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Influence of *Limosilactobacillus fermentum* IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 on the growth of spoilage fungi in bakery products

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ABSTRACT

Fungi are the main microorganisms responsible for the spoilage of bakery products, and their control and subsequent reduction of food waste are significant concerns in the agri-food industry. Synthetic preservatives are still the most used compounds to reduce bakery product spoilage. On the other hand, studies have shown that biopreservation can be an attractive approach to overcoming food and feed spoilage and increasing their shelf-life. However, limited studies show the preservation effects on real food matrices. Therefore, this study aimed to investigate the influence of microorganisms such as lactic acid bacteria (LAB) and yeasts on the growth of spoilage filamentous fungi (molds) on bread and panettones. In general, on conventional and multigrain bread, treatments containing *Limosilactobacillus fermentum* IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 showed similar results when compared to the negative control (calcium propionate) in delaying the fungal growth of the tested species (*Aspergillus chevalieri*, *Aspergillus montevidensis*, and *Penicillium roqueforti*). Different from bread, treatments with *W. anomallus* in panettones delayed the *A. chevalieri* growth up to 30 days, 13 days longer than observed on negative control (without preservatives). This study showed that biopreservation is a promising method that can extend bakery products' shelf-life and be used as an alternative to synthetic preservatives.

1. Introduction

Bread comprises one of the earliest foods prepared by humans and has been an integral part of the human diet. Breads are prone to chemical, physical, and microbial spoilages, demanding strategies from food industries to ensure the shelf-life and quality of the product. Bread shelf-life depends on the formulation and storage conditions, reaching 2–4 days when storage is done at room temperature or up to 14 days at refrigeration conditions (Cauvain and Young, 2011). Amongst the spoilage agents, fungi are the most critical causes of shelf-life reduction and considerable losses in the bakery industry.

Contrary to bread, panettones are bakery products with a relatively long shelf life, and their consumption increases during Christmas. Panettones are products with an upward trend because of their sensorial features, which appeal to different types of people in different countries (Consumidor Moderno, 2023; Stefanello et al., 2019a, 2019b). During the industrial production of panettone, the main challenges comprise the production of the dough with the capacity to hold fruits and raisins during proofing and baking and the achievement of a shelf-stable product formulation (Benejam et al., 2009).

Deterioration caused by spoilage fungi is a ubiquitous problem for bread and bakery products (Pitt and Hocking, 2009; Garcia and Copetti,

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2019). In addition to the economic losses, which are around 5 % in the United States of America (USA) and 10 % in countries like Brazil (Freire, 2011), it may cause the depreciation and rejection of the brand that commercializes the product, as moldy bread represents 2/3 of complaints about moldy food by Brazilian consumers (Hawkins et al., 2007; Lemos et al., 2018).

The contamination of bakery products by filamentous fungi in the industry may occur mainly in the cooling, slicing, and packaging steps (Legan, 1993; Garcia et al., 2019), where the environmental air has been described as an essential source of fungal spores (Andrade and Salustiano, 2008; dos Santos et al., 2016; Garcia et al., 2019). The mold spores spread through the air as aerosols and are mainly responsible for the spoilage of baked goods like bakery products.

One of the ways to avoid spoilage of bakery products is by using food-grade preservatives, such as acetic and sorbic acid and, mainly, propionic acid and its salts (Gioia et al., 2017). However, their use can be limited to the appearance of resistant strains such as *Penicillium roqueforti*, which can cause bread spoilage at sub-lethal levels of preservative concentrations (Suhr and Nielsen, 2004). Furthermore, the use of these preservatives has called attention to the concerns about their solubility and toxicity (Dengate and Ruben, 2002). As such, several alternative strategies have been studied to extend the shelf-life of bakery products. One of the most promising strategies for application in bakery products comprises microorganisms, given that several are subjected to fermentation.

Several studies have used microorganisms from natural sources, such as dough fermentation, as novel alternatives to avoid bread spoilage (Valerio et al., 2009; Coda et al., 2013; Sun et al., 2020). This process, called biopreservation, consists of using endogenous lactic acid bacteria (LAB) and yeasts isolated from natural sourdoughs, which possess the ability to produce compounds (mainly organic acids) with fungistatic or fungicidal properties (Stefanello et al., 2019a). Lactic, acetic, formic, phenyllactic, and citric acids comprise the primary acids produced by these microorganisms during fermentation (Valerio et al., 2009; Axel et al., 2015; Rizzello et al., 2011).

Although the studies using microorganisms isolated from natural sourdoughs demonstrate satisfactory results on in vitro tests, reports demonstrating the effectiveness of these microorganisms in natural (in situ) food matrices, such as bakery products, are less frequent. Therefore, this study aimed at ascertaining the impact of the individual LAB *Limosilactobacillus fermentum* IAL 4541 and yeast *Wickerhamomyces anomalus* IAL 4533 on the growth inhibition of spoilage fungi isolated from bakery products as a means towards the extension of the shelf-life of conventional and multigrain bread and panettones.

2. Material and methods

2.1. Sourdough microorganisms

Limosilactobacillus fermentum IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 strains were chosen as both were isolated from a dried sourdough (Stefanello et al., 2018) and deposited at Núcleo de Coleção de Microorganismos (NCMO) of Instituto Adolfo Lutz, São Paulo, Brazil.

