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Combined effect of water activity and pH on the growth of food-related ascospore-forming molds

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Abstract

Purpose: The contamination of raw materials, packaging, or processing environments by fungal ascospores is a real concern for food industries, where variable rates of spoilage can be reached in pasteurized acidic products such as fruit juices, fruit jams, or soft drinks. The aim of this work was to assess the combined effect of a_w and pH on the growth of six isolates from three genera of ascospore-forming molds that may occur in raw materials and in food industrial environments, in order to determine the environmental conditions that prevent the spoilage of pasteurized foods and beverages.

Methods: Growth tests were carried out on 60-day-old ascospores from *Aspergillus hiratsukae* (≡*Neosartorya hiratsukae*), *Aspergillus thermomutatus* (≡*Neosartorya pseudofischeri*), *Chaetomium flavoviride*, *Chaetomium globosum*, *Talaromyces bacillisporus,* and *Talaromyces trachyspermus*. The tests were performed up to 90 days at 25 °C, using sucrose solutions at different a_w (0.85, 0.88, 0.92, 0.95) and pH (3.20, 3.50, 3.80, 4.20, 4.60) values. Growth was characterized by fitting an ordinary logistic regression model to the collected growth data.

Results: The explained percentage of the growth/no growth models ranged between 81.0 and 99.3%: a_w exerted the largest influence on the growth of all tested species, while pH was significant only for *Chaetomium* isolates. The minimum conditions for germination and growth were a_w 0.92 and pH 3.50 or 3.80, respectively, for *C. flavoviride* (46 days) and *C. globosum* (39 days), a_w 0.92 and pH 3.20 for *T. trachyspermus* (13 days), a_w 0.88 and pH 3.20 for *T. bacillisporus* (39 days), and a_w 0.88 and pH 3.20 for the two aspergilli (33 and 27 days, respectively, for *A. hiratsukae* and *A. thermomutatus*).

Conclusions: Most of the spoiling mycetes tested were well-adapted to the formulations considered; therefore, foods strategies aiming to inhibit their growth should explore also the hurdle effect exerted by other factors (e.g., antioxidants, organic acids, oxygen levels).

Keywords: Water activity, pH, Growth tests, *Talaromyces*, *Neosartorya*, *Chaetomium*

Introduction

About one third of the food produced for human consumption every year is lost or wasted (EU 2016; FAO 2012). Although losses due to microbial contamination or spoilage by bacteria, yeasts, and molds are not well-documented, this is a real concern for the food industry (Elkhishin et al. 2017). For pasteurized high acidic fruit products, where only some microorganisms can grow, most of the juice processor members from the American Juice Products Association (JPA) and the European International Fruit and Vegetable Juice Association (IFU) declared to be forced to discard ingredients or product at least once a year due to microbial spoilage, and that the presence of *Alicyclobacillus* species or Heat-Resistant Molds (HRM) is a serious threat to food quality, so much that 64% of them have experienced

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HRM spoilage of finished products (Snyder and Worobo 2018). Among the abovementioned microorganisms, HRM can be considered more adaptable than alicyclobacilli because they grow across a wider range of temperature and pH, as well as at minimal oxygen headspace concentrations (dos Santos et al. 2020; Pitt and Hocking 2009; Samson et al. 2010). This means that, once ascospores are activated by pasteurization treatments, their germination and growth can hardly be hindered, leading to relevant incidental spoilage cases (Rico-Munoz 2017). Apart from accurate monitoring of ingredients, processing environments and packaging, most of the industrial processing steps do not significantly reduce ascospores presence (dos Santos et al. 2018); additionally, the use of preservatives such as sorbate, benzoate, and sulfur dioxide (King et al. 1969) or of technological aids such as chitosan (Manusia and Berni 2017) proved only partially effective or completely ineffective against HRM.

To prevent or limit contamination, acting on physico-chemical parameters such as water activity (a_w), hydrogen ion concentration (pH), or dissolved oxygen levels can achieve the so-called "hurdle-effect". Unfortunately, the literature data concerning this topic are limited to only a few fungal species: the a_w influence on ascospore germination and growth was studied on *Byssochlamys* species (Panagou et al. 2010; Roland and Beuchat 1984; Valík and Piecková 2001), *Neosartorya fischeri* (Valík and Piecková 2001; Zimmermann et al. 2011), *Eurotium* species (Greco et al. 2018), or *Talaromyces avellaneus* (Valík and Piecková 2001); the effect of oxygen levels was investigated on *Byssochlamys* species by Taniwaki et al. (2001) and on *Byssochlamys* and *Neosartorya* isolates by dos Santos et al. (2019). The combined influence of different parameters on ascospore-forming species was explored only on *Monascus ruber* (Panagou et al. 2003) and *Neosartorya fischeri* (dos Santos et al. 2020; Nielsen et al. 1988; Nielsen 1991).

