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RESEARCH ARTICLE

Antagonist activity of yeasts and lactic acid bacteria against phytopathogenic strains of economic importance in agriculture

Hanna Cáceres[1*], Maritza Barriga-Sánchez[1], Lucero Bendezú[2], Elio Huamán[1], Bladimir Becerra-Canales[1], Aybel Almanza[3], Javiera Ortiz-Campos[4], and Lorena Barra-Bucarei[4]

[1]Universidad Autónoma de Ica, Chincha Alta 11702, Perú.
[2]Bio Levasa Perú S.A.C., Provincia y Departamento de Ica 11003, Perú.
[3]Universidad Nacional Agraria la Molina, La Molina 15024, Lima, Perú.
[4]Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Chillán, Chile.
[*]Corresponding author (hanna.caceres@autonomadeica.edu.pe).
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ABSTRACT

Agriculture requires new alternatives to control pests and diseases; biological controls can be a sustainable alternative for the continued success of this sector. This study was thus carried out to identify yeasts and lactic acid bacteria that have the antagonistic capacity to control three phytopathogens as *Botrytis cinerea*, *Lasiodiplodia theobromae* and *Alternaria brassicae* that have caused significant economic losses in agriculture. Thirteen strains of yeast and seven strains of lactic acid bacteria (LAB) were used to measure the percentage of growth inhibition, production of volatile organic compounds, production of biofilms, and the production of enzymes through a completely randomized design. The yeasts *Hanseniaspora opuntiae* and *Saccharomyces cerevisiae* and the LAB *Lactococcus lactis* and *L. brevis* stood out for their antagonistic capacity to inhibit the growth of phytopathogens by 66%, 58%, 65% and 39%, respectively. Pest and disease control highly depend on chemical phytosanitary inputs with negative economic, environmental, and social effects. This study demonstrated that the yeast *H. opuntiae* and the LAB *L. brevis* have potential as biological controls and has been observed to inhibit growth by more than 39%, providing a sustainable alternative that is less harmful to the environment and human health. To guarantee their effectivity under field conditions, their individual application, consortium application, and concentration, timing, and proper application method must be considered.

Key words: Biofilms, biological agents, biopesticide enzymes, lactic acid bacteria, volatile organic compounds, yeast.

INTRODUCTION

Looking to the year 2050, it will be necessary to increase agricultural production to satisfy the growing global demand for food, derived from both the increase in population and certain changes in consumption habits. In the next decade, the growth of agricultural production worldwide must be sufficient to cover the increase in demand and keep real prices relatively stable, or even with a downward trend (ECLAC, FAO, IICA, 2019). On the other hand, it has been reported that every year 1300 t food produced for human consumption is wasted and during post-harvest alone 25% to 50% can be lost due to plant diseases induced by microorganisms or suboptimal handling and storage conditions (FAO, 2022). Most of these losses are caused by plant pathogenic fungi of the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Penicillium*, *Mucor* and *Rhizopus*. *Lasiodiplodia theobromae* in particular has been associated with serious damage in diverse crops of economic importance worldwide, and is considered a dangerous pathogen that can cause plant death. Some diseases reported in crops of agricultural importance, in association with this fungus, are root rot, gummosis, cancer, downy dieback, leaf blight and cob rot. Among the internal damage, a reduction in chlorophyll *a* and *b*

contents has been observed (Dwiastuti and Aji, 2021). *Alternaria brassicae* usually affects all stages of plant growth, its symptoms are manifested on stems, fruits, and leaves. The fungus enters the leaves through the stomatal orifice; 3 d after the infection takes hold, grayish spots begin to form producing phytotoxins such as homodestruxin B and destruxin B that can cause damage to mustard and rapeseed leaves (Blagojević et al., 2020). *Botrytis cinerea* is a fungus that causes gray rot disease, affecting plant organs, including flowers, stems, leaves and fruits and causing great economic losses in the post-harvest period. This fungus can secrete oxalic acid to decrease the pH of the host plant tissues in order to stimulate the production and activity of fungal enzymes such as laccases, proteases and pectinases. The accumulation of this oxalic acid causes Ca^{2+} chelation and, in turn, inhibits callose deposition and weakens the pectin structures of plant cell walls, eventually resulting in the death of infected plants (Shi and Sun, 2017). Synthetic fungicides are the most widely used solution to control these phytopathogens. However, increasing regulatory policies worldwide and the consumer's demand to reduce their application due to potentially harmful side effects to the environment and humans, have led to the search for more sustainable alternatives (Ferreira-Saab et al., 2018). Biological control by antagonistic microorganisms such as yeasts and/or lactic acid bacteria (LAB), have become an emerging and very promising alternative (Taroub et al., 2019). Yeasts stand out as highly efficient antagonists compared to other microorganisms. They have simple nutritional requirements, can rapidly colonize the host for long periods of time, can grow in adverse conditions with no special nutrient requirements, and they produce no compounds that are harmful to human health. They also show different antagonistic mechanisms, such as competing for space and nutrients, producing hydrolytic enzymes, producing volatile organic compounds (VOCs) inducing host resistance, changing the pH at the plant surface, producing ethanol, and biosynthesizing antifungal killer toxins known as mycocins. In addition, yeasts favor adhesion and biofilm formation, directly intervening in competitiveness, which improves their activity as a biocontrol of pathogens and aids in their permanence in the environment (Rossouw et al., 2018). The LAB have shown a great inhibitory effect on plant pathogenic bacteria and fungi, making them important natural biological control agents that do not harm the surrounding ecosystem. They have the ability to produce a variety of antimicrobial compounds and effective substances, such as organic acids (lactic, acetic and propionic acids), bacteriocin antibiotics, bacteriocin-like substances, as well as hydrogen peroxide, and carbon dioxide. The LAB also produce major VOCs, including diacetyl, acetic acid and acetoin (Diaz et al., 2021). Some published references have reported that the yeast *Saccharomyces cerevisiae* has been able to suppress the growth of *B. cinerea* on Thompson seedless grapes (Wang et al., 2018), while the LAB *Enterococcus lactis* has proven to suppress the growth of the fungus *Alternaria alternata*, isolated from stems, leaves, roots and fruits of tomatoes and carrots (Zabouri et al., 2021).

