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Bio-protective potential of lactic acid bacteria isolated from fermented wax gourd

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Abstract The antifungal activities of 85 lactic acid bacteria strains isolated from fermented wax gourd against the four fungal species, *Penicillium oxalicum*, *Aspergillus flavus*, *Aspergillus sydowii*, and *Mucor racemosus*, were determined. Inhibitory activity against at least one or more fungal species was observed with 27 *Weissella cibaria* and 11 *Weissella paramesenteroides* strains. Among these strains, *W. cibaria* 861006 and *W. paramesenteroides* 860509 showed greater inhibitory activities and were therefore selected for further analysis. The results suggested that the antifungal activities were originated from the organic acids produced by *W. cibaria* 861006 and *W. paramesenteroides* 860509. The application tests indicated that the growth of *P. oxalicum* could be effectively inhibited by *W. cibaria* 861006 for 6 days on grape surfaces. However, *W. paramesenteroides* 860509 could only remain its inhibition effect for 48 h. The findings obtained in this study suggest the potential use of *W. cibaria* 861006 as a bio-protective agent against fungi for agricultural purposes or ready-to-eat fresh fruit and vegetable products.

Introduction

Phytopathogenic and spoilage fungi cause economic losses of agricultural products and are responsible for certain human diseases. In the case of agricultural products, various fungal species such as *Penicillium oxalicum*, *Botrytis cinerea*, and *Monilinia laxa* have been described as common spoilage microorganisms that cause the blue mold rot of fresh fruits and vegetables (Kwon et al. 2008; Trias et al. 2008; Umemoto et al. 2009). In particular, *P. oxalicum* causes *Penicillium* rots, the most common and destructive type of post-harvest diseases that occur on fruits and vegetables primarily during storage or transport (Kwon et al. 2008; Umemoto et al. 2009). To control fungal decay, chemical fungicides are frequently used. However, many chemical fungicides are not authorized for postharvest treatment due to the possible toxicological risks (Trias et al. 2008).

Bio-preservation has attracted special interest as an innovative method of extending the shelf life of food products through the use of lactic acid bacteria (LAB). Bio-preservation involves the inoculation of food products with selected LAB strains that are able to inhibit the growth of undesirable microorganisms. This mechanism of inhibition might involve various LAB-produced antimicrobial compounds, such as organic acids, hydrogen peroxide (H_2O_2), cyclic dipeptides, and bacteriocins (Lavermicocca et al. 2003; Matamoros et al. 2009; Ström et al. 2002).

Various LAB were isolated and tested for their activity against phytopathogenic fungi, and the results support the potential use of LAB as biocontrol agents (Gourama 1997; Ström et al. 2002; Lavermicocca et al. 2003; Sathe et al. 2007; Trias et al. 2008). In our previous study, a total of 85 LAB strains which included 27 *Weissella cibaria* and 58 *Weissella paramesenteroides* were isolated from the fermentation process of fermented wax gourd (*yan-dong-gua*), a traditional fermented food in Taiwan. Fermentation of wax gourd usually continues for at least 1 month, but some producers maintain a fermentation time of 2 months or even longer. The *yan-dong-gua* has a long preservation time which may due to the addition of NaCl (approximately 7–10%) and alcohol (a small quantity) at the beginning of fermentation process.

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Five *W. cibaria* strains showed bacteriocin-like inhibitory substance (BLIS) producing abilities against *Lactobacillus sakei* JCM 1157^T. However, inhibitory ability against other microorganism remains unknown in the previous study. In a study by Trias et al. (2008), *W. cibaria* TM128 was found to have a high ability to effectively decrease fungal infection levels and have the potential to prevent fresh fruit and vegetable spoilage. Antifungal activities of all 85 *Weissella* strains were therefore determined in this study. Furthermore, strains with high antifungal activity were selected among 85 LAB isolated from *yan-dong-gua*, and the mechanisms of the antifungal activity were investigated. In addition, these antifungal strains were applied as bio-preservatives on fresh grape, and their antifungal abilities against the *P. oxalicum* were evaluated.

