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NATIVE KAZAKH AISULU YEASTS AS BIOCONTROL AGENTS AGAINST THREE MAJOR POSTHARVEST PATHOGENS: FIRST W AND B TRITROPHIC SCREEN

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ABSTRACT

Postharvest fungal pathogens cause substantial losses of fresh fruit, highlighting the need for sustainable biological alternatives to sulfur dioxide (SO_2)-based preservation. This study evaluated native epiphytic yeasts isolated from the Kazakh *Aisulu* table-grape cultivar using a two-stage screening framework followed by testing in two postharvest systems: artificially wounded berries (W system) and unwounded grape bunches (B system). Antagonistic activity was assessed against *Botrytis cinerea* F-RKM 1032, *Aspergillus niger* ATCC 6275, and *Rhizopus stolonifer* ATCC 6227b. Initially, 34 yeast isolates were screened for inhibition of fungal growth (PIRG: 23.76–71.76%; selection threshold >50%) and biofilm-forming capacity (OD_{550} > 0.245), resulting in 11 candidates for further evaluation. In vivo screening identified five strains with high protective activity: *Metschnikowia pulcherrima* A2, *Hanseniaspora uvarum* HU15, *Aureobasidium pullulans* AP11 and AP13, and *Clavispora lusitaniae* CL03. Against *B. cinerea*, biocontrol efficacy in wounded berries (BEW) reached 77.47% for A2, 78.56% for HU15, 73.43% for AP11, 71.65% for AP13, and 65.06% for CL03. Importantly, *M. pulcherrima* A2 and *H. uvarum* HU15 retained high efficacy in the more restrictive unwounded-bunch system, with B-system biocontrol efficacy (BEB) of 78.02% and 72.83%, respectively. Their W-to-B efficacy ratios were close to 1 (0.99–1.20), indicating that their protective activity remained relatively stable between wounded berries and intact grape bunches. In contrast, *A. pullulans* AP11 and AP13 showed substantially reduced efficacy in the B system, with W-to-B efficacy ratios of 1.79–3.07. *C. lusitaniae* CL03 was excluded from further consideration because of biosafety concerns. Comparative evaluation indicated that A2 performed better than the previously reported *Metschnikowia* sp. 291 in the B system, whereas HU15 showed efficacy comparable to the previously reported *H. uvarum* P2. Overall, *M. pulcherrima* A2 and *H. uvarum* HU15 emerged as the most promising indigenous biocontrol candidates because they combined high antifungal efficacy with stable performance in both wounded and unwounded grape systems. These strains warrant further evaluation under cold-storage and commercial conditions, individually and as a defined microbial consortium, to develop sustainable postharvest grape-protection strategies.

***Keywords*:** *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Botrytis cinerea*, *Aspergillus niger*, *Rhizopus stolonifer*, Kazakh Aisulu cultivar.

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1. INTRODUCTION

More than one-third of harvested fruits and vegetables, valued for their well-documented nutritional and health benefits, are lost before reaching consumers, primarily due to postharvest rot driven by fungal pathogens (Wang et al., 2016; Aune et al., 2017; Zakrevskii, 2018; Öztekin et al., 2023; Nepal et al., 2025). The most prevalent fungal agents responsible for this decay, both before and after harvest, include *Botrytis cinerea*, *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Rhizopus*, and *Cladosporium* spp., and they remain the subject of intensive ongoing research (Freire et al., 2018; Lovato et al., 2019; De Simone et al., 2020; Zhang et al., 2020b; Tziros et al., 2022; Liu et al., 2024). *Botrytis cinerea* causes gray mold and infects more than 200 plant species, including grapevines. This necrotrophic fungus kills plant cells by releasing toxins and reactive oxygen species, triggering an oxidative burst in the host. *B. cinerea* synthesizes the sesquiterpene botrydial and the polyketide botcinic acid, both critical for damaging host cells and colonizing plant tissues (Lovato et al., 2019). It also produces cell wall-degrading enzymes and oxalic

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acid during infection, and recent evidence shows that the pathogen can trigger host programmed cell death as a defensive strategy (De Simone et al., 2020; Tziros et al., 2022).

Aspergillus niger, another economically important postharvest pathogen, produces ochratoxin A (OTA), a highly toxic secondary metabolite frequently detected in grapes, wine, and grape juice. OTA contamination poses a significant food safety concern because of its well-documented immunosuppressive, teratogenic, neurotoxic, genotoxic, mutagenic and carcinogenic effects, particularly on the liver, kidneys, and hematopoietic system (Freire et al., 2018). During fungal colonization, OTA may accumulate in both fresh fruits and processed grape products, thereby compromising product quality and posing substantial health risks to consumers (Freire et al., 2018). Rhizopus stolonifer, commonly known as black bread mold, is another major postharvest pathogen characterized by prolific airborne dispersal of conidia and rapid growth under warm, humid conditions. These biological characteristics facilitate extensive postharvest spoilage, accelerating fruit deterioration and substantially reducing the shelf life, marketability, and commercial value of affected produce (Zhang et al., 2020b; Liu et al., 2024).

Chemical fungicides remain the primary tools for managing major postharvest pathogens. In vineyards, agrochemicals are mainly applied before harvest, while sulfur dioxide (SO_2) is predominantly used to control grape mildew after harvest. However, growing concerns about environmental pollution, residue accumulation, and potential human health risks from synthetic fungicides have shifted attention toward biological control agents and other innovative, integrated pest-management strategies. A recent assessment of integrated management strategies for gray mold concluded that despite decades of intensive research, only a small number of BCAs have reached the market as registered products (e.g., Shemer™, Candifruit™, BoniProtect™, Yield Plus™, Nexy™, Pantovital™, Biosave™), and several early products such as Aspire™ (based on *Candida oleophila*) and Yieldplus™ (based on *Cryptococcus albidus*) were ultimately withdrawn because of low or inconsistent efficacy under commercial conditions, small market size, and the high cost of registration (Romanazzi et al., 2015; Droby et al., 2016; Cignola et al., 2024; Rai et al., 2026). These experiences have reinforced the need for improved selection criteria and a more detailed understanding of BCA mechanisms and stability (Wilson & Wisniewski, 1989; Elena & Köhl, 2021).

Among the antagonistic microorganisms proposed as BCAs, specific antagonistic yeasts are the most commonly reported because of a unique combination of advantageous traits (Hernandez-Montiel et al., 2021; Öztekin et al., 2023). Yeasts can withstand the extreme environmental conditions that occur before and after harvest, including the high sugar levels, elevated osmotic pressure, and low pH typical of the fruit microenvironment (Hernandez-Montiel et al., 2021; Öztekin et al., 2023). They grow on inexpensive substrates and can therefore be mass-produced in fermenters. Unlike filamentous fungi, yeasts do not produce allergenic mycotoxins and have simple nutritional requirements, which allow them to colonize dry fruit surfaces for long periods (Di Canito et al., 2021; Reyes-Bravo et al., 2021; Salerno et al., 2024). A recent screening of native grape-associated yeasts from Malbec berries in Argentina found that 82% of 111 epiphytic isolates corresponded to the yeast-like organism *Aureobasidium pullulans*, followed by non-*Saccharomyces* genera such as *Hanseniaspora uvarum* (6.3%), *Metschnikowia pulcherrima* or spp. (5.4%), *Cryptococcus laurentii*, *Starmerella bacillaris*/*Candida zemplinina*, and *Rhodotorula* spp. (Prendes et al., 2017). Similar surveys of muscadine and *Vitis vinifera* cultivars have repeatedly identified *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Wickerhamiella*, and *Aureobasidium* as the dominant and most biotechnologically promising genera (Vargas et al., 2012; Cordero-Bueso et al., 2017; Ayogu et al., 2024; Agarwal & Sheikh, 2025). Importantly, all evaluated *Metschnikowia* (pulcherrima or spp.), *S. bacillaris*, and almost all *H. uvarum* strains completely prevented *Alternaria alternata* infection when applied two hours before pathogen inoculation on grape berry wounds, supporting the value of locally isolated epiphytic yeasts as biocontrol candidates (Prendes et al., 2017).

The mechanisms by which yeast BCAs suppress postharvest pathogens are now recognized to be multifactorial. The principal modes of action include competition for nutrients and space, mycoparasitism and direct pathogen interaction, secretion of cell wall–degrading enzymes and antimicrobial metabolites, biofilm formation, induction of host resistance, and, more recently, competition for iron through siderophore-like compounds (Romanazzi et al., 2016; Freimoser et al., 2019; Dukare et al., 2019; Köhl et al., 2019). Competition for nutrients and space is considered the cornerstone of yeast-mediated biocontrol: as single-celled organisms, yeasts multiply rapidly in fruit wounds that are rich in carbohydrates (disaccharides and monosaccharides) and nitrogenous compounds, depleting the resources required for pathogen germination and growth; this principle underlies wound protection of fruits by fast-colonizing yeasts such as *Pichia guilliermondii*, *A. pullulans*, and *Metschnikowia* spp. against *Penicillium*, *Botrytis*, and *Colletotrichum* spp. (Köhl et al., 2019; Cignola et al., 2024). Mycoparasitism involves direct yeast-pathogen hyphal interactions accompanied by the secretion of lytic enzymes such as chitinases, β-1,3-glucanases, and proteases that degrade fungal cell walls (Freimoser et al., 2019). Antibiosis includes the production of volatile organic compounds and diffusible antifungal metabolites: for example, *Aureobasidium pullulans* L47 produces aureobasidins that inhibit inositol phosphoryl ceramide synthase, *Pichia membranifaciens* CYC 1106 and *Saccharomyces cerevisiae* CBS8112 produce killer toxins that damage pathogen membranes and cause cell-cycle arrest, and *Rhodotorula glutinis* ySL 30 produces the dihydroxamate siderophore rhodotorulic acid, which delays spore germination and reduces apple decay

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by 72% (Roca-Couso et al., 2021). Biofilm formation has emerged as a particularly important mechanism: dense microbial communities encased in a polymeric extracellular matrix adhere to fruit surfaces and create a mechanical barrier that limits pathogen colonization (Klein & Kupper, 2018). Only *S. cerevisiae* cells collected during the biofilm phase were able to suppress *Penicillium expansum* on apples; similarly, biofilms of *Pichia kudriavzevii* improved biocontrol of *B. cinerea* and *Colletotrichum gloeosporioides* on pear fruit, and the morphology transition of *Candida diversa* from single cells to pseudohyphal/biofilm forms improved biocontrol of *B. cinerea* in apple and kiwifruit (Klein & Kupper, 2018). Among the most distinctive mechanisms described in recent years is iron depletion by *Metschnikowia pulcherrima*, which secretes pulcherriminic acid that non-enzymatically chelates ferric ions to form the red pigment pulcherrimin in the surrounding medium. Because iron is essential for fungal growth and pathogenicity, this sequestration starves pathogens such as *B. cinerea*, *Alternaria alternata* and *P. expansum*; remarkably, when iron is added back to the medium, both the inhibitory halo and the biocontrol efficacy of *M. pulcherrima* disappear, and pathogen hyphae entering the pigmented zones crack, confirming iron starvation as a dominant antagonistic mechanism (Sipiczki, 2006; Saravanakumar et al., 2008; Roca-Couso et al., 2021; Wang et al., 2023a). Finally, induction of host resistance, manifested as the upregulation of pathogenesis-related genes and accumulation of defense metabolites such as chitinase and β-1,3-glucanase, is now recognized as the principal long-term mechanism for enhancing fruit resistance after pathogen attack (Romanazzi et al., 2016; Freimoser et al., 2019; Fernandez-San Millan et al., 2023). A practical demonstration of integrated mechanisms comes from field trials in which *M. pulcherrima* MPR3 combined with on-farm biological treatments reduced gray mold (*Botrytis cinerea*) incidence and severity on organic table grapes by 67.8–86.2%, with the protective effect persisting into postharvest storage where disease reduction reached approximately 98%—without negative impacts on grape microbial communities (Lombardo et al., 2023; Puyo et al., 2023).