In the continuous search for new antagonists that can achieve high levels of protection in cultures, no studies have been carried out regarding the antagonistic capacity of other yeast genera (*Candida*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Zygosaccharomyces*, *Tausonia*, *Kluyveromyces* and *Torulaspora*) and LAB (*Lactococcus*, *Enterococcus*, *Lactobacillus* and *Gluconacetobacter*) as biological control agents of the phytopathogenic fungi *A. brassicae*, *B. cinerea* and *L theobromae*.

This study was thus carried out to identify yeasts and lactic acid bacteria with the antagonistic capacity to control three phytopathogens *Botrytis cinerea*, *Lasiodiplodia theobromae* y *Alternaria brassicae* that have caused significant economic losses in agriculture crops.

MATERIALS AND METHODS

This research was carried out in the facilities of the Microbiology Laboratory of the Universidad Autónoma de Ica, located in the district of Chincha Alta, province of Chincha, Peru. In this province, agriculture is important for domestic consumption as well as foreign exportation.

Yeasts and lactic acid bacteria

We worked with 13 yeast strains and seven lactic acid bacteria (LAB) acquired from germplasm banks; each had a MycoBank and American Type Culture Collection (ATCC) code. These were isolated from different sources of food and alcoholic beverages (Table 1), and were conserved in the Agricultural Microbiology Culture Collection of the Microbiology Laboratory of the Environment and Sustainable Development Research Line (CCMA) of the Universidad Autónoma de Ica, Peru. The yeast strains were reactivated in a yeast peptone dextrose (YPD) broth

(BD, Franklin Lakes, New Jersey, USA) (composition in g L^{-1}: Glucose 20, yeast extract 10, casein peptone 20). Their pH was adjusted to 5-8 ± 0.2 with the use of HCl, then they were placed in a shaker at 200 rpm for 48 h at room temperature. Similarly, the 13 strains were seeded in duplicate in Petri dishes with glucose peptone yeast (GPY) agar (Difco) (composition in g L^{-1}: Glucose 20, yeast extract 10, casein peptone 20, bacteriological agar 20). The LAB were reactivated in a MRS broth (Man's, Rogosa's and Sharpe's) (composition in g L^{-1}: Glucose 20, yeast extract 5, meat peptone 10, meat extract 8, dipotassium phosphate 2, sodium acetate trihydrate 5, triammonium citrate 2, magnesium sulfate 0.2, manganese sulfate 0.05, Tween 80 polysorbate 1). Their pH was adjusted to 6.2 with the use of HCl, subsequently they were shaken at 200 rpm for 24-48 h at room temperature. Similarly, the seven strains were seeded in duplicate on plates with MRS agar (composition g L^{-1}: Glucose 20, yeast extract 5, meat peptone 10, meat extract 8, dipotassium phosphate 2, sodium acetate trihydrate 5, triammonium citrate 2, magnesium sulfate 0.2, manganese sulfate 0.05, Tween 80 polysorbate 1, bacteriological agar 20).

Table 1. Description of the yeasts and lactic acid bacteria used in this study.

Code	Species	Treatment	Isolation	°C	Atmosphere	pH
27439	*Candida incommunis*	1	Grape must	24	Aerobic	6.2 ± 0.2
253819	*Candida parapsilosis*	2	Puerto Rico drinking fountain	30-35	Aerobic	6.0 ± 0.2
296478	*Debaryomyces hansenii*	3	Japanese sake	24-26	Aerobic	5.6 ± 0.2
488270	*Hanseniaspora opuntiae*	4	Grape must	24-26	Aerobic	5.6 ± 0.2
513463	*Pichia guilliermondii*	5	Rancid butter	24-26	Aerobic	5.6 ± 0.2
227217	*Pichia membranifaciens*	6	German wine	24-26	Aerobic	5.6 ± 0.2
263896	*Saccharomyces ellipsoideus* subsp. *fulliensis*	7	Wine	24-26	Aerobic	5.6 ± 0.2
492348	*Saccharomyces cerevisiae*	8	Distillery	24-26	Aerobic	5.6 ± 0.2
325702	*Zygosaccharomyces rouxii*	9	Grape must	24-26	Aerobic	5.6 ± 0.2
258102	*Zygosaccharomyces bailii*	10	USA wine	24-26	Aerobic	5.6 ± 0.2
812190	*Tausonia pullulans*	11	Environment	24-26	Aerobic	5.6 ± 0.2
316067	*Kluyveromyces thermotolerans*	12	Fermentation of plum jam from the USSR	24-26	Aerobic	5.6 ± 0.2
812190	*Torulaspora delbrueckii*	13	French grape must	24-26	Aerobic	5.6 ± 0.2
ATCC 7963	*Lactococcus lactis*	14	Curd	37	Microaerophilia 95% air 5% CO_2	7.4
ATCC43186	*Enterococcus mundtii*	15	Environment	37	Microaerophilia	7.0
ATCC3272	*Lactobacillus reuteri*	16	Pig manure	37	Aerobic	6.5 ± 0.2
ATCC 8014	*Lactobacillus plantarum*	17	Fermented pickled cabbage	30	Microaerophilia	6.5 ± 0.2
ATCC14869	*Lactobacillus brevis*	18	Pig manure	30	Microaerophilia	6.5 ± 0.2
ATCC49037	*Gluconacetobacter diazotrophicus*	19	Sugar cane root	30	Microaerophilia	6.8
ATCC14835	*Gluconacetobacter liquefaciens*	20	Fermented persimmon from Japan	26	Aerobic	6.8

Phytopathogenic fungi

The phytopathogenic strains of *Alternaria brassicae*, *Botrytis cinerea* and *Lasiodiplodia theobromae* were taken from the strain collection of the Universidad Autónoma de Ica, Peru, and were first molecularly identified. The refreshing of the strains was performed with a Petri dish containing the initial culture, by spiking in the center of the Petri dish; a total of eight plates were reactivated with potato dextrose agar (PDA) (composition in g L^{-1}: Potato extract: 4, dextrose 20 and agar 20) and left to incubate for 7 d at room temperature (Cheng et al., 2019).