Therefore, the aim of this work was to assess the combined effect of a_w and pH on the growth of isolates from three different genera of ascospore-forming molds (*Aspergillus* with *Neosartorya* morphs; *Talaromyces*; *Chaetomium*) commonly detected in raw materials and in industrial environments (dos Santos et al. 2018; Rico-Munoz and dos Santos 2019; Sato and Takei 2000; Tranquillini et al. 2017), in order to find the best conditions to avoid fungal spoilage of pasteurized foods and beverages.

Materials and methods

Microorganisms

This study was carried out using the following fungal strains:

- *Aspergillus hiratsukae* (≡ *Neosartorya hiratsukae*) SSICA 3913, isolated from a spoiled tea beverage
- *Aspergillus thermomutatus* (≡*Neosartorya pseudofischeri*) SSICA 121014, isolated from spoiled strawberry jam
- *Chaetomium globosum* DSM 1962, isolated from stored cotton in the USA
- *Chaetomium flavoviride* ATCC 32404, isolated from dead *Juncus* stems in Hungary
- *Talaromyces bacillisporus* SSICA 10915, isolated from heat-treated blueberries
- *Talaromyces trachyspermus* SSICA 15007, isolated from heat-treated berries

Talaromyces and *Aspergillus* were tested because their presence in raw materials used for food and beverage production is well known. *Chaetomium* were assessed because they are resistant to the chemical agents used for sanitation of industrial food plants (Scaramuzza et al. 2020a; Scaramuzza et al. 2020b) and are responsible for spoilage in foods packaged by aseptic filling machines (Sato and Takano 2000).

Preparation of ascospore suspensions

Ascospore suspensions were prepared according to Scaramuzza et al. (2020a). Briefly, each isolate was purified, spread on potato dextrose agar (PDA, Oxoid, Cambridge, UK) in Petri dishes, and incubated at 30 °C up to 60 days to enhance ascospore production and to increase resistance (Conner and Beuchat 1987; Dijksterhuis and Teunissen 2004; King and Whitehand 1990; Tournas and Traxler 1994). Mycelium and ascomata were collected into a sterile glass bottle containing a 0.1% (v/v) Tween 80 solution and sterile glass beads (3 mm diameter), shaken for 5 min using a mixer (Vortex, Continental Instruments), and filtered through sterile glass wool. Spore concentration was assessed by means of a differential interference contrast (DIC) microscope (Eclipse 80i, Nikon, Tokyo, Japan), to confirm that each was a suspension with free ascospores. Filtered spores suspensions were stored at − 20 °C until use.

Growth tests

All tests were carried out using sucrose solutions to attain a wide range of a_w values (0.85–0.95). Sucrose solutions, prepared according to Grover and Nicol (1940), at a_w 0.85, 0.88, 0.92, and 0.95 showed 65.6, 61.0, 51.4, and 39.8 °Bx, respectively. All solutions were sterilized at 115 °C for 10 min, and their pH was aseptically adjusted with 5.0% citric acid to obtain pH values equal to 3.20, 3.50, 3.80, 4.20, and 4.60.

The a_w was measured using an a_w meter (LabMaster Novasina GmbH, Pfäffikon, Switzerland). The total soluble solids were measured by means of PAL-3 a

refractometer (Atago, Tokio, Japan) as degrees Brix that corresponds to 1 g of sucrose in 100 g solution. The pH was measured using a pH meter (Seven Compact S220, Mettler Toledo, Columbus, OH, USA) equipped with an "EasyFerm Bio HB-MS 160" electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland).

Physico-chemical analyses were carried out on uninoculated solutions at the beginning and at the end of the tests, in order to check the maintenance of initial a_w, °Bx, and pH.