The activation of *Li. fermentum* IAL 4541 was performed by the resuspension of the lyophilized strain in 3 mL of de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany), followed by shaking for 30 s in a vortex mixer and incubation at 36 °C for 24 h under microaerophilic conditions (candle jar, about 3–5 % CO_2 and 8–10 % O_2). An aliquot of 100 μL of *Li. fermentum* was transferred to Petri dishes containing MRS agar using the spread-plate technique. The plates were incubated at 36 °C for 48 h under microaerophilic conditions (candle jar, about 3–5 % CO_2 and 8–10 % O_2). The activation of the *W. anomalus* IAL 4533 strain was done by the subculture of cells in a GYP (glucose 20 g/L; yeast extract 5 g/L; peptone 10 g/L; agar 20 g/L) plate and incubated for 48 h at 30 °C under aerobic conditions. Both microbial cultures grown on plates were scraped with a sterile inoculation loop and resuspended to a sterile flask with 3 mL of sterile 0.1 % peptone solution (Merck, Germany).

For inoculum obtention, 1 mL of each peptone suspension of *Li. fermentum* IAL 4541 and *W. anomalus* IAL 4533 were singly transferred into Erlenmeyer flasks containing 100 mL of YM or MRS broth and incubated for 24 h at 25 and 30 °C, respectively (Paramithiotis et al., 2005). Twelve-hour cells were harvested (5000 x g, 15 min, 4 °C), washed twice with distilled sterile water, and resuspended in distilled water to an optical density at 620 nm corresponding to approximately 10^{10} colony-forming unit (CFU) per mL of *Li. fermentum* IAL 4541 and 10^8 CFU/mL of *W. anomalus* IAL 4533. These inoculums were used as microbial starter cultures in sourdough preparation.

2.2. Sourdough preparation

A seven-stage technique derived from a traditional procedure (Paramithiotis et al., 2005) and adjusted to this experiment was applied to prepare the sourdough. Dough made with each microorganism (*L. fermentum* or *W. anomallus*) was prepared by mixing 50 mL of each microbial starter culture with 100 g of wheat flour (Renata®, Selmi, Brazil). After 24 h fermentation at 28 °C, the first sourdough was formed. It was followed by successive mixing and fermentation until the final sourdough on day 7 (168 h).

2.3. Bread and panettone ingredients and breadmaking process

Three products were produced using the same equipment: conventional bread, multigrain bread, and panettones. Four different formulations were tested for each product, the F1 composed of *Li. fermentum* IAL 4541 and F2 by *W. anomalus* IAL 4533. Also, formulations 3 and 4 were composed by control trials of commercial yeast *Saccharomyces cerevisiae* with calcium propionate (CP) (0.3 % w/w) as a preservative (positive control, PC) and in the absence of the artificial preservative (negative control, NC).

Loaves of bread were prepared using the formulation and straight-dough method according to the AACCI method 10–10.03 with modifications (AACCI, 2010). For each formulation, 1 kg of wheat flour and the weight of other ingredients was calculated based on % flour weight (base flour, b.f.), with 2 % refined sugar, 3 % shortening, 1.5 % salt, 1 % dry yeast or 30 % sourdough and water addition until optimal absorption. The dough was then mixed in a spiral mixer (A-30, Progresso, Brazil) for 8 min (7 min at low speed, 1 min at high-speed), divided into pieces of 220 g, molded, placed in pans [(18 × 8.0 × 4.5) cm], proofed at 36 °C and 75–85 % relative humidity (RH) in a proofer (Super Freezer, Brazil) for 1 h (for positive and negative control) and 2 h (for F1 and F2). After this, the fermented doughs were baked at 220 °C for 15 min using an electric convection oven (Vipinho 0448, Curitiba, Brazil). The loaves were cooled to room temperature under sterile conditions for approximately 60 min. After cooling the bread, they were packed in plastic polypropylene packages and closed carefully to minimize cross-contamination.

Multigrain bread loaves were also produced for these experiments. The breadmaking process was the same as that of conventional bread. The difference consisted of adding a mix of 15 % grains (sesame, oat, and linseed) in the formulation (b.f.).

The panettone formulation used and processing parameters employed were the same as previously described using the sponge and dough method (Stefanello et al., 2019b). For panettones F1 and F2, the sponge was done by kneading the ingredients for 7 min (5 min at low speed, 2 min at high speed), proofing at 26 °C and 75–85 % RH for 16 h (overnight). For the treatments with commercial yeast (PC and NC), the proofing time was shortened to 15 min. Afterward, the ingredients of the dough and the fermented sponge were mixed for 7 min (5 min at low speed, 2 min at high speed), rested for 10 min, and portioned into 250 g. Then, this amount was placed into paper forms following fermentation

for 5 h at 36 °C and 75–85 % RH. The proofing time was 5 h for panettones F1 and F2 and 1 h for PC and NC, which was the time required for the doughs to reach the top of the form. The panettones were baked at 180 °C for 35 min in an electric convection oven (Vipinho 0448, Perfecta, Brazil), cooled down, and then packed in polypropylene packages, following storage until further analysis.

2.4. Fungal strains and inoculum preparation

A mix of three different mold strains from the same species was used in the experiments: *Penicillium roqueforti* (PR06, PR11, PR67) isolated from moldy bread, *Aspergillus chevalieri* (formerly *Eurotium chevalieri*) (AC32, AC33, AC35) and *Aspergillus montevidensis* (formerly *Eurotium amstelodami*) (AM53, AM55, AM57) isolated from moldy panettones.