Evaluation of the percentage of pathogen radial growth inhibition

For the selection of inhibitory strains of the three phytopathogenic fungi, confrontations were carried out by dual culture of all the yeast strains. Agar disks with mycelium of the phytopathogenic fungi (*A. brassicae*, *B. cinerea* and *L. theobromae*) were placed in the center of plates with a PDA medium, and two lines of yeast were sown on each end at 3 cm. For the control, only the disks of the phytopathogens were inoculated with the disks of the phytopathogens. The medium YPD was used in the confrontations with LAB. Subsequently, the Petri dishes were incubated at 28 ± 2 °C and radial growth was observed on the third, fifth and seventh days; a total of 26 replicates were performed for each yeast and bacterial strain. The inhibition percentage of pathogen radial growth inhibition (PPRGI) of mycelium was evaluated according to the following formula (Barra-Bucarei et al., 2020):

$$PPRGI = [((R1 - R2)/R1)] \times 100$$

where R1 is the pathogen growth on the control plate and R2 is the pathogen growth, controlled with yeast or LAB seeded in parallel.

The strains able to effectively control against the three phytopathogens were selected for further tests, such as analyzing the volatile organic compound (VOC) production, biofilm production and hydrolytic enzyme production.

Production of volatile organic compounds

Aliquots of 50 µL suspensions were seeded on YPD plates for the selected yeasts and incubated at 28 ± 2 °C. For the selected LAB, MRS agar plates were used and incubated at 30 °C for 24 h. Then, the technique of overlapping plates was used with the help of Parafilm, the edges were covered in order to avoid air leakage where discs with fungal mycelium were placed in the center of the plates with PDA and covered with plates containing yeast or LAB cultures. For the control, only PDA plates seeded with the phytopathogen were used, they were incubated at 26 ± 2 °C (Figure 1). The mycelial diameter was measured after 7 d, and the mycelial growth inhibition rate was calculated using the equation published by Gao et al. (2018). Also, four replicates were performed for each treatment and the experiment was repeated twice.

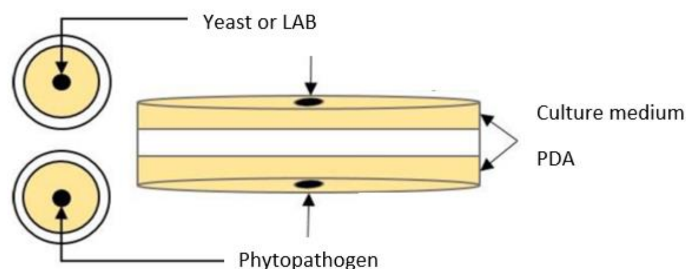

Figure 1. Diagram representing the confrontation system to determine the production of volatile compounds, where the inoculum of the phytopathogen was placed on one Petri dish base and the antagonist on the other. Source: Vázquez-Gómez et al., 2019; Ruiz-Moyano et al., 2020. LAB: Lactic acid bacteria; PDA: potato dextrose agar.

Biofilm production

An essential first step of biofilm formation is the initial binding of microorganisms. Therefore, the ability to form biofilms was evaluated by measuring yeast adherence to a polystyrene surface, with some modifications. The method included the following steps: Selected strains were grown for 12 h in the dark at 28 °C in a tube containing 3 mL YPD medium for yeasts and a tryptone soy broth (TSB) for lactic acid bacteria, with a pH of 5.6. The yeast and LAB suspensions (approximately 1 mL) were poured into Eppendorf tubes and adjusted to an optical density of 0.2 measured at 660 nm. The cultures were then centrifuged for the removal of their supernatants. Cells were resuspended in 1mL YPD and TSB medium; then, from the adjusted suspensions, 160 µL aliquots were inoculated into each well of microplates. For the control, only the culture media were used; plates were sealed and incubated at 30 °C for 24 h. Five wells were used for each strain and condition set, and

two wells were used as controls. After incubation, the wells with the adjusted media were washed three times with a saline phosphate buffer (pH 7.2) to remove free cells. The control well was washed with sterile water to remove cells that were not attached to the wells. Finally, aliquots of 160 µL 0.1% (w/v) Coomassie blue were added to all wells and allowed to stand for 20 min and then washed again with phosphate-buffered saline three times to remove the staining. Cell adherence was quantified by solubilizing the retained Coomassie blue with 160 µL 10% sodium dodecyl sulfate (SDS) for 30 min. The resulting solution was measured using a microplate spectrophotometer (Vis spectrophotometer, KV 1200, UK) at an optical density of 570 nm (Arnaouteli et al., 2021). The variable evaluated was the cell turbidity of the solution, which either contained or did not contain the cells that were attached.

Enzyme production

Tests were performed on the strains selected after the PPRGI trial. A completely randomized design was used. These were designed to assess whether these strains had good enzyme production, specifically lipases, chitinases and proteases because these enzymes alter the composition of the cell wall of pathogenic microorganisms, inducing the suppression of their growth (Pretscher et al., 2018). The results obtained were classified according to the following scale: No activity, mild activity, medium activity, and good activity.

Lipases. This trial was carried out in a modified medium composed (% w/v) mainly of: 0.2% Peptone, 0.5% yeast extract, 2% agar supplemented with 0.1% $CaCl_2$ and 0.1% Tween 80, with pH 6. Each strain was streaked in Petri dishes and incubated for 3 d at 30 °C. This methodology is based on the hydrolysis of Tween 80 by lipases because it possesses oleic acid esters. In addition, Ca-bound fatty acids were released into the medium, creating a Ca complex expressed as crystals around the site of the beneficial colony (Dukare and Paul, 2021). Finally, as a result of lipase production, a zone of opacity was observed around the microorganism colony.

Proteases. This trial was carried out in 10% skim milk in a minimal medium (0.003% NaCl, 0.03% $MgSO_4$ and 0.015% K_2HPO_4) with 2% agar, and pH 6. A streaking of each selected strain was performed in Petri dishes, which were subsequently incubated at 30 °C for 3 d. Finally, the production of a transparent halo around the area closest to the colony was observed, while the rest of the medium remained white because the microorganisms that produce the enzyme caseinase to hydrolyze casein form soluble N components, shown as a clear zone around the colony in the Petri dish (Kedar et al., 2018).

