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# Application of Microbial Antagonists in Combination with Sodium Bicarbonate to Control Post-Harvest Diseases of Sweet Cherry (*Prunus avium* L.) and Plums (*Prunus salicina* Lindl.)

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**Abstract:** Postharvest diseases cause considerable losses of fruits and vegetables during transportation and storage, and synthetic fungicides are the first option for their prevention. However, promising alternatives to chemical fungicides are currently available, and several post-harvest diseases can be controlled using microbial antagonists. This study utilised a comprehensive methodology to assess the antagonistic and synergistic interactions between four yeasts and two bacteria in conjunction with sodium bicarbonate (SB) during the treatment of sweet cherries and plums. The aim of this study was to evaluate the effects of microbial antagonists and sodium bicarbonate on fruits. The in situ treatments showed a protective effect exerted by the antagonists *P. guillermondii* and *H. uvarum,* and their combination with SB. However, in vivo studies did not indicate enhanced efficacy when combined with this compound. In conclusion, the use of microbial antagonists in conjunction with SB has been successful in preventing post-harvest rot of cherries and plums. Tests conducted on live organisms have shown that microbial antagonists are viable alternatives to synthetic fungicides for the control of stone fruit rot.

**Keywords:** biological control; hyperparasitism; microbial antagonists; post-harvest rot; stone fruit

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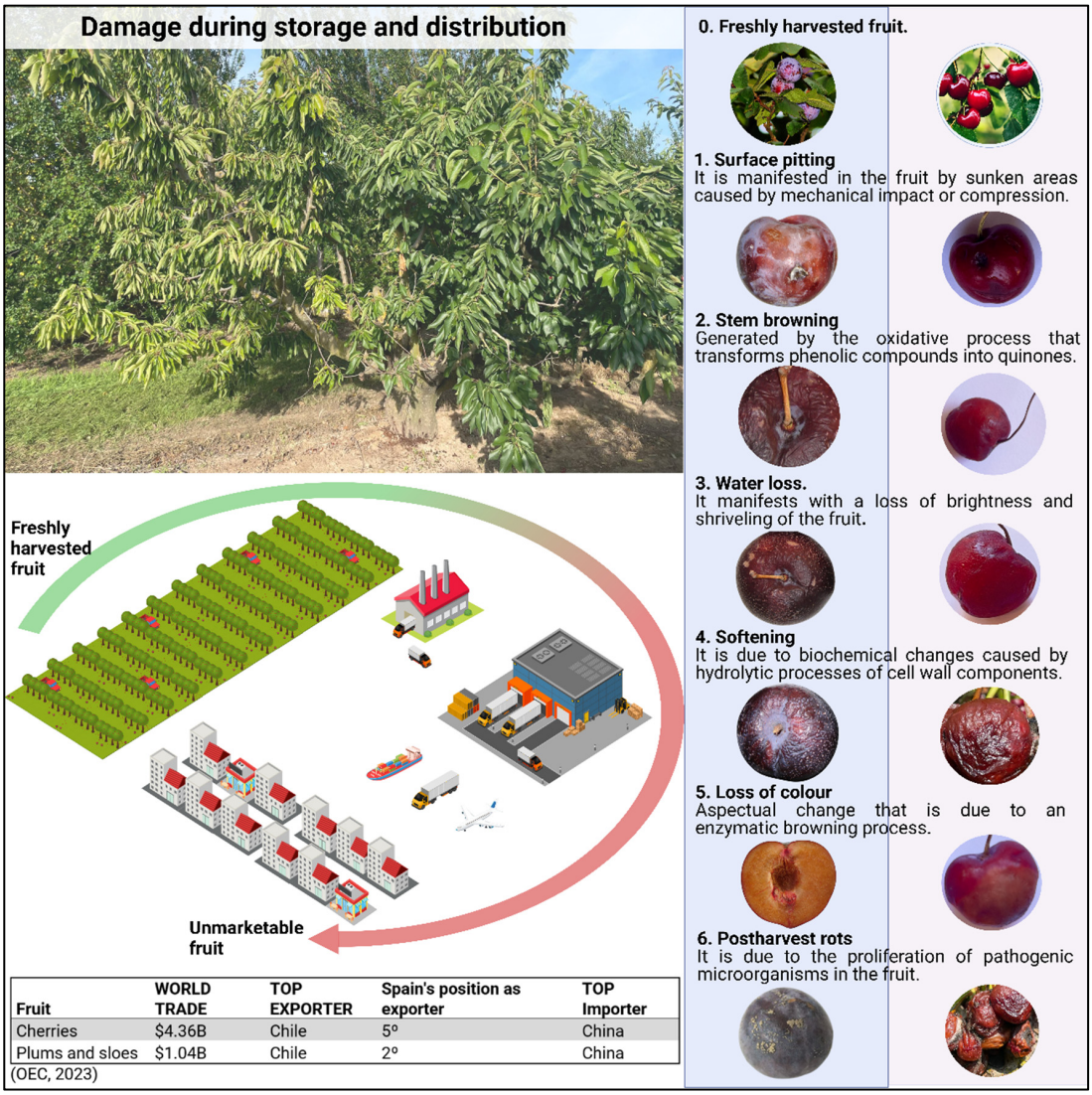
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## 1. Introduction