P. roqueforti strains were grown in MEA (Malt Extract Agar, Merck, Germany) for 7 days at 25 °C under aerobic conditions, and the strains of *A. chevalieri* and *A. montevidensis* in CY20S (Czapek Yeast Extract Agar with 20 % Sucrose) for 14 days at 25 °C, according to the methodology described by Pitt and Hocking (2009). For the obtention of the inoculum, after the incubation period, the conidial suspensions were prepared by adding 10 mL of a sterile solution of Tween 80 at a concentration of 0.01 % (v/v) in each tube previously inoculated, then scraping the surface with a sterile loop, shaking in the vortex and filtering with sterile gauze to remove hyphae and conidiophores. Conidia suspensions were adjusted using a Neubauer chamber to 10^5 conidia/mL (Stratford et al., 2009).

2.5. Influence of sourdoughs on the growth of spoilage species

After baking and cooling, baking products were inoculated aseptically on their surfaces (breads and panettone). To that purpose, a drop (10 μL) of the suspension mix (10^5 conidia/mL) of each fungal species was inoculated separately on the surface of the products, thus resulting in the inoculation of approximately 10^3 conidia per sample. Afterward, bread and panettones were packed in low-density polyethylene packaging and stored at controlled room temperature (25 °C) during the entire experiment.

Thirty-six experimental conditions were performed, as follows: 3 types of food products (conventional bread, multigrain bread, and panettone); 4 formulations {negative control, positive control [with calcium propionate], F1 [*Li. fermentum*] and F2 [*W. anomallus*]}; and 3 fungal species (*P. roqueforti*, *A. chevalieri* and *A. montevidensis*). Four repetitions were performed independently per experimental condition.

The influence of sourdoughs on the growth of the inoculated fungal spoilage species was verified by assessing the time (in days) required to observe the first visible colony (t_{obs}) for each single product. As soon the colony started to appear on the visible eye (>3 mm), the colony diameter (mm) was measured with the aid of a universal caliper (Graduation: 0.02 mm, Accuracy: 0.01 mm) (MTX, Brazil), as a function of time (three times per day).

2.6. Modeling of fungal growth

The Baranyi and Roberts (1994) growth model, adapted to describe fungal growth (Eqs. (1) and (2)) (Garcia et al., 2009; Marín et al., 2009), was fitted to colony diameter data as a function of time, using the add-in Excel package DMFit. This model assumes that the fungal colony grows constantly after a lag until reaching a maximum diameter (Panagou et al., 2010).

$$Y(t) = \mu_{max}A(t) - ln\left[1 + \frac{exp(\mu_{max}\mathrm{A(t)}) - 1}{exp(Y_{max})}\right] \quad (1)$$

$$A(t) = t + \left(\frac{1}{\mu_{max}}\right) ln[exp(-\mu_{max}t) + exp(-\mu_{max}\lambda) - exp(-\mu_{max}t - \mu_{max}\lambda)]\Big) \quad (2)$$

where: t: time of storage [day]; Y(t): diameter of the colony as a function of time [mm]; μ_{max}: maximum growth rate [mm/day]; λ: duration of lag phase [day]; Y_{max}: maximum diameter of the colony [mm].

For conditions where the colony reached its maximum diameter, the n and m curvature fitting parameters in DMFit software were fixed as 0 and 2, respectively. For the other conditions, n = m = 0. Mycelial growth rate (μ) was determined for each experimental condition's single replicate ($n = 4$).

2.7. Statistical analysis

For each of the matrices under study (conventional bread, multigrain bread, and panettone), it was evaluated whether the formulations containing microorganisms from sourdough (F1 and F2) compared to the control formulations (PC and NC) were capable of affecting the μ_{max} and t_{obs} parameters of three spoilage fungi. To this end, a study with a complete factorial design was conducted, resulting in 36 different trials and carried out in quadruplicate. For μ_{max} and t_{obs} data from each matrix × fungi combination, one-way analysis of variance (ANOVA) followed by a mean comparison test (Schott-Knott) was performed to assess the existence of statistically significant differences ($p < 0.05$) from the four formulations (F1, F2, PC and NC). The Sisvar software (version 5.6, 2015, Brazil) was used for all performed statistical analyses (Ferreira, 2011).

3. Results

Fig. 1 shows the time required to observe the three fungal species' first visible colony (t_{obs}) on the baked suitable matrices. In general, on conventional and multigrain bread (Fig. 1A and B, respectively), F1 (*Li. fermentum*) and F2 (*W. anomallus*) showed similar results when compared to negative control (NC) in delaying the fungal growth of the three tested species and regarding the inhibition of *P. roqueforti* and *A. montevidensis*, F1 and F2 significantly increased t_{obs} compared to NC ($p < 0.05$). However, such values are significantly lower than those promoted by PC. For *A. chevalieri*, however, none of the two tested formulations (F1 and F2) were able to prolong t_{obs} against NC ($p > 0.05$).

Regarding the effect of treatments on panettone (Fig. 1C), it can be observed that for *A. chevalieri*, F1 resulted in a significant increase ($p < 0.05$) of t_{obs} in 13 days compared to NC, while for *A. montevidensis*, both F1 and F2 were able ($p < 0.05$) to delay the appearance of the mold compared to NC. However, PC presented significantly higher tobs for both fungi than all other formulations. For *P. roqueforti*, no formulation tested, including PC, resulted in an increase in tobs compared to NC.

