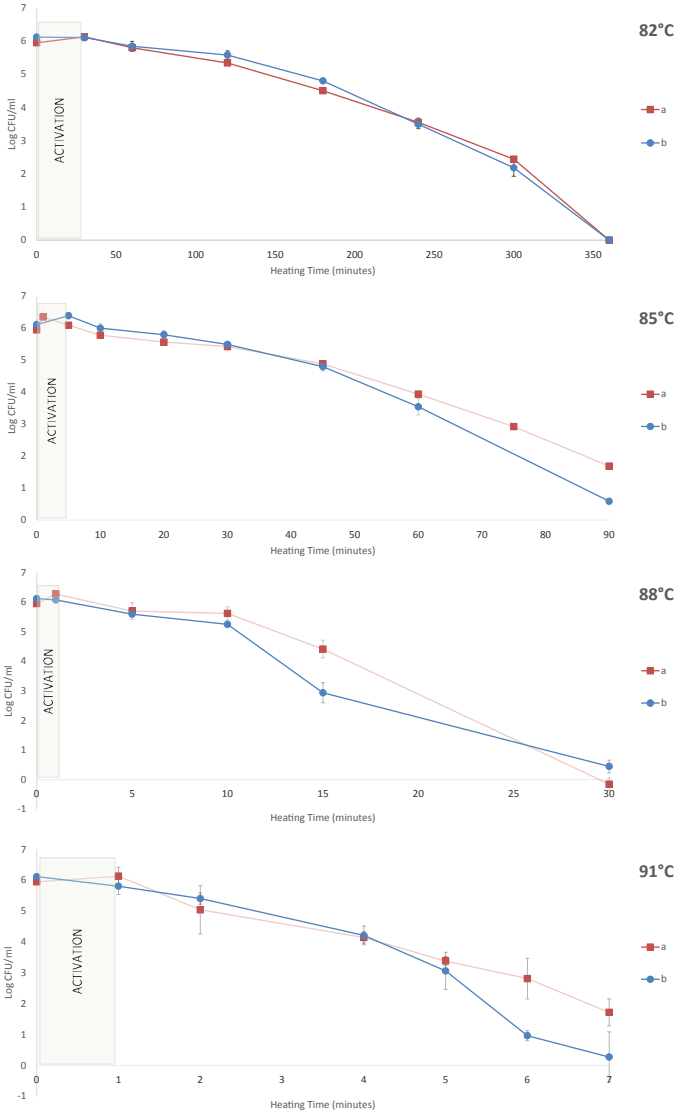

Figure 2: Inactivation curves for *T. bacillisporus* in blueberry and grape juice in its unaltered formulation (a) or supplemented with chitosan (b). Vertical error bars indicate standard deviation for mean values.

both media investigated, a marked difference was detected for other strains tested. In particular, the MIC for *B. fulva, M. floridanus* and *T. macrosporus* was respectively equal to 100 (*B. fulva, M. floridanus*) and 1000 mg/L in MEB, while it noticeably increased up to 2500 (*B. fulva, M. floridanus*) or 5000 (*T. macrosporus*) mg/L in blueberry and grape juice. This effect could be due to the well-known metal binding properties of chitosan [31-33].

Synergic effect of heat treatment and chitosan on spores: The combined effect of heat-treatment (85°C for 5') and chitosan on survival and growth of HRM spores was assessed. Results are reported in Figure 1. As graph shows, the heat treatment applied completely activated the spores, without giving any decimal reduction to their concentration. Then, just after five days, each of the heat-resistant strains tested spoiled inoculated samples. Therefore, it can be concluded that a combined effect between thermal treatment and chitosan was not observed for HRM.

Thermal resistance studies on *Talaromyces bacillisporus*

Inactivation curves for *T. bacillisporus* ascospores heated at 82°C, 85°C, 88°C and 91°C are shown in Figure 2. Since a linear correlation between heating time and logarithm of surviving ascospores has been observed after maximum spore activation, the regression analysis on the best fit (a straight line that best represents the data on a scatter plot) has been carried out and the death rate curve parameters for each temperature have been obtained. As Table 3 shows, the 95% confidence intervals calculated for any D value showed no statistically significant differences when heat resistance values were compared, except for treatments at 85°C, where a lower heat resistance was registered in fruit juice supplemented with chitosan.

Decimal reduction time curves have been modeled, too (Figure 3). Since a linear correlation between heating temperatures and logarithm of D values could be expected, the regression analysis on the best fit has been carried out and the z values for each heating medium were calculated (Table 3).

Based on these results, it can be stated that all thermal death curves for the strain tested followed the same trend of other heat resistant moulds. In particular, a maximum activation was reached after 30 minutes at 82°C and just after one minute at higher temperatures. Data obtained from thermal death curves and statistical elaboration of raw data showed that *D* values of *T. bacillisporus* ranged between 47.6 and 71.4 minutes at 82°C; 13.3 and 23.3 minutes at 85°C; 3.6 and 5.9 minutes at 88°C; 0.9 and 1.8 minutes at 91°C. Also, *z* values ranged in a very restricted interval, being equal to 5.5°C (R^2=0.995) for fruit juice in its unaltered formulation and 5.2°C (R^2=0.997) in the one supplemented with chitosan.