Each a_w-pH combination was transferred into 20-ml Pyrex® round-bottom sterile tubes with screw cap (7 ml per tube, in order to keep a sufficient headspace) and separately inoculated with 0.05 ml of each ascospore suspension. These tubes permit a homogeneous distribution of the inoculated spores in the solution by means of a vortex apparatus, whereas screw caps allow to maintain sterility without altering the composition of the inoculated medium during the test. *Chaetomium* suspensions were not heat-treated, since their reproductive structures are heat sensitive, albeit resistant to chemical stresses. On the contrary, *Talaromyces* and *Aspergillus* ascospores were heat-treated at sub-lethal temperatures (75 °C) for 30 min, in order to break their dormant state and start the germination (Dijksterhuis 2007). Both inoculated and uninoculated (negative controls) tubes were incubated at 25 °C up to a maximum of 90 days and checked daily to assess the development of hyphal filaments. In case of mycelial growth, the filaments detected were transferred on acidified PDA plates for confirmation. All combinations were tested in triplicate.

Statistical analysis

The results of the growth data at each a_w underwent the analysis of variance (ANOVA) considering pH as factor and, when significant, Fisher's least significant difference tests (LSD) at $p \leq 0.05$ were performed. The analyses were done using the statistical program STATGRAP HICS® Centurion. Mean values, standard error, and coefficient of variation were calculated using the Excel program (Microsoft® Office Excel 2016).

Development of growth/no growth models

Growth data were used to develop models for all the fungi tested, with a_w, pH, and time of incubation as explanatory variables. A total of 180 data for each isolate (60 combinations of a_w, pH, and time with three replicates) was considered for the construction of each model. An ordinary logistic regression model (Gysemans et al. 2007) consisting of a polynomial (right-hand side) and $\text{logit}(p) = \ln \frac{p}{(1-p)}$ (left-hand side), where logit is the *log*istic un*it* and p is the probability that growth occurs ($0 \leq p \leq 1$), was used to describe the data. The logistic regression model (below) included the main factors; their interactions and the quadratic expression of main factors, b_i (i = 0,..., 8) are the parameters to be estimated:

$$\begin{aligned}\text{logit}(p) = {} & b_0 + b_1 a_w + b_2\ \text{pH} + b_3\ \text{time} + b_4 a_w \text{pH} \\ & + b_5 a_w\ \text{time} + b_5\ \text{pH time} + b_6\ {a_w}^2 \\ & + b_7\ \text{pH}^2 + b_8\ \text{time}^2\end{aligned}$$

The models were fitted with the statistical program STATGRAPHICS® Centurion; the terms were selected by the forward stepwise procedure, based on the significance of the likelihood-ratio criterion ($p < 0.001$).

Results

High acidic foods are usually heat-treated and stored at room temperature; in order to mimic the commercial life of such products, our tests were therefore carried out at 25 °C. Furthermore, in high acidic products, the real concern for food industries is the visible spoilage by fungal mycelium that unavoidably leads to consumer rejection; thus, the tests were focused on mycelial growth rather than on ascospores germination. In this study, the fungal growth was tested in sucrose solutions under different conditions.

The growing ability of six fungal isolates was studied under combinations of four a_w (0.85, 0.88, 0.92, and 0.95) and five pH values (3.20, 3.50, 3.80, 4.20, and 4.60) at 7, 30, and 90 days of incubation. Figures 1, 2, and 3 show an overview of the results regarding the growth/no growth conditions of the different strains. The estimated parameters ($p \leq 0.001$) with their standard errors are summarized in Table 1. The variance explained by the models ranged between 81.0 and 99.3% and the adjusted percentages between 77.8 and 96.7%.

Among the physico-chemical factors considered, a_w exerted the largest influence on the growth of all strains; a significant pH effect was observed only on *C. flavoviride* and *C. globosum* growth, where the interaction with incubation time was significant as well (Table 1).

The growth/no growth models concerning *Chaetomium* strains (Fig. 1) recorded the lowest values for germination and growth at a_w 0.92 and pH 3.50 or 3.80, respectively, for *C. flavoviride* and *C. globosum*, whereas the highest growth rate was observed for both strains at a_w 0.95 and pH down to 4.20. For *Talaromyces* strains (Fig. 2), a marked interspecific difference was observed: the lowest values for germination and growth were recorded at a_w 0.92 and pH 3.20 for *T. trachyspermus* and at a_w 0.88 and pH 3.20 for *T. bacillisporus*. On the contrary, the highest growth rate occurred at higher a_w (0.95) regardless of the pH. For *Aspergillus* strains within the group *Neosartorya* (Fig. 3), the minimum a_w and pH values for germination and growth were 0.88 and 3.20