It is estimated that more than one-third of harvested fruits and vegetables worldwide are lost during postharvest handling [1]. As summarised in Figure 1, among the factors contributing to these losses, intrinsic physiological senescence and fungal pathogen infection are the most significant [2]. Specifically, the post-harvest storage period of stone fruits, such as sweet cherries and plums, is limited by various factors, including water loss, softening, surface pitting, stem browning, colour loss, and post-harvest rot [3].




| Fruit | WORLD TRADE | TOP EXPORTER | Spain's position as exporter | TOP Importer |
|---|---|---|---|---|
| Cherries | $4.36B | Chile | 5º | China |
| Plums and sloes | $1.04B | Chile | 2º | China |

(OEC, 2023)

**Figure 1.** Factors contributing to the deterioration of plums and cherries before they reach the market [4].

Additionally, stone fruits are susceptible to the development of fungal infections throughout the supply chain, as their tissues deteriorate during prolonged storage periods, making them more susceptible to infections during shelf life (SL) [5]. In this regard, post-harvest rot is mainly caused by fungal pathogens, such as *Botrytis cinerea* Pers.:Fr, *Monilinia* spp., *Alternaria alternata* (Fr.:Fr.) Keissl, *Penicillium expansum* Link, *Rhizopus stolonifer* (Erhenb.:Fr) Vuill, *Cladosporium* spp. and *Mucor* spp. [6].

For many years, synthetic fungicides have proven to be the most effective treatment for controlling post-harvest diseases in fruits and vegetables. However, their use is increasingly restricted owing to public concerns about the environmental and human health risks associated with the use of pesticides, as well as the development of resistance in fungal pathogens to fungicides. Since the 2010s, the use of synthetic fungicides to control post-harvest diseases in stone fruit has been limited to a few authorised products, namely fludioxonil (Scholar®), (DeccoPyr Pot®), and (Xedathane 20®), the first of which was registered in 2015 [7].

Extremadura is a major contributor to the national plum production in Spain, accounting for more than 40% of the total output. It is also one of the primary regions for cherry production in Europe, ranking second in the national scene after the Aragon. In 2022, the Extremadura sector produced almost 4% of the cherry output in the European Union [4,8,9].

Determining the precise extent of fruit spoilage caused by biotic and abiotic factors is a complex task. Nevertheless, several factors, such as the volume and quality of production, weather events, different types of growers, and sales destinations can influence the occurrence of spoilage [10]. A pilot study by INE from 2003 to 2006 found that approximately 20% of harvested fruits spoilt before reaching consumers, showing that microorganisms are a major cause [11].

If 20% of Extremadura's losses are due to biological agents, then in 2022, about 4.5 metric tons of cherries and 15 metric tons of plums were lost. These losses are significant, as they represent produce that is unable to reach its intended points of sale due to deterioration caused by these agents.

Owing to these restrictions, there is a global trend towards the development of safer and more eco-friendly alternative methods to reduce or replace the use of synthetic fungicides. In this context, the use of microbiological control agents, particularly microbial antagonists, for the treatment of post-harvest diseases shows promise as an alternative to chemical fungicides for stone fruits [12].

The use of microbial antagonists offers several advantages, including safety, persistence, low impact on the ecological balance, and compatibility with other methods. However, the major limitations of biocontrol are the lack of eradication activity and an activity spectrum narrower than that of synthetic fungicides [13]. Currently, many microbial antagonists against post-harvest diseases have been identified, and commercially available biocontrol formulations include AMYLO-X® WG (*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747; Mitsui Agriculture International S.A./N.V, Woluwe-Saint-Pierre, Belgium), NEXY® (*Candida oleophila* strain 0: Agrauxine S.A., Marcq en Baroeul, France), TUSAL® (*Trichoderma asperellum* strain T25 and *Trichoderma atroviride* strain T11, Timac Agro España S.A., Navarra, Spain), and POLYVERSUM® (*Pythium oligandrum* strain M1: Agrichem, S.A., Madrid, Spain) [14]. Despite the progress made in post-harvest biocontrol over the past 30 years, commercial applications of microbial antagonists remain limited. Economic and regulatory constraints are the main obstacles to the successful introduction of these products into the market [15,16].

