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Yeast Mixtures for Postharvest Biocontrol of Diverse Fungal Rots on *Citrus limon* var Eureka

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Abstract: Mexico is among the most important citrus fruit producers in the world. However, during storage, several problems related to fungi can arise. The most common fungal postharvest diseases detected on *Citrus limon* var Eureka (Italian lime) produced in the Tamaulipas state are green/blue mold (*Penicillium* spp.), fusarium rot (*F. oxysporum*, *F. solani*, *F. proliferatum*, among others), and anthracnose (*Colletotrichum* spp.). In this work, we selected yeasts, occurring as the natural epiphytic mycoflora of lemons or from fermented traditional products, to be tested as part of a formulation for protecting stored lemons against fungal diseases. The best-performing yeasts, labeled as LCBG-03 (*Meyerozyma guilliermondii*), LCBG-30 (*Pseudozyma* sp.), and LCBG-49 (*Saccharomyces cerevisiae*), were selected to test their compatibility and biocontrol performance against strains of *Penicillium digitatum* (AL-38), *Fusarium* sp. (AL-21), *Colletotrichum gloeosporioides* (AL-13), and *Epicoccum sorghinum* (H3A). Based on their in vitro performance regarding the percentage of radial growth inhibition, both applied individually or as two yeasts mixed at equal cellular concentrations, the best combinations (containing *M. guilliermondii* formulated with either *Pseudozyma* sp. or *S. cerevisiae*) were selected with efficacies higher than 95% in both in vitro fungal radial growth rate inhibition and on stored lemon fruits. This work contributes to the search for compatible yeast combinations with the aim to diminish the fungal losses of citrus fruits using biocontrol for citrus postharvest protection.

Keywords: yeasts; antifungal formulation; Meyerozyma; Pseudozyma; *Saccharomyces cerevisiae*; Penicillium; Colletotrichum; Fusarium; Epicoccum

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1. Introduction

The most important postharvest diseases of *Citrus* species are anthracnose, sour rot, and green/blue mold caused by *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Penicillium digitatum*, and *P. italicum*, respectively [1,2]. Fungal diseases can be controlled by applying ortho-phenylphenate, imazalil, and thiabendazole, but pathogens develop resistance to these chemicals, which progressively diminishes their efficacy [3,4]. Synthetic chemicals can cause carcinogenicity, teratogenicity, and high and acute residual toxicity [5,6]. Biological control using microbial antagonists has received a great deal of attention as a promising alternative to chemicals. Yeast is a major component of the epiphytic microbial community on the surfaces of fruits and vegetables [1]. In *Citrus* spp., the most reported epiphytic yeasts are: *Candida famata*, *Candida oleophila*, *Candida saitona*, *Candida sake*, *Debaryomyces hansenii*, *Kloeckera apiculate*, *Metschnikowia pulcherrima*, *Metschnikowia fruticola*, and *Pichia guilliermondii* [7–12], which display antagonistic behavior toward filamentous fungi.

Yeasts have been extensively studied as promising biocontrol agents because of their simple nutritional requirements adapting to the fruit's environment; their ability to colonize dry surfaces for long periods of time; their high tolerance to a wide range of temperature, pH, and oxygen levels [12]; and their easy and rapid growth on bioreactors. Moreover, they do not produce allergenic spores, mycotoxins, or antibiotics, as many fungi or bacteria do [12]. As active ingredients, yeasts are a very convenient microorganism, as they can satisfactorily use a wide range of carbohydrates, which include disaccharides, monosaccharides, and nitrogen sources; hence, competition for space and nutrients is one of the main mechanisms of action of most postharvest microbial biocontrol agents. For effectiveness, the initial populations of microbial antagonists are usually applied at a concentration of 10^7–10^8 CFU/mL (Colony Forming Units) of formulation for controlling postharvest decay on fruits and vegetables [13]. While developing the mixed cultures of microbial antagonists, their compatibility should be considered. The combined use of biocontrol agents was suggested by [1] to increase the biocontrol product strategy's efficacy. Although several yeasts with antifungal properties have been successfully identified on fruits, few studies are available about mixed microorganisms' antagonistic activities, such as by Janisiewicz and Korsten [1], and for citrus fruits, such as by Panebianco et al. [14] using a combination of a bacteria (*Pseudomonas* sp.) and a filamentous fungus (*Trichoderma* sp.) for the biocontrol of *P. digitatum* on oranges in postharvest storage. In this work, we explored the use of combining different yeast genera in a formulation containing a lemon extract, first characterizing their biological compatibility as growth, and then testing them in vitro and on simulated postharvest storage conditions on Italian lemon fruit (*Citrus limon* var Eureka) to assess their potential as a postharvest biocontrol formulated product for this important crop.