Several complementary strategies have been developed to improve the often-variable efficacy of yeast BCAs. These include combining antagonistic yeasts with plant hormones such as salicylic acid (Farahani & Etebarian, 2012; Qin et al., 2003; Xu et al., 2008; Cheng et al., 2019) or β-aminobutyric acid (Cheng et al., 2019); with other chemicals and additives (Gramisci et al., 2018; Shao et al., 2019; Zheng et al., 2019); with food-compatible coatings such as chitosan (Zhou et al., 2016); with physical treatments such as hot water (Zong et al., 2010); with other yeast strains in designed mixtures or synthetic microbial consortia (Maciag et al., 2023; Öztekin & Karbancıoğlu-Güler, 2023; Chen et al., 2025); and by genetic modification or yeast surface display of antifungal proteins (Zhao et al., 2020; Fernandez-San Millan et al., 2023). Despite these advances, the commercial application of yeast BCAs still faces substantial bottlenecks: low and inconsistent efficacy under non-sterile, real-world conditions, the requirement of at least six months of shelf life in formulated products, and the limited market for biopesticides that discourages registration of new entities (Droby et al., 2016; Romanazzi et al., 2015; Cignola et al., 2024). Furthermore, the gap between promising in vitro and in vivo laboratory results and successful scale-up in storage facilities remains a major unresolved challenge (Droby et al., 2016; Ayogu et al., 2024).

Recent advances in genomics, transcriptomics, and other omics technologies have substantially improved understanding of yeast–host–pathogen interactions and accelerated the identification of novel BCAs (Blanco-Ulate et al., 2015, 2017; Crandall et al., 2020; Sarethy & Saharan, 2021). However, despite the rapidly expanding literature on yeast-mediated biocontrol, three critical knowledge gaps limit the development of robust, locally adapted BCAs for table grapes. First, most screening programs rely on a small number of laboratory or commercial strains, while the native epiphytic mycobiome of specific grape cultivars, particularly underrepresented varieties from Central Asia, remains largely unexplored as a source of region-specific antagonists (Cordero-Bueso et al., 2017; Prendes et al., 2017; Zhang et al., 2020a; Ayogu et al., 2024; Agarwal & Sheikh, 2025*).* Second, although key mechanisms (competition, biofilm formation, iron depletion, induced resistance) have been described individually in model systems on non-grape hosts, their relative contributions under the harsh, nutrient-poor conditions of intact grape bunches compared with artificial wounds remain poorly characterized (Köhl et al., 2019; Zhang et al., 2020a; Maciag et al., 2023). Third, few studies systematically compare the same yeast strains across matched wounded-berry (W) and unwounded-bunch (B) tritrophic systems. against multiple pathogens—an approach that is essential for identifying BCAs whose efficacy is stable in the stressful microenvironments typical of commercial postharvest storage (Schena et al., 2000; Droby et al., 2016; Zhang et al., 2020a).

To address these gaps, the present study aimed to (1) isolate and characterize epiphytic yeast strains naturally associated with the local Kazakh table grape variety *Aisulu*; (2) implement a two-stage screening that combines in vitro antagonism tests and biofilm-formation assays with in vivo testing in both W (artificially wounded berries) and B (unwounded bunches) model postharvest tritrophic systems; (3) quantify biological control efficacy against the three most damaging pathogens of stored grapes—*B. cinerea*, *A. niger*, and *R. stolonifer*—using standardized $BE_{(pat)}$ and $R_{(pat)}$ metrics; and (4) identify candidate BCAs whose performance remains stable under the harsher conditions of the B system, providing a foundation for subsequent optimization through combinations with phytohormones, yeast mixtures, or genetic modifications.

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2. MATERIALS AND METH/ODS

2.1. Yeast Isolation

We collected berries of the Aisulu table grape variety (*Vitis vinifera*) from the Talgar district of the Almaty region, specifically from the village of Almalyk. We collected the berries at the experimental site of the Talgar branch of the Kazakh Research Institute of Fruit and Vegetable Growing, located within a pomological garden in southeastern Kazakhstan. The experimental site is situated in the foothill zone of the Trans-Ili Alatau (Fig. 1).

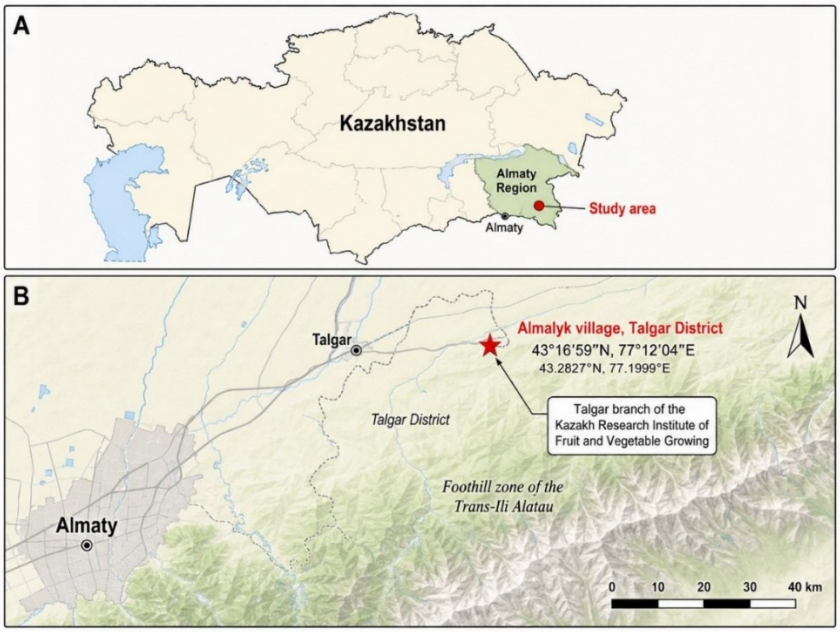

Fig. 1: Location of the study area in southeastern Kazakhstan. (A) Location of Almaty Region within Kazakhstan and the general position of the study area. (B) Enlarged map showing Almalyk village, Talgar District, Almaty Region, where berries of Vitis vinifera cv. Aisulu were collected at the experimental site of the Talgar branch of the Kazakh Research Institute of Fruit and Vegetable Growing.

The Talgar area lies at approximately 1,000–1,500 m above sea level. The archived field documentation did not preserve the exact berry-sampling date or plot-level meteorological records. Under local production conditions, Aisulu grapes reach commercial maturity in late August; in a related trial conducted by the same institute, Aisulu harvest maturity was recorded between 22 and 28 August. Therefore, April–August was used to characterize the principal grape-growing period, and late August was used to characterize the likely harvest and sampling window. Long-term climatic normals for Talgar indicate mean monthly air temperatures of 9.4, 15.0, 19.4, 21.7, and 20.6°C from April through August, respectively, averaging approximately 17.2°C over this period. Mean monthly maximum/minimum temperatures increase from 15.6/3.3°C in April to 27.2/12.2°C in August. Mean monthly precipitation totals for April–August are 40.6, 48.3, 30.5, 22.9, and 15.2 mm, respectively, corresponding to approximately 158 mm during the characterized growing period. August is predominantly clear, with clear, mostly clear, or partly cloudy conditions occurring during approximately 86% of the month. These values represent regional climatic normals and should be interpreted as proxies rather than as measurements obtained directly at the experimental plot during the sampling season.

Four berries were placed in each of five Erlenmeyer flasks containing 125 mL of sterile water. The samples were incubated in an incubator shaker (New Brunswick Scientific Innova 44, Eppendorf) at 200 rpm for 30 minutes. After shaking, transfer the wash solution to 50 mL Falcon tubes and centrifuge at 3000 × g for 15 minutes in a refrigerated centrifuge (5810 R, Eppendorf) with a swing-bucket rotor (A-4-62). The pellets were resuspended in a liquid medium composed of yeast extract, peptone, and dextrose (YPD). We serially diluted this suspension in YPD from 10^-1 to 10^-6. Next, 0.1 mL of each dilution was plated onto nutrient yeast dextrose agar (NYDA) (Sigma-Aldrich, US) using a sterile glass spatula. All plates were incubated at 28 ± 2°C for 48 hours. After incubation, the plates with higher dilutions were examined under a microscope (OPTIKA B-510LD4) to identify the yeast.

The isolates were recultured on fresh NYDA agar to obtain pure cultures. These pure cultures were then incubated in YPD medium at 28 ± 2°C for 48 hours. After incubation, the cultures were stored at -80°C in liquid YPD containing 30% (v/v) glycerol (Sigma-Aldrich, USA).

2.2. Preparation of Yeasts for Experiments

One hundred microliters of the stock frozen yeast culture were subcultured twice in 10 mL of YPD medium at 28 ± 2°C for 48 hours in a test tube. Subsequently, we inoculated 0.5 mL of this culture into 50 mL of nutrient yeast

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extract dextrose broth (NYDB) in 125 mL Erlenmeyer flasks. This culture was incubated in an Innova 44 Incubator Shaker (Eppendorf) at 150 rpm for an additional 48 hours at 28 ± 2°C. The yeast cells were then collected by centrifugation at 3000 × g for 15 minutes using a refrigerated Centrifuge 5810 R with a swing-bucket rotor A-4-62 (Eppendorf). After centrifugation, the cells were washed twice with sterile deionized water and resuspended in distilled water to reach an initial concentration of approximately 5×10^8 cells/mL. The yeast cell count in the suspension was determined using a hemocytometer.

2.3. Preparation of Fungal Pathogens for Experiments

Three fungal pathogen strains were used throughout the study: Botrytis cinerea F-RKM 1032, obtained from the Republican Collection of Microorganisms LLC, Astana, Kazakhstan, and Aspergillus niger ATCC 6275 and Rhizopus stolonifer ATCC 6227b, obtained from the American Type Culture Collection, Manassas, VA, USA. F-RKM 1032, ATCC 6275, and ATCC 6227b are culture-collection catalogue numbers. We grew the pathogen cultures on Potato Dextrose Agar (PDA, Sigma-Aldrich, US), supplemented with streptomycin sulfate at 300 mg/L (Sigma-Aldrich, US). These cultures were incubated in the dark at 22 to 25°C for 7 to 10 days before use. To prepare the spore suspension, we washed the Petri dish surfaces with sterile distilled water containing 0.05% Tween 80. The suspension was then filtered through eight layers of sterile cheesecloth and shaken to ensure uniform mixing. Finally, we diluted the suspension to a final concentration of 1×10^5 conidia/mL. The number of conidia in the suspension was determined using a hemocytometer.

2.4. *In Vitro* Screening of Yeast Isolates for Antagonistic Activity against *B. cinerea* using the Dual-Culture Method

The experiments used a dual-culture method with slight modifications (Hassan et al., 2021). To assess the effectiveness of yeast isolates against a pathogen, we cultured them together on potato dextrose agar (PDA). We took a 6 mm mycelial plug from a seven-day-old culture of *B. cinerea* (F-RKM 1032) and placed it at the center of an agar plate. Then we streaked a 2-day-old yeast culture onto the same plate, positioning it 1.0 cm from the edge. We also prepared a separate plate with only the fungus as a control. All plates were incubated at 28 ± 2°C in a thermostat (BINDER RI 115, Germany) for seven days. Finally, we measured the percent inhibition of radial growth (PIRG) using the formula provided in equation (1).

$$PIRG = [(R1 - R2) / R1] \times 100 \quad (1)$$

R1 indicates the fungus's radial growth in the control group without yeast, while R2 shows the radial growth in samples with yeast.

The dual-culture assay comprised 35 conditions: 34 yeast isolates and one fungus-only control. Each condition was represented by three independently inoculated Petri dishes (n = 3), and one Petri dish was considered the experimental unit. Thus, 105 Petri dishes were evaluated in total.

2.5. *In Vitro* Screening of Yeast Isolates for Biofilm Formation Activity

We used a standard method with slight modifications (Wang et al., 2023b). Yeasts were cultured in 1 mL of YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose. The culture was incubated for 20 hours at 30°C in a BINDER RI 115 incubator (Germany) and the cell concentration was adjusted to a final optical density (OD600) of approximately 1.0 (approximately 3×10^7 cells/mL). After incubation, we mixed 20 µL of the yeast cell suspension with 180 µL of YPD medium in the wells of a sterile 96-well plate (Corning, Sigma-Aldrich, US). This mixture was incubated for 72 hours at 28°C. We then removed the medium and washed the wells twice with 200 µL of phosphate-buffered saline (PBS) to remove any free cells. To stain biofilm-forming cells, we added 200 µL of 0.1% crystal violet and incubated for 10 minutes at room temperature. The wells were then rinsed three times with PBS and air-dried. We added 200 µL of 33% acetic acid to each well and incubated for 30 minutes at room temperature with shaking at 150 rpm using an Innova 44 Shaker-incubator (Germany). OD550 readings for the sample and control were obtained with a reader (EZ-Read 400, Biochrom, USA). YPD medium without yeast cells served as a negative control. The biofilm screen comprised 20 conditions: 19 yeast isolates and one YPD-only negative control. Each condition was measured in three wells of the same 96-well plate (n = 3 technical replicates), corresponding to 60 wells in total. Because the three wells were prepared from the same culture batch, they were treated as technical rather than independent biological replicates.