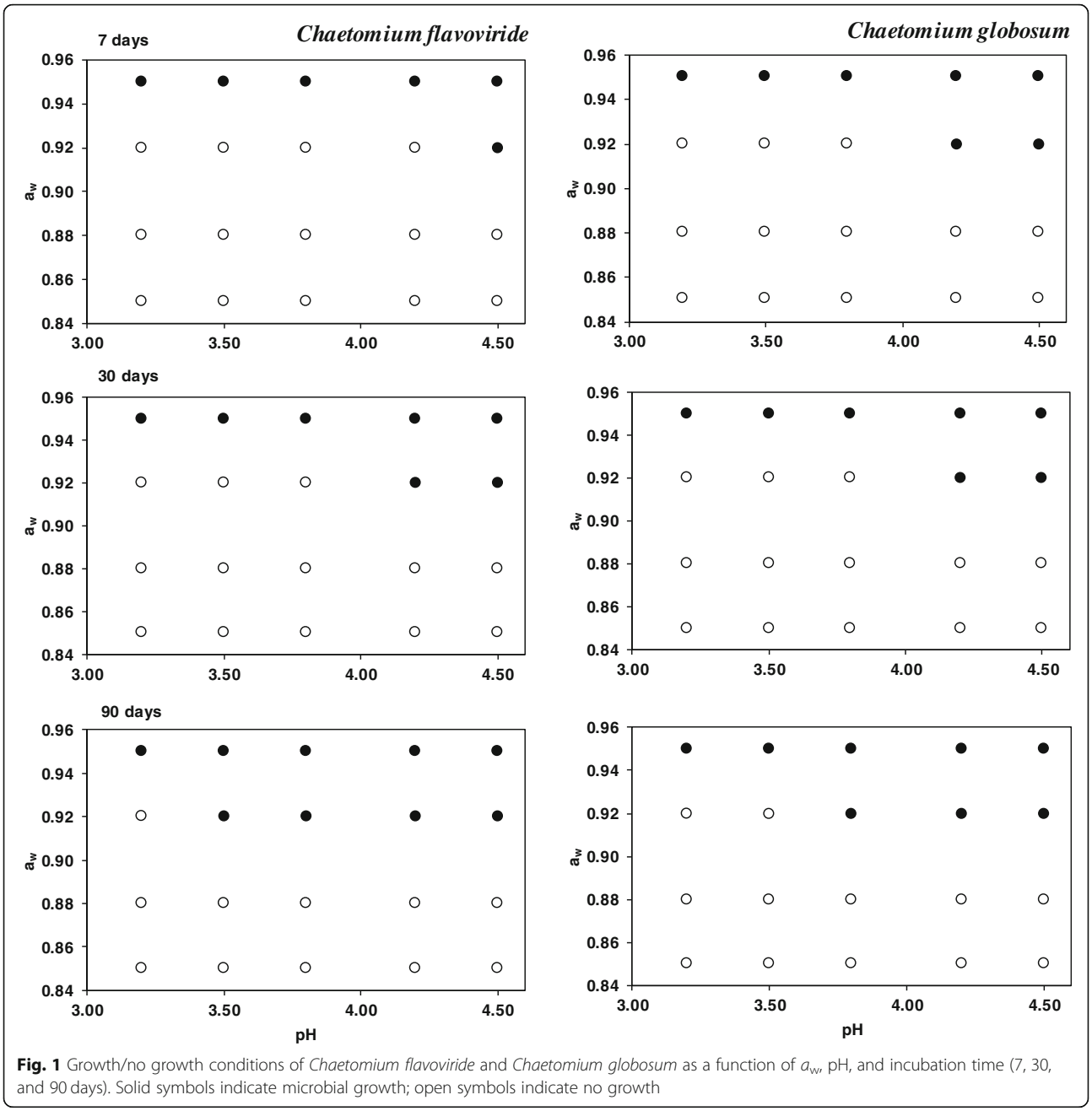

Fig. 1 Growth/no growth conditions of *Chaetomium flavoviride* and *Chaetomium globosum* as a function of a_w, pH, and incubation time (7, 30, and 90 days). Solid symbols indicate microbial growth; open symbols indicate no growth

for both strains. For such isolates, the fastest growth was observed at a_w 0.95, regardless of the pH considered.

The average growth time of the fungi tested in different sucrose solutions is shown in Table 2. The time increase needed for mycelium formation was a function of a_w decrease. Specifically, the combined effect of a_w and pH mostly induced an increment in the number of days needed for micro-mycelia formation in all the strains tested, when increasing concentrations of sucrose were considered. At high a_w values (0.95), the number of days for growth was always not significantly different ($p > 0.05$) at the various pH and for all tested isolates, except for *Chaetomium* strains. On the contrary, when lower a_w values were tested, the optimum growth conditions greatly differed for each strain and among replicates. At a_w 0.88, a first hurdle effect (no growth) was recorded for three out of six tested fungi; at a_w 0.85, no strain germinated and consequently did not develop micro-mycelia (Figs. 1, 2, and 3).