2. Materials and Methods

2.1. Fungal Strains

The phytopathogenic fungal strains of *Colletotrichum gloeosporioides* (AL-13) [15], *Fusarium* sp. (AL-21), and *Penicillium digitatum* (AL-38) were isolated from infected lemons in a packing facility from Tamaulipas (Mexico). The pathogenic strain *Epicoccum sorghinum* (H3A) was isolated from diseased *Agave tequilana* Weber var azul leaves [16] and was used for its highly broad phytopathogenic profile, as we tested it in detached lemon leaves where it was 100% infectious, which is probably due to its capability of producing tenuazonic acid, a potential herbicide [17]. All the strains belong to the Laboratory of Industrial Biotechnology fungal culture collection (LBI-CBG) and are preserved on glycerol at −70 °C (Table 1). The working cultures were prepared by inoculating a frozen loop from these preserved cryovials, streaking on potato dextrose agar (PDA, BD Bioxon, Becton Dickinson de Mexico, Ciudad de Mexico, Mexico) plates to visualize any contamination. The individual colonies were picked to be grown on a fresh PDA plate at 29 °C in the dark for five to eight days depending on the fungus. The produced spores were harvested by adding 5 mL of sterile saline solution, liberating the spores using a sterile glass rod, and collecting the liquid with the spores in a clean vial. The solutions with final spore concentrations of 1×10^5 spores/mL were prepared as the working inoculum.

2.2. Yeast Strains

The yeast strains used in this work were from *C. limon* var Eureka epiphytic mycoflora (*Meyerozyma guilliermondii* (LCBG-03), *Macalpinomyces* sp. (LCBG-27), and *Pseudozyma* sp. (LCBG-30)) and from agave mezcal must (*Saccharomyces cerevisiae* Sc3D6 (LCBG-49)) [18]. All belong to the LBI-CBG fungal culture collection (CBG-IPN) and were preserved on glycerol at −70 °C (Table 1). For the inoculum preparation, the strain was streaked on PDA (BD Bioxon, Becton Dickinson de Mexico, Ciudad de Mexico, Mexico) to verify its purity, and, after 24 h of growth, a random colony was taken and propagated on 200 mL of yeast extract, peptone, and dextrose broth (YPD, BD Bioxon, Becton Dickinson de Mexico, Ciudad de Mexico, Mexico), incubating for 18 h at 29 °C and 200 rpm, and used as inoculum in the experiments.

Table 1. Microorganisms used in this work, belonging to the Laboratory of Industrial Biotechnology-Center for Genomic Biotechnology (LBI-CBG) fungal collection, indicating their vegetable tissue of isolation.

Code	Accession Number	Identity	Tissue of Isolation
Fungal phytopathogens			
AL-13	KC341958.1	*Colletotrichum gloeosporioides*	Pericarp and flowers of *Citrus limon* var Eureka
AL_21	KC341966.1	*Fusarium* sp.	
AL-38	KC341982.1	*Penicillium digitatum*	
H3A	MK041914.1	*Epicoccum sorghinum H11_1*	Surface of *Agave tequilana* leaf
Biocontrol yeasts			
LCBG-03	HM991450.1	*Meyerozyma guilliermondii*	Pericarp of *Citrus limon* var Eureka
LCBG-27	OQ850308	*Macalpinomyces* sp.	
LCBG-30	OQ850309	*Pseudozyma* sp.	
LCBG-49 (Sc3D6)	JQ824876	*Saccharomyces cerevisiae*	Agave mezcal must

2.3. Compatibility among Yeasts

To assess the initial viability and whether the physical contact of two different yeasts induced competition among them, which would cause a drop in the whole population viability, the mixtures were prepared from freshly harvested samples from the YPD individual cultures at 18 h of growth. The whole cell population for each yeast was calculated from the Neubauer chamber data, and the yeasts were mixed 1:1 to a total initial concentration of 1×10^8 yeasts/mL. The mixtures were allowed to interact for one hour without mixing; then, the viable cell concentrations were briefly quantified using the micro-drop technique [18], and 10 µL of the appropriate dilution of the yeast mixture (and individual samples, for comparison) was carefully placed as drops in 50% PDA (BD Bioxon, Becton Dickinson de Mexico, Ciudad de Mexico, Mexico) plates and incubated for 24 h at 29 °C. The colony counts were recorded, and the concentration of colony forming units (CFU/mL) were calculated from six different drops.