Chitinases. In this trial, a sterilized basal medium (BM) was used, composed of 1.0 g L^{-1} citric acid monohydrate, 0.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 200 µL L^{-1} Tween 80, 3.0 g L^{-1} $(NH_4)2SO_4$, 2.0 g L^{-1} KH_2PO_4, 15 g L^{-1} agar, 0.15 g L^{-1} bromocresol purple, and 4.5 g L^{-1} colloidal chitin, with a pH adjusted to 4.7. A replating of each selected strain was performed in Petri dishes. This medium was observed to have a bright yellow coloration. Finally, it was incubated for 3 d at 30 °C. The trial was evaluated by observing a shift from lemon yellow to intense violet (Aoki et al., 2020), indicating that the microorganism under evaluation was producing extracellular chitinases.

Cellulases. A carboxymethyl cellulose (CMC) agar was used in this trial, composed of 10.0 g L^{-1} CMC; 2.0 g L^{-1} KH_2PO_4; 2.0 g L^{-1} K_2HPO_4 and 15.0 g L^{-1} agar-agar, with pH 6. In Petri dishes, each selected strain was streaked, then incubated at 30 °C for 3 d. As a developer, Congo Red was added at 1% (w/v) after 15 min, the excess was removed and 0.1 M NaCl was added and left to stand for 15 min. Finally, clear zones were observed around the colony of the microorganisms, as a result of the production and subsequent disappearance of cellulases in these zones; on the contrary, the rest of the surface was red (Gharied et al., 2020).

Statistical analysis

The PPRGI data regarding the dual confrontation technique carried out to identify VOCs performed by yeasts and LAB were analyzed separately using InfoStat statistical software version 2020 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). One-way ANOVA tests were performed to identify the difference between treatments and Tukey tests were used to determine the differences in means. Different letters indicate significant differences between groups at $P \leq 0.05$.

RESULTS AND DISCUSSION

Percentage of pathogen radial growth inhibition

Considering the 13 yeasts applied in this study, *Hanseniaspora opuntiae* presented 58% growth inhibition for *B. cinerea* and 62% growth inhibition for *A. brassicae* (Table 2). These data agree with the results obtained for the control of *Corynespora cassiicola*, where *H. opuntiae* inhibited approximately 50% of its growth by the action of VOCs, which induced the plant defense response in a dose-dependent manner, and could be induced after 24 h pretreatment and maintained with non-significant reduction for up to 5 d. Nonetheless, yeast VOCs may diffuse throughout the plant and/or, once inside the plant cell, they may induce defense responses as true elicitors (Ferreira-Saab et al., 2018). Moreover, Gómez-Albarrán et al. (2021) reported that *Hanseniaspora uvarum* U1 significantly reduced the growth rate of *Alternaria carbonarius*, *A. parasiticus* and *A. flavus*, by 34%, 31% and 19%, respectively. The growth rates of *A. steynii* and *A. welwitschiae* were reduced by 11%. The growth rate of *A. westerdijkiae* was not affected by the presence of *H. uvarum* U1, but its dormancy phase was extended by 75%, suggesting that this biological control agent has potential to control the growth of the fungus. *Hanseniaspora uvarum* U1 also behaved as an effective detoxifying agent of aflatoxin B1 and ochratoxin A, mediated by cell wall adsorption mechanisms as an active mechanism (Gómez-Albarrán et al., 2021). This also agrees with the results of Romanens et al. (2019), where *A. flavus* growth was inhibited after 10-14 d by the four selected antifungal strains (*Lactobacillus fermentum* M017, *L. fermentum* 223, *H. opuntiae* H17 and *S. cerevisiae* H290). When these strains were applied as a single culture, they inhibited fungal growth from 51%-95% and when they were combined in four co-cultures, each consisting of the LAB and the two yeast strains, they achieved 100% inhibition.

Table 2. Percentages of growth inhibition (PGI) in evaluated strains. [1]Data obtained from the evaluation of three pre-screening replicates. [2]Data obtained from 26 replicates (of the strains that had the best PGI in the pre-screening). *Tausonia pullulans* and *Lactococcus lactis* had problems with growth, thus the 26 replicates were not carried out. *L. theobromae*: F = 294.65, gl = 19, p < 0.0001; *B. cinerea*: F = 1803.14, gl = 19, p < 0.0001; *A. brassicae*: F = 762.81, gl = 19, p < 0.0001.

Strains	Species	[1]Pre-screening *Lasiodiplodia theobromae*	[2]*L. theobromae* Day 7	[1]Pre-screening *Botrytis cinerea*	[2]*B. cinerea* Day 7	[1]Pre-screening *Alternaria brassicae*	[2]*A. brassicae* Day 7
1	*Candida incommunis*	4.33^{abc}		1.00^{a}		24.00^{c}	
2	*Candida parapsilosis*	1.33^{ab}		49.00^{hi}		55.00^{gh}	
3	*Debaryomyces hansenii*	0.33^{a}		52.00^{ij}		60.00^{j}	
4	*Hanseniaspora opuntiae*	5.67^{bc}		80.00^{n}	58.07^{a}	59.00^{ij}	62.39^{a}
5	*Pichia guilliermondii*	11.00^{de}		58.00^{kl}		52.00^{g}	
6	*Pichia membranifaciens*	7.00^{cd}		27.00^{f}		48.00^{f}	
7	*Saccharomyces ellipsoideus* subsp. *fulliensis*	2.00^{ab}		37.00^{g}		55.00^{gh}	
8	*Saccharomyces cerevisiae*	52.00^{g}	55.56^{b}	61.00^{l}	54.99^{a}	56.00^{hi}	66.07^{b}
9	*Zygosaccharomyces rouxii*	2.67^{abc}		17.00^{d}		40.00^{e}	
10	*Zygosaccharomyces bailii*	2.00^{ab}		49.00^{h}		34.00^{d}	
11	*Tausonia pullulans**	12.00^{e}		70.00^{*h}		59.00^{ij}	
12	*Kluyveromyces thermotolerans*	2.00^{ab}		53.00^{j}		57.00^{hi}	
13	*Torulaspora delbrueckii*	5.00^{abc}		2.00^{ab}		39.00^{e}	
14	*Lactococcus lactis**	7.00^{cd}		12.00^{c}		61.00^{*jk}	
15	*Enterococcus mundtii*	7.00^{cd}		28.00^{f}		19.00^{b}	
16	*Lactobacillus reuteri*	7.00^{cd}		4.00^{ab}		20.00^{b}	
17	*Lactobacillus plantarum*	30.00^{f}		5.00^{b}		14.00^{a}	
18	*Lactobacillus brevis*	50.00^{g}	49.00^{a}	56.00^{jk}	64.53^{b}	64.00^{k}	59.79a
19	*Gluconacetobacter diazotrophicus*	7.00^{cd}		35.00^{g}		45.00^{f}	
20	*Gluconacetobacter liquefaciens*	7.00^{cd}		24.00^{e}		39.00^{e}	