Comparing the results presented by the three fungal species on conventional bread with each other, the treatments with calcium propionate (positive control, PC) showed the best results on the growth control of *A. chevalieri* and *A. montevidensis*. This preservative could avoid the fungal growth of these species for up to 30 days. On the other hand, *P. roqueforti* growth was observed from the ^{seventh} day of the experiment. For F1, F2, and NC, the highest t_{obs} values were presented by *A. montevidensis*. Very similar results were observed on the multigrain bread. On panettones, PC still presented the best results to avoid the fungal growth for the *Aspergillus* spp. However, *P. roqueforti* could still grow on the 17th day of the experiment. Furthermore, for the F1 and F2 treatments, the highest t_{obs} values were presented by *A. chevalieri*.

Fig. 2 shows the means of mold species' colony diameters (mm) plotted against the time (days). The growth rate was estimated after fitting the Baranyi and Robert model to the data. This model was satisfactorily fitted to the growth rate of the tested fungal species, as most of the R^2 values obtained were above 0.95 (Table 1). This model is usually used for the evaluation of fungi growth in different studies and is also the most suitable for the evaluation of fungi growth (Garcia et al., 2009; Baranyi et al., 2014; Santos et al., 2017).

According to Fig. 2A, the maximum diameter size on conventional

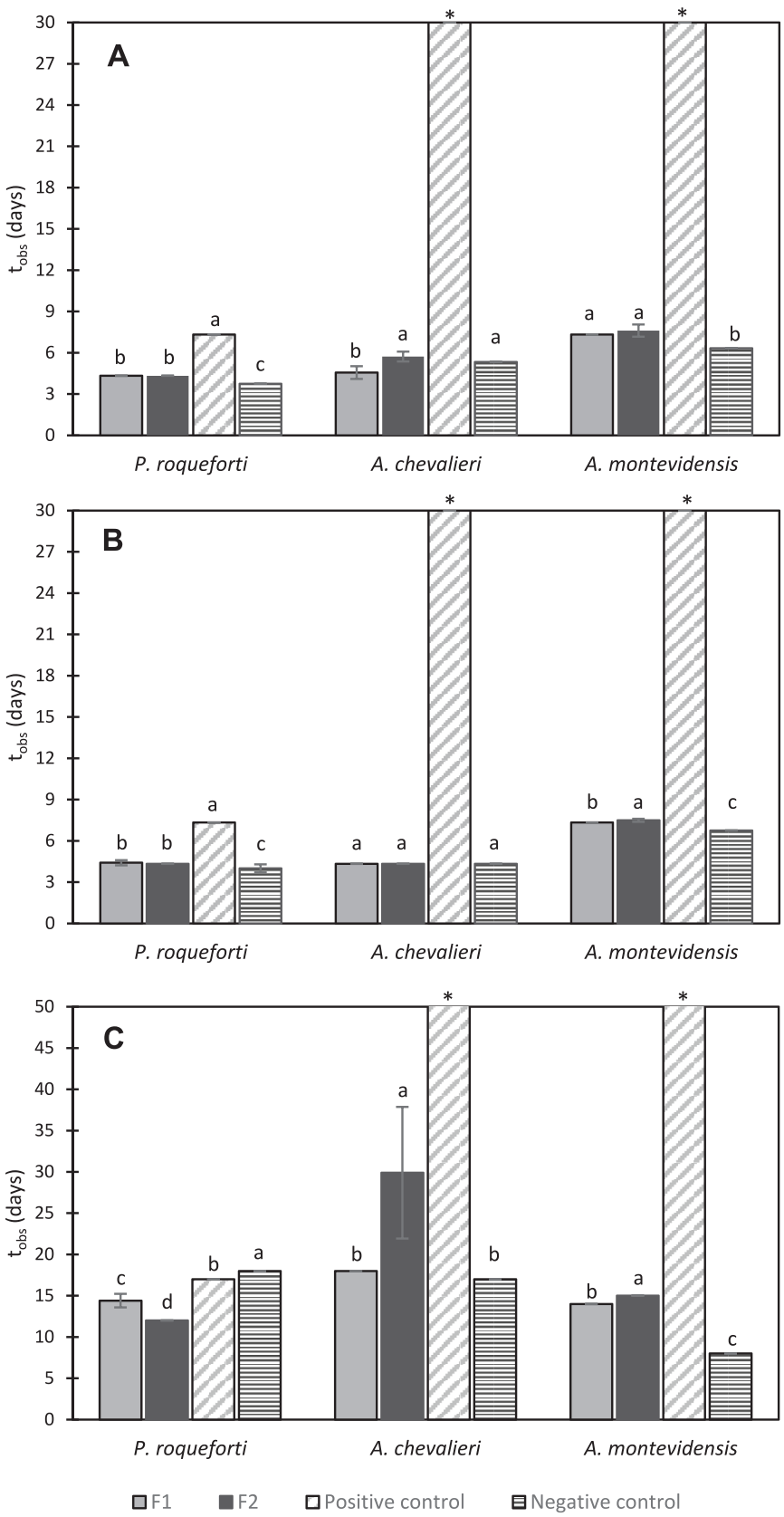

Fig. 1. Time required for the observance of the first fungal colony (t_{obs}) of *Penicillium roqueforti*, *Aspergillus montevidensis* and *Aspergillus chevalieri* on conventional bread (A), multigrain loaves of bread (B) and panettones (C) made with L. *fermentum* IAL 4541 (F1), *W. anomalus* IAL 4533 (F2), calcium propionate (positive control) or without preservative (negative control). The standard deviation is represented by error bars. Different lowercase letters for the same mold indicate significant differences from formulations according to the Scott-Knott test ($p < 0.05$). *No fungal growth was observed during the whole experiment time, i.e., $t_{obs} > 100$ days (for panettones) and $t_{obs} > 30$ days (for breads).