Methods and materials

Cultures and media

Four spoilage fungal cultures, *Mucor racemosus* (I082801, isolated from moldy breads), *P. oxalicum* (I092302, isolated from moldy grapes), *Aspergillus flavus* (I092903, isolated from moldy breads), and *Aspergillus sydowii* (I092904, isolated from moldy breads), were previously isolated. All fungal cultures were identified basing on their physiological and genetic characteristics, such as cell morphology and its rDNA sequences. These fungal cultures were maintained on PD broth (potato 4% and D-glucose 20%) with glycerol solution (200 mg/mL) at −80°C.

LAB strains were cultured in de Mann–Rogosa–Sharpe (MRS) broth (Difco Lactobacilli MRS Broth; Sparks, MD, USA) for 24–48 h at 30°C under anaerobic conditions (Mitsubishi AnaeroPak System, Pack-Anaero, Mitsubishi Gas Chemicals, Tokyo, Japan).

Screening for antifungal activity of LAB isolates

The *yan-dong-gua* LAB isolates were screened for antifungal activity by agar streak assay using the fungal cultures described above as indicators. Briefly, the MRS plates were overlaid with MRS soft agar (7 mg/mL) seeded with 10^4 spores/mL of each fungal culture, respectively. The cultured MRS broth of each LAB strain was mixed well by vortexing and then streaked onto the surface of the fungal pre-seeded MRS soft agar plates. The streak dimension was approximately 3×10 mm. The plates were incubated aerobically at 30°C for 48–72 h. Clear zones of inhibition were recorded by determining the distance from the bacterial streak to the edge of visible fungal hyphae. The results were scored as follows: −, no visible inhibition; w, weak inhibition; ++, inhibition area from 2 to 5 mm; and +++, inhibition area >5 mm. The inhibition size and the number of inhibited fungal cultures of each LAB strain were determined and then compared. The LAB strains which showed the higher antifungal activities and had inhibitory abilities against all fungal cultures used in this study were selected from both the *W. cibaria* and *W. paramesenteroides* groups and were used for further analysis and in the application test as bio-preservatives on fresh grape.

Effect of proteinase K and pH on cell-free supernatant antifungal activity

To obtain cell-free supernatants (CFS) of selected strains, overnight cultures of individual antifungal LAB were centrifuged at 12,000 rpm for 5 min, and supernatant were filter-sterilized (0.45 μm pore size; Millipore, USA). The antifungal activity of CFS (in absence of any treatment or pH adjustment), neutralized CFS (adjust pH to 7.0 with 4 N NaOH), and neutralized CFS treated with proteinase K (10 mg/mL of 20 mmol/L sodium phosphate buffer, pH 7, 37°C for 5 h) were determined using the agar-well diffusion assay. Briefly, the inhibitory zone was determined by pouring each treated CFS fluid into the different holes (8 mm diameter) made on the fungal pre-seeded MRS agar plates, as described by Yanagida et al. (2005). In addition, Gourama (1997) indicated that *Penicillium* species can cause food spoilage at low temperature and is an important contaminant of foods and agricultural commodities. *P. oxalicum* I092302 was therefore selected as the indicator strain in this assay.

Antifungal activity of concentrated CFS

Twenty-fold concentrated CFS of selected LAB strains were prepared following the methods described by Rouse et al. (2008). Briefly, 20 mL of CFS was freeze-dried overnight without any treatment and resuspended in 1 mL of 10 mmol/L acetic acid. The final pH values of dissolved concentrated CFS solutions were approximately 4.6–4.8. In addition, neutralized CFS of selected strains were treated with proteinase K. The concentrated CFS solutions were also tested against the proteolytic enzyme proteinase K (10 mg/mL of 20 mmol/L sodium phosphate buffer, pH 7, 37°C for 5 h) using the agar-well diffusion assay. Acetic acid (10 mmol/L) was used as the control, and *P. oxalicum* I092302 was used as the indicator strain in this assay.

Assessment of selected LAB strains in the prevention of fungal spoilage of fruits

To determine whether the antifungal LAB had potential application in bio-preservation, a food trial was undertaken using grapes as model. The grapes were washed and

subsequently air-dried for more than 2 h before tests. *P. oxalicum* I092302 was previously isolated from the moldy grapes, and evaluation was therefore performed using *P. oxalicum* I092302 on fresh grapes. *P. oxalicum* I092302 was pre-cultured on PDA plates, the spores collected and then adjusted to a final concentration of 10^5 spores/mL in 3 mL glycerol–water (0.2 mg/mL). The selected antifungal LAB strains were cultured overnight in MRS broth at 30°C. The LAB cultures were adjusted to an A_{600} value of 1 (approximately 10^9 cells/mL) using a MRS blank broth prior to use.