2.4. Liquid Formulation Preparation

The yeast obtained from the YPD broth cultures after 24 h had a population concentration of approximately from 1 to 3×10^9 yeasts/mL, depending on the strain evaluated. The cell pellet was obtained by centrifuging the broths at 3000 rpm for 10 min at 4 °C and resuspending the yeast pellet in a small volume of distilled sterile water. The flavedo (lemon peel, 70 g/L) base formulation was supplemented by adding 0.02% ascorbic acid and 2.5% galactose, as stress protectants. The yeast was added to an initial concentration of 1×10^8 CFU/mL in the formulation. All formulations were stored at 8 °C to evaluate their shelf life.

2.5. Biocontrol Effect of the Formulations Tested In Vitro

The formulations prepared with either individual or yeast combinations were tested in vitro periodically to assess the shelf life for the biocontrol activity against fungi AL-13, AL-21, AL-38, and positive control H3A (*C. gloeosporioides*, *Fusarium* sp., *P. digitatum*, and *E. sorghinum*, respectively). At an initial inoculum concentration of 10^8 cells/mL, 100 µL aliquots of each tested yeast formulation were evenly distributed on plates of 50% PDA using a glass rod. A fungal agar plug of 5 mm diameter, obtained from the edge of a 7-day colony of each of the tested fungi grown on PDA, was placed in the center of the plate with mycelia facing the agar surface and incubated at 29 °C. The colony radius was recorded periodically for 5 days, and the radial growth rates of the fungi were calculated as µm/h and compared with the fungal controls' rates without formulation to calculate the radial growth rate inhibition percentage. Each treatment was replicated at least three times. The formulations' shelf life was tested from day 1 to 180 to assess the cells' viabilities and their biocontrol effect against each fungus.

2.6. Biocontrol Performance of the Formulations on Fruits

The mature lemons (used to accelerate infection process) were surface-disinfested with a 1% solution of commercial bleach (sodium hypochlorite) for ten minutes and then drained and air-dried for 4 h prior to wounding. The formulation suspensions were sprayed on to the respective fruit surfaces and allowed to dry for one hour. Six fruits per tray were placed on sanitized plastic trays. For fungi inoculation, the fruits were wounded at five equatorial equidistant points; then, 5 µL of a suspension of spores of the tested fungus was placed on each wound, and the trays were stored at 24 ± 1 °C and 85% relative humidity. The disease incidence was evaluated by counting the number of infected wound spots and decayed wounds over 10 days of storage. Each treatment consisted of 5 trays, and sterile water and imazalil (0.4 g/L) treatments were used as controls. The results were displayed as a heat map, drawn using R software (R-2.15.3-win), and the normalization method was performed using a scale package.

2.7. Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted followed by a Fisher's Least Significant Difference test to separate means ($p \leq 0.05$) using the Analyze-it software for Microsoft Excel (version 2.20) and the JMP routine of the SAS software for ANOVA analysis.

3. Results

3.1. Compatibility of Yeasts

The yeasts' compatibilities were tested in all combinations by evaluating the mixtures' viabilities and comparing with the population values attained by the individual cultures. The test indicated (Figure 1) that yeasts LCBG-03 (*M. guilliermondii*), LCBG-30 (*Pseudozyma* sp.), and LCBG-49 (*S. cerevisiae* strain 3D6) are compatible between each other, as the population counts increased after one hour of contact, indicating that the yeasts continued growing normally after contact.

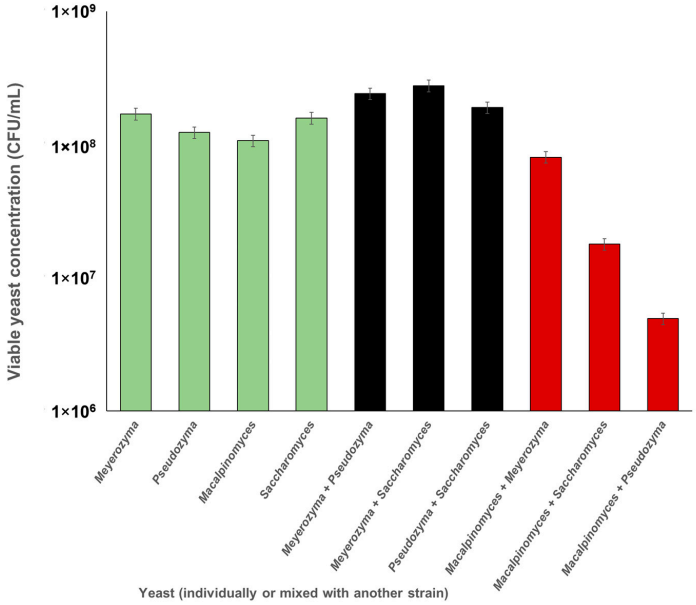

Figure 1. Compatibility tests shown as yeasts' viabilities on individual and mixed suspensions after one hour of contact. Red bars are individual strains referred just by their genera (*Meyerozyma guilliermondii* LCBG-03, *Pseudozyma* sp. LCBG-30, *Macalpinomyces* sp. LCBG-27, and *Saccharomyces cerevisiae* LCBG-49). Black bars indicate those compatible genera combinations, as they grew better than the individual strains; red bars indicate those genera combinations that were not compatible, specifically those including *Macalpinomyces* sp. Three replicates were analyzed for each combination, and the standard deviation was below 10% for all data.