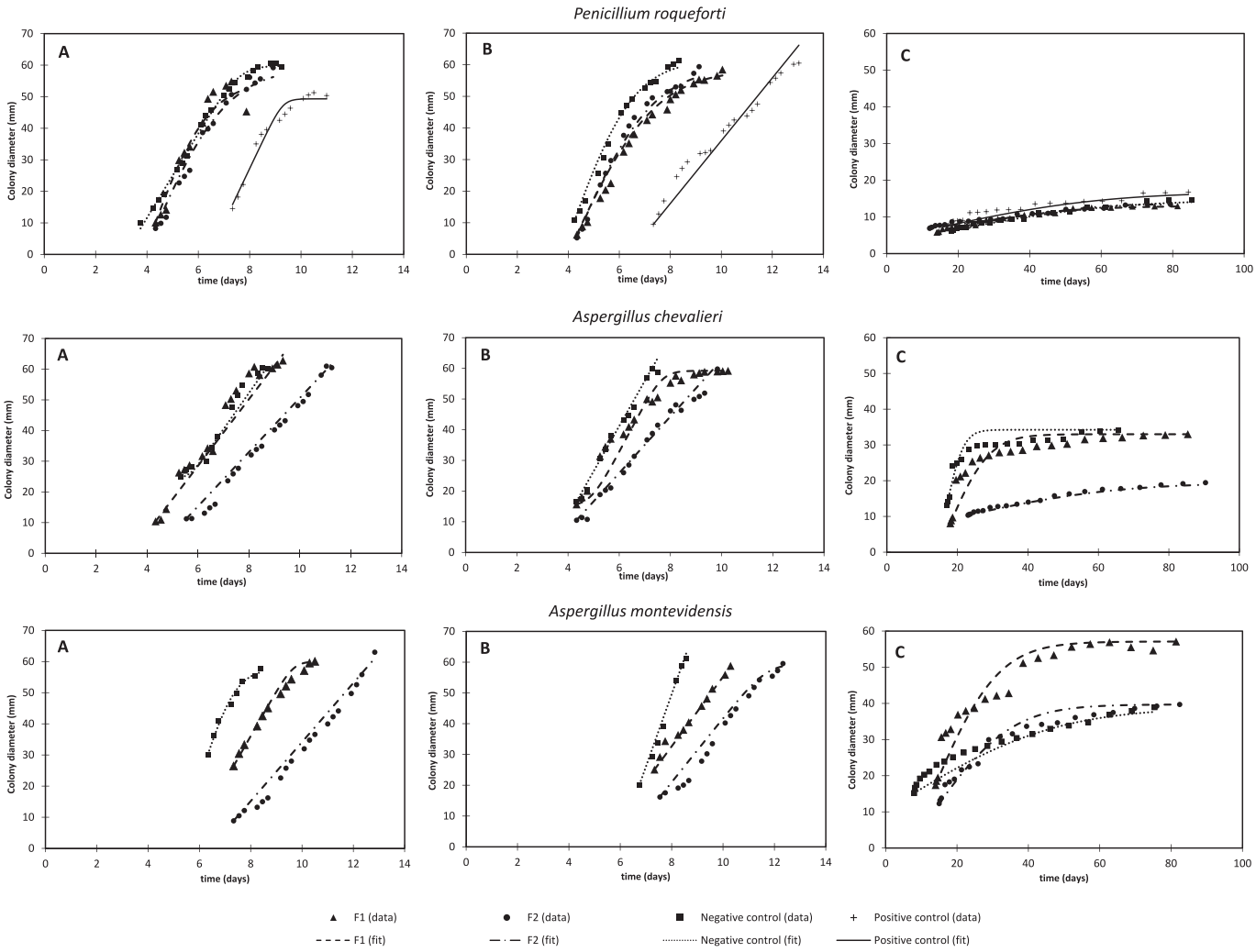

Fig. 2. Mycelial growth curves of *Penicillium roqueforti*, *Aspergillus montevidensis* and *Aspergillus chevalieri* on conventional bread (A), multigrain bread (B), and panettones (C) made with *L. fermentum* IAL 4541 (F1), *W. anomalus* IAL 4533 (F2), calcium propionate (positive control) or without preservative (negative control). Symbols represent the mean value of colony diameter data from four replicates, and lines represent the fitted Baranyi and Roberts model.

Table 1
Mycelial growth rate (μ_{max})[1] of three spoilage fungi on different bakery products made with or without microorganisms isolated from sourdough.