In addition to microbial antagonists, low-toxicity chemical alternatives such as sodium bicarbonate (SB) have been evaluated for controlling post-harvest [17]. SB exhibits adequate antifungal activity, low risk of phytotoxicity, and minimal environmental impact, and it is readily available and inexpensive, with a lack of restrictions for many applications [17]. Furthermore, SB has been approved as a food additive and is generally recognised as a safe (GRAS) substance by the United States Food and Drug Administration (FDA). As a result, it has become the most commonly used food preservative for controlling decay in citrus fruits [18,19]. It has also been successfully used to reduce post-harvest decay in carrots, mangoes, avocados, bell peppers, melons, and sweet cherries [20–24].

The activity spectrum of these alternative control methods is not as broad as that of chemical fungicides, and many of them cannot achieve maximum effectiveness, even under optimal conditions. Therefore, a combination of treatments must be used to develop a multibarrier control strategy that is suitable for commercial applications. In this regard, microbial antagonists and $NaHCO_3$ have been combined to achieve effects similar to those of chemical fungicides in reducing the post-harvest decay of several fruits, such as sweet cherry, pears, mandarin, and grapes [25–28].

The objective of this study was to investigate the biocontrol activities of different microbial antagonists and SB, both in situ and to control post-harvest diseases in sweet cherries (*Prunus avium* L. cv. Sweethearts) and plums (*Prunus salicina* Lindl. cv. 'Angeleno').

## 2. Materials and Methods

### 2.1. Chemicals and Fruit Material

Sweet cherries (*Prunus avium* L. cv. 'Sweethearts') and plums (*Prunus salicina* Lindl. cv. 'Angeleno') were harvested at commercial maturity, following standards established in the packing house in Extremadura, Spain. The fruits were disinfected by immersion in 2%

sodium hypochlorite (NaClO) (Sigma-Aldrich, Darmstadt, Germany) for 2 min, washed with tap water, and air-dried before use.

Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Germany) was used at a concentration of 2% (*w*/*v*).

### 2.2. Antagonists

Microbial antagonists were acquired from the Spanish-type culture collection (CECT). *Cryptococcus laurentii*, *Trichosporon pullulans*, *Hanseniaspora uvarum*, and *Pichia guillermondii* were cultured in potato dextrose broth (BioCult Laboratories, Madrid, Spain) for 48 h at 25 °C. *Bacillus subtilis* and *Pseudomonas syringae* were cultured in Mueller–Hinton broth (Oxoid, Basingstoke, UK) for 24 h at 37 °C. To obtain microbial cell suspensions, cells were centrifuged at 6000 rpm (3365× *g*) for 10 min, resuspended in sterile peptone water, and centrifuged a second time. Washed cells (pellets) of the four yeasts and two bacteria were suspended in peptone water and adjusted to a concentration of $1 \times 10^8$ cells $mL^{-1}$ using a Neubauer haemocytometer. (Brand GmbH + Co KG, Wertheim, Germany).

### 2.3. Pathogen Culture

The pathogens used in this study were obtained from the INTAEX Collection (Badajoz, Spain). Initially, these pathogens were isolated from infected sweet cherries and plums obtained from fruit-packaging houses in Extremadura. Subsequently, they were identified using PCR. The pathogenicity of these isolates was evaluated, and the strains with the highest capacity to cause rot were selected. Spore suspensions of *Botrytis cinerea*, *Penicillium expansum*, *Aspergillus niger*, *Fusarium oxysporum*, and *Rhizopus stolonifer* were cultured for one week on potato dextrose agar (PDA) (Oxoid, Basingstoke, UK) enriched with malt extract at 25 °C, whereas *Monilia laxa* was cultured on PDA enriched with nectarine macerated at 25 °C to induce sporulation. Spores were removed from the surface of the cultures, suspended in 5 mL of sterile deionised water, and filtered through four layers of sterile gauze to remove any adhered mycelia. Spore concentrations were adjusted to $1 \times 10^4$ spores $mL^{-1}$ using a Neubauer haemocytometer.