In this study, the yeast *S. cerevisiae* was found to achieve a growth inhibition of 56%, 55% and 66% for *L. theobromae*, *B. cinerea* and *A. brassicae*, respectively. These results were higher than those obtained by Oro et al. (2018), who used the yeasts *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *S. cerevisiae* as biocontrol agents in sweet cherries on postharvest brown rot, mainly caused by *Monilinia laxa*. In this study, the yeast *S. cerevisiae* reduced brown rot when applied at a concentration of 1×10^8 CFU mL^{-1} with only a 21% infection rate, while the control had a 100% infection rate. None of these yeasts produced phytotoxic substances, both in the intact fruit and in the inoculated wound (Oro et al., 2018). The results obtained by Liu et al. (2017) showed higher fungal inhibition than those obtained in the present investigation. They evaluated 216 *S. cerevisiae* yeast strains isolated from wine to control the phytopathogen *Colletotrichum gloeosporioides*, a causal agent of grape anthracnose prior to harvest. Three of the evaluated strains were antagonistic to this phytopathogen, producing antifungal compounds, inhibiting the germination of *C. gloeosporioides* conidia, and producing β-1,3-glucanase and chitinase. All the isolates studied in Liu et al. (2017) colonized grape berries in large numbers and controlled the targeted phytopathogen when artificially inoculated on grape berries. The application of the *S. cerevisiae* GA8 isolate resulted in a 70% reduction of *C. gloeosporioides* disease on grape berries (Liu et al., 2017).

In the present study, when evaluating the yeast *Candida parapsilosis*, 49% and 55% of radial growth inhibition was obtained for *B. cinerea* and *A. brassicae*, respectively. These results are in agreement with those obtained by Jaibangyang et al. (2020), who evaluated 366 antagonistic yeast strains (epiphytic and endophytic) isolated from rice, sugarcane and maize leaves from Thailand, which are potentially capable of producing VOCs active against the aflatoxin-producing fungus *A. flavus* A39. Only 49 of the 366 evaluated yeast strains were able to produce antifungal VOCs. *Candida nivariensis* DMKU-CE18 was the most effective yeast strain for inhibiting both mycelial growth (64.9% + 7.0% inhibition) and the conidial germination (49.3% + 3.3%) of *A. flavus* A39, and for reducing aflatoxin production (74.8 ± 6.5%) in maize kernels. The primary VOC produced by this yeast strain was closest to 1-pentanol (Jaibangyang et al., 2020). When considering the seven LAB evaluated in our study, *L. brevis* presented 49%, 60% and 65% growth inhibition for *L. theobromae*, *A. brassicae* and *B. cinerea* respectively. These results are lower than that obtained by Al-Shammari and Majeed (2016) who determined the in vitro antifungal activity of LAB (*L. fermentum*, *L. reuteri*, *Lactobacillus* sp. No. 1, *Lactobacillus* sp. No. 2 and *Lactococcus* sp.) against *Fusarium oxysporum*, *Phytophthora infestans*, *Pythium ultimum* and *Alternaria* sp., where all LAB achieved a 100% inhibition rate against the phytopathogens on MRS agar than on PDA agar at 37 ºC for 5 d.

Here we found that the growth inhibition of LAB *Enterococcus mundtii* was 7%, 19% and 28% against *L. theobromae*, *A. brassicae* and *B. cinerea*, respectively. These results were similar to those obtained by Zabouri et al. (2021) who determined the in vitro antifungal activity of LAB identified as *E. lactis* and *E. faecium* against five strains of the phytopathogenic, toxigenic and deteriorating fungal species *A. alternata* isolated from stems, leaves, roots and fruits of tomatoes and carrots. The 15 evaluated LAB showed between 13% and 100% inhibition against the five strains of *A. alternata*, among the best species were *E. lactis* with the code BL12 and *E. faecium* with the code BL35, suggesting a possible application in food technology as biopreservatives against phytopathogenic and food spoilage fungi (Zabouri et al., 2021).

Production of volatile organic compounds

Recently, VOCs, produced by microorganisms as biological control agents, have received increasing attention. For example, 3-methyl-1-butanol and 2-methyl-1-butanol produced by *S. cerevisiae* could inhibit the development of *Phyllosticta citricarpa*, which causes citrus black spot (Toffano et al., 2017). The VOCs produced by *Lachancea thermotolerans* have revealed their potential to protect tomatoes inoculated with *Fusarium oxysporum* (Zeidan et al., 2018). Furthermore, Grzegorczyk et al. (2017) hypothesized that VOCs could be one of the main mechanisms of *Debaryomyces hansenii* KI2a and *Wickerhamomyces anomalus* BS91 against *Monilinia fructigena* and *M. fructicola*, which cause considerable economic losses in stone fruit crops (Jaibangyang et al., 2020).

In the results of the present investigation (Table 3), *H. opuntiae* showed a 12%, 39% and 39% growth inhibition of *L. theobromae*, *B. cinerea* and *A. brassicae*, respectively. These results are in agreement with those reported by Galván et al. (2022), where *H. uvarum* and *H. opuntiae* produced VOCs, such as 2-phenylethyl acetate (2PEA) and furfuryl acetate (FA), which inhibited growth, germination, gene expression, and aflatoxin and ochratoxin A production. The VOCs 2PEA and FA effectively controlled *Aspergillus flavus* M144 and *A. niger* M185 using at least 50 µL for FA and 100 µL for 2PEA in dried figs (Galván et al., 2022). In this case, the action of both compounds repressed the expression of the genes involved from early on in the biosynthesis of the aflatoxin and ochratoxin A of this