2.6. Identification of Antagonistic Yeasts Using Molecular Taxonomy and Phylogenetic Analysis

We identified yeast isolates with the highest antagonistic activity and strong biofilm- forming abilities at the species level. Following the manufacturer's protocol, DNA was extracted from the yeasts using the Thermo

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Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). All reagents were from Thermo Fisher Scientific, USA. We amplified the ITS 1 - 5. 8 S - ITS 2 region of nuclear ribosomal DNA (nrDNA) from yeast isolates using the method described by White et al. (1990). Amplification was performed with universal primers, "ITS 1" (5'- TCCGTAGGTGAACCTGCGG- 3') as the forward primer and "ITS 4" (5'- TCCTCCGCTTAATTGATATGC- 3') as the reverse primer, in a total reaction volume of 30 µl. The PCR mixture contained 5 µl of DNA (57.5 ng), 1 U of Taq DNA Polymerase, 0.2 mM of each dNTP, 10 × KCl buffer, 2.5 mM MgCl2, and 10 pmol of each primer. The PCR program was as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 40 seconds, and 72°C for 50 seconds. We performed a final elongation step at 72°C for 7 minutes during PCR using a Proflex amplifier (Applied Biosystems). After amplification, the PCR products were purified following the ExoSAP- IT ™ PCR Product Cleanup protocol. For sequencing, we prepared a 10 µL reaction mixture containing 6 µL of Master Mix, 1 µL of primer, and 3 µL of PCR product. The Master Mix consisted of 2 µL of BigDye Terminator v3.1; 1 µL of BigDye sequencing buffer, and 3 µL of nuclease- free water. The sequencing protocol involved heating the mixture to 94°C for 1 minute, followed by 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. We purified the PCR products according to the manufacturer's instructions using the BigDye XTerminator Purification Kit. We then performed sequencing on an Applied Biosystems 3500 Genetic Analyzer. The sequences obtained were aligned and identified with BLAST from the National Center for Biotechnology Information (NCBI). For multiple sequence alignment, ClustalW was used, and we conducted phylogenetic analysis using the Neighbor- Joining method in MEGA 11 software. We calculated bootstrap support values for the phylogenetic tree from 1000 replicates.

The 11 study isolates were designated Metschnikowia pulcherrima A2, Hanseniaspora uvarum HU15, Aureobasidium pullulans AP04, AP05, AP06, AP08, AP11, AP12, and AP13 and Clavispora lusitaniae CL01 and CL03. These labels are internal study strain codes, not sequence accession numbers. No public GenBank accession numbers are currently linked to the ITS sequences of the 11 study isolates; the corresponding author can provide their consensus sequences upon reasonable request. The accession numbers displayed in Fig. 2 refer exclusively to external GenBank reference sequences used for phylogenetic comparison. The correspondence among original isolate number, study code, taxonomic identification is summarized in Table S11, and the external reference accessions used in Fig. 2 are listed in Table S12.

Table S11: Study microorganisms, original isolate numbers and internal strain codes

Microorganism	Study code	Original isolate no.	Source
Metschnikowia pulcherrima	A2	1	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Hanseniaspora uvarum	HU15	34	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP04	5	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP05	6	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP06	7	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP08	9	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP11	12	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP12	13	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Aureobasidium pullulans	AP13	14	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Clavispora lusitaniae	CL01	17	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Clavispora lusitaniae	CL03	19	Surface of Aisulu grape berries, Almalyk, Kazakhstan
Botrytis cinerea	F-RKM	-	Republican Collection of Microorganisms LLC
Aspergillus niger	ATCC 6275	-	American Type Culture Collection
Rhizopus stolonifer	ATCC 6227b	-	American Type Culture Collection

Table S12: External GenBank ITS reference sequences used in Fig. 2

Species	External GenBank reference accessions shown in Fig. 2
Aureobasidium pullulans	AM160630.1; EF690466.1; OW987078.1; HM992505.1; LC317470.1; OR584262.1; M55639.1
Hanseniaspora uvarum	KC254055.1; KM402041.1
Metschnikowia pulcherrima	OQ550248.1; OR662633.1
Clavispora lusitaniae	LC412737.1; KF959833.1

2.7. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Artificial Wounds in Grape Berries within Postharvest Biocontrol Models: W Systems

The tritrophic W System is a model postharvest biological control system in which the host component is an artificially wounded grape berry. We prepared the host organism by first depriving a whole, healthy grape berry of its natural epiphytic microbiota with a sodium hypochlorite solution, then applying an artificial wound. The other two components were yeast and a fungal pathogen. We used the method, with some modifications, as outlined in Schena et al. (2000). We purchased ripe Bull's Eye (Vitis Sensation) table grapes from a local market. Because these fruits

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were obtained through retail, their vineyard of origin, harvest date, cultivation history, and growing-season meteorological data were unavailable. Consequently, the climatic characterization reported in Section 2.1 applies to the Aisulu grapes collected at the Almalyk experimental site and not to the Bull's Eye grapes used in the W system. For each fungal pathogen, the W-system experiment comprised 12 conditions: 11 yeast treatments and one water-treated control. We used four replicate units per condition, and each replicate comprised 18 berries distributed between two covered plastic boxes containing nine berries each. We averaged the infection percentages from the two boxes and treated each pair as one experimental unit. Thus, 864 berries were used for each pathogen and 2,592 berries across the three pathogen assays. The berries were carefully separated from the bunches to prevent mechanical damage or spoilage and were selected for uniformity in size, color, and shape.

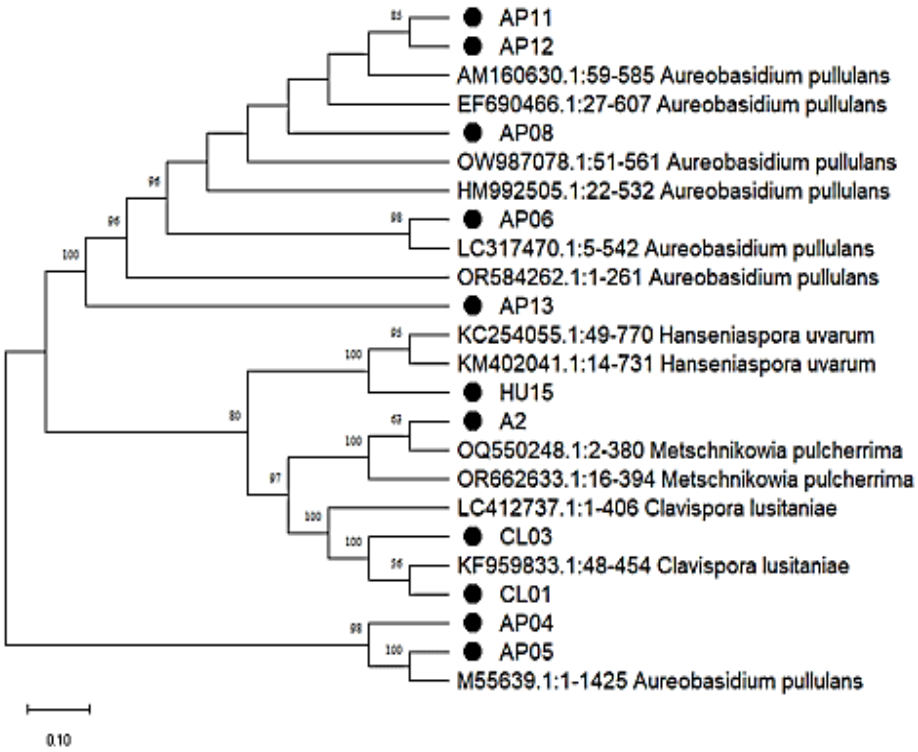

Fig. 2: Neighbor-joining analysis of antagonistic yeasts based on ITS1–5.8S–ITS2 nucleotide sequences. Numbers above branches indicate bootstrap support from 1,000 replicates, and the scale bar represents 0.1 substitutions per site. Black circles identify the 11 study isolates. GenBank accession numbers shown beside species names identify external reference sequences used for comparison; none belongs to the study isolates A2, HU15, AP04, AP05, AP06, AP08, AP11, AP12, AP13, CL01, or CL03. The correspondence between study codes and original isolate numbers is provided in Table S11, and the external reference accessions are listed in Table S12.

We then soaked the berries in a 2% sodium hypochlorite solution for two minutes to eliminate natural epiphytic microflora. After thoroughly rinsing the berries with sterile water in a laminar flow cabinet, we dried them. Using a sterile dissecting needle, we punctured the surface of each berry once, creating a two-millimeter-deep wound in the equatorial zone. We then inoculated each wound with 20 µl of an antagonistic yeast cell suspension containing 1.0×10^7 cells/ml. After two hours, we added a 20 µl aliquot of a fungal spore suspension containing 5.0×10^4 conidia/ml to the same wound. For the control, we used sterile distilled water instead of the yeast suspension.

To prevent contamination, we securely attached nine berries to the bottoms of plastic boxes using adhesive tape. For each yeast isolate, we prepared two boxes, each containing water-soaked paper to maintain high humidity, and then sealed them with lids. We stored the boxes at 20°C and measured the percentage of infected wounds after five days. We analyzed four independent replicate units for each yeast × pathogen condition (n = 4), with each replicate comprising 18 berries distributed between two boxes.

2.8. Evaluation of Biological Control Efficacy against Pathogens in the W System

We converted the percentage of infected grape berries in the W system into biological control efficacy, BEW(pat), against fungal pathogens using formula (2).

$$BEW(pat) = (PIWC - PIWS) / PIWC \quad (2)$$

BEW (pat) indicates the biological control efficacy against the pathogen in the W system.

PIWC indicates the percentage of infected artificial wounds in the bitrophic "host-pathogen" system within the control group.

PIWS indicates the percentage of infected artificial wounds in the tritrophic W system (i.e., the "host-pathogen-antagonist" relationship) within the sample group.

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2.9. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Unwounded Berries in Grape Bunches within Postharvest Biocontrol Models: B Systems

By our definition, a tritrophic System B is a model postharvest biological control system that uses bunches of grapes as the host component, which have been previously stripped of their natural epiphytic microbiota by treatment with sodium hypochlorite. The antagonistic yeast and fungal pathogen are the second and third components of this tritrophic System B. Here, we used, with minor modifications, the method described in the other article (Schena et al., 2000). For the experiment, 324 bunches of 10 healthy table grape berries of the Aisulu variety (Vitis vinifera) were selected and provided by the Kazakh Research Institute of Fruit and Vegetable Growing. The Aisulu bunches were supplied by the Talgar branch of the institute in Almalyk and were evaluated at commercial maturity. Accordingly, the regional climatic context and the local late-August harvest window described in Section 2.1 are applicable to the source area of the fruit used in the B system, although the exact harvest date of this experimental lot was not retained in the archived records.

We selected bunches that were similar in size and weight. We surface-sterilized them by soaking them in a 2% sodium hypochlorite solution for two minutes to eliminate natural epiphytic microflora. After thoroughly rinsing the bunches with sterile water in a laminar flow cabinet, we dried them. We immersed them for two hours in a suspension of antagonistic yeasts at a concentration of 1.0 × 10^7 cells/mL or in sterile distilled water for the control group.

Two hours later, we inoculated the bunches by spraying them with pathogen spore suspensions of *B. cinerea*, *A. niger*, and *R. stolonifer* at a concentration of 5.0×10^4 conidia/mL. The B-system experiment comprised six antagonist conditions—five yeast strains and one water-treated control—crossed with three fungal pathogens. Each antagonist × pathogen combination was represented by three independent replicate boxes (n = 3). Each box contained six bunches of ten berries, corresponding to 60 berries, and the box was considered the experimental unit. The complete experiment therefore included 54 boxes, 324 bunches, and 3240 berries. Water-soaked paper was used to maintain high humidity, and the boxes were sealed with lids. Inside each box, the bunches were arranged to ensure adequate spacing to prevent contact between them. The treated bunches were stored at 20°C, and the percentage of infected berries was assessed after five days.