### 2.4. Fruit Inoculation and Selection of Microbial Antagonists

The effects of antagonism, SB, or a combination of both on the pathogen population were studied using the in situ assay previously established by [29]. Sweet cherries and plums were wounded (3 mm deep and 3 mm wide) with a sterile nail in the equatorial zone. Each wound was inoculated with 20 µL of the following treatment suspensions: antagonist ($1 \times 10^8$ cells $mL^{-1}$), SB (2%), or antagonist ($1 \times 10^8$ cells $mL^{-1}$) in combination with SB (2%). Fruits treated with sterile distilled water were used as controls. When the wounds were air-dried, sweet cherries and plums were challenge-inoculated with 15 µL of a conidial suspension of *M. laxa*, *B. cinerea*, *P. expansum*, *A. niger*, *F. oxysporum* or *R. stolonifer* at $1 \times 10^4$ spores $mL^{-1}$ independently and stored in air at 25 °C with ≈90% relative humidity (RH). Disease incidence was measured after four days of storage. Fruits that developed rot in the pathogen-inoculated area were quantified, which allowed the establishment of the rot inhibition rate expressed as a percentage of inhibition.

### 2.5. Assessment of Microbial Antagonists and Sodium Bicarbonate for Effective Post-Harvest Disease Control in Sweet Cherries: In Vivo Evaluation and Industrial Application Simulation

For the in vivo assay, the fruits were divided into blocks and the selected microbial antagonist and SB were applied individually and in combination to evaluate their efficacy against pathogens. The antagonists chosen for the in vivo assays were *H. uvarum* and *P. gullermondii*, which showed significant effectiveness in the in situ assays.

To simulate an industrial application, the fruit that typically exhibited the most rapid deterioration in commercialisation conditions, namely the cherry, was selected from among the two specimens tested. Subsequently, the fruits were immersed in a solution containing antagonists.

Post-harvest storage conditions were designed to replicate those commonly used in fruit-processing plants. The typical storage conditions used were 0–4 °C for temperature and 90–95% relative humidity (RH). Furthermore, the study also considered shelf-life conditions of 25 °C and a relative humidity of 90%.

Thirty cherries were sampled at harvest; after 30 days of cold storage, they were maintained for 3 and 5 days of shelf life. These time points were chosen to simulate the duration required to transport the fruit from Spanish crop fields to distant destinations. The viability of the antagonist treatments was assessed by comparing the inoculated fruits with control fruits subjected to the same storage conditions.

### 2.6. Statistical Analysis

Data were analysed using Statistical Package SPSS 17.0 version for Windows (SPSS Inc., Chicago, IL, USA). The mean values were compared using ANOVA, and when significant differences ($p < 0.05$) were observed between the mean values, Tukey's test was applied. Mean values with standard deviations are reported.

## 3. Results

### 3.1. Efficacy of Biocontrol Agents on In Situ Assay

Figure 2 (combining some data published in previous works by [30] and data from this study) depicts the antagonist activity of four yeast strains and two bacteria (*C. laurentii*, *T. pullulans*, *H. uvarum*, *P. guillermondii*, *B. subtilis*, and *P. syringae*, respectively) against five filamentous pathogenic fungi (*M. laxa*, *B. cinerea*, *P. expansum*, *A. niger*, and *F. oxysporium*) in "Sweetheart" cherries and "Angeleno" plums.

Regarding *M. laxa*, which is responsible for brown rot, the four antagonistic yeasts caused partial rot inhibition in sweet cherries, reaching total inhibition when combined with SB (Figure 2D). Regarding antagonistic bacteria, *B. subtilis* achieved total inhibition when combined with SB, as shown in Figure 2.

For *P. expansum*, none of the antagonists demonstrated effectiveness against this mould, which causes blue rot in sweet cherries (Figure 2C). Similarly, the addition of 2% SB did not significantly reduce the decay caused by the mould. However, in "Angeleno" plums (Figure 2C), an antagonistic effect was observed with the combined application of *H. uvarum* and *P. guillermondii* along with SB. Only when combined with SB did *C. lauretii*, *T. pullulans* and *P. guillermondii* inhibited *P. expansum* growth.

In contrast to *F. oxysporum*, only *H. uvarum* exhibited growth inhibition owing to a synergistic effect, similar to that observed in *M. laxa*.

When evaluating antagonist activity in sweet cherries against *A. niger* (Figure 2E), it was observed that *H. uvarum* and *P. guillermondii* partially inhibited rot development caused by these fungi, reaching total inhibition in both cases (total antagonist effect against *A. niger*). The same inhibition was observed for *A. niger* and *H. uvarum* combined with SB in plums (Figure 2E), although all antagonists partially inhibited *A. niger* growth, with the exception of *B. subtilis*.