In contrast, mixing any of these yeasts with LCBG-27 (*Macalpinomyces* sp.) sharply decreased the viable yeast populations by approximately 50% (mixed with LCBG-03), 80% (mixed with L49), and 95% (mixed with LCBG-30), thus indicating the unfeasibility of using such mixtures for biocontrol purposes. Hence, yeasts LCBG-03, -30, and -49 were selected as active ingredients to be included in a liquid formulation, individually or in dual mixes; their biocontrol performances were tested along 6 months in vitro; and the viability was followed up to 24 months.

3.2. Biocontrol Effect of Formulations In Vitro

The yeast formulations were tested in half-diluted PDA against *C. gloeosporioides* (AL-13), *Fusarium* sp. (AL-21), *P. digitatum* (AL-38), and *E. sorghinum* (H3A), assessing the inhibition on their radial growth with storage time, up to 180 days (Table 2).

Table 2. Viability of the yeasts in the formulations and in vitro biocontrol performance as percentage of radial growth rate inhibition for the four tested fungi throughout the storage time of the formulations at 8 °C.

Days of Storage of the Formulation	Yeasts Present on Formulation	Radial Growth Rate Inhibition (%)				Viable Yeast Cell Count
		C. gloeosporioides AL-13	*Fusarium* sp. AL-21	*P. digitatum* AL-38	*E. sorghinum* H3A	($\times 10^8$) CFU/mL
1	LCBG 03+30	93	92	48	38	2.68 ^{A}
	LCBG 03+49	93	86	56	39	2.42 ^{A}
	LCBG 30+49	80	84	34	58	1.97 ^{A}
	LCBG03	88	70	37	49	1.68 ^{B}
	LCBG30	79	64	32	47	1.43 ^{B}
	LCBG49	79	68	22	47	1.35 ^{B}
25	LCBG 03+30	93	93	48	50	2.77 ^{A}
	LCBG03+49	96	87	62	48	2.37 ^{A}
	LCBG30+49	81	81	43	64	1.93 ^{A}
	LCBG03	84	70	44	42	1.58 ^{B}
	LCBG30	88	73	50	42	1.37 ^{B}
	LCBG49	88	69	32	42	1.27 ^{B}
100	LCBG03+30	96	92	56	57	2.95 ^{A}
	LCBG03+49	96	89	56	54	2.45 ^{A}
	LCBG30+49	86	83	54	64	1.95 ^{A}
	LCBG03	89	73	47	48	1.65 ^{B}
	LCBG30	89	73	42	48	1.45 ^{B}
	LCBG49	89	73	47	48	1.33 ^{B}
180	LCBG03+30	94	94	57	61	2.98 ^{A}
	LCBG03+49	94	92	57	61	2.48 ^{A}
	LCBG30+49	88	84	54	72	1.98 ^{A}
	LCBG03	88	78	51	52	1.68 ^{B}
	LCBG30	88	78	51	52	1.48 ^{B}
	LCBG49	88	78	51	48	1.37 ^{B}

All percentage data show a maximum standard deviation of 3% or less, from six replicates. Different upper letters in the last column show significant differences according to ANOVA testing at a $p \leq 0.05$ (LSD).

The results show that *C. gloeosporioides* (AL-13) and *Fusarium* sp. (AL-21) were the more affected fungi in all the formulation treatments, with inhibition percentages higher than 75%, particularly *Fusarium* sp. (AL-21), which was more affected by the mixed formulations. For *C. gloeosporioides* (AL-13) and *Fusarium* sp. (AL-21), the two formulations of LCBG-03 mixed with both LCBG-30 and LCBG-49 resulted in the highest biocontrol effect, above 93% and 83%, respectively. The fungal strains of *Penicillium* and *Epicoccum* were the least affected on their radial growth rates; however, the formulation with LCBG-03 mixed with LCBG-49 was the most effective to lower the radial growth rate of fungus *P. digitatum* (AL-38) in about 55%, and the formulation with the mixed LCBG-30 mixed with LCBG-49 controlled fungus *E. sorghinum* (H3A) at approximately 60%. The viable yeasts' concentration and biocontrol activity remained approximately the same during the 180 days of storage for all the formulations (Table 2); hence, the next step was to test their activity on mature lemon fruits at simulated conditions of a packing facility.