2.10. Evaluation of Biological Control Efficacy against Pathogens in the B Systems

We converted the percentage of infected grape berries in system B into biological control efficacy (BEB; pat) against fungal pathogens using the standard formula (3).

$$BEB(pat) = (PIBC - PIBS)/ PIBC \quad (3)$$

BEB (pat) indicates the biological control efficacy against the pathogen in the B system.
PIBC indicates the percentage of infected berries in the bitrophic "host-pathogen" system within control groups.

PIBS shows the percentage of infected berries in the tritrophic B system, which includes "host-pathogen-antagonist" within the sampled group.

2.11. Evaluation of the B and W Systems' Selectivity for Protection Against Fungal Pathogens

We evaluated the selectivity for protection against one of the two fungal pathogens (pat) in B and W systems by using a statistical method called the "overlap rule for standard error (SE) columns" (Cumming et al., 2007) (see paragraph 2.12).

2.12. Data Analysis and Statistical Assessment of Results

Data are presented as means ± standard errors (SE) calculated across the experimental units defined above. Separate one-way analyses of variance (ANOVAs) were performed for PIRG, OD550, and infection incidence. For the W- and B-system experiments, each fungal pathogen and experimental system were analyzed separately, with yeast treatment as the fixed factor. Percentage variables were converted to proportions and arcsine-square-root transformed before analysis, whereas OD550 values were analyzed on the original scale. The ANOVA model assumed independent experimental units, approximately normally distributed residuals, and homogeneous residual variance. Independence was defined at the Petri-dish level for the dual-culture assay, at the paired-box level for the W system, and at the box level for the B system. The arcsine-square-root transformation was used to improve residual normality and variance homogeneity. Statistical calculations were performed using the ASTATSA One-Way ANOVA with Tukey HSD online calculator, 2016 web release, developed by Navendu Vasavada and accessed on 14 August 2025. When the omnibus ANOVA was significant, treatment means were compared using Tukey's honestly significant difference test at $\alpha = 0.01$. BEW, BEB, and R values were calculated from untransformed treatment means and were treated as derived descriptive indices rather than as independent observations in ANOVA. Residual-level diagnostic output was not retained from the legacy web analysis, which is recognized as a limitation of the study.

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Phylogenetic analysis was performed using MEGA version 11.

We assessed the statistical significance of selectivity for protection against fungal pathogens in the B and W systems using the overlap rule for standard error (SE) bars (Cumming et al., 2007). This method was applied using the percentage of infected berries in grape bunches (PIBS) in the B system and the percentage of infected wounds in grape berries (PIWS) in the W system (see Tables 1A and 2A). According to this rule, a "Gap" is the number of arms of the average SE bar that would fit between the bottom of the error bar of the sample with the higher (H) mean value and the top of the error bar of the sample with the lower (L) mean value. A gap of 2 means that the distance between the H and L error bars equals twice the average length of the error bar arms for both samples, and the probability (P) is roughly 0.05. For example, a Gap of 2 indicates that the distance between the SE bars is equal to twice the length of the average SE bar arms for the two samples. When n = 3 or 4 and twice the lengths of the SE bar arms touch (meaning the Gap distance is two average SE bar arms), P is approximately 0.05. Suppose the Gap distance exceeds two, and n equals 3 or 4. In that case, the infection rate of grape berries after treatment with one yeast against two fungal pathogens differs significantly ($P < 0.05$). Tables 4, as well as Tables 5, illustrate the method for calculating the gap distance.

Table 1A: *In vivo* screening of antagonistic yeast strains for the percentage of infected artificial wounds in W systems against *B. cinerea* F-RKM 1032, *A. niger* ATCC 6275, and *R. stolonifer* ATCC 6227b

Fungal pathogens	Values	Control / Patho-gens	Yeast strains ♯										
			A2	AP13	AP06	CL03	AP11	AP04	AP08	AP12	AP05	CL01	HU15
		Percentage of infected artificial wounds (PIW) on grape berries §											
		PIWC	PIWS										
B.cinerea	$Mean_{cin}$ §	68.81	15.50	19.51	58.35	24.04	18.28	60.35	59.98	53.80	59.36	51.1	14.75
F-RKM 1032	Groups ¥		A	B	D	B	B	D	D	C	C	C	A
A.niger	Meaning §	82.29	18.3	24.24	63.28	35.42	30.09	60.49	60.24	51.13	59.30	63.38	23.58
ATCC 6275	Groups ¥		A	B	D	B	B	C	C	C	C	D	A
R.stolonifer	$Mean_{st}$ §	83.10	21.96	26.03	74.13	26.46	24.20	71.8	70.27	66.11	72.67	68.71	19.67
ATCC 6227b	Groups ¥		A	B	D	B	A	D	C	C	D	C	A

§ Values are taken from Tables S3A to S5A in the Supplementary materials; ¥ Data come from the Main Tables S3B to S5B in the Supplementary materials; ♯ A2 - *M. pulcherrima* A2; AP – *A. pullulans* strains 13, 06, 11, 04, 08, 12, and 05; CL – *C. lusitaniae* strains 03 and 01; HU15 – *H. uvarum* strain 15; Note: For each yeast × pathogen condition, n = 4 replicate units were analyzed; each replicate comprised 18 berries distributed between two boxes containing nine berries each. The two box-level percentages were averaged before statistical analysis.

Table 2A: *In vivo* screening of antagonistic yeast strains for the percentage of infected berries in grape bunches challenged by *B. cinerea* F-RKM 1032, *A. niger* ATCC 6275, and *R. stolonifer* ATCC 6227b within the B systems

Fungal pathogens	Values	Control / Pathogens	Yeast strains ♯				
			A2	CL03	AP 11	AP 13	HU 15
		Percentage of infected berries, PIB in grape bunches §					
		PIBC	PIBS				
B. cinerea	$Mean_{cin}$ §	60.55	13.31	20.79	36.49	36.37	16.45
F-RKM 1032	Groups ¥	C	A	A	B	B	A
niger	$Mean_{nig}$	46.38	16.40	23.6	30.35	35.73	16.44
ATCC 6275	Groups ¥	C	A	B	B	B	A
R. stolonifer	$Mean_{st}$	63.53	32.46	42.96	41.30	41.79	30.18
ATCC 6227b	Groups ¥	C	A	B	B	B	A

♯ A2 - *M. pulcherrima* strain; AP11 and AP13 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; HU15 – *H. uvarum* strain 15; § Values were sourced from Tables S6A through S8A in the Supplementary materials; ¥ Data we collected from the Main Tables in the Supplementary materials, specifically from Tables S6B through S8B; Note: For each yeast × pathogen condition, n = 3 independent replicate boxes were analyzed; each box contained six bunches of ten berries, corresponding to 60 berries per experimental unit.

3. RESULTS

3.1. Objects of Study

The objects of this study were isolates of epiphytic yeasts collected from the surface of berries of the local Kazakh table grape variety, Aisulu. The yeast cultures formed large, round colonies with a shiny surface, ranging from beige to white, and had a pasty consistency when grown on potato dextrose agar (PDA). In the broth medium, yeast growth formed a significant sediment at the bottom of the test tube. We selected thirty-four yeast isolates based on their cultural and morphological characteristics.

3.2. In Vitro Screening of Yeast Isolates for Antagonistic Activity against Botrytis cinerea using the Dual-Culture Assay

Fig. 3 summarizes the antagonistic activity of 34 epiphytic yeast isolates recovered from the surface of the local Kazakh Aisulu table grape cultivar against Botrytis cinerea. Considerable variation in antagonistic potential was observed among the isolates, as reflected by differences in the percentage inhibition of radial mycelial growth (PIRG)

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(Fig. 3). The highest inhibitory activity was recorded for isolate #29, which achieved a PIRG of 71.76%, whereas isolate #10 exhibited the lowest antagonistic effect, with a PIRG of 23.76%.

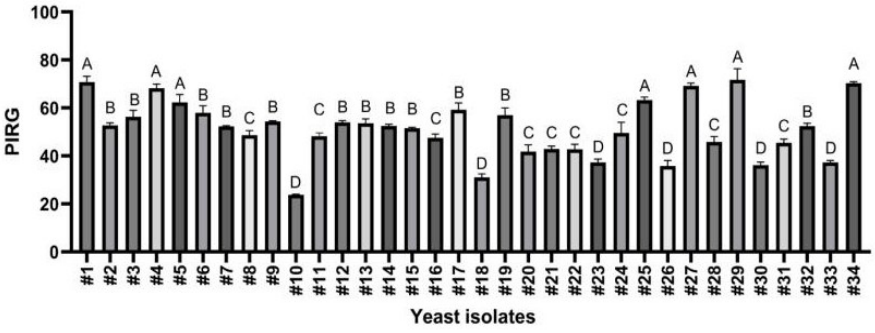

Fig. 3: Effects of yeast isolates on the radial growth of *Botrytis cinerea* (F-RKM 1032) in a dual-culture assay after seven days of incubation at 28 ± 2°C. PIRG indicates the percentage inhibition of fungal radial growth. Vertical bars show means ± SE from three independently inoculated Petri-dish experimental units (n = 3). Columns with different letters indicate significant differences according to one-way ANOVA with post-hoc Tukey Honestly Significant Difference multiple range tests (P<0.01).

Based on PIRG values, the isolates were categorized into four statistically distinct groups (A–D) using multiple-comparison analysis (Fig. 3). Nineteen isolates assigned to groups A and B demonstrated comparatively strong antagonistic activity, each exceeding the predefined selection threshold of 50% PIRG, and were therefore advanced to the next stage of screening. In contrast, the remaining 15 isolates belonging to groups C and D exhibited significantly lower inhibitory activity and were excluded from further evaluation. This initial screening effectively reduced the candidate population to isolates with the greatest potential for postharvest biocontrol of B. cinerea.

3.2. *In Vitro* Screening of Yeast Isolates for Biofilm Formation Activity

After screening 34 epiphytic yeast isolates from local grape berries for antagonistic activity, we excluded 15 isolates for their lower effectiveness. The next step in identifying BCAs was to test whether the 19 selected antagonistic yeast isolates could form biofilms in vitro. Fig. 4 shows the results of testing these 19 isolates for biofilm formation. After 72 hours of incubation, most yeast isolates adhered well to the wells of the microtiter plate, even after two washes with sterile water. The stained biofilms from 11 isolates had significantly higher optical density than the controls, which had an average OD550 of 0.245. Isolates #1 and #34 showed the highest optical density values (Fig. 4). Based on biofilm-forming capacity, 11 isolates (#1, #5–#7, #9, #12–#14, #17, #19, and #34) were selected for further analyses, whereas the remaining eight isolates were excluded because of their comparatively low biofilm production.