Fungi	Formulation[2]	Bakery product					
		Conventional bread		Multigrain bread		Panettone	
		μ_{max} (mm/day)	R^2	μ_{max} (mm/day)	R^2	μ_{max} (mm/day)	R^2
P. roqueforti	NC	15.1 ± 2.2^{a}	0.99	26.1 ± 3.1^{a}	0.98	0.2 ± 0.0^{a}	0.98
	PC	19.4 ± 3.1^{a}	0.96	9.9 ± 1.8^{c}	0.95	0.3 ± 0.1^{a}	0.99
	F1	25.6 ± 5.5^{a}	0.95	20.4 ± 0.8^{b}	0.98	0.3 ± 0.1^{a}	0.98
	F2	22.0 ± 4.2^{a}	0.98	22.2 ± 1.4^{b}	0.99	0.1 ± 0.0^{b}	0.96
A. chevalieri	NC	11.8 ± 1.0^{a}	0.96	14.7 ± 1.5^{a}	0.98	5.3 ± 1.2^{a}	0.84
	PC[3]	–	–	–	–	–	–
	F1	10.9 ± 1.0^{a}	0.93	15.9 ± 2.3^{a}	0.96	2.7 ± 0.6^{b}	0.83
	F2	8.8 ± 0.5^{b}	0.98	9.2 ± 0.6^{b}	0.98	0.3 ± 0.1^{c}	0.98
A. montevidensis	NC	28.3 ± 1.8^{a}	0.98	23.5 ± 0.5^{a}	0.98	0.7 ± 0.1^{a}	0.90
	PC[3]	–	–	–	–	–	–
	F1	14.4 ± 3.5^{b}	0.97	11.3 ± 1.3^{b}	0.98	2.7 ± 1.5^{a}	0.91
	F2	9.5 ± 0.1^{c}	0.97	10.4 ± 0.7^{b}	0.95	1.6 ± 0.3^{a}	0.96

[1] Values represent mean ± standard deviation of the growth rate for each condition. Different lowercase letters in the same column for the same mold indicate significant differences in the kinetic parameter according to the Scott-Knott test ($p < 0.05$).

[2] NC: negative control (formulation with commercial yeast and without preservative); PC: positive control (formulation with commercial yeast and calcium propionate 0.3 % w/w); F1: formulation with *Li. fermentum* IAL 4541; F2: formulation with *W. anomalus* IAL 4533.

[3] No fungal growth was observed during the whole experiment time (100 days for panettones or 30 days for breads).

bread were 60.6 mm for *P. roqueforti* (NC), 62.8 mm for *A. chevalieri* (F1), and 63 mm for *A. montevidensis* (F2). On multigrain bread (Fig. 2B), these values were 61.5 mm for *P. roqueforti* (NC), 59.9 mm for *A. chevalieri* (NC) and 61.1 mm for *A. montevidensis* (NC). Finally, on panettones (Fig. 2C), the maximum diameter size observed for *P. roqueforti* and *A. chevalieri* on the PC and NC treatment was 16.8 mm and 34.2 mm, respectively. The F1 treatment showed the highest value for *A. montevidenses* (55.6 mm).

For conventional bread, *A. chevalieri* and *A. montevidensis* showed the lowest growth rate ($p < 0.05$) on F2 treatment, respectively, 8.8 and 9.5 mm/day, compared to the NC. On the other hand, these fungi could not grow in PC treatment (Table 1). Although *P. roqueforti* was able to grow in all the treatments, no statistical differences were observed in the growth rate of this fungus on conventional bread.

On multigrain bread, *Aspergillus* spp. did not show colonies on PC, while on F2, this fungus presented the lowest growth-rate ($p < 0.05$) on F2. However, *P. roqueforti* also showed a significantly low growth rate on PC (9.9 mm/day).

On panettones, the lowest growth rate values were observed on F2 treatment for *P. roqueforti* and *A. chevalieri* (0.1 and 0.3 mm/day, respectively, $p < 0.05$), although *A. chevalieri* did not grow on PC. No statistical differences were observed between treatments for *A. montevidensis*.

4. Discussion

In recent years, studies evaluating lactic acid bacteria (LAB) and yeasts and their ability to produce a range of active compounds that can be responsible for inhibiting fungal growth have been highlighted (Rizzello et al., 2011; Coda et al., 2013; Cortés-Zavaleta et al., 2014; Hong et al., 2017; Sun et al., 2020). This activity results from an effect between the low pH due to the production of organic acids (lactic and acetic acids and others) and other antifungal metabolites produced by these microorganisms (Axel et al., 2015; Coda et al., 2011).

The antifungal effects of *Li. fermentum* in in vitro studies were controversial. For example, the capacity to inhibit the mycelium growth of *Fusarium verticillioides* by *Li. fermentum* strains but, conversely, were not effective in avoiding the growth of *Penicillium* spp. under the same conditions (Kharazian et al., 2017). On the other hand, Gerez et al. (2013) related that *Li. fermentum* CRL 251 was able to produce fungal inhibitory peptides, and it was able to retard above 80 % the growth of *Aspergillus niger*, *Penicillium* spp., and *Fusarium graminearum*. In our study, this LAB was not a promising agent for extending the shelf-life of the studied baked products, as it was the least efficient in avoiding the growth of the tested strains in both types of bread and panettones. *Li. fermentum* is known as responsible for producing a range of different organic acids, such as lactic, acetic, and phenyllactic acids (Gerez et al., 2013; Kharazian et al., 2017), Cyclo (L-Tyr-L Pro), phenylpyruvic, caffeoylhexose-deoxyhexoside, 3,5-Di-O-caffeoylquinic acid (Yépez et al., 2017). This last one is known not to have antifungal properties as far as we know. Lactic acid is the main compound produced by this LAB, but in small quantities, as Yépez et al. (2017) observed. When compared to other organic acids, lactic acid showed a lower antifungal in vitro effect against *Penicillium nordicum* and ochratoxin A (OTA) production, in comparison to acetic acid (Guimarães et al., 2018). Indeed, fungicidal traits are first attributed to acetic acid rather than lactic acid (Novotni et al., 2021; Rocha and Malcata, 2016). Also, a higher concentration of this acid was required (548 mM) to inhibit the growth of the yeast *S. cerevisiae*, 40 % more than the necessary acetic acid (Thomas et al., 2002).