phytopathogen. The application of 2PEA and FA in the early post-harvest stages of dried figs is thus recommended to control mycotoxin accumulation (Galván et al., 2022). Accordingly, Tejero et al. (2021) and Ruiz-Moyano et al. (2020) also identified several VOCs produced by *H. uvarum* and *H. opuntiae* that, in vitro, decreased the growth and aflatoxin production by *A. flavus*. In the present study, *S. cerevisiae* showed a 2%, 37% and 39% growth inhibition for *B. cinerea*, A*. brassicae* and *L. theobromae* respectively. These results were lower than those obtained by Oro et al. (2018), who also used *S. cerevisiae* as a biological control agent on some postharvest rot-causing fungi in strawberry (*Fragaria ×ananassa* 'Alba') fruits. Oro et al. (2018) concluded that VOCs reduced the mycelial growth of *B. cinerea* by 69%. They also identified ethyl acetate vapor as the main VOC produced by yeasts, which completely inhibited *B. cinerea* at 8.97 mg cm^{-3} and suppressed gray mold on strawberry fruit at 0.72 mg cm^{-3}. In the results obtained by Błaszczyk et al. (2017), two tested killer yeasts produced agar-diffusible antifungal metabolites. Both *Wickerhamomyces anomalus* and *Pichia membranifaciens* strains significantly reduced the mycelial growth of *Penicillium italicum* and *B. cinerea*. After 10 d of incubation, volatile compounds produced by *P. membranifaciens* inhibited the growth of *P. italicum* and *B. cinerea* by 21% and 40%, respectively (Błaszczyk et al., 2017). The results obtained by Zhou et al. (2018) also determined that the yeast *Debaryomyces nepalensis* induced peroxidase (POD), phenylalanine ammonium lyase, chitinase and β-1,3-glucanase activities and the VOCs produced by *D. nepalensis* reduced mycelial growth compared to control plates. This same study found that VOCs inhibited *Colletotrichum gloeosporioides* by 40% to 42% after 48 h.

Table 3. Percentage of growth inhibition of phytopathogenic strains by volatile organic compound (VOC) production of yeasts and lactic acid bacteria. Data obtained from the evaluation of four measurements. *L. theobromae*: $F = 23.92$, gl = 2, $p < 0.0003$; *B. cinerea*: $F = 18.71$, gl = 2, $p < 0.0006$; *A. brassicae*: $F = 0.70$, gl = 2, $p < 0.5203$.

Strains	Species	*Lasiodiplodia theobromae*	*Botrytis cinerea*	*Alternaria brassicae*
4	*Hanseniaspora opuntiae*	12[a]	39[b]	39[a]
8	*Saccharomyces cerevisiae*	39[b]	2[a]	37[a]
18	*Lactobacillus brevis*	2[a]	52[b]	25[a]

Biofilm production

After the evaluation, it was observed that LAB (*Lactococcus lactis* and *Lactobacillus brevis*) had a good biofilm production activity (Table 4), in accordance with Limanska et al. (2019) where *Lactobacillus plantarum* strains had a great capacity to adhere to the shoots and leaves of *Lepidium sativum* L. seedlings, and the lactobacilli were able to form biofilm in the absence of other microorganisms. The lactobacilli were also able to compete with phytopathogens, protecting the plant surface and altering the mature biofilm of the pathogen. The results of Mechmeche et al. (2022) also revealed that the levels of total coliforms, yeasts and fungi decreased significantly with the inoculation of *L. plantarum*. The observations of the same study also revealed that the biofilm formed after 3 to 7 d of air-drying had the same morphological characteristics and its volume increased during storage.

Table 4. Biofilm production and enzymatic activity of the strains evaluated. +: Slight activity; ++: medium activity; +++: good activity; -: no activity.

Strains	Chitinase	Protease	Cellulose	Lipase	Biofilm
Hanseniaspora opuntiae	++	+	-	++	++
Saccharomyces cerevisiae	-	+	-	-	+
Debaryomyces hansenii	++	++	-	-	-
Pichia guilliermondii	-	+	-	++	++
Lactococcus lactis	-	++	-	-	+++
Lactobacillus brevis	-	+++	-	-	+++

Enzyme production

Another mechanism of yeasts to inhibit phytopathogens is the production of hydrolytic enzymes. In the present study, *D. hansenii* was observed to have medium enzyme activity, producing chitinases and proteases (Table 4). This partially agrees with Hernández-Montiel et al. (2018), who reported that *D. hansenii* showed β-1,3-glucanase and protease activities and did not produce chitinase. Both β-1,3-glucanase and protease were reported to act directly on the cell wall of the phytopathogen, which is composed mainly of chitin (*ca*. 20%), β-glucans (*ca*. 50%-60%) and proteins (*ca*. 20%-30%). Specifically, β-1,3-glucanase hydrolyzes β-1,3-glucan produced smaller oligosaccharides and glucose, at random sites along the polysaccharide chain, which were used as a carbon source by the yeast. Proteases have proven to directly degrade proteins contained within the cell membrane of the phytopathogen, facilitating the yeast to feed primarily on a source of N and amino acids (Liu et al., 2017). Marsico et al. (2021) also reported that *H. uvarum* Ale5 showed β-1,3-glucanase, protease, and lipase activity, and none of the evaluated yeast isolates could hydrolyze chitin, partially coinciding with our results, where *H. opuntiae* did show a mild chitinase production.

CONCLUSIONS

Of the 20 strains studied, two yeasts and one lactic acid bacteria exerted high percentages of inhibition of mycelial growth to the phytopathogens studied. Thus, *Lasiodiplodia theobromae* and *Alternaria brassicae* had their growth inhibited by contact with *Saccharomyces cerevisiae*, *Hanseniaspora opuntiae*, and *Lactobacillus brevis*. *Botrytis cinerea* was inhibited by H. *opuntiae* and L. *brevis*. These strains, when applied individually or in microbial consortia, become new alternatives as biological control agents.

Author contribution

Conceptualization: H.C. Methodology: H.C., L.B. Software: E.H. Validation: M.B-S., B.B-C., A.A. Formal analysis: E.H. Investigation: H.C., L.B. Resources: H.C. Data curation: E.H. Writing-original draft: H.C. Writing-review & editing: H.C., L.B., L.B-B., J.O. Supervision: M.B-S., B.B-C. Project administration: H.C. Funding acquisition: H.C. All co-authors reviewed the final version and approved the manuscript before submission.