Discussion

Chaetomium isolates were hydrophilic, i.e., did not germinate and grow at $a_w < 0.90$; furthermore, while at a_w 0.95, they were able to grow within 7 days regardless of

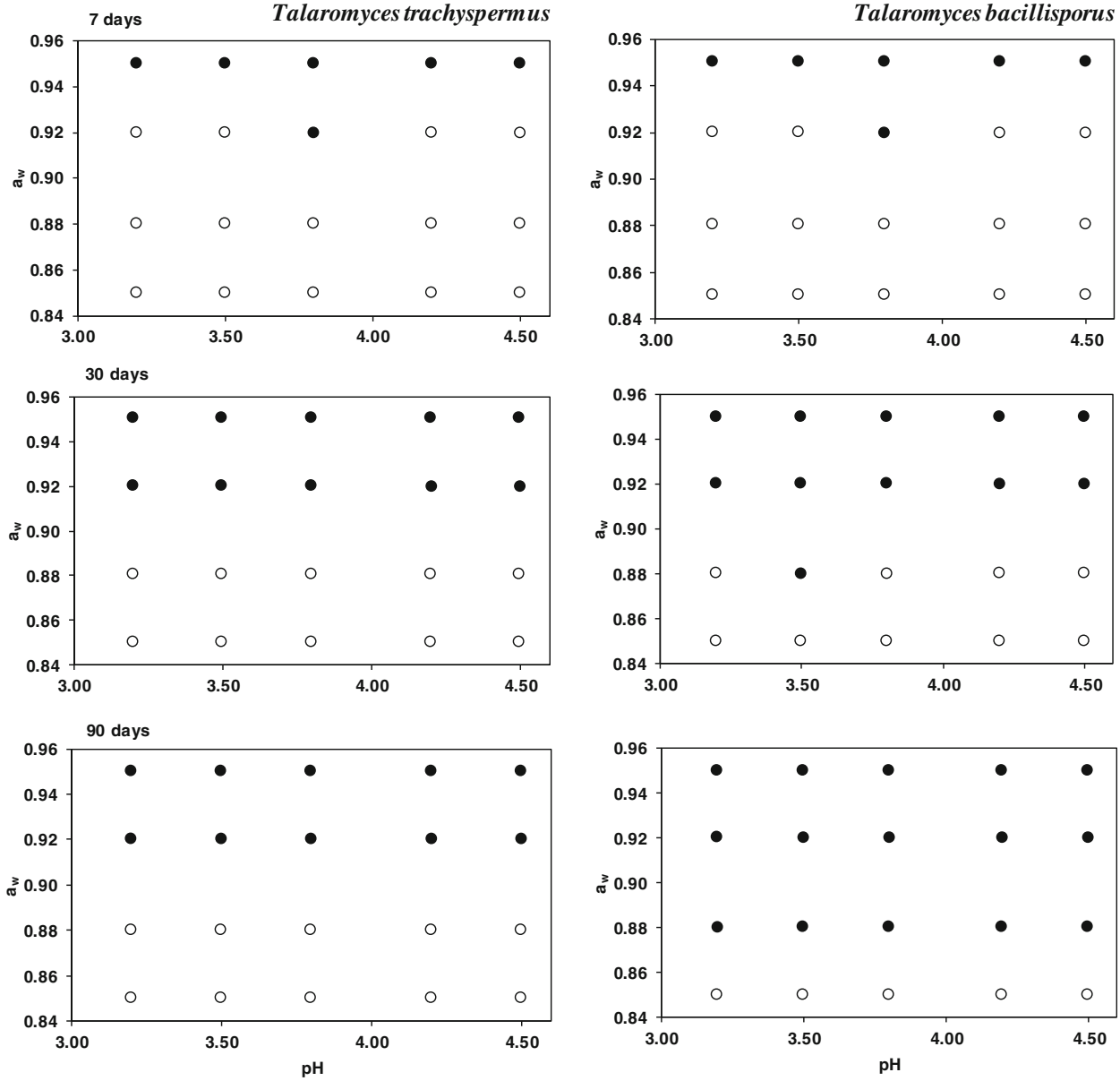

Fig. 2 Growth/no growth conditions of *Talaromyces trachyspermus* and *Talaromyces bacillisporus* as a function of a_w, pH, and incubation time (7, 30, and 90 days). Solid symbols indicate microbial growth; open symbols indicate no growth

the pH considered, at a_w 0.92 their growth was inhibited if the pH was lower than 3.50. Under this a_w-pH combination, the germination time varied from 40 to 52 days for *C. flavoviride*, whereas *C. globosum* did not germinate at all. This could be due to the fact that *C. globosum* has both a hydrophilic and a neutrophilic nature, mainly growing at a_w > 0.90 and pH between 4.3 and 9.4 (Pitt and Hocking 2009); only recently it showed reduced growth even at pH 3.51, although an anomalous morphology was observed (Straus 2011).