In cherries, the inhibition of *B. cinerea* (Figure 2A) was similar to that observed for *A. niger* in the same fruit. *H. uvarum* and *P. guillermondii* presented partial rot inhibition, reaching total inhibition in combination with SB. However, treatment with 2% SB and SB combined with *C. laurentii*, *T. pullulans*, and *B. subtilis* resulted in the partial inhibition of *B. cinerea* growth, resulting in a lower incidence of losses caused by this mould.

All antagonistic microorganisms were effective in the plums, showing an increase when the treatments were combined with SB.

Antagonist activity varied significantly depending on the fruit. *H. uvarum* and *P. guillermondii* exhibited an effect in plums when combined with SB, and also for *P. guillermondii* alone. In the cherries, no antagonistic effects were observed for any of the tested microorganisms.

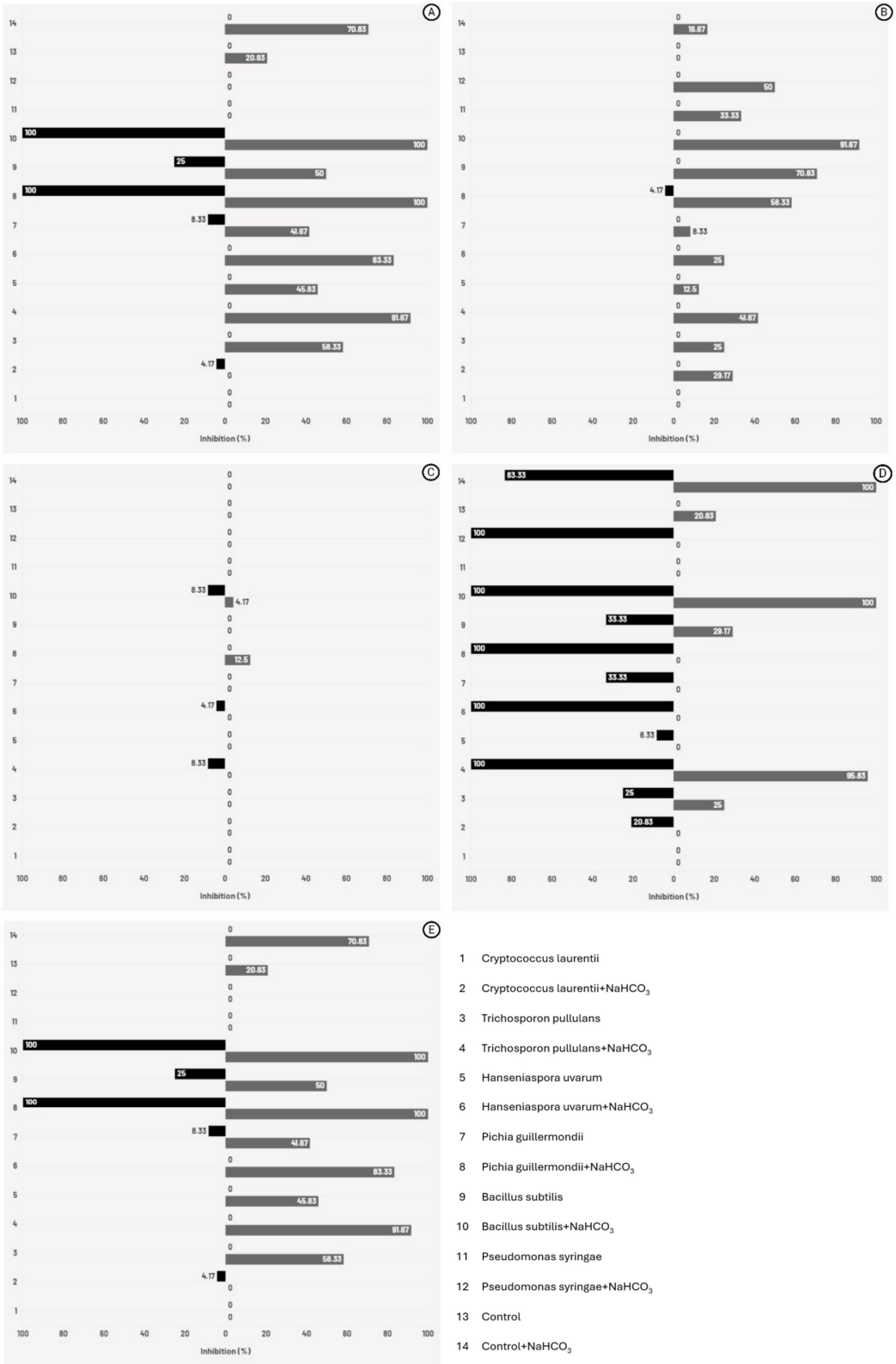



**Figure 2.** Antagonistic impact of four yeasts (*C. laurentii*, *T. pullulans*, *H. uvarum*, and *P. guilliermondii*) and two bacteria (*B. subtilis* and *P. syringae*) both individually and when combined with 2% sodium bicarbonate against five cherry (in black) and plum (in grey)-damaging fungi: ((**A**) *B. cinerea*, (**B**) *F. oxysporum*, (**C**) *P. expansum*, (**D**) *M. laxa*, and (**E**) *A. niger*). The data represent the mean. Data with the mean ± standard error of the mean of 40 independent experiments are included in the Supplementary Materials.

### 3.2. Efficacy of Biocontrol Agents on In Vivo Assay

To evaluate the effect of the antagonist *P. guilliermondii* when subjected to distribution and marketing conditions, we conducted an experiment to examine the influence of various antagonist concentrations on highly perishable cherry fruit. Owing to its rapid deterioration during the distribution process, it is considered the most challenging fruit to maintain in terms of quality and freshness.