As the results showed in Table 1, strain *W. cibaria* 860106 showed no inhibition activity against *P. oxalicum* I092302. However, strain *W. cibaria* 860106 was found to have BLIS producing ability in the previous study (Lan et al. 2009). Strain 860106 was therefore used as a contrast and

Table 1 Inhibition spectra for antifungal activity of LAB strains

Strain no.	Species	Indicator strains			
		M. racemosus I082801	*P. oxalicum* I092302	*A. flavus* I092903	*A. sydowii* I092904
860101	*W. cibaria*	–	–	w	–
860102	*W. cibaria*	–	–	w	–
860103	*W. cibaria*	–	–	w	–
860104	*W. cibaria*	–	–	w	–
860105	*W. cibaria*	–	–	w	–
860106	*W. cibaria*	–	–	w	–
860108	*W. cibaria*	–	–	w	–
860109	*W. cibaria*	–	–	w	–
860110	*W. cibaria*	–	–	w	–
860111	*W. cibaria*	–	–	w	–
860112	*W. cibaria*	w	–	w	–
860301	*W. cibaria*	–	–	w	–
860302	*W. cibaria*	–	–	w	–
860303	*W. cibaria*	–	–	w	–
860304	*W. cibaria*	–	–	w	–
860305	*W. cibaria*	–	–	w	–
860306	*W. cibaria*	–	–	w	–
860307	*W. cibaria*	–	–	w	–
860308	*W. cibaria*	–	–	w	–
860309	*W. cibaria*	–	–	w	–
860310	*W. cibaria*	–	–	w	–
860311	*W. cibaria*	–	–	w	–
860312	*W. cibaria*	w	w	w	++
860504	*W. paramesenteroides*	–	w	w	–
860506	*W. cibaria*	w	w	w	+++
860509	*W. paramesenteroides*	w	++	w	+++
860512	*W. paramesenteroides*	w	–	–	–
861003	*W. paramesenteroides*	w	–	–	–
861005	*W. cibaria*	w	++	w	+++
861006	*W. cibaria*	w	++	w	+++
861007	*W. paramesenteroides*	w	++	w	++
861008	*W. paramesenteroides*	w	–	–	–
861011	*W. cibaria*	w	++	w	++
861404	*W. paramesenteroides*	w	++	w	++
862103	*W. paramesenteroides*	w	w	w	+++
862104	*W. paramesenteroides*	w	w	w	+++
862105	*W. paramesenteroides*	w	w	w	++
862107	*W. paramesenteroides*	w	++	w	++

– no visible inhibition, *w* weak inhibition, ++ inhibition area from 2 to 5 mm, +++ inhibition area >5 mm