Talaromyces isolates varied in their xerotolerance. At a_w 0.95, the tested isolates grew within 3 (*T. bacillisporus*) or 6 (*T. trachyspermus*) days; at lower a_w values, both strains showed an inflection point in days needed for growth at pH 3.80 (a_w 0.92) or at pH varying from 3.50 to 3.80 (a_w 0.88). At the latter a_w value, *T. bacillisporus* was the only strain that produced micromycelia at all pH tested in 23–41 days, whereas *T. trachyspermus* did not grow at all. Our results are hardly comparable with literature results since, to the best of our knowledge, studies concerning lowest a_w for germination and growth of *Talaromyces* species are missing. The only exception is an early study by Hocking and Pitt (1979) where minimum a_w values of 0.84, 0.86, 0.88, and

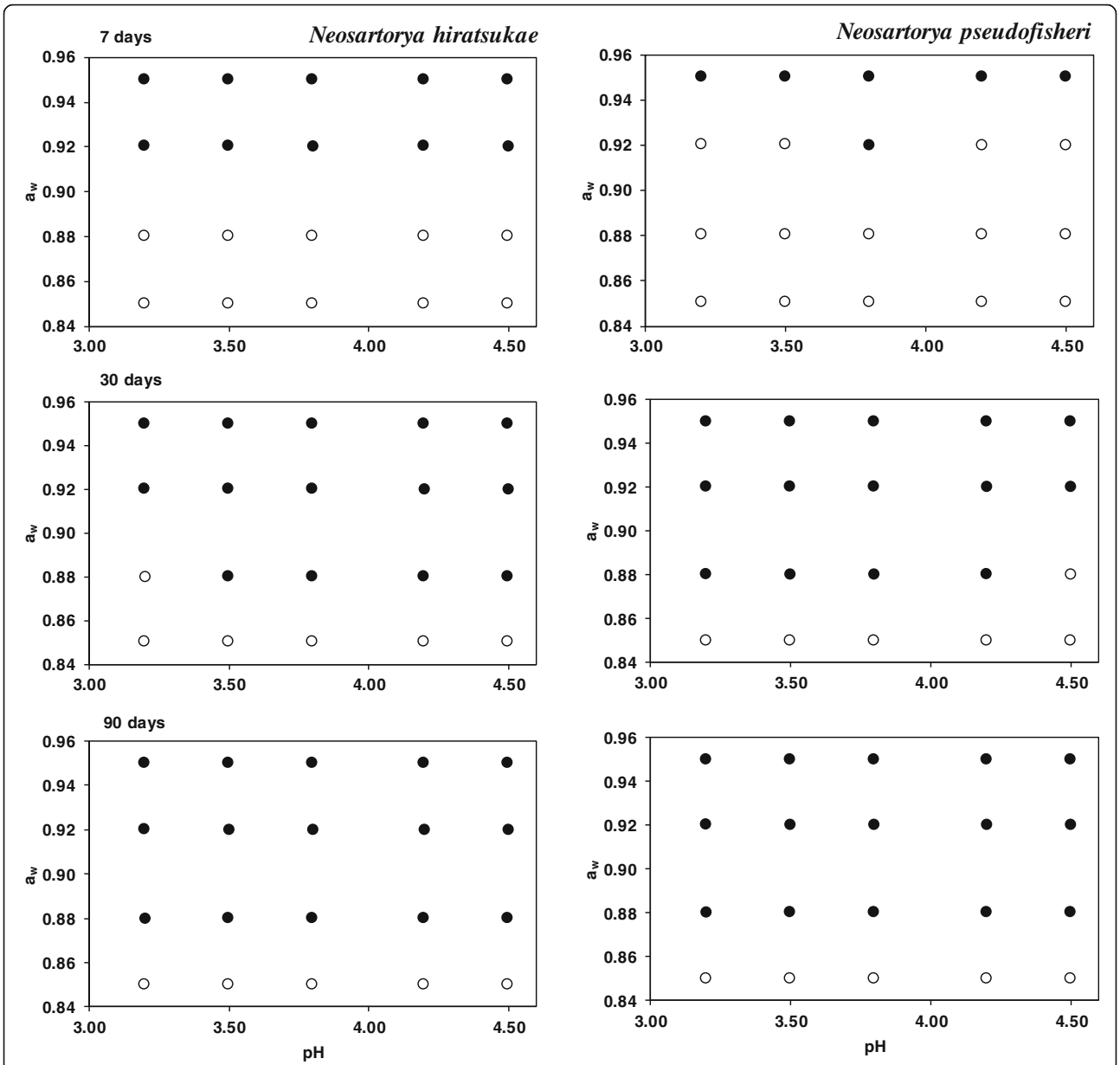

Fig. 3 Growth/no growth conditions of *Aspergillus hiratsukae* (*Neosartorya hiratsukae*) and *Aspergillus thermomutatus* (≡*Neosartorya pseudofischeri*) as a function of a_w, pH, and incubation time (7, 30, and 90 days). Solid symbols indicate microbial growth; open symbols indicate no growth

0.90 (14 days at 25 °C) were reported for *T. purpurogenus*, *T. islandicus*, *T. wortmannii*, and *T. funicoulosus*, respectively. Such data refer to glycerol-based media and conidia, because at that time the abovementioned species were still identified as *Penicillium*.

Aspergillus isolates with *Neosartorya* morphs were found the most xerotolerant, with little differences between isolates. At a_w 0.95, both *A. hiratsukae* and *A. thermomutatus* were able to develop mycelium within 3 or 4 days, respectively. At a_w 0.92, growth times of *A. hiratsukae* varied from 3 to 5 days, whereas those of *A. thermomutatus* were twice those at 0.95, excepted at pH 3.80. At a_w 0.88, the number of days for growth increased from 18 