The incorporation of antagonists in cherries led to a marked decrease in the incidence of rotting after 10 days of shelf life (Figure 3). Specifically, treatment with *P. guillermondii* resulted in a rot incidence approximately 85% lower than that of the control samples. In addition, *H. uvarum* treatment reduced rotting by approximately 78%.

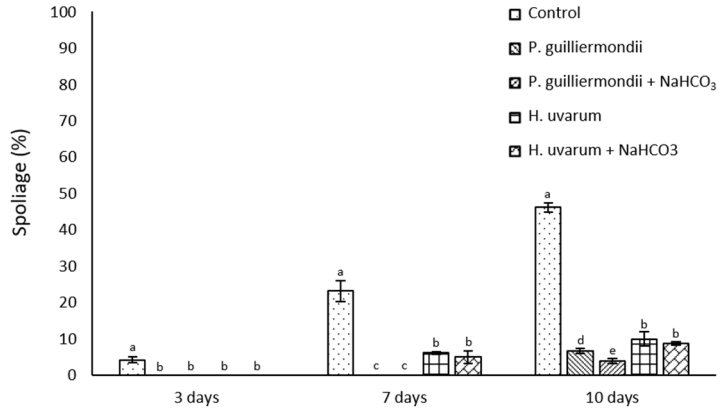



**Figure 3.** Influence of different inoculation concentrations of microbial antagonists (*H. uvarum* and *P. guillermondii*), either alone or in combination with sodium bicarbonate, on post-harvest sweet cheery under optimal conditions to induce biotic damage (25 °C and 90–100% HR). Means ± SD presented in each bar followed by the same letters are not significantly different (Tukey's test, $p < 0.05$).

Figure 4 demonstrates that after 30 days of cold storage at 0 °C, inoculation with $10^8$ colony-forming units (CFUs) per mL effectively reduced cherry spoilage to virtually zero under refrigerated conditions. Concentrations of $10^6$ CFUs/mL or lower did not exhibit any significant differences compared to the untreated control cherries. However, the data revealed that when refrigeration conditions were combined with marketing conditions, concentrations of $10^8$ CFUs/mL during the marketing phase provided a significant fourfold protection against deterioration resulting from biotic damage. Although lower concentrations maintained a positive effect, their incidence was much lower.