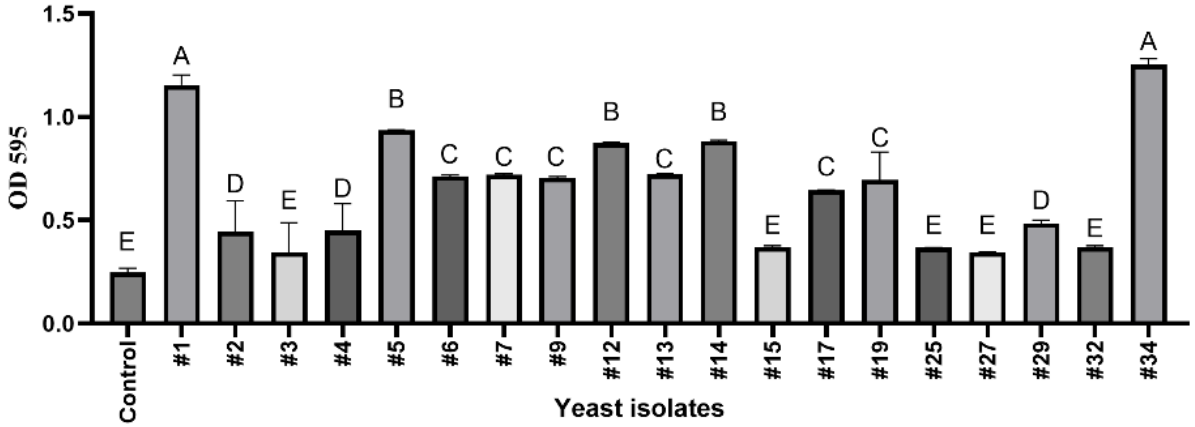

Fig. 4: In vitro biofilm-forming activity of yeast isolates. Vertical bars show means ± SE from three technical well replicates prepared from the same culture batch (n = 3). Columns labeled with different letters indicate significant differences, as determined by one-way ANOVA with post hoc Tukey honestly significant difference multiple-range tests (P<0.01). We categorized the yeast isolates by the OD550 values shown in Figure 2 into five groups: A, B, C, D, and E, and then analyzed these groups for statistical differences in their OD550 values. Finally, we selected 11 yeast isolates with the relatively highest biofilm-forming activity from groups A, B, and C

3.3. Identification of Antagonistic Yeasts using Molecular Taxonomy and Phylogenetic Analysis

Fig. 2 shows a phylogenetic tree that clusters 11 antagonistic yeasts, selected based on their performance in a previous in vitro biofilm formation assay (Fig. 4). The tree groups these yeasts into proposed clades representing phylogenetically related genera. Phylogenetic analysis based on the ITS region assigned the 11 selected

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antagonistic isolates to four genera. Isolate #1 was identified as *Metschnikowia pulcherrima* (A2), isolate #34 as *Hanseniaspora uvarum* (HU15), isolates #17 and #19 as *Clavispora lusitaniae* (CL01 and CL03), whereas isolates #5, #6, #7, #9, #12, #13, and #14 belonged to *Aureobasidium pullulans* (AP04, AP05, AP06, AP08, AP11, AP12, and AP13, respectively). Bootstrap support reached 63% for the *M. pulcherrima* clade and 56% for the *C. lusitaniae* clade (Fig. 2). The correspondence between the original isolate numbers and the internal strain codes is summarized in Table S11; the GenBank accession numbers of the external reference sequences used in Fig. 2 are listed separately in Table S12.

3.4. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Artificial Wounds in Grape Berries within W Systems

Table 1A presents the results of screening 11 selected antagonistic yeast strains in W systems. We divided these yeast strains into four groups: A, B, C, and D, based on their ability to protect wounds from fungal infections in W systems. Strains in groups A and B did not differ from each other ($P>0.01$), but differed from strains in groups C and D ($P<0.01$). Strains in groups C and D did not differ from each other ($P>0.01$).

In experiments with six yeast strains—A. pullulans (AP05, AP04, AP06, AP08, and AP12) and C. lusitaniae (CL01)—wound infection rates, comparable to the control variant, were observed, with the pathogen present in the wound of a grape berry. Infection rates for gray mold ranged from 51.1 to 60.35%, for black mold from 51.13 to 63.38%, and for Rhizopus rot from 66.11 to 74.13% (Table 1A). These six yeast strains were classified as C and D groups (Table 1A). The control groups showed wound infection rates of 68.81 to 83.10% and were also classified as group C or D (Table 1A). In the W systems that used five yeast strains—M. pulcherrima (A2), H. uvarum (HU15), A. pullulans (AP11, AP13), and C. lusitaniae (CL03)—pathogens resulted in low wound infection rates. Specifically, the infection rates for gray mold ranged from 14.75 to 24.04%, for black mold caused by *A. niger* from 18.13 to 35.42%, and for Rhizopus rot from 19.67 to 26.46%, as shown in Table 1A. These yeast strains were classified as group AB, as shown in Table 1A. Accordingly, these five strains (*M. pulcherrima* A2, *H. uvarum* HU15, *A. pullulans* AP11 and AP13, and *C. lusitaniae* CL03) were selected for subsequent experiments, whereas the remaining strains were excluded because of their substantially lower protective activity. Biological control efficacy (BEW) for the selected strains was subsequently calculated according to Eq. (2).

3.5. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Berries in Grape Bunches within the B Systems

Table 2A presents the results of experiments conducted in the B systems, which mimic the natural microenvironment of the grape berry bunch. In this step, we tested five highly effective antagonistic yeast strains previously identified in the W systems (as shown in Table 1A). In the control variants of the pathogen-host interaction, the percentage of infected berries in the grape bunches due to pathogen contamination ranged from 46.38% to 63.53%.

We observed relatively high levels of grape berry infection in the B models. The incidence of black mold ranged from 23.6 to 35.73%, while *Rhizopus* rot ranged from 41.30 to 42.96%. These results were associated with the B systems, which included the antagonistic yeast strains *A. pullulans* (AP11 and AP13) and *C. lusitaniae* (CL03). We categorized these yeast strains into group B (Table 2A). Regarding gray mold infection, strains AP11 and AP13 were classified as group B. However, strain CL03 was assigned to group A because it had a 20.79% gray mold infection rate (Table 2A). The infection rates of grapes in bunches with strains AP11 and AP13 were at least twice as high for gray and black mold and significantly higher for Rhizopus rot than those of two promising strains, A2 and HU15, which belong to group A (Table 2A). Biological control efficacy (BEB) was subsequently calculated according to Eq. (3) (Table 2B). The highest BEB values were obtained for strains A2 and HU15, whereas AP11 and AP13 consistently exhibited lower efficacy. Based on their overall performance, A2 and HU15 were identified as the most promising candidates for further postharvest evaluation.

3.6. Evaluation of Biological Control Efficacy of W and B Systems Using Targeted Antagonistic Yeast Strains against *B. cinerea* F-RKM 1032, *A. niger* ATCC 6275, and *R. stolonifer* ATCC 6227b

To standardize data for the postharvest biocontrol system, we calculate the "biological control efficacy" (BE) presented in Tables 1B and 2B.

3.7. Comparing the Biocontrol Efficacies of W and B Systems Using the Same Antagonistic Yeast Strains against Fungal Pathogens

This study conducted a quantitative comparison of the biocontrol efficacies of the W and B systems using the

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same target antagonistic yeast strains against fungal pathogens (Table 3). The coefficient R(pat) represents the ratio of the biocontrol efficacies of the W and B systems, calculated as BEW/BEB. We used this coefficient to assess differences in biocontrol efficacy between the W and B systems shown in Table 3. Notably, its value ranges from about 1 for strains A2, HU15, and CL03 to 3.07 for strain AP13 (Table 3).

Table 1B: Biological control efficacy of W systems with antagonistic yeast strains against *B. cinerea* F-RKM 1032, *A. niger* ATCC 6275, and *R. stolonifer* ATCC 6227b

Fungal strains	Groups¥	Yeast strains ♯										
		A2	AP13	AP06	CL03	AP11	AP04	AP08	AP12	AP05	CL01	HU15
		Biocontrol efficacy, BEW(pat), % ♠										
B.cinerea F-RKM 1032		77.47	71.65	15.2	65.06	73.43	12.29	12.83	21.81	13.73	25.74	78.56
	Groups	A	B	D	B	B	D	D	C	C	C	A
A.niger ATCC 6275		77.76	70.54	23.1	56.96	63.43	25.74	26.8	37.87	27.94	22.98	71.35
	Groups	A	B	D	B	B	C	C	C	C	D	A
R.stolonifer ATCC 6227b		73.57	68.68	10.8	68.16	70.88	13.6	15.44	20.45	12.55	17.32	76.33
	Groups	A	B	D	B	A	D	C	C	D	C	A

♠ Values were calculated using Formula (2) from Section 2.8 in the Materials and Methods; ¥ Groups are sourced from Table 1A; ♯ A2 - *M. pulcherrima* A2; AP – *A. pullulans* strains 13, 06, 11, 04, 08, 12, and 05; CL – *C. lusitaniae* strains 03 and 01; HU – *H. uvarum* strain 15.

Table 2B: Biological control efficacy of B systems with targeted antagonist yeast strains against *B. cinerea* F-RKM 1032, *A. niger* ATCC 6275, and *R. stolonifer* ATCC 6227b

Fungal pathogens	Yeast strains ♯				
	A2	CL03	AP 11	AP 13	HU 15
	Biocontrol efficacy, BEB(pat) ♠				
B. cinerea F-RKM 1032	78.02	65.66	39.74	39.93	72.83
Groups ¥	A	A	B	B	A
A.niger ATCC 6275	64.64	49.12	34.56	22.96	64.55
Groups ¥	A	B	B	B	A
R. stolonifer ATCC 6227b.	48.91	32.38	34.99	34.22	52.49
Groups ¥	A	B	B	B	A

♯ A2 - *M. pulcherrima* strain; AP11 and AP13 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; HU15 – *H. uvarum* strain; ♠ Values were calculated using Formula (3) from Section 2.10 in the Materials and Methods; ¥ Data are sourced from Table 2A.

Table 3: Comparison of the biological control efficacy against three pathogens between the W and B systems for each of the target strains of antagonistic yeasts

	Postharvest biological control system models								
Fungal pathogens	*Botrytis cinerea* F-RKM 1032			*Aspergillus niger* ATCC 6275			*Rhizopus stolonifer* ATCC 6227b		
Yeast strains ♯	W	B	R(pat) ♠ = BEW/BEB	W	B	R(pat) ♠ = BEW/BEB	W	B	R(pat) ♠ = BEW/BEB
	Biocontrol efficacy, %			*Biocontrol efficacy, %*			*Biocontrol efficacy,%*		
	BEW(cin)	*BEB(cin)*	R(cin)	*BEW(nig)*	*BEB(nig)*	R(nig)	*BEW(st)*	*BEB(st)*	R(st)
A2	77.47	78.02	0.99	77.76	64.64	1.20	73.57	48.91	1.50
HU15	78.56	72.83	1.08	71.35	64.55	1.11	76.33	52.49	1.45
AP11	73.43	39.74	1.85	63.43	34.56	1.84	70.88	34.99	2.03
AP13	71.65	39.93	1.79	70.54	22.96	3.07	68.68	34.22	2.01
CL03	65.06	65.66	0.99	56.96	49.12	1.16	68.16	32.38	2.11

♯ A2 - *M. pulcherrima* strain; HU15 – *H. uvarum* strain; AP11 and AP13 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; ♠ The coefficients R (pat) indicate the ratio of the biological control efficacy of system W to that of system B.

The five antagonistic yeasts differed markedly in the stability of their biocontrol performance between the two experimental systems. The lowest R values were observed for *Metschnikowia pulcherrima* A2 and *Hanseniaspora uvarum* HU15 against *Botrytis cinerea* and *Aspergillus niger*, indicating that their biocontrol efficacy remained largely unchanged between the W and B systems. In contrast, *Aureobasidium pullulans* strains AP11 and AP13 exhibited substantially higher R values against all three pathogens, indicating a pronounced reduction in biological control efficacy under B-system conditions. Intermediate values were obtained for *Clavispora lusitaniae* CL03. For *Rhizopus stolonifer*, R values were consistently higher than those observed for *B. cinerea* and *A. niger* across all antagonistic strains, indicating that biocontrol efficacy decreased in the B system irrespective of the yeast strain used.

3.8. Evaluation of the Selectivity of B and W Systems for Protection against a Fungal Pathogen

***3.8.1. Evaluation of the B System's Selectivity for Protection against Fungal Pathogens*:** We evaluated the B

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system's selectivity against fungal pathogens by calculating the average percentage of infected grapes per bunch for each pathogen. Table 4 presents the mean values and standard errors. It also includes the Gap values (cin/nig, cin/st, and nig/st), which we calculated using the method described in Table 4. It also includes the Gap values (cin/nig, cin/st, and nig/st), which we calculated using the overlap-rule method for standard errors described by Cumming et al. (2007).