3.3. Biocontrol Effect of Formulations on Fruits

The formulations containing individual or mixed yeast combinations were tested on mature wounded lemons and inoculated with spores of the phytopathogenic fungi (*C. gloeosporioides* (AL-13), *Fusarium* sp. (AL-21), *P. digitatum* (AL-38), and *E. sorghinum* (H3A)). The treatments' biocontrol performances were compared to three control treatments: formulation without yeast, sterile water without yeast, and imazalil solution, during 10 days of storage. In general, all the mixed formulations had a higher inhibition percentage than the single yeast ones. At 4 days of storage, the fruits showed some infection symptoms (Figure 2) such as mycelia and some sporulation, and, after 10 days of storage, the fruits just treated with water or with an imazalil solution were thoroughly covered by spores from the fungi, while those with the formulation, with and without yeasts, had various degrees of protection, as shown in Figure 2 (lower panel) for *Fusarium* sp. (AL-21) and *E. sorghinum* (H3A) as examples.

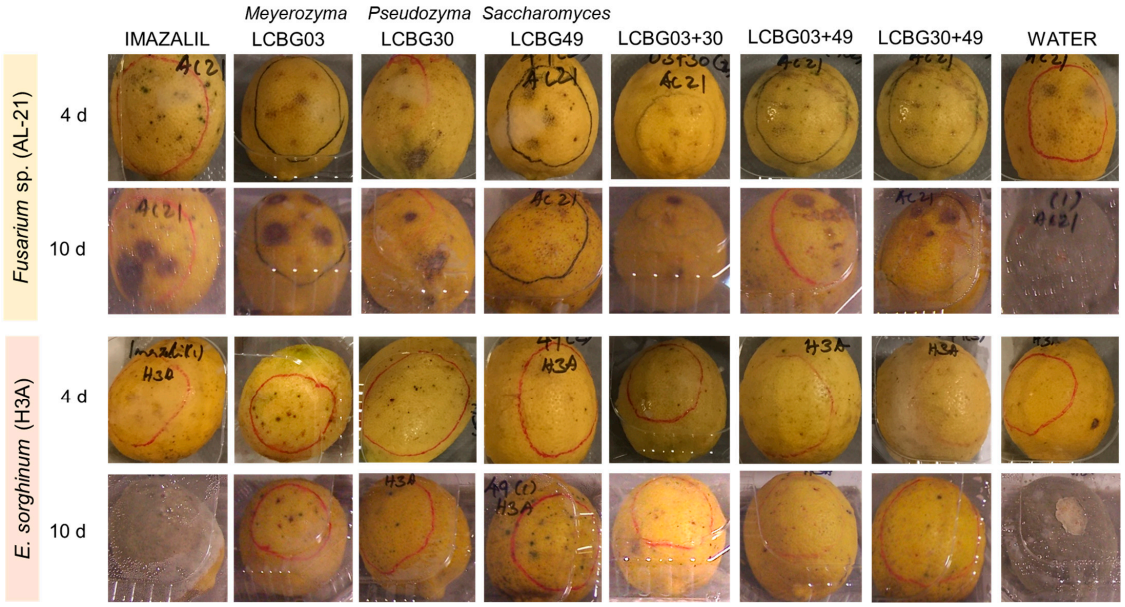

Figure 2. Representative results on lemon fruits showing the single and mixed formulations' effects against fungi AL-21 (*Fusarium* sp., **upper panel**) and H3A (*Epicoccum sorghinum*, **lower panel**), respectively, after 4 and 10 days of inoculation. Negative control is sterile water and positive control is imazalil. Formulation base is not shown in the figure but had a similar control level as imazalil. Each formulation was tested on 30 lemon fruits.

The analysis of all the data at 10 days of treatment is shown as a heatmap (Figure 3), and the formulations with mixed yeast were more efficient on preventing fungal growth and sporulation on fruits.

Regarding the overall response to the formulations against all the tested fungi, using imazalil and the formulation base without yeast both provided 46% protection, the formulation with one yeast provided 63% protection, and the formulations with two mixed yeasts provided an average of 90% protection for the lemons. The response was fungus-specific. The infections caused by *P. digitatum* and *E. sorghinum* were controlled in the highest percentage of 97% with the mixed yeasts *M. guilliermondii* (LCBG-03) and *Pseudozyma* sp. (LCBG-30) formulations.