Although the Baranyi and Roberts model, adopted in this study, was initially developed to describe the bacterial population growth, it has proved to be one of the best alternatives to satisfactorily describe the bidimensional mycelial growth of fungi (Baranyi et al., 2014; Garcia et al., 2009). However, some considerations need to be made. When a single fungal spore finds a favorable environment, it must pass through some developmental stages, such as germination, hyphal elongation, and mycelium elongation, to become a visible colony. It is assumed that in the hyphal elongation phase, the radial growth of the colony, not yet visible, is exponential. In contrast, in the mycelium elongation phase, this growth becomes a linear function of time. In this third stage, after some time, the colony reaches a minimum diameter, around 3 mm, to be visually observed (t_{obs}) (Dagnas et al., 2015). Thus, the duration of the lag phase parameter (λ), identified by the Baranyi and Roberts model, is a virtual value obtained by estimating the intersection of the linear region of the graph with the time axis in the growth curve. Therefore, this parameter corresponds to the germination period plus a period of the hyphal elongation stage. In turn, the maximum growth rate parameter (μ_{max}) will express the linear speed with which the colony size increases during the mycelium elongation phase. In this stage, if the environment is favorable, the colony tends to grow indefinitely or until it encounters some physical obstacle. In this way, the Y_{max} parameter is often not associated with an intrinsic behavior of the microorganism under analysis (Garcia et al., 2009; Gougouli and Koutsoumanis, 2013).

Considering these issues, the main kinetic parameter used in this work to compare the different treatments was μ_{max}. Based on this parameter, it was possible to evaluate which formulations promoted a delay in the mycelial growth rate of the deteriorating fungi under study, which directly impacted t_{obs}. The geometric difference between the analyzed samples and other factors discussed below meant that only some curves had a higher asymptote (Fig. 2), with the establishment of a supposed Y_{max}. In addition, such factors contributed to the initial size of visible colonies varying between treatments and replicates. Therefore, in this study, it was decided to fit the growth curves, disregarding the lag phase of the model ($n = 0$).

Since the present study evaluated the behavior and growth rate of different microorganisms on the surface of different bakery products, some experimental points must be considered. For example, as breads and panettones have different sizes, it could be challenging to compare the growth of the microorganisms on their surfaces. Also, the problematic uniform spreading of the drop containing the spores of the microorganism on the surface of the products on the surface of the products. All these parameters must be considered when comparing the D_{max} observed in our studies, not just the behavior of the microorganism itself. Therefore, there are better ways to compare the effect of each formulation on fungal development than this parameter.

The time required for the observance of the first fungal colony (t_{obs}) is an important parameter, as from a consumer's point of view, the first visible sight of mold growth on bakery products is a critical point of shelf-life evaluation (Axel et al., 2015). The t_{obs} observed in this study were higher on panettones than both types of bread (Fig. 1). It could be related to the pH of these products. The pH of bread is usually around 5.5 (Moro et al., 2022), while the pH of panettones is slightly more acidic (5.0) (Stefanello et al., 2019b). It could explain the higher efficacy of the LAB, yeast, and CP on the panettone assays. Stratford et al. (2009) mentioned that preservatives, such as organic acids, have increased efficacy in lower pH values. For example, Guynot et al. (2005) observed that at pH 4.5, the concentration of potassium sorbate in bakery-based products could be reduced to some extent only at low water activity levels (a_w).

In contrast, at pH 5.5, fungal growths were observed even by adding 0.3 % of potassium sorbate. The spectrum of action and antimicrobial action of the organic acids produced by LAB is based on penetrating cell membranes in their undissociated form, leading to a drop in the intercellular pH and consequently disrupting metabolic activities (Brul and Coote, 1999). Since pH influenced the antifungal activity significantly, it was assumed that secreted organic acids were primarily responsible for the described antifungal effect.

CP is a fungistatic food-grade preservative used worldwide in different types of foods. In countries of the European Union (EU), the maximum concentration allowed is 0.3 % (w/w) (Valerio et al., 2009), while in Brazil, it is considered *Quantum satis* (Garcia et al., 2021). In our

study, CP (0.3 % w/w) was the most effective treatment, as it inhibited the growth of both *Aspergillus* species (Fig. 2). Similar results were obtained by Marin et al. (2003), where CP was more effective towards *Aspergillus niger* and *A. montevidensis* than it was against *Aspergillus pseudoglaucus* (formerly *Eurotium repens*) and *Aspergillus ruber* (formerly *Eurotium rubrum*). On the other hand, it could have been more effective in avoiding the growth of *P. roqueforti*. This species is known as one of Brazil's primary fungi responsible for spoiling bread (Garcia et al., 2009), and *P. roqueforti*'s resistance to food preservatives, such as CP, has been previously highlighted. Suhr and Nielsen (2004) observed that *P. roqueforti* isolates were stimulated at high a_w (0.97) and high propionate concentrations (0.3 %). However, it was observed that the stimulation of growth occurred relatively late during growth, as the lag phase was prolonged for approximately one week in the formulation with 0.3 % propionate. Similarities with our results were observed in the study of Ryan et al. (2008), where sourdough with *Lactiplantibacillus plantarum* and *Fructolactobacillus sanfranciscensis* were not able to avoid the outgrowth of *P. roqueforti* on bread. On the other hand, when this dough was combined with CP (0.3 % w/w), a remarkable increase in bread shelf-life was observed.