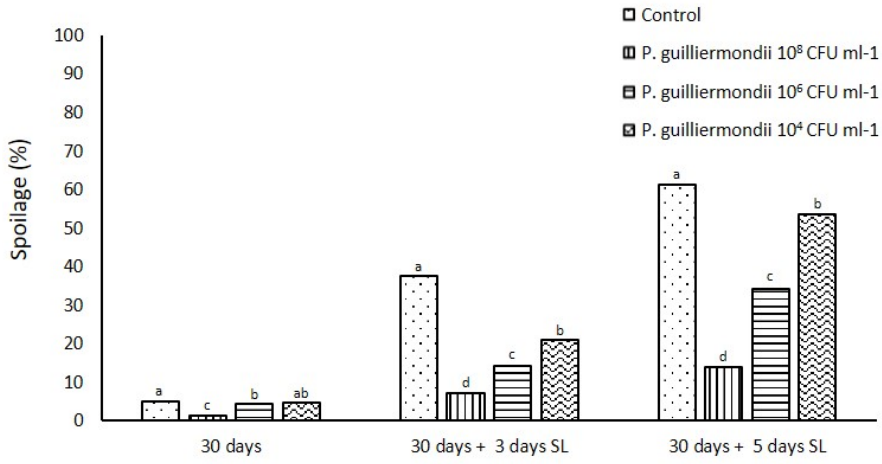



**Figure 4.** Influence of different inoculation concentrations of *P. guilliermondii* antagonist on post-harvest sweet cheery at refrigeration temperature (0 °C). Means ± SD presented in each bar followed by the same letters are not significantly different (Tukey's test, $p < 0.05$).

## 4. Discussion

To develop potential microbial antagonists, it is crucial to ensure that the selected organisms are suitable and effective in reducing post-harvest diseases compared to synthetic fungicides. Biocontrol agents must be capable of colonising, surviving, and multiplying in an environment favourable to the pathogen [31]. Another relevant factor is the viable population of microbial antagonists, which must exhibit efficiency at reasonable microbial concentrations to become viable and marketable biocontrol products [12]. By meeting these criteria, potential microbial antagonists can be developed that offer efficacy comparable to that of synthetic fungicides, while thriving in the target environment and achieving significant disease reduction at practical cell concentrations.

To reduce post-harvest decay without relying on fungicides, promising alternatives based on biological control and SB were tested alone and in combination as a strategy to provide satisfactory control of *M. laxa*, *B. cinerea*, *P. expansum*, *A. niger* and *F. oxysporum* on sweet cherries and plums. In situ and in vivo assays were conducted to determine the most suitable fungal control strategy.

### 4.1. In Situ Assays

A synergistic effect between the antagonist and SB was proposed, as neither the effect of any stand-alone treatment nor the sum of SB and the antagonist reached 100% inhibition. This finding aligns with those of [32,33], who reported that a combination of the yeast antagonists *Candida oleophila* and SB improved the efficacy of the antagonist. The inhibitory effect of SB on microorganisms may be attributed to a reduction in cell turgidity pressure, which leads to collapse and shrinkage of hyphae and spores, resulting in fungistasis [34]. This is consistent with the results obtained after treatment with "Angeleno" plums (Figure 2A), where the effect of antagonists against *M. laxa* increased when combined with SB.

Previous studies have found that SB has an inhibitory effect on the growth of *P. expansum* [35], and a synergistic effect has been observed when studying the combined effect of antagonists and SB. In previous studies using the antagonists *C. laurentii* and *T. pullulans*, both significantly reduced the severity and incidence of decay caused by *P. expansum* in sweet cherries [36]. A similar observation was reported by [29], who used the antagonists *C. laurentii* and *T. pullulans*.

Similar behaviour was observed by [37], where *C. laurentii* was effective in controlling *B. cinerea* infection in pears. This effect is potentiated by the addition of salt ($CaCl_2$), mainly because of its capacity to induce natural resistance [38].

Although [39] found that in situ *Bacillus subtilis* fmbJ exhibited a strong antifungal effect on mycelium growth, sporulation, and germ tube elongation of *R. stolonifer* when applied to fruits, this mould showed a mild sensitivity to the antagonistic effects of the microorganisms included in the study.

### 4.2. In Vivo Assay

*H. uvarum* and *P. guilliermondii* were the two antagonists that exhibited better capabilities for biological control in the in vitro assay and were thus selected for the following in vivo assay in this study.

Previous studies have demonstrated that the effectiveness of antagonists depends on the fruit from which they are applied. For example, [40] found that while a biofilm-forming strain of *Pichia fermentans* proved most effective in controlling brown rot in apple fruit, it exhibited unexpected pathogenic behaviour in peaches, causing rapid decay of fruit tissues, even in the absence of inoculated pathogens (*M. fruticola*).

This aligns with our results, as we observed different behaviours of the antagonist in cherries compared to those in plum fruits.

Research indicates that coatings containing high concentrations of *P. guillermondii* can reduce the incidence of damage to cherries by more than four-fold during marketing. This finding is consistent with that of [41], who reported that applying a high dose of an antagonist suspension to the surface of the fruit could result in a significant decrease in the

damage caused by biotic factors. However, it is important to note that the effectiveness of this strategy is greater when applied as a pre-harvest treatment rather than as a post-harvest treatment. Nonetheless, even when applied as a post-harvest treatment, the benefits remain significant.