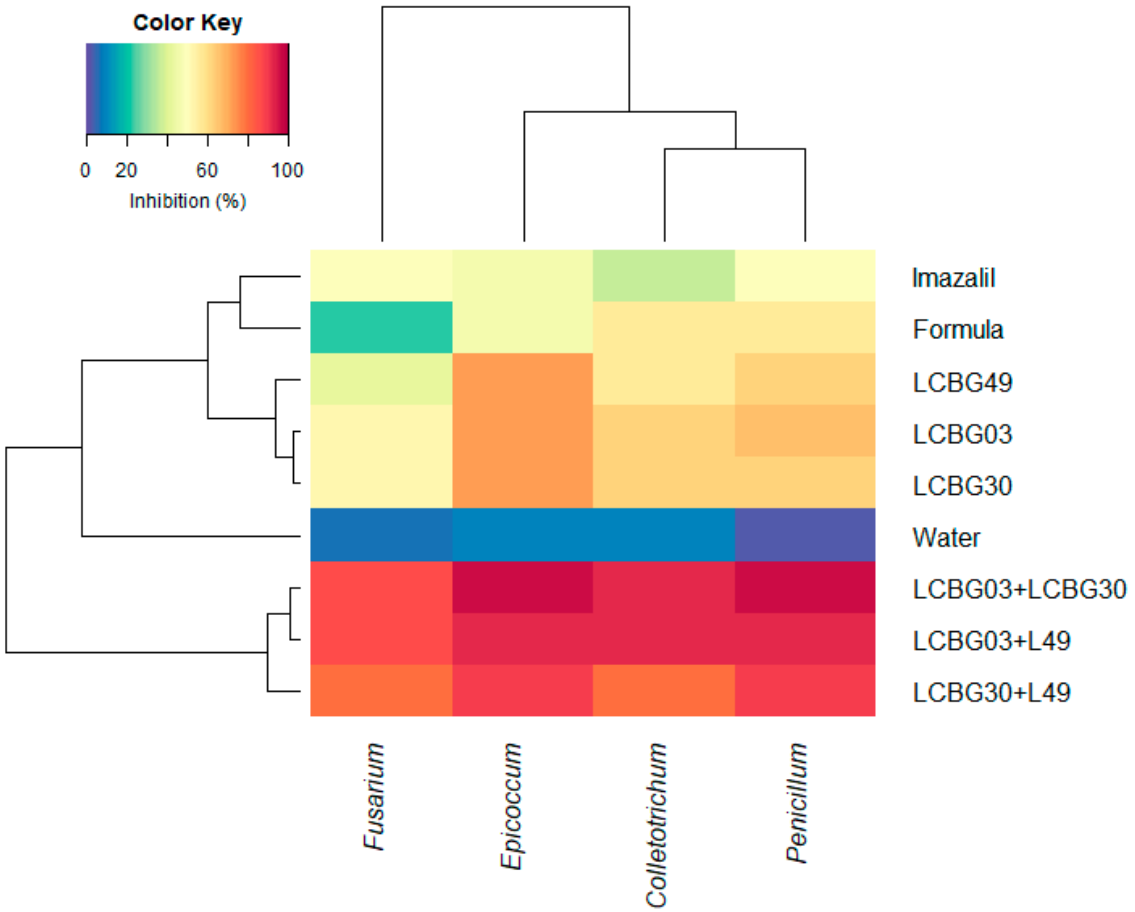

Figure 3. Heat map of the formulated single and mixed yeast products' biocontrol performances, expressed as a percentage of inhibition of infection on fruits after ten days of puncture inoculation. Controls are sprayed with water (Water), sprayed with the base formulation without any yeasts (Formula), and sprayed with an imazalil solution (Imazalil). Fungi tested are *Fusarium* sp. (AL-21), *Epicoccum sorghinum* (H3A), *Colletotrichum gloeosporioides* (AL-13), and *Penicillium digitatum* (AL-38).

4. Discussion

Diverse studies have reported various yeasts' biocontrol capabilities, mainly belonging to genera *Pichia*/*Meyerozyma* [12,19,20], *Candida* sp. [9,21], *Cryptococcus* sp. [1,22], *Pseudozyma* sp. [23–25], *S. cerevisiae* [26,27], and, more recently, *Clavispora lusitaniae* [28], among others. The species used in this work have been reported as antagonists for several phytopathogenic fungi; for example, [20] studied the antagonistic potential of the epiphytic yeasts of grapes belonging to four species *M. guilliermondii*, *Hanseniaspora uvarum*, *Hanseniaspora clermontiae*, *S. cerevisiae*, and *P. kluyveri* against *Botrytis cinerea*, *Aspergillus carbonarius*, and *P. expansum* fungal strains, which were isolated from wild vines, and concluded that *P. kluyveri* was the most effective for controlling the fungal infections. Additionally, [24] suggested that antagonist yeast genera *Metschnikowia*, *Pichia*, *Candida*, *Pseudozyma*, *Kazachstania*, *Issatchenkia*, *Hanseniaspora*, and *Barnettozyma*, which are frequently found on leaves and fruits in the Beibei Chongqing orangery, have the potential for inhibiting citrus green mold. However, these yeasts are usually reported as individual biocontrol agents, as using a mixture of such yeasts is not straightforward, because they can present antagonistic interactions that preclude their use in an antifungal formulation; hence, the first step in this work was evaluating their biological compatibility and then assessing if an additive and/or synergistic effect could be obtained by their mixed use.