to 33 (*A. hiratsukae*) and from 22 to 29 (*A. thermomutatus*) with decreasing pHs. These results are comparable with those obtained by Berni et al. (2017) in strawberry-based media inoculated with *Aspergilli* showing *Neosartorya* morphs, where the reported number of days needed for growth were similar at a_w 0.92, but no growth was observed on one strain of *A. hiratsukae* and the same strain of *A. thermomutatus*, maybe due to the preserving effect exerted by the citric acid present in strawberries that proved able to retard and/or inhibit ascospores growth (Amaeze 2013; Campo

Table 1 Estimated coefficients ± standard errors from the second logistic regression model ($p < 0.001$)

Parameter	*C. flavoviride*	*C. globosum*	*T. trachyspermus*	*T. bacillisporus*	*A. hiratsukae*	*A. thermomutatus*
Constant	− 2161.7 ± 505.9	− 1798.4 ± 517.0	− 1268.9 ± 528.8	− 515.7 ± 455.6	− 4129.8 ± 431.5	− 711.9 ± 474.4
a_w	2136.2 ± 511.8	1752.1 ± 524.6	1373.5 ± 573.6	548.4 ± 485.8	4642.8 ± 485.2	756.9 ± 505.4
pH	42.5 ± 17.7	44.0 ± 28.7	ns	ns	ns	ns
a_w × time	ns	ns	0.593 ± 0.549	1.606 ± 1.417	ns	2.316 ± 1.617
pH × time	0.187 ± 0.090	0.079 ± 0.074	ns	ns	0.439 ± 0.055	ns
time^2	ns	ns	ns	− 0.011 ± 0.010	ns	− 0.015 ± 0.016
Deviation (%)						
Explained	98.4	98.4	93.9	81.0	99.3	87.7
Adjusted	95.0	95.0	91.5	77.8	96.7	84.4

and Santos 2006; dos Santos et al. 2019; Sturm et al. 2003). Analogously, our data largely overlap those by dos Santos et al. (2020) which observed a minimum number of days for *Neosartorya fischeri* growth ranging from 18 to 40 under similar conditions (30 °C; 0.88 a_w; 0.8% oxygen levels). On the contrary, Valík and Piecková (2001) reported *N. fischeri* ability to grow, at reduced rates, even at a_w 0.85, the little discrepancy with our study being probably attributable to the different experimental conditions applied.