Table 4: The necessary data to evaluate the B systems' selectivity for protection against fungal pathogens, along with the corresponding Gap values (cin/nig, cin/st, and nig/st)

Fungal pathogens[¥]	values[§]	Yeast strains [♯]				
		A2	CL03	AP 11	AP 13	HU 15
		Percentage of infected grape berries (PIBpat), PIBB				
B.cinerea	Mean(cin)	13.33	25.55	34.57	36.30	16.30
	SD(cin)	2.94	5.56	2.04	3.40	3.90
	SE(cin)	1.70	3.21	1.18	1.96	2.26
		Percentage of infected grape berries (PIBpat), PIBA				
A.niger	Mean(nig)	16.30	23.70	30.00	35.56	16.67
	SD(nig)	3.57	3.40	2.22	2.94	2.23
	SE(nig)	2.07	1.96	1.28	1.70	1.29
Gap (cin/nig)		-0.4265[Nd]	-2.7152	-5.7089	-2.404	-1.7911
Gap (nig/cin)		-3.5735	-1.2848	1.70888	-1.596	-2.2089
		Percentage of infected grape berries,(PIBpat), PIBR				
R.stolonifer	Mean(st)	32.22	42.96	41.11	41.85	30.00
	SD(st)	2.22	3.40	1.11	2.79	2.22
	SE(st)	1.28	1.96	0.64	1.61	1.28
Gap (cin/st)		10.6583	4.72939	5.18676	1.10467	5.74299
Gap (nig/st)		7.50917	7.81143	9.54372	1.79626	8.3787

[♯] A2 - *M. pulcherrima* strain; AP11 and AP13 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; HU15 – *H. uvarum* strain; [¥] *B. cinerea* (F-RKM 1032) from the Republican Collection of Microorganisms LLC, *A. niger* (ATCC 6275), and *R. stolonifer* (ATCC 6227b) from the American Type Culture Collection; [§] Values are taken from Tables S6A through S8A in the Supplementary materials; [Nd] - not determined, a value was not determined due to a non-significant difference between means; Gap(cin/nig) > 2 indicates that the B system is selective for protection against *B. cinerea* over *A. niger*; Gap(cin/st) > 2 suggests that the B system is selective for protection against *B. cinerea* over *R. stolonifera*; Gap(nig/st) > 2 indicates that the B system is selective for protection against *A. niger* over *R. stolonifer*.

***3.8.2. Evaluation of the W Systems' Selectivity in Protecting against Fungal Pathogens*:** We evaluated the W systems' selectivity in defending against fungal pathogens by calculating the average percentage of infected artificial wounds on grape berries for each pathogen. Table 5 displays the mean values and standard errors and includes the Gap values (cin/nig, cin/st, and nig/st). Table 6 presents selectivity data showing that systems W and B differ in their selectivity for pathogen protection. Systems B with strains A2, HU15, CL03, and AP11 are selective in protecting against *B. cinerea* and *A. niger* but not against *R. stolonifer*. Systems W with strains CL03 and AP11 are selective in protecting against *B. cinerea* and *R. stolonifera* but not against *A. niger.* In the B system, strains A2, HU15, CL03, and AP11 provided selective protection against *Botrytis cinerea* and *Aspergillus niger*, whereas protection against *Rhizopus stolonifer* was significantly lower (Table 4 and 6). Under identical pathogen inoculum concentrations and antagonist treatments, *R. stolonifer* consistently produced higher berry infection levels than the other two pathogens. In contrast, no comparable selectivity was observed in the W system, where strains A2, HU15, CL03, and AP11 provided similar levels of protection against all three pathogens (Table 5 and 6).

4. DISCUSSION

The decision to source biological control agents from the autochthonous epiphytic community of the local *Vitis vinifera* cultivar Aisulu is consistent with the now prevailing view that indigenous, site-adapted yeasts possess important ecological advantages over laboratory or commercial strains. Native epiphytic yeasts are better adapted to the berry microenvironment, allowing more efficient colonization of grape surfaces and more stable persistence under postharvest conditions. Previous studies demonstrated that yeasts isolated directly from grape berries suppress *Botrytis cinerea* and other postharvest pathogens more effectively than reference strains maintained in culture collections (Prendes et al., 2017; Kasfi et al., 2018; Li et al., 2024). Interestingly, the taxonomic composition of the antagonistic yeasts identified in the present study closely resembles that reported for grape berries from other viticultural regions. Surveys conducted on Malbec grapes in Argentina recovered predominantly *Aureobasidium pullulans*, followed by *Hanseniaspora uvarum* and *Metschnikowia pulcherrima*, whereas our isolates from the Kazakh cultivar Aisulu were dominated by the same three genera together with *Clavispora lusitaniae* (Prendes et al., 2017). This agreement across geographically distinct vineyards supports the view that *Metschnikowia*, *Hanseniaspora*, and *Aureobasidium* constitute a conserved core of the grape epiphytic microbiota and represent particularly promising sources of biological control agents (Prendes et al., 2017; Reyes-Bravo et al., 2021). Sequential

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screening under both laboratory and biologically relevant conditions is essential because strong antagonism observed in vitro does not necessarily translate into effective disease suppression on intact fruit. The two-stage W/B screening strategy proposed by Schena et al. (2000) addresses this limitation by distinguishing performance under nutrient-rich wounded tissues from that under the more restrictive conditions of intact grape bunches. The present results support this concept: although many isolates showed promising antagonistic activity in vitro and in W systems, only *M. pulcherrima* A2 and *H. uvarum* HU15 maintained high efficacy under the more stressful conditions of the B system.

Table 5: The necessary data to evaluate the W system's selectivity for protection against fungal pathogens, along with the corresponding gap values (cin/nig, cin/st, and st/nig)

Fungal pathogens	Values[§]	Yeast strains[♯]				
		A2	CL03[♠]	API1[♠]	API3	HU15[♠]
		Percentage of infected artificial wounds (PIWpat), PIWB				
B. cinerea	$Mean_{cin}$	15.28	25.00	18.06	19.45	16.67
	SD_{cin}	5.32	3.21	2.78	5.56	0.00
	SE_{cin}	2.66	1.61	1.39	2.78	0.00
		Percentage of infected artificial wounds (PIWpat), PIWA				
A.niger	$Mean_{nig}$	16.67	34.72	30.56	23.61	22.22
	SD_{nig}	4.54	2.78	3.20	2.78	9.07
	SE_{nig}	2.27	1.39	1.60	1.39	4.54
	Gap (cin/nig)	-1.4358[Nd]	4.49074	6.36052	-0.0024	0.44728
		Percentage of infected artificial wounds (PIWpat), PIWR				
R. stolonifer	$Mean_{st}$	22.22	26.39	23.61	26.39	18.06
	SD_{st}	4.54	2.78	2.78	2.78	2.78
	SE_{st}	2.27	1.39	1.39	1.39	1.39
	Gap (cin/st)	0.82	-1.07	2.00*	1.33	0.00
	Gap (st /nig)	-4.4495	3.99281	2.64215	-4	-0.5945
	Gap (nig/st)	0.45	-7.99	-6.64	0.00	-3.41

[♯] A2 - *M. pulcherrima* strain; API1 and API3 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; HU15 – *H. uvarum* strain; [¥] *B. cinerea* (F-RKM 1032) from the Republican Collection of Microorganisms LLC, *A. niger* (ATCC 6275), and *R. stolonifer* (ATCC 6227b) from the American Type Culture Collection; [§] Values are taken from Tables S3A through S5A in the Supplementary Materials; [Nd] - not determined, a value was not determined due to the impossibility of calculation; [♠] In this case, the Gap (st/nig) was calculated based on the fact that Mean(nig) was higher than Mean(st) (see Table 6); * An asterisk sign indicates that the W system protects equally against both *B. cinerea* and *R. stolonifer* with a probability of P=0.95; Gap (cin/nig) > 2 indicates that the W system is selective for protection against *B. cinerea* over *A. niger* with a probability of P > 0.95; Gap (cin/st) > 2 demonstrates that the W system is selective for protection against *B. cinerea* over *R. stolonifer* with a probability of P > 0.95; Gap (st/nig) > 2 indicates that the W system is selective for protection against *R. stolonifer* over *A. niger* with a probability of P > 0.95.

Table 6: Combined data on the selectivity of W and B systems for fungal pathogens

Systems	Gap	Antagonistic yeasts [♯]				
		Group A			Group B	
		A2	HU15	CL03[♪]	API1	API3
B	Gap (cin/st)	+[§]	+	+	+	
	Gap(nig/cin)					
	Gap (nig/st)	+	+	+	+	
W	Gap (cin/st)				*	
	Gap(cin/nig)			+[§]	+	
	Gap (st /nig)			+	+	

[♯] A2 - *M. pulcherrima* strain; API1 and API3 – *A. pullulans* strains; CL03 – *C. lusitaniae* strain; HU15 – *H. uvarum* strain; [¥] *B. cinerea* (F-RKM 1032) from the Republican Collection of Microorganisms LLC, *A. niger* (ATCC 6275), and *R. stolonifer* (ATCC 6227b) from the American Type Culture Collection; [♪] Strain CL03 is classified as Group A based on PIBS measurements after infection with *B. cinerea* in the B system (see Table 2A); [§] A plus sign (+) indicates that the Gap distance exceeds 2; *An asterisk sign (*) indicates that the Gap distance equals 2; A gap (cin/st) of 2 indicates that the W system protects equally against both *B. cinerea* and *R. stolonifera;* A gap (cin/st) > 2 indicates that the W and B systems are selective for protection against B. cinerea rather than *R. stolonifera;* Gap (cin/nig) > 2 indicates that the W system is selective for protection against *B. cinerea* over *A. niger*; Gap (st/nig) > 2 indicates that the W system is selective for protection against *R. stolonifer* over *A. niger*; Gap (nig/st) > 2 indicates that the B system is selective for protection against *A. niger* over *R. stolonifer.*

4.1. *In Vitro* Screening of Yeast Isolates for Antagonistic Activity against *B. cinerea* using the Dual-Culture Method

Identifying effective BCAs against diseases requires appropriate screening methods to assess potential antagonists for the development of biocontrol products. Testing candidate biocontrol organisms on plants *in vivo* is often necessary. However, large-scale screening studies can be slow and labor-intensive.

The inhibition of mycelial growth and zone formation in dual cultures, as illustrated in Fig. 3, is likely due to the release of potential diffusible antifungal compounds, as reported in other studies. For instance, one study reported that the growth of *Rhizoctonia solani* colonies in dual cultures was reduced by 57.3% when using strains L1 and L8 of *A. pullulans.* The yeast produced several identified non-volatile organic compounds, primarily complex carbohydrates (such as pullulan and other β-glucans), degrading enzymes, cyclic depsipeptides, and siderophores, all

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of which contributed to inhibiting the pathogen's mycelial growth (Di Francesco et al., 2021). The dual-culture assay evaluates only one component of the antagonist–pathogen interaction, namely the production and diffusion of soluble or semi-volatile metabolites capable of suppressing fungal mycelial growth. The wide variation in PIRG values observed among our isolates (23.76–71.76%) therefore most likely reflects strain-specific differences in the production of antifungal secondary metabolites, a phenomenon previously described for both *Aureobasidium pullulans* and *Metschnikowia* species (Reyes-Bravo et al., 2021; Roca-Couso et al., 2021).

Several classes of diffusible metabolites have been proposed to explain this antagonistic activity. *A. pullulans* produces aureobasidins, cyclic depsipeptides that inhibit fungal sphingolipid biosynthesis through inositol phosphorylceramide synthase inhibition, thereby disrupting membrane integrity (Roca-Couso et al., 2021). Other yeasts produce killer toxins that form pores in pathogen cell membranes or arrest cell-cycle progression, while volatile organic compounds, including 2-phenylethanol, ethyl acetate and isoamyl acetate derivatives, further contribute to fungal growth inhibition (Reyes-Bravo et al., 2021; Roca-Couso et al., 2021). The strong antagonistic activity observed for isolate #29 and several *A. pullulans* isolates in the present study is likely the result of the combined action of several of these mechanisms. Nevertheless, the exact metabolites responsible for inhibition remain to be identified and should be investigated in future studies using metabolomic approaches such as HPLC-MS.

4.2. *In Vitro* Screening of Yeast Isolates for Biofilm Formation Activity

Biofilms are dense microbial communities that adhere to fruit surfaces and are encased in a polymeric extracellular matrix (Costa-Orlandi et al. 2017). Biofilm formation by yeasts is considered one of several potential methods for inhibiting fungi and managing postharvest fruit diseases (Dukare et al., 2019; Freimoser et al., 2019; Zhang et al., 2020a). Antagonistic yeasts use this ability to compete for space and nutrients (Rendueles & Ghigo, 2015; Klein & Kupper, 2018; Freimoser et al., 2019). Scherm et al. (2003) demonstrated a direct link between the formation of S. cerevisiae M25 biofilm and its ability to suppress pathogens. Only S. cerevisiae cells collected during biofilm formation were effective at preventing P. expansum from growing on apples (Scherm et al., 2003). Biofilm formation is a crucial strategy for Metschnikowia citriensis to defend against Penicillium digitatum and Penicillium italicum on citrus fruit (Liu et al., 2019). The results shown in Fig. 4 are consistent with previous research (Parafati et al., 2015). In this study, the researchers found that M. pulcherrima cells formed significant biofilms after 48 hours of incubation, reaching an optical density (OD) at 590 nm of 1.15. After 72 hours, the OD measured 1.12 units. In a separate study, strains L1 and L8 of A. pullulans formed biofilms on polystyrene plates, with ODs ranging from 1.0 to 1.5 after 48 hours under similar incubation conditions. The data presented in Fig. 4 closely align with the results from these cited studies, confirming the validity of our findings.

4.3. Identification of Antagonistic Yeasts Using Molecular Taxonomy and Phylogenetic Analysis

Identification of antagonistic yeasts provides an important basis for understanding their ecology, mechanisms of action, and potential practical application as biological control agents (Wilson & Wisniewski, 1994). The relatively moderate bootstrap support observed for the *Metschnikowia pulcherrima* (63%) and *Clavispora lusitaniae* (56%) clades is consistent with previous reports describing substantial ITS variability within these taxa. In *M. pulcherrima*, heterogeneous rDNA repeats, frequent nucleotide polymorphisms, and ambiguous barcode boundaries may reduce phylogenetic support values (Sipiczki, 2020). The predominance of *Aureobasidium pullulans* among the selected isolates is not unexpected because this species is one of the most widespread epiphytic yeasts inhabiting grape berries and has repeatedly been reported as an effective antagonist of postharvest pathogens. However, previous studies have also demonstrated considerable genetic diversity and phenotypic variability within this species (Gostinčar et al., 2019). Such variability may influence the stability of biocontrol performance and therefore should be considered during strain selection and commercial development.

4.4. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Artificial Wounds in Grape Berries within W Systems

The tritrophic W system (see paragraph 2.7) contains a rich supply of nutrients and maintains an acidic pH (Spadaro & Droby, 2016). The interactions among the W system's components are complex and not fully understood. Researchers are actively investigating these mechanisms, but a comprehensive understanding of their interactions is still evolving. The W system represents a nutrient-rich, acidic microenvironment that closely resembles wounded grape tissues after harvest and therefore provides a stringent model for evaluating postharvest biological control agents (Spadaro & Droby, 2016). The high protective activity observed for *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Aureobasidium pullulans*, and *Clavispora lusitaniae* is likely explained by the combined action of several complementary biocontrol mechanisms, including competition for nutrients and space, biofilm formation, secretion of antifungal metabolites, and induction of host resistance, rather than by any single mechanism alone (Romanazzi et al., 2016; Dukare et al., 2019; Zhang et al., 2020a).

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The infection levels obtained with *M. pulcherrima* are comparable to those previously reported for *Metschnikowia* spp. in wounded fruit systems (Schena et al., 2000). Likewise, the performance of *A. pullulans* falls within the range reported for other strains evaluated against postharvest pathogens (Schena et al., 1999). Differences among studies are expected because biocontrol efficacy depends on the specific pathogen strain, host cultivar, antagonist genotype, and environmental conditions (Rovenich et al., 2014; Romanazzi et al., 2016; Spadaro & Droby, 2016; Tian et al., 2016; Lovato et al., 2019; Zhang et al., 2020b; Reyes-Bravo et al., 2021; Tziros et al., 2022). The seven *A. pullulans* isolates recovered in the present study – AP04, AP05, AP06, AP08, AP11, AP12, and AP13 – were identified by ITS sequencing; however, ITS markers alone do not capture the substantial genomic and phenotypic diversity reported within this species. Comparative genomic analyses have revealed extensive intraspecific recombination and trait heterogeneity among *A. pullulans* strains, resulting in pronounced differences in their biocontrol potential (Gostinčar et al., 2019; Reyes-Bravo et al., 2021). This diversity is reflected in our results, where some isolates exhibited only weak protection, whereas AP11 and AP13 ranked among the most effective strains in the W system. These findings reinforce previous observations that species-level identification alone is insufficient for selecting superior biological control agents. Consequently, future screening programs would benefit from higher-resolution approaches, including microsatellite analysis or whole-genome sequencing, to distinguish elite *A. pullulans* genotypes from less effective isolates. The concept of biological control efficacy (BE) provides a standardized quantitative framework for comparing antagonist performance across different host–pathogen systems and therefore facilitates evaluation of candidate BCAs under experimentally comparable conditions.

4.5. *In Vivo* Screening of Antagonistic Yeast Strains for the Percentage of Infected Berries in Grape Bunches within the B Systems

The B system represents intact grape bunches and therefore provides a substantially more challenging environment for biological control than artificial wounds. In contrast to wounded tissues, intact berries offer limited nutrient availability and reduced access to leakage products, requiring antagonistic yeasts to compete successfully for scarce resources while maintaining stable populations on the berry surface (Spadaro & Droby, 2016). Consequently, this model more closely reflects commercial postharvest storage conditions. Competition for nutrients and space is widely regarded as one of the principal mechanisms underlying yeast-mediated biological control of postharvest diseases (Zhang et al., 2020a; He et al., 2024). Under wound conditions, leakage of carbohydrates, amino acids and other nutrients promotes rapid colonization by antagonistic yeasts, allowing them to occupy infection sites before pathogen establishment. By rapidly consuming available nutrients and excluding pathogens from colonization sites, antagonistic yeasts suppress spore germination and mycelial growth without necessarily killing the pathogen. The protection levels observed for *Metschnikowia pulcherrima* A2, *Hanseniaspora uvarum* HU15, *Aureobasidium pullulans* AP11 and AP13 and *Clavispora lusitaniae* CL03 are therefore consistent with previously reported biological control efficacies for these genera under nutrient-rich wound conditions (Zhang et al., 2020a; Reyes-Bravo et al., 2021).

The superior performance of *M. pulcherrima* A2 and *H. uvarum* HU15 in the B system suggests that these native epiphytic yeasts are well adapted to the grape surface microenvironment. Their effectiveness is likely attributable to the combined action of several complementary mechanisms previously described for postharvest BCAs, including rapid colonization of the berry surface, competition for nutrients and space, biofilm formation, production of antifungal metabolites, and induction of host defense responses (Romanazzi et al., 2016; Dukare et al., 2019; Zhang et al., 2020a). Such multifunctional antagonism is particularly advantageous under the nutrient-limited conditions of intact grape bunches.

The performance of *M. pulcherrima* A2 was generally consistent with previous reports describing *Metschnikowia* spp. as highly effective postharvest BCAs (Schena et al., 2000). Although protection against *Rhizopus* rot was lower than reported in some earlier studies, efficacy against gray and black mold was comparable or higher. These differences are expected because both antagonist performance and pathogen susceptibility vary considerably among individual strains (Reyes-Bravo et al., 2021). Similarly, the efficacy of *H. uvarum* HU15 agrees well with previous studies demonstrating strong suppression of gray mold on grape berries by *Hanseniaspora* isolates (Liu et al., 2010). This consistency supports the growing evidence that naturally occurring *H. uvarum* populations represent promising biological control agents for postharvest protection of grapes. In contrast, the two *Aureobasidium pullulans* isolates evaluated in the present study showed substantially lower efficacy than some strains previously described in the literature (Schena et al., 1999). This observation illustrates the pronounced strain-dependent variability that characterizes this species and indicates that taxonomic identity alone is insufficient to predict biological control performance. Genetic diversity within *A. pullulans* populations has previously been reported as a major source of variation in antagonistic activity (Gostinčar et al., 2019).

An additional advantage of the dominant antagonists identified in this study is that they belong to the non-*Saccharomyces* epiphytic yeast community naturally associated with grape berries. Unlike filamentous fungal

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pathogens such as *Aspergillus* spp., which are capable of producing mycotoxins including ochratoxin A during postharvest storage (Freire et al., 2018), non-*Saccharomyces* wine yeasts generally lack mycotoxin biosynthetic pathways and are therefore considered intrinsically safer for food-related applications (Reyes-Bravo et al., 2021). The recovery of *M. pulcherrima*, *H. uvarum*, and *A. pullulans* from Aisulu berries is consistent with this food safety advantage and further supports their suitability as biological control agents.

Although *Clavispora lusitaniae* CL03 demonstrated good antagonistic activity in both the W and B systems, its practical application as a postharvest biocontrol agent is limited by biosafety considerations. *C. lusitaniae* is increasingly recognized as an opportunistic human pathogen and has been associated with resistance to several clinically important antifungal drugs (Mendoza-Reyes et al., 2022). Consequently, despite its promising biological performance, its use in food applications would likely face regulatory restrictions and therefore requires careful safety assessment before practical implementation.

Overall, differences observed among antagonistic yeasts most likely reflect the combined influence of yeast genotype, pathogen aggressiveness, host characteristics and environmental conditions, all of which are recognized determinants of postharvest biocontrol success (Romanazzi et al., 2016; Dukare et al., 2019; Zhang et al., 2020a). Considering their consistently high efficacy under both wounded and intact berry conditions, *M. pulcherrima* A2 and *H. uvarum* HU15 emerged as the most promising candidates for the biological control of major postharvest diseases of table grapes.

4.6. Comparing the Biocontrol Efficacies of W and B Systems Using the Same Antagonistic Yeast Strains against Fungal Pathogens

Comparison of the W and B systems provides insight not only into the absolute efficacy of antagonistic yeasts but also into the stability of their performance under contrasting postharvest microenvironments. Whereas the W system represents nutrient-rich artificial wounds, the B system mimics intact grape bunches, where nutrient availability is considerably lower and environmental conditions are more restrictive (Spadaro & Droby, 2016). Consequently, the ratio between biological control efficacies in the two systems (R) serves as an indicator of antagonist robustness under environmentally stressful conditions. Among the strains evaluated, *Metschnikowia pulcherrima* A2 and *Hanseniaspora uvarum* HU15 showed the greatest stability. Their R values remained close to 1 against *Botrytis cinerea* and *Aspergillus niger*, indicating that their antagonistic activity was largely maintained despite the transition from nutrient-rich wounds to intact berries. Such stability agrees well with current screening criteria for biological control agents, which emphasize consistent performance under variable environmental conditions as an essential characteristic of effective BCAs (Wilson & Wisniewski, 1989; Nunes, 2012; Jiménez & Köhl, 2020).

The marked reduction in biological control efficacy observed for several antagonists after transfer from the W to the B system is consistent with the ecological differences between these two experimental models. Unlike wounded berries, intact grape bunches provide only limited nutrient leakage, forcing antagonistic yeasts to survive and compete under substantially more restrictive conditions. Rapidly colonizing species with high affinity for scarce nutrients, such as *M. pulcherrima* and *H. uvarum*, are therefore expected to maintain higher efficacy than slower-growing or more substrate-dependent antagonists, such as some *Aureobasidium pullulans* strains (Zhang et al., 2020a; He et al., 2024). This interpretation is fully consistent with the R values obtained in the present study, where strains A2 and HU15 remained comparatively stable across both experimental systems, whereas AP11 and AP13 showed a pronounced loss of efficacy under nutrient-limited conditions. In contrast, *A. pullulans* strains AP11 and AP13 exhibited approximately twofold lower efficacy in the B system than in the W system, indicating that although both strains performed well in artificial wounds, their antagonistic activity declined substantially under the more stressful conditions of intact grape bunches. Similar strain-dependent variation has been reported previously for *A. pullulans*, whose considerable genetic diversity is reflected in marked differences in biological control performance (Gostinčar et al., 2019). Therefore, our findings suggest that AP11 and AP13 do not satisfy the stability criteria expected of commercially useful BCAs.

The consistently superior performance of *M. pulcherrima* A2 may additionally be explained by one of the best-characterized mechanisms of this species, namely iron depletion through pulcherrimin production. Pulcherriminic acid secreted by *M. pulcherrima* chelates ferric ions to form the insoluble pigment pulcherrimin, thereby depriving competing fungi of iron required for respiration, oxidative-stress defence and pathogenic development (Reyes-Bravo et al., 2021; Roca-Couso et al., 2021; He et al., 2024). Previous studies have demonstrated that supplementation with exogenous iron almost completely abolishes the antagonistic activity of pulcherrimin-producing strains, confirming iron competition as one of the principal mechanisms responsible for disease suppression. The low R values obtained for strain A2 against *B. cinerea* and *A. niger* suggest that this mechanism remained effective even under the nutrient-limited conditions represented by the B system.

Besides direct antagonism, antagonistic yeasts may also enhance host resistance by priming defence responses

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before pathogen invasion. Previous studies have reported increased accumulation of pathogenesis-related proteins, β-1,3-glucanases, chitinases, stilbenes and reactive oxygen species following yeast colonization of grape berries (Zhang et al., 2020a; Reyes-Bravo et al., 2021; He et al., 2024). Recent transcriptomic and metabolomic analyses further demonstrated that yeast-mediated protection is accompanied by extensive reprogramming of host defence pathways (Lovato et al., 2019). Although host defense activation was not investigated in the present study, future transcriptomic, metabolomic, and integrative multi-omics analyses of *Aisulu* grape berries could elucidate the extent to which induced resistance contributes to the biocontrol efficacy of the selected yeast antagonists. The consistently higher R values observed for *Rhizopus stolonifer* compared with *Botrytis cinerea* and *Aspergillus niger* indicate that suppression of Rhizopus rot was inherently more challenging under B-system conditions. Notably, this trend was also observed for the two most stable antagonists, *Metschnikowia pulcherrima* A2 and *Hanseniaspora uvarum* HU15, whose antagonistic performance against *B. cinerea* and *A. niger* remained largely unchanged across the two experimental systems. The elevated R values recorded for *R. stolonifer* (1.45–2.11) therefore suggest that the residual reduction in biocontrol efficacy was primarily attributable to the greater aggressiveness and infection dynamics of the pathogen, rather than to diminished ecological fitness or antagonistic capacity of the yeast strains.

Rhizopus stolonifer possesses several biological characteristics that contribute to its exceptional pathogenicity, including rapid mycelial growth, prolific conidial production and dispersal, and the secretion of extracellular hydrolytic enzymes that promote host tissue penetration and maceration (Baggio et al., 2016). Moreover, successful phytopathogens can manipulate or suppress host immune responses during infection, thereby facilitating colonization and disease progression (Rovenich et al., 2014). These attributes likely account for the comparatively lower biocontrol efficacy observed against R. stolonifer, despite the stable antagonistic performance of the selected yeast strains against Botrytis cinerea and Aspergillus niger. This interpretation is further supported by the selectivity analysis presented in the following section, which revealed consistently higher infection levels for R. stolonifer than for the other two pathogens. Collectively, the comparative evaluation of the W and B systems demonstrates that screening based solely on artificially wounded fruit may overestimate the practical performance of biological control agents (BCAs). Although several yeast isolates exhibited satisfactory antagonistic activity in the W system, only Metschnikowia pulcherrima A2 and Hanseniaspora uvarum HU15 consistently retained high biocontrol efficacy under the more physiologically relevant and nutritionally restrictive conditions of intact grape bunches. These findings identify A2 and HU15 as the most promising indigenous yeast candidates for the development of effective postharvest BCAs for table grapes. The pathogen-dependent differences in efficacy and stability are further explored through the selectivity analysis presented in the subsequent section.

4.7. Evaluation of the Selectivity of B and W Systems for Protecting Against a Fungal Pathogen

The selectivity analysis provides additional evidence supporting the interpretation proposed in Section 4.6 that *Rhizopus stolonifer* strain 6227b is more difficult to suppress under the stressful conditions represented by the B system. Although the antagonistic yeasts maintained high efficacy against *Botrytis cinerea* and *Aspergillus niger*, protection against *R. stolonifer* was consistently weaker. Because pathogen inoculum concentrations, antagonist concentrations, berry size, and bunch weight were kept constant throughout the experiments, the observed differences are most likely attributable to biological characteristics of the pathogen rather than to experimental variation.

The absence of pathogen selectivity in the W system contrasts with the clear selectivity observed in the B system. This difference can be explained by the distinct ecological conditions represented by the two experimental models. Artificial wounds provide abundant nutrients released from damaged tissues, allowing antagonistic yeasts to colonize rapidly and compete effectively with invading pathogens. Under these nutrient-rich conditions, the protective activity of the yeasts appears sufficient to suppress all three pathogens to a comparable extent. In contrast, intact grape bunches represent a nutrient-limited environment in which antagonists must establish and persist under considerably greater ecological stress (Romanazzi et al., 2016; Spadaro & Droby, 2016; Zhang et al., 2020a). The selective reduction in protection against *R. stolonifer* observed in the B system therefore supports the hypothesis that this pathogen possesses a greater capacity to infect intact berries than either *B. cinerea* or *A. niger*. The aggressive behaviour of *R. stolonifer* has previously been associated with rapid colonization, extensive secretion of extracellular hydrolytic enzymes, and efficient invasion of host tissues, characteristics that enable the pathogen to overcome host barriers and compete successfully under restrictive environmental conditions (Baggio et al., 2016; Rovenich et al., 2014). These biological features likely explain why *R. stolonifer* remained comparatively difficult to control even when highly effective antagonistic yeasts were present. Importantly, the selectivity analysis also corroborates the conclusions drawn from the comparison of W and B systems in the previous section. The reduced biological control efficacy against *R. stolonifer* is more plausibly explained by the exceptional aggressiveness of the pathogen than by a loss of antagonist activity. This interpretation is supported by the stable performance of *Metschnikowia pulcherrima* A2 and *Hanseniaspora uvarum* HU15 against *B. cinerea* and *A. niger* under the same environmental conditions.

Taken together, these findings indicate that *M. pulcherrima* A2 and *H. uvarum* HU15 satisfy an important

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requirement for biological control agents—the ability to maintain antagonistic activity under environmentally challenging postharvest conditions. Their reduced efficacy against *R. stolonifer* most likely reflects the inherent aggressiveness of this pathogen rather than instability of the yeast strains themselves, further supporting the selection of A2 and HU15 as the most promising candidates for biological control of postharvest grape diseases.

4.8. Combination strategies to enhance BCA efficacy

A common and well-documented bottleneck of single-yeast BCAs is the moderate efficacy under non-sterile, real-world conditions. Several recent strategies have been validated to overcome this limitation. First, combination with the plant hormone β-aminobutyric acid was tested by Cheng et al. (2019), who showed that *H. uvarum* + BABA significantly improved control of postharvest kiwifruit diseases compared with either agent alone, presumably by priming host resistance. Second, salicylic acid combined with *Pichia membranaefaciens* or with mixed antagonistic yeasts has consistently elevated biocontrol efficacy on peach, sweet cherry and mandarin fruit, through the joint action of SA-induced systemic acquired resistance and yeast competition/iron depletion (Zhang et al., 2020a; Reyes-Bravo et al., 2021). Third, food-grade coatings (chitosan) applied together with *P. membranaefaciens* on citrus reduced green and blue mould incidence by 66% and 83% respectively after four days of storage at 20°C (Reyes-Bravo et al., 2021). Fourth, physical treatments such as hot water, cold acclimatization and sublethal oxidative stress applied as preadaptation to yeast inocula raised biocontrol efficacy in apple, tomato and citrus (Reyes-Bravo et al., 2021; He et al., 2024). Fifth, multi-strain or synthetic microbial consortia yield more consistent suppression than single-strain applications, because niche complementarity stabilizes the consortium under fluctuating environmental conditions (Prendes et al., 2017; Reyes-Bravo et al., 2021; He et al., 2024). Sixth, genetic modification, exemplified by surface-displayed flagellin on *S. cerevisiae* cells, increased biocontrol of *B. cinerea* on tomato (Zhang et al., 2020a; Reyes-Bravo et al., 2021). In our setting, the most readily adoptable next steps are: (1) combination of A2 or HU15 with BABA or SA on Aisulu bunches; (2) formulation with chitosan or pullulan-based coatings; (3) construction of a defined A2 + HU15 consortium for *B. cinerea* and *A. niger* control, leveraging the complementary iron-depletion mechanism of A2 and the strong competitive colonization of HU15.

4.9. Limitations and future directions

Three limitations should be acknowledged. First, the *in vivo* data in W and B systems were generated on detached berries and bunches; field-aged berries under actual vineyard storage conditions could alter the efficacy landscape. Second, although ITS region identification is the established gold standard for yeast taxonomy, finer resolution achieved by whole-genome sequencing would substantially improve strain tracking, biosafety evaluation and intellectual property protection. Third, omics-level dissection of the host response (transcriptomics of berry tissue, metabolomics of stilbenes and PR proteins) under A2 and HU15 protection would mechanistically validate the induced-resistance component of our interpretation (Lovato et al., 2019; Zhang et al., 2020a; Roca-Couso et al., 2021). Future research priorities are therefore: (1) field validation of A2 and HU15 in commercial vineyard trials across multiple Kazakh growing regions; (2) combination testing with BABA, SA, chitosan coatings and the A2 + HU15 consortium to optimize field efficacy; (3) whole-genome sequencing of A2 and HU15 for strain tracking and to enable future genetic improvement; (4) targeted mutagenesis or surface-display engineering of pulcherrimin production in A2 to enhance iron-depletion potency; and (5) biosafety assessment and residue analysis to support future regulatory submissions in the Eurasian Customs Union.

5. CONCLUSION

We employed a rigorous two-stage screening strategy to identify indigenous epiphytic yeast strains with strong potential as postharvest biological control agents (BCAs) from the local Kazakh *Aisulu* table grape cultivar. In the first stage, 34 epiphytic yeast isolates were evaluated for their antagonistic activity against *Botrytis cinerea* and their biofilm-forming ability under in vitro conditions. Based on predefined selection criteria, 23 isolates exhibiting comparatively low antagonistic or colonization potential were excluded, resulting in the selection of 11 promising candidates for subsequent in vivo evaluation. In the second stage, the selected isolates were assessed against the three principal postharvest fungal pathogens—*Botrytis cinerea*, *Aspergillus niger*, and *Rhizopus stolonifer*—using two complementary postharvest bioassay systems: wounded grape berries (W system) and intact grape bunches (B system). Six isolates were excluded because of their limited ability to suppress pathogen infection in the W system. Of the remaining five candidates, three were subsequently eliminated due to reduced efficacy under the more physiologically relevant conditions of the B system or, in the case of *Clavispora lusitaniae* CL03, because of biosafety concerns despite its antagonistic potential. This sequential screening strategy ultimately identified *Metschnikowia pulcherrima* A2 and *Hanseniaspora uvarum* HU15 as the two most promising biological control agents. Both strains consistently maintained high biocontrol efficacy under intact bunch conditions and exhibited performance comparable to, or exceeding, that reported for previously characterized yeast antagonists. Their stable activity across contrasting

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postharvest microenvironments highlights their potential for practical application in commercial grape storage and transportation. The comparative evaluation further revealed distinct pathogen-dependent differences in biocontrol performance. Although all three pathogens were effectively suppressed in wounded berries, *Rhizopus stolonifer* exhibited markedly greater aggressiveness under the nutritionally restrictive conditions of intact grape bunches, resulting in lower control efficacy than that observed for *B. cinerea* and *A. niger*. These findings underscore the importance of incorporating both wounded- and unwounded-fruit assays into screening programs, as reliance on wounded-fruit assays alone may overestimate the practical performance of candidate BCAs under commercial postharvest conditions. Overall, the indigenous yeast strains *M. pulcherrima* A2 and *H. uvarum* HU15 represent promising sustainable alternatives to sulfur dioxide (SO_2)-based preservation and synthetic fungicides for the postharvest protection of table grapes. Future research should focus on elucidating the molecular and ecological mechanisms underlying their antagonistic activity, persistence, and colonization using transcriptomic, metabolomic, and other multi-omics approaches. In addition, their efficacy should be evaluated as a defined microbial consortium, in combination with resistance-inducing compounds such as β-aminobutyric acid or salicylic acid, and through advanced strategies including yeast surface engineering to further improve biocontrol performance and extend cold-chain shelf life.

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