4.1. Compatibility among Yeasts

In the current study, although the compatible yeast mixtures' final species compositions were not verified after the initial one-hour contact, we suppose, as supported by the high cell concentrations (Figure 1), that the yeasts did not inhibit each other, and that their viable initial numbers were higher than the pure-strain formulations; additionally, we supposed that the colony counts remained practically the same in the formulated mixtures (Table 2) throughout time, as well as their biocontrol performance, thus indicating that the dual mixtures of LCBG-03 *(M. guilliermondii*), LCBG-30 *(Pseudozyma* sp.), and LCBG-49 (*S. cerevisiae*) were all compatible.

The yeast LCBG-03 *(M. guilliermondii*) was previously characterized and the probable production of metabolites or enzymes implied in the cell/cell interaction (*M. guilliermondii* in contact with *P. digitatum* heat-inactivated mycelia or with *Agaricus bisporus* cell walls) was documented [29]. The competition for sugars and nitrates plays a key role in the interactions of *M. guilliermondii* with other fungi, such as *B. cinerea* in apples [30]. In this crop, the pentose phosphate pathway (PPP) may supply the yeast with an efficient consumption of apple nutrients, which favors the competitive colonization of apple wounds by the yeast against *B. cinerea* [31]. The yeast LCBG-27 (*Macalpinomyces* sp.) was the only one that affected the viability of all three other species (*M. guilliermondii*, *Pseudozyma* sp., and *S. cerevisiae*) used. As pointed out by [32], competition among the yeasts can be evidenced by several behaviors, such as toxin production, resource competition, and growth and fitness changes, and usually cell-to-cell contact is needed to display the competence and dominance behavior. In this work, a growth change was only observed when the strain LCBG-27 (*Macalpinomyces* sp.) was present, hence indicating that this yeast establishes an antagonistic interaction with the other three yeasts. The *Macalpinomyces* genus belongs to the Ustilaginaceae family, which is known for their plant pathogenicity and ability to infect economically essential crops, including barley, sugarcane, wheat, and oats [33]. The Ustilaginaceae yeasts show a wide range of secondary metabolites including organic acids, polyols, and extracellular glycolipids, which have potential applications [34]. However, the specific mechanism implied in such yeast-yeast antagonisms is still to be elucidated. Hence, the yeast LCBG-27 was excluded from the formulations for the in vitro and in vivo experiments.

4.2. Performance of the Formulations In Vitro

Using a Petri dish as a screening methodology for fungal biocontrol agents is well established, and it relies on adequately selecting the culture medium and incubation conditions. In our experiments, we observed that, although the individual formulated yeasts had a good performance inhibiting the radial growth rate of the tested fungi, the use of a mixed inoculum consistently increased the biocontrol activity (Table 2). The strains of *P. digitatum* (AL-38) and *E. sorghinum* (H3A) were clearly more resistant to the presence of the biocontrol yeasts, individually or in a mixed formulation, when tested in vitro. *E. sorghinum* reportedly produces tenuazonic acid, which was formulated as a bioherbicide [17]; however, it has not been reported as being antagonistic to other fungal species. On the other hand, the radial growth rates of *Fusarium* sp. (AL-21) and *C. gloeosporioides* (AL-13) were very susceptible to the presence of the yeasts. Pereyra et al. [28] evaluated citrus epiphytic yeasts' performances on a modified dual culture assay against a pathogenic strain of *P. digitatum* using an in vitro technique. These authors were able to test 43 isolates that were able to inhibit the fungus' growth and, from those, the most effective were tested on wounded fruits. Six isolates (belonging to the *Clavispora lusitaniae* species) could restrict the mycelial growth of *P. digitatum* on *C. limon* fruits in the in vivo microscale assay. Finally, four isolates were the most effective on lemons in the macroscale test, where the fruits were protected by submerging fruits on the yeast suspensions, with a 60–80% protection efficiency after 5 days, which is a time lower than the one tested by us. In our work, we were also interested in the shelf life of the formulations, and, as can be clearly seen in Table 2, the in vitro viability and biocontrol performance of the formulations are preserved after 6 months of storage.