A direct comparison between our results and literature data was possible only with the paper by Tranquillini et al. [34], where heat-resistance of *T. bacillisporus* was assessed in the same heating medium, but using a culture medium not supplemented with tartaric acid for counting the survivors. In that case, the heat resistance of *T. bacillisporus* proved significantly lower at almost all the temperatures tested, except for 91°C where no difference was found using unacidified PDA. In general, heat resistance of the tested strain proved comparable with that of most HRMS usually found to spoil pasteurized products, even if lower than that of some emerging spoiling species such as *Hamigera avellanea* and *Thermoascus crustaceus,* that respectively exhibited a D_{90}=19.23-23.81 and 35.71-55.56 minutes in the same heating media considered for this study [28], or of some other *Thermoascus* strains that showed a D_{90}=20.5-56.2 in a glucose-tartrate solution [35].

Heating medium	Heating temperature (°C)	Unstandardized coefficients		R^2	*D* value (min)	*z* value (°C)
		a (min^{-1})	b (-)			
BGJ	82	-0.017	7.111	0.96	58.8^a (50.0-71.4)	5.5
	85	-0.048	6.540	0.98	20.8^b (18.5-23.3)	
	88	-0.225	7.116	0.97	4.4^d (3.6-5.7)	
	91	-0.678	6.699	0.97	1.5^e (1.3-1.8)	
CHIT	82	-0.018	7.253	0.96	55.6^a (47.6-71.4)	5.2
	85	-0.065	7.077	0.97	15.4^c (13.3-18.2)	
	88	-0.204	6.557	0.98	4.9^d (4.1-5.9)	
	91	-0.959	7.284	0.96	1.0^e (0.9-1.3)	

Note: a: Slope of the best fit; b: Intercept in the linear function: y=ax+b. Data in parentheses indicate a 95% confidence interval for the corresponding D values. Within each column, values not sharing a common superscript letter are significantly different (p<0.05).

Table 3: Thermal death rate curve parameters and heat resistance values for ascospores of *T. bacillisporus* in blueberry and grape juice in its unaltered formulation (BGJ) or supplemented with chitosan (CHIT) evaluated by means of a SPSS linear regression.

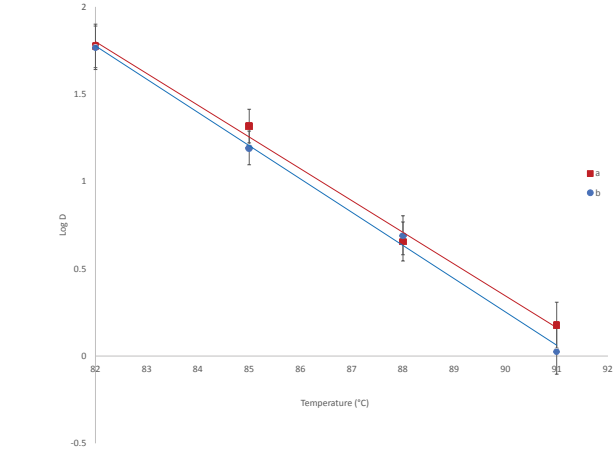

Figure 3: Decimal reduction time curves for *T. bacillisporus* in blueberry and grape juice in its unaltered formulation (a) or supplemented with chitosan (b). Vertical error bars indicate standard deviation for mean values.

Conclusion

Heat treatments normally applied to fruit products are sufficient to inactivate the non-spore forming microorganisms that can spoil these products. Despite their low pH can prevent germination of most spore-forming microorganisms surviving to thermal treatment, HRM spores are an exception, due to their acidophilic nature that allows them to germinate and cause great economic losses to food producers. Silva [36] have proposed these microorganisms as a "target" for the heat treatment of fruit products, although this could lead to drastic heat treatments to achieve their complete inactivation and a subsequent loss in both nutritional and sensory characteristics of fruit products. For this reason, the use of brand new natural molecules (such as chitosan) alone or in combination with a pasteurization process has been assessed to counteract growth and spoilage of heat-resistant microorganism in acid products.

With regard to chitosan effect on HRM, its inhibitory concentration on strains inoculated in blueberry and grape juice varied in a wide range. Although *A. neoglaber, T. bacillisporus* and *A. niger* proved

most resistant to chitosan, their MIC being higher than 5000 mg/L, a marked difference was detected for other strains tested. In particular, the MIC for *B. fulva, M. floridanus* and *T. macrosporus* was respectively equal to 2500 (*B. fulva, M. floridanus*) or 5000 (*T. macrosporus*) mg/L in blueberry and red grape juice. With regard to a synergic effect with thermal treatment, chitosan did not prove to affect survival of HRM just after the heat shock. In line with these findings, also heat resistance parameters of a mold such as *T. bacillisporus* were not significantly affected by supplementation of blueberry and grape juice with 500 mg/L of chitosan, D values ranging between 47.6 and 71.4 minutes at 82°C; 13.3 and 23.3 minutes at 85°C; 3.6 and 5.9 minutes at 88°C; 0.9 and 1.8 minutes at 91°C (z=5.2°C to 5.5°C).

In conclusion, chitosan did not prove effective alone or combined with thermal treatment either to inactivate all heat resistant Fungi tested or to modify heat-resistance parameters of a heat resistant mould such as *T. bacillisporus*. So, its use cannot be considered as an interesting strategy to apply milder thermal treatments and to achieve stability, thus preserving the sensory and nutritional characteristics of acid products.

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