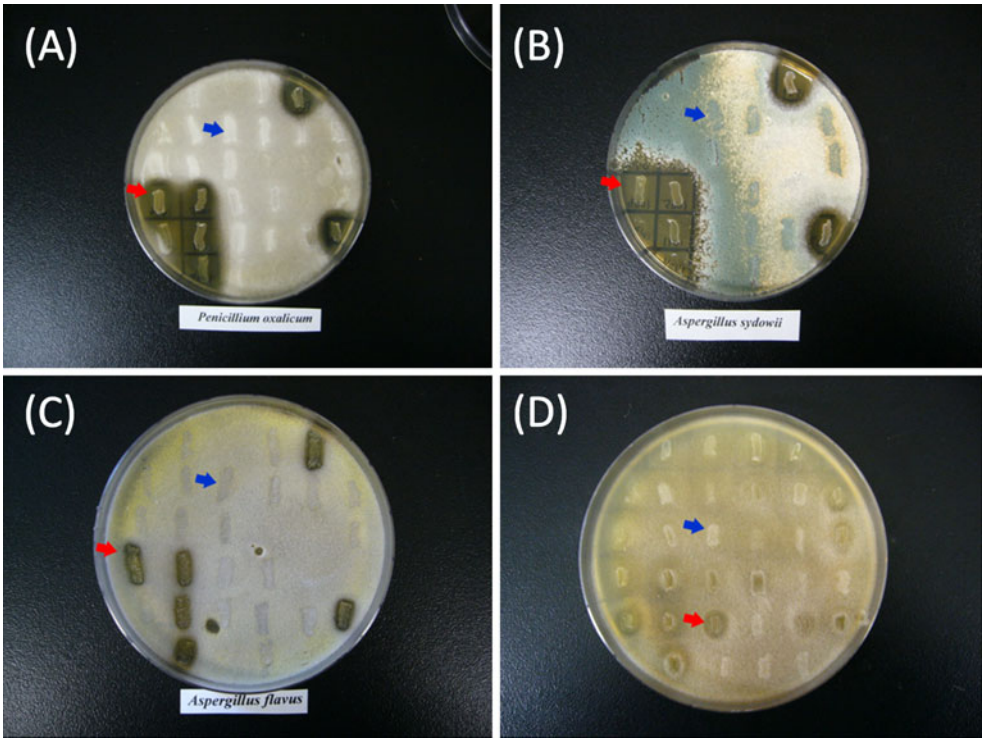

Fig. 1 Agar streak assay with LAB strains on MRS agar overlaid with (**a**) *P. oxalicum* I092302, (**b**) *A. sydowii* I092904, (**c**) *A. flavus* I092903, and (**d**) *M. racemosus* I082801. *Red arrows* an example of the strain with antifungal activity; *blue arrows* an example of the strain without antifungal activity

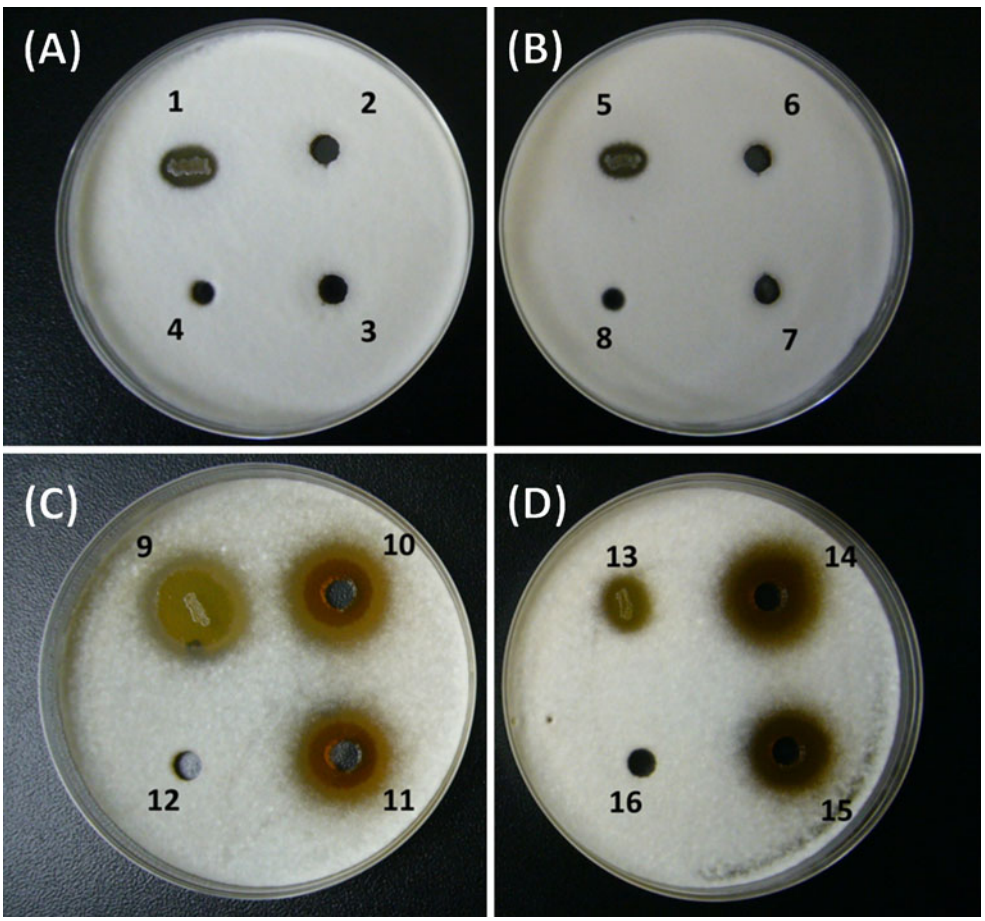

Fig. 2 Effects of various treatments on antifungal activity. Plates (**a**) and (**c**) are treatments of *W. paramesenteroides* 860509; plates (**b**) and (**d**) are treatments of *W. cibaria* 861006. Nos. *1*, *5*, *9*, and *13* are bacterial streaks; nos. *2* and *6* are neutralized CFS; nos. *3* and *7* are neutralized CFS with proteinase K treatment; nos. *4* and *8* are normal CFS. Nos. *10* and *14* are concentrated CFS; nos. *11* and *15* are concentrated CFS with proteinase K treatment; nos. *12* and *16* are the control treatment (10 mmol/L acetic acid solution)