In general, a_w effect proved strain-dependent for *Talaromyces* and *Chaetomium* strains that displayed different behaviors when the same physico-chemical conditions were applied. Considering the same pH value, optimal growth always occurred at the highest a_w value (0.95) for all tested fungi. On the contrary, the pH influence proved to be genus-dependent: considering the same a_w value, mild acid conditions (pH 4.50) were optimal for *Chaetomium* and *Aspergilli* with *Neosartorya* ascospores, whereas lower values (pH 3.80) were optimal for *Talaromyces* isolates growth. These findings can be considered a confirmation of the fact that a_w is one of the dominant environmental factors governing food stability and spoilage by molds, whereas pH usually exerts minor effects over a broad range (3–8) (Pitt and Hocking 2009).

Conclusions

During the last decades, studies concerning the combined effects of different physico-chemical parameters on ascospore-forming species have been sporadically carried out on a limited number of fungal species. Nevertheless, their possible presence in raw materials, packaging, or processing environments is a real concern for food industries, where variable rates of spoilage can be reached in pasteurized acid products such as fruit juices, fruit jams, or sugar-added beverages. Therefore, the search for punctual data and predictive models by food producers is increasing due to the need to avoid microbial-related spoilage incidents and reputation damages. This study was carried out to provide the food industry with a reference point in the early steps of the production process, when target spoilage microorganisms and thermal parameters must be defined.

Our results indicate the optimal and limiting growth conditions for the fungi examined and highlight the synergistic effects between a_w and pH in sucrose-added models mimicking acid-pasteurized beverages. Considering the influence of hydrogen ion concentration, the optimal growth conditions for the ascospore-forming molds occurred when pH values were between 3.80 and 4.50, even if values down to 3.20 did not always inhibit or inactivate them, meaning that these mycetes are well-

Table 2 Growth time (days) ± standard deviation ($n = 3$) for ascospore-forming molds at various a_w and pH values

Strain	a_w 0.88					a_w 0.92					a_w 0.95				
	3.20	3.50	3.80	4.20	4.50	3.20	3.50	3.80	4.20	4.50	3.20	3.50	3.80	4.20	4.50
Cf	-	-	-	-	-	-	46 ± 6	39 ± 0	11 ± 2	9 ± 3	7 ± 0	6 ± 1	5 ± 0	2 ± 0	2 ± 0
Cg	-	-	-	-	-	-	-	39 ± 0	9 ± 3	6 ± 1	5 ± 0	5 ± 0	4 ± 2	2 ± 0	2 ± 0
Tt	-	-	-	-	-	13 ± 2	13 ± 2	5 ± 0	11 ± 0	12 ± 1	4 ± 0	4 ± 0	5 ± 1	5 ± 1	4 ± 0
Tb	39 ± 2	26 ± 0	29 ± 3	37 ± 4	34 ± 0	11 ± 0	10 ± 0	7 ± 0	10 ± 0	15 ± 1	4 ± 1	3 ± 0	4 ± 1	3 ± 0	3 ± 0
Nh	33 ± 1	23 ± 2	17 ± 1	17 ± 0	18 ± 2	5 ± 0	5 ± 0	5 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0
Np	27 ± 6	23 ± 3	23 ± 4	23 ± 1	29 ± 6	9 ± 1	9 ± 1	5 ± 0	8 ± 0	11 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0

Growth time is the time to visible fungal growth. *Cf*, *Chaetomium flavoviride* ATCC 32404; *Cg*, *Chaetomium globosum* DSM 1962; *Nh*, *Aspergillus hiratsukae* (= *Neosartorya hiratsukae*) SSICA 3913; *Np*, *Aspergillus thermomutatus* (= *Neosartorya pseudofischeri*) SSICA 121014; *Tb*, *Talaromyces bacillisporus* SSICA 10915; *Tt*, *Talaromyces trachyspermus* SSICA 15007. The symbol (-) indicates that fungal growth was not observed after 90 days

adapted to pH of pasteurized products. Considering the effect of a_w, the optimal growth conditions were recorded at the highest value (0.95). Although fungal growth was observed at a_w values as low as 0.88 (*Talaromyces* and *Aspergillus* with *Neosartorya* morphs) or 0.92 (*Chaetomium*), none of the tested isolates proved xerophile, i.e., able to grow below 0.85 in at least one set of tested environmental conditions.

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N/A

Authors' contributions
IR carried out all experiments. NS made contributions to acquisition, analysis, and interpretation of data. AH performed both the statistical analysis and the development of growth/no growth models, being actively involved in drafting and revision of the manuscript. EB has made substantial contribution to the study design of the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The authors declare that all materials and data are available.

Ethics approval and consent to participate
This research does not contain any studies with human participants or animals.

Consent for publication
Informed consent is not applicable in this work.

Competing interests
The authors declare that they have no competing interests.

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