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References

Al-Shammari, R.H., Majeed, H.Z. 2016. Efficiency of lactic acid bacteria as biological control agents against some fungi. Al-Mustansiriyah Journal of Science 27(2):35-40.

Aoki, Y., Haga, S., Suzuki, S. 2020. Direct antagonistic activity of chitinase produced by *Trichoderma* sp. SANA20 as biological control agent for grey mould caused by *Botrytis cinerea*. Microbiology, Parasitology & Virology 6(1):1747903. doi:10.1080/23312025.2020.1747903.

Arnaouteli, S., Bamford, N.C., Stanley-Wall, N.R., Kovács, Á. 2021. *Bacillus subtilis* biofilm formation and social interactions. Nature Reviews Microbiology 19:600-614. doi:10.1038/s41579-021-00540-9.

Barra-Bucarei, L., France, A., Gerding, M., Silva, G., Carrasco-Fernández, J., Castro, J.F., et al. 2020. Antifungal activity of *Beauveria bassiana* endophyte against *Botrytis cinerea* in two Solanaceae crops. Microorganisms 8:65. doi:10.3390/microorganisms8010065.

Blagojević, J., Vukojević, J., Ivanović, B., Ivanović, Ž. 2020. Characterization of *Alternaria* species associated with leaf spot disease of *Armoracia rusticana* in Serbia. Plant Disease 104(5):1378-1389.

Błaszczyk, U., Sroka, P., Satora, P., Duliński, R. 2017. Effect of *Wickerhamomyces anomalus* and *Pichia membranifaciens* killer toxins on fermentation and chemical composition of apple wines produced from high-sugar juices. Journal of Food and Nutrition Research 56(2):189-199.

Cheng, L., Nie, X., Jiang, C., Li, S. 2019. The combined use of the antagonistic yeast *Hanseniaspora uvarum* with β-aminobutyric acid for the management of postharvest diseases of kiwifruit. Biological Control 137:104019. doi:10.1016/j.biocontrol.2019.104019.

Diaz, D.G.G., Pizzolitto, R.P., Vázquez, C., Usseglio, V.L., Zunino, M.P., Dambolena, J.S., et al. 2021. Effects of the volatile organic compounds produced by *Enterococcus* spp. strains isolated from maize grain silos on *Fusarium verticillioides* growth and fumonisin B1 production. Journal of Stored Products Research 93:101825. doi:10.1016/j.jspr.2021.101825.

Dukare, A., Paul, S. 2021. Biological control of Fusarium wilt and growth promotion in pigeon pea (*Cajanus cajan*) by antagonistic rhizobacteria, displaying multiple modes of pathogen inhibition. Rhizosphere 17:100278. doi:10.1016/j.rhisph.2020.100278.

Dwiastuti, M.E., Aji, T.G. 2021. Citrus stem rot disease (*Lasiodiplodia theobromae* (Pat.) Griff. & Maubl) problem and their control strategy in Indonesia. IOP Conference Series: Earth and Environmental Science 752(1):012030. doi:10.1088/1755-1315/752/1/012030.

ECLAC, FAO, IICA. 2019. The outlook for agriculture and rural development in the Americas: A perspective on Latin America and the Caribbean 2019-2020. Economic Commission for Latin America and the Caribbean (ECLAC), Food and Agriculture Organization of the United Nations (FAO), Inter-American Institute for Cooperation on Agriculture (IICA), San José, Costa Rica.

FAO. 2022. 1.3 billion tons of food are lost every year. News. Available at https://www.fao.org/venezuela/noticias/detail-events/fr/c/1472356/ (accessed June 2024).

Ferreira-Saab, M., Formey, D., Torres, M., Aragon, W., Padilla E., Tromas, A., et. al. 2018. Compounds released by the biocontrol yeast *Hanseniaspora opuntiae* protect plants against *Corynespora cassiicola* and *Botrytis cinerea*. Frontiers in Microbiology 9:01596. doi:103389/fmicb.2018.01596.

Galván, A., Hernández, A., Córdova, M., Martín, A., Serradilla M., López-Corrales, M., et. al. 2022. Control of toxigenic *Aspergillus* spp. in dried by volatile organic compounds (VOCs) from antagonistic yeasts. International Journal of Food Microbiology 376:109772. doi:10.1016/j.ijfoodmicro.2022.109772.

Gao, H., Li, P., Xu, X., Zeng, Q., Guan, W. 2018. Research on volatile organic compounds from *Bacillus subtilis* CF-3: Biocontrol effects on fruit fungal pathogens and dynamic changes during fermentation. Frontiers in Microbiology 9:456. doi:10.3389/fmicb.2018.00456.

Gharied, M., Abo-Zaid, G., Bashir, S., Hafez, E. 2020. Screening and molecular identification of cellulase-producing *Bacillus* spp. from agricultural soil: Its potential in biological control. Middle East Journal of Applied Sciences 10(2):272-278. doi:10.36632/mejas/2020.10.2.26.

Gómez-Albarrán, C., Melguizo, C., Patiño, B., Vázquez, C., Gil-Serna, J. 2021. Diversity of mycobiota in Spanish grape berries and selection of *Hanseniaspora uvarum* U1 to prevent mycotoxin contamination. Toxins 13(9):649. doi:10.3390/toxins13090649.

Grzegorczyk, M., Zarowska, B., Restuccia, C., Cirvilleri, G. 2017. Postharvest biocontrol ability of killer yeasts against *Monilinia fructigena* and *Monilinia fructicola* on Stone fruit. Food Microbiology 61:93-101. doi:10.1016/j.fm.2016.09.005.

Hernández-Montiel, L.G., Gutierrez-Perez, E.D., Murillo-Amador, B., Vero, S., Chiquito-Contreras, R., Rincon-Enriquez, G. 2018. Mechanisms employed by *Debaryomyces hansenii* in biological control of anthracnose disease on papaya fruit. Postharvest Biology and Technology 139:31-37. doi:10.1016/j.postharvbio.2018.01.015.

Jaibangyang, S., Nasanit, R., Limtong, S. 2020. Biological control of aflatoxin-producing *Aspergilus flavus* by volatile organic compound-producing antagonistic yeasts. BioControl 65(3):377-386. doi:10.1007/s10526-020-09996-9.