also as a positive control in this test. In addition, sterilized water was used as a blank. The surface of the test fruit was either initially sprayed with LAB culture or sterilized distilled water. After air-drying for 30 min, 2 mL of *P. oxalicum* I092302 spore solution was sprayed onto the surface of the fruit. Each treatment contained five to six berries and was performed in an independent capped sterilized container. All treatments were incubated at 30°C for 4 days, and variations were observed and recorded per 24 h. This experiment was repeated twice.

Results

Inhibitory activities against at least one or more fungal species were observed with 27 *W. cibaria* and 11 *W. paramesenteroides* strains. Multi-inhibitory abilities (against all four fungal cultures) were observed with strains 860312, 860506, 860509, 861005, 861006, 861007, 861011, 861404, 862103, 862104, 862105, and 862107 (Table 1; Fig. 1). Some of the strains showed excellent inhibitory abilities against *P. oxalicum* and *A. sydowii* (Table 1; Fig. 1a, b).

As the results showed in Table 1, several *W. cibaria* and *W. paramesenteroides* strains showed excellent inhibitory activities against the fungal cultures. Strains *W. cibaria* 861006 and *W. paramesenteroides* 860509 were randomly selected for further analysis.

When assessing the effect of proteinase K and pH on the antifungal activities of *W. cibaria* 861006 and *W. paramesenteroides* 860509, no antifungal activity was observed with the CFS, neutralized CFS, or neutralized CFS with proteinase K treatment. Antifungal activity was only observed with the bacterial streak (Fig. 2a, b). However, when determining the antifungal activity against *P. oxalicum* I092302, inhibition was observed with the bacterial streaks, concentrated CFS, and also proteinase K treated concentrated CFS (Fig. 2c, d). No inhibitory activity was observed in the control treatment (10 mmol/L acetic acid solution).

The results of the grape spoilage prevention test showed that *P. oxalicum* I092302 grew well on the surface of the contrast and control treatments, and hyphal growth was obviously observed after 48 h. No fungal hyphae could be found on the surface of *W. cibaria* 861006-treated grapes until 144 h (Fig. 3). However, hyphal growth was observed *W. paramesenteroides* 860509-treated grapes after incubation for 72 h. The similar results were observed in the repeated experiment. Moreover, no obvious fermentation occurred by LAB strains were observed in the grape treatments, judging by its appearance and smell.

Discussion

The fungal species investigated in this study have been previously linked to the spoilage of various foods and fruits: *M.*