The species *W. anomalus* (formerly *Pichia anomala*) can grow under high osmotic pressure, low pH, and a broad range of temperatures (Fredlund et al., 2002). In this study, this species showed the second-best results on inhibition of the growth between the tested fungal strains. Its antifungal effects have been mainly related to producing the organic acid ethyl-acetate (Plata et al., 2003; Druvefors et al., 2005; Coda et al., 2011). Compared to *Saccharomyces cerevisiae* or other yeasts, the synthesis of ethyl acetate by *W. anomalus* occurs via an inverse esterase from acetate rather than from acetyl-coenzyme A (acetyl-CoA) via ethanol acetyltransferase. The capacity of *W. anomalus* to synthesize ethyl-acetate was considered part of a general stress response mechanism, which mainly prevents the intracellular accumulation of toxic acetic acid. *W. anomalus* exhibits a broad spectrum of antifungal activity, delaying the growth of several fungi, which contaminate foods and food-related environments (Coda et al., 2011; Masih et al., 2000; Stefanello et al., 2019a). It was observed that considerable levels of ethyl-acetate were synthesized by *W. anomalus* during breadmaking (Coda et al., 2013). In general, in our study, the treatment containing *W. anomallus* (F2), despite not presenting results as satisfactory as the PC, was able to inhibit mainly the growth of *A. montevidensis*, helping to increase the shelf-life of such baking products, especially panettone (Fig. 1C). An average of 7 days for conventional and multigrain bread and 15 days for panettones were observed in the treatments containing this yeast.

Regarding modifications caused by the combined use of LAB and yeast in bakery products, Coda et al. (2011) observed that bread manufactured with the combination of *Lp. plantarum* 1A7 and *W. anomalus* LCF1695 showed the most loaf volume and softness, and in sensory analysis, it was appreciated for its elasticity, color, and overall taste. Moreover, although the concentration of ethyl-acetate decreased in part during breadmaking under pilot plant conditions, this bread showed a prolonged shelf-life. In the study by Stefanello et al. (2019b), panettones manufactured with the combination of LAB and yeast maintained their softness better during storage when compared with the controls. However, regarding microbial stability, control panettones with and without preservatives became moldy faster. In contrast, sourdough panettones (with *W. anomallus* or *W. anomallus and Li. fermentum*) remained stable throughout all the monitored storage (>90 days).

Another factor that must be considered is the effect of abiotic factors on the production of antifungal compounds by LAB and yeasts. For example, Roy et al. (1996) studied the effect of incubation time and temperature on the production of antifungal compounds by *Lactococcus lactis* subsp. lactis, and they reported an optimum production at 30 °C after 48 h of incubation. Corsetti et al. (1998) also reported the maximum production of organic acids, with antifungal activity, by *Fr. sanfranciscensis* after 48 h of incubation at 30 °C, when the initial pH of the medium was 6.0.

Another study was found to evaluate the continuous production of organic acid by sourdough during storage. However, it is essential to highlight the importance of the storage temperature to maintain the shelf-life of baked goods. Garcia et al. (2021) observed that the antifungal effects of CP and potassium sorbate against spoilage of bakery products by the fungi *Hyphopichia burtonii* and *Paecilomyces variotii* was increased in lower pH (5.0) and lower temperatures (25 °C). On the other hand, *P. roqueforti* was inhibited to a greater extent at 30 °C and pH 5.5. In this study, the temperature of 25 °C was used in the experiments because that is the condition at which bakery products are usually marketed. Also, it is the optimum growth temperature for *Aspergillus* spp. and *Penicillium* tested (Pitt and Hocking, 2009).

5. Conclusions

The results obtained in this study can help to understand the behavior of spoilage fungi responsible for the spoilage of bakery products. This study observed that even though the use of microorganisms such as *W. anomallus* in the formulation of the products did not reach the effectiveness of traditional preservatives, this yeast can extend the shelf life of bakery products, mostly panettones. Finally, future studies could help elucidate if combining biopreservation with traditional preservatives can assist in developing more robust bakery product formulations with a longer shelf-life and fewer preservatives.

CRediT authorship contribution statement

Marcelo V. Garcia: Conceptualization, Methodology, Formal Analysis, Software, Validation, Visualization, Investigation, Data Curation, Resources, Writing - Original Draft, Writing - Review & Editing. **Raquel F. Stefanello:** Investigation, Methodology, Formal Analysis, Visualization. **Arthur K.R. Pia:** Investigation, Methodology, Formal Analysis, Visualization, Software, Resources, Writing - Original Draft, Writing - Review & Editing. **Jessica G. Lemos:** Investigation, Methodology, Formal Analysis, Visualization. **Elizabeth H. Nabeshima:** Investigation, Methodology, Formal Analysis, Visualization, Resources, Writing - Original Draft. **Elena Bartkiene:** Investigation, Methodology, Formal Analysis, Visualization. **João Miguel Rocha:** Investigation, Methodology, Formal Analysis, Visualization. **Marina V. Copetti:** Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **Anderson S. Sant'Ana:** Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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