Kedar, S., Arun-Vashistht, M., Prashanth, A., Parveen, N., Chakraborty, S., Sindhu, S. 2018. Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from seawater samples. Journal of Genetic Engineering and Biotechnology 16(1):39-46. doi:10.1016/j.jgeb.2017.10.001.

Limanska, N., Merlich, A., Galkin, M., Vasylieva, N., Choiset, Y., Ivanytsia, T., et al. 2019. Biofilm formation and genetic diversity of *Lactobacillus plantarum* strains originated from France and Ukraine. Journal of Microbiology, Biotechnology and Food Sciences 8(6):1326-1331. doi:10.15414/jmbfs.2019.8.6.1326-1331.

Liu, Z., Du, S., Ren, Y., Liu, Y. 2017. Biocontrol ability of killer yeasts (*Saccharomyces cerevisiae*) isolated from wine against *Colletotrichum gloeosporioides* on grape. Journal of Basic Microbiology 58(1):60-67. doi:10.1002/jobm.201700264.

Marsico, A., Velenosi, M., Perniola, R., Bergamini, C., Sinonin, S., David-Vaizant, V., et al. 2021. Native vineyard non-*Saccharomyces* yeasts used for biological control of *Botrytis cinerea* in stored table grape. Microorganisms 9:457. doi:10.3390/microorganisms9020457.

Mechmeche, M., Ksuntini, H., Setti, K., Hamdi, M., Kachouri, F. 2022. Dried tomato slices: An approach to increase safety and shelf-life of by the use of *Lactobacillus plantarum*. Journal Food Science, Nutrition and Public Health 2:1-13.

Oro, L., Ferliziani, E., Ciani, M., Romanazzi, G., Comitini, F. 2018. Volatile organic compounds from *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* inhibit growth of decay causing fungi and control postharvest diseases of strawberries. International Journal of Food Microbiology 256:18-22. doi:10.1016/j.ijfoodmicro.2017.10.027.

Pretscher, J., Fischkal, T., Branscheidt, S., Jäger, L., Kahl, S., Schlander, M., et al. 2018. Yeasts from different habitats and their potential as biocontrol agents. Fermentation 4(2):31. doi:10.3390/fermentation4020031.

Romanens, E., Freimüller, S., Volland, A., Stevens, M. Krähenmann, Isele, D., et al. 2019. Screening of lactic acid bacteria and yeast strains to select adapted antifungal co-cultures for cocoa bean fermentation. International Journal of Food Microbiology 290:262-272. doi:10.1016/j.ijfoodmicro.2018.10.001.

Rossouw, D., Meiring, S.P., Bauer, F.F. 2018. Modifying *Saccharomyces cerevisiae* adhesion properties regulates yeast ecosystem dynamics. mSphere 3(5):e00383-18. doi:10.1128/msphere.00383-18.

Ruiz-Moyano, S., Hernández, A., Galván, A., Córdoba, M., Casquete, R., Serradilla, M.J., et al. 2020. Selection and application of antifungal VOCs-producing yeasts as biocontrol agents of grey mould in fruits. Food Microbiology 92:103556. doi:10.1016/j.fm.2020.103556.

Shi, J.F., Sun, C.Q. 2017. Isolation, identification, and biocontrol of antagonistic bacterium against *Botrytis cinerea* after tomato harvest. Brazilian Journal Microbiology 48:706-714.

Taroub, B., Salma, L., Manel, Z., Ouzari, H.I., Hamdi, Z., Moktar, H. 2019. Isolation of lactic acid bacteria from grape fruit: Antifungal activities, probiotic properties, and in vitro detoxification of ochratoxin A. Annals of Microbiology 69:17-27. doi:10.1007/s13213-018-1359-6.

Tejero, P., Martín, A., Rodríguez, A., Galván, A.I., Ruiz-Moyano, S., Hernández, A. 2021. In vitro biological control of *Aspergillus flavus* by *Hanseniaspora opuntiae* L479 and *Hanseniaspora uvarum* L793, producers of antifungal volatile organic compounds. Toxins 13:663. doi:10.3390/toxins13090663.

Toffano, L., Fialho, M.B., Pascholati, S.F. 2017. Potential of fumigation of orange fruits with volatile organic compounds produced by *Saccharomyces cerevisiae* to control citrus black spot disease at postharvest. Biological Control 108:77-82. doi:10.1016/j.biocontrol.2017.02.009.

Vázquez-Gómez, E.I., Soria-Leal, L.Y., Chávez-Avilés, M.N. 2019. Chapter 3. Análisis del efecto antagonista de los compuestos orgánicos volátiles (COV's) producidos por Trichoderma spp. y Bacillus subtilis de manera individual y en co-cultivo sobre el crecimiento de *Botrytis cinerea* causante de la pudrición de fresa. In p. 17-26. Seguridad alimentaria: Aprovechamiento integral y calidad microbiológica de los alimentos. Universidad Autónoma de Chihuahua, Chihuahua, México.

Wang, X., Dean, A., Glave, E., Weller, D., Okubara, A. 2018. Biological control of *Botrytis cinerea*: Interactions with native vineyard yeasts from Washington State. Phytopathology 108(6):691-701. doi:10.1094/PHYTO-09-17-0306-R.

Zabouri, Y., Cheriguene, A., Chougrani, F., Merzouk, Y., Marchetta, A., Urzí, C., et al. 2021. Antifungal activity of lactic acid bacteria against phytopathogenic *Alternaria alternata* species and their molecular characterization. Journal of Food and Nutrition Research 60(1):18-28.

Zeidan, R., Ul-Hassan, Z., Al-Thani, R., Balmas, V., Jaoua, S. 2018. Application of low-fermenting yeast *Lachancea thermotolerans* for the control of toxigenic fungi *Aspergillus parasiticus*, *Penicillium verrucosum* and *Fusarium graminearum* and their mycotoxins. Toxins 10(6):242. doi:10.3390/toxins10060242.

Zhou, Y., Lib, W., Zeng, J., Shaoa, Y. 2018. Mechanisms of action of the yeast *Debaryomyces nepalensis* for control of the pathogen *Colletotrichum gloeosporioides* in mango fruit. Biological Control 123:111-119. doi:10.1016/j.biocontrol.2018.05.014.