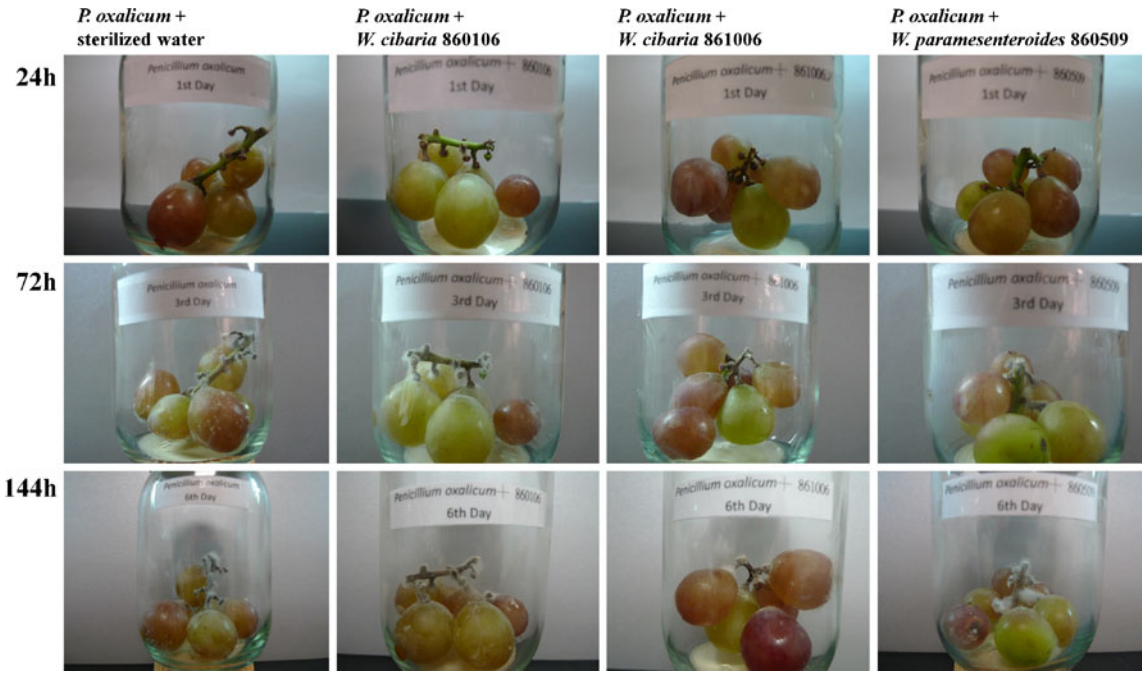

Fig. 3 Evaluation of bio-protection effects on grape. The fruits were treated with *P. oxalicum* I092302 spores and sprayed with sterilized distilled water or LAB cultures *W. cibaria* 860106, *W. cibaria* 861006, and *W. cibaria* 860509, respectively. All treatments were incubated at 30°C for 4 days and recorded per 24 h

racemosus causes spoilage of cheese, cheesecake, and meats (Pitt and Hocking 1997); *A. flavus* causes spoilage of jams and certain fresh fruits and vegetables (Katsumata et al. 2002; Pitt and Hocking 1997); *A. sydowii* causes spoilage of breadfruits, cakes, and other foods (Fustier et al. 1998; Pitt and Hocking 1997); and *P. oxalicum* is a pathogen of yams, greenhouse cucumbers, tomato, and maize (Askun 2006; Kwon et al. 2008; Pitt and Hocking 1997; Umemoto et al. 2009).

The antifungal activity of certain LAB is less well characterized, but organic acids, as still un-characterized proteinaceous compounds, and cyclic dipeptides can inhibit the growth of some fungi (Rouse and van Sinderen 2008). Organic acids produced by *Lactobacillus plantarum*, such as 3-hydroxy fatty acids and phenyllactic acid, have been reported to inhibit the growth of some fungal cultures (Lavermicocca et al. 2003; Sjögren et al. 2003).

In a study by Trias et al. (2008), *W. cibaria* TM128 was found to have a high ability to effectively decrease fungal infection levels and had the potential to prevent fresh fruit and vegetable spoilage. Organic acids were considered to be the main antifungal substances, based on the observation that pH-neutralized supernatants lost inhibition ability.

A total of 27 *W. cibaria* and 58 *W. paramesenteroides* strains were isolated from *yan-dong-gua* in our previous study (Lan et al. 2009). All *W. cibaria* strains showed inhibitory activities against at least one or more fungal species. However, inhibitory activities against fungi were only observed in 11 *W. paramesenteroides* strains. In addition, *W. cibaria* strains 860312, 860506, 861005, 861006, and 861011 inhibited the growth of all four fungal cultures used in this study, while the remaining strains could only inhibit one or two fungal cultures (Table 1). Similar results were also observed with 11 *W. paramesenteroides* strains. Individual differences among species and strains could be obviously observed in the present study.

In the present study, both concentrated CFS and proteinase K-treated CFS of *W. cibaria* 861006 and *W. paramesenteroides* 860509 showed inhibitory activities against *P. oxalicum* I092302. The results suggest that the main antifungal substance is non-proteinaceous. However, no inhibitory activity could be observed with non-concentrated CFS or its treatments, indicating that antifungal activity could not be revealed until the antifungal substances reached a specific concentration. Both the bacterial streak of *W. cibaria* 861006 and *W. paramesenteroides* 860509 maintained their inhibitory activities against *P. oxalicum* I092302. A possible explanation may be the constant organic acid production by the live cultures. However, more scientific data become necessary to clarify the true mechanism of their inhibition activities.

When applying the LAB strains to