4.3. Performance of the Formulations on Stored Lemons

The idea of using a mixture of biocontrol agents with complementary, even synergistic, mechanisms is technologically attractive; however, due to the yeasts' competitive natures, there is little published information related to using mixed yeast genera or mixed strains of yeasts as biocontrol agents with a proved increase in biocontrol efficiency. In our work, using a formulation that included a lemon flavedo extract also reduced the fungal infection without yeast, for example, being around 57% on *Penicillium* sp. (AL38) and thus being more effective than using the chemical fungicide (50%) for these phytopathogenic fungi. When a formulation using the mixture of LCBG-03 and LCBG-30 was used (*M. guilliermondii* plus *Pseudozyma* sp.), the infection was controlled by 97% (Figure 3).

The LCBG-03 and LCBG-30 formulations were the most effective for the four tested phytopathogenic fungi. For the genus *Pseudozyma*, to which LCBG-30 belongs, Kohl et al. [35] reported that *P. flocculosa* is an efficient biocontrol agent in part due to producing both flocculosin and 6-methyl-9-heptadecanoic acid, which cause fungal cell death. As reported, the biocontrol mechanisms for *M. guilliermondii*, which is the identity for the yeast LCBG-03, are mainly competition for nutrients and space, as well as the production of some hydrolytic enzymes, such as β-glucanases. More importantly, for the mixed interaction with *Pseudozyma* sp., it has an inducible (by contact with *P. digitatum* cell walls) production of two ABC transporters, which might serve as a defense against toxic compounds produced by the fungus [19] and/or by *Pseudozyma* sp., as tested in this work. For formulations containing the strain *S. cerevisiae* LCBG-49, one of the main effects of including this strain was delaying the germination of all four tested fungi, which is probably due to several antifungal volatile compounds being produced, such as ethanol, acetaldehyde, ethyl acetate, isoamyl acetate, and ethyl decanoate, as has been observed also by [26] for their *S. cerevisiae* strains to control sour and gray rot grapes.

Regarding developing formulations as biocontrol products, Sui et al. [36] reviewed all the possible stress factors that yeasts must overcome to be successfully used in a liquid product, with oxidative stress being one of the most important; hence, using an antioxidant such as ascorbic acid, as well as a protective sugar, is recommended. In our case, the developed formulations followed such guidelines, and, additionally, using a flavedo extract also resulted in an effective formulation that preserved the biocontrol characteristics of the yeast strains used. Some authors have also reported formulations containing talc as the carrier and sodium alginate (1.5%) as the adjuvant, observing high viability, and the addition of sucrose (1%) and yeast extract (1%) improved the biocontrol efficacy and shelf life. Klein and Kupper [37] showed the importance of adding nutrients in *A. pullulans*-based formulations when aiming for their use on a commercial scale. The micronutrients (boric acid, cobalt chloride, and ammonium molybdate) favored the antagonistic action of *A. pullulans* against *Geotrichum citri aurantii*, which is the causal agent of sour rot in citrus. Ammonium sulfate 1% and sucrose 0.5% favored the yeast during the competition between the microorganisms. Adding ammonium sulfate (1%) in the yeast culture stimulated biofilm production and increased the antagonistic activity against the disease and allowed for the better survival of yeast in wounded sites of citrus fruit. In our work, using a flavedo extract probably provided the microelement supply, thus resulting in a very stable formulation with a long shelf life developing, which was adequate for industrial purposes. Additionally, the formulations were able to control several genera of fungi, which is one of the limiting factors of the commercial biocontrol products, aimed for specific pathogens, as reviewed by Droby et al. [38]. As such, these authors also propose using a consortia of microorganisms to increase the performance and resilience to environmental stresses and complement their biocontrol capabilities.

5. Conclusions

The mixing of two compatible yeasts increased their viability and improved their effectiveness in controlling the radial growth of fungi in in vitro assays and on stored lemon fruits. A liquid formulation that included a lemon flavedo extract also reduced the fungal

infection without yeast, being more effective than the commercial chemical fungicide. The best yeast combination to include in the formulation to control the fungal infection for all phytopathogens assayed was *M. guilliermondii* (LCBG-03) and *Pseudozyma* sp. (LCBG-30). There are few reports in the literature assessing biocontrol products' performances, including yeast mixtures in horticulture crops and *Citrus* sp. This work contributes to the search for those formulations, including compatible yeast combinations that aim to diminish the postharvest fungal losses of citrus fruits.

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