### 4.3. Synergistic Effect of $NaHCO_3$-Antagonistic Microorganism Treatments

The osmotolerance described in species of the genus *Pichia* [42] may be the first cause of the synergistic effect observed in our study between sodium bicarbonate and the applied antagonistic yeasts, which may allow for adaptation to the hyperosmotic environment generated by foliar application of the tested treatment, without affecting its antagonistic behaviour.

Although, as explained by [43], all microorganisms have positive responses and can withstand high pressures in changing osmotic environments, and sensitivity to osmotic stress is responsible for the cell viability of many microorganisms. Permeabilisation of cytoplasmic membranes is a critical event related to cell viability temperature, and the kinetics of membrane metabolite flux regulation are the main factors that determine osmotolerance as they allow correct dehydration and rehydration, avoiding rupture of cell membranes due to sudden osmotic adaptations [44]. The adaptive mechanism of yeasts and bacteria in hyperosmotic environments, as pointed out by [45], consists of the synthesis of osmolytes such as intracellular glycerol, which can counteract up to 95% of the external osmotic pressure generated by exposure to metabolites at high concentrations such as NaCl. In view of our results, the effect of $NaHCO_3$ may be similar to those observed with NaCl and may explain the results obtained.

However, the hyphal mechanism that penetrates host epidermal cells has been extensively detailed. As described [46,47], it is based on the internal hydrostatic pressure (turgor) of the hyphae, which ranges between 0.8 and 1.2 MPa, and, together with enzymatic events, allows the invasion of host tissues. The application of sodium bicarbonate may induce a change in the osmotic environment, leading to the loss of turgor through the release of intracellular water, thereby decreasing the observed post-harvest damage associated with fungal pathogen growth.

Finally, regarding the effect of the 'chemical' treatment tested, the effects described by [48] in pears by the exogenous application of sodium bicarbonate at a concentration of 1–3% in combination with low storage temperatures are similar to those found in our study, where it was applied at 2%. As in the aforementioned study, an improvement in fruit firmness was observed, which can be attributed to the reduction in the activity of enzymes, such as cellulase, pectin methylesterase, and polyphenol oxidase.

## 5. Conclusions

The synergy achieved between $NaHCO_3$ and yeast (especially *P. guillermondii* and *H. uvarum*) represents an agronomically significant solution, providing a multi-target effect against postharvest pathogens. The osmotic alteration of sodium bicarbonate serves as the primary tool of the treatment, enhanced by the antagonistic capacity of yeasts in their antifungal activity against the evaluated postharvest fungi.

The application of 2% sodium bicarbonate not only improved the storage time in the simulation of the cold shelves of the fruit plants but also improved sensory qualities. Additionally, the use of *P. guillermondii* and *H. uvarum* as microbial antagonists to protect plums and cherries from pathogenic moulds has shown promising results. Through in situ and in vivo assays, these antagonists demonstrated the highest antagonistic activity, effectively controlling post-harvest diseases and extending the shelf life of fruits. However, a comprehensive understanding of the modes of action of antagonists is essential for effective disease control, risk assessment, and prevention of resistance development, including the impact of antimicrobial metabolites, although metabolites from natural microbe–plant interactions are generally considered insignificant.

In summary, the synergistic efficacy of the studied treatment in 'in vivo' assays offers a promising alternative to synthetic products with similar activity, providing an environmentally friendly solution for combating post-harvest damage. Further research is necessary to determine the optimal conditions for employing these techniques in fruit storage and investigate the health implications of metabolites produced by antagonistic microbes and their impact on consumers.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app142310978/s1.

**Author Contributions:** Conceptualisation: J.D.-A.; data curation: B.N.-P., J.D.-A. and J.R.-P.; formal analysis: B.N.-P., S.M. and J.R.-P.; funding acquisition: J.D.-A.; investigation: J.R.-P., N.I.-P. and A.G.; methodology: J.D.-A., S.M. and B.N.-P.; project administration: J.D.-A.; resources: J.D.-A.; software: B.N.-P., J.R.-P. and N.I.-P.; supervision: J.D.-A.; validation: J.R.-P., S.M. and J.D.-A.; visualisation: B.N.-P. and A.G.; writing—original draft: B.N.-P. and S.M.; writing—review and editing: B.N.-P., A.G. and J.D.-A. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The original contributions presented in the study are included in the articleand Supplementary Materials, further inquiries can be directed to the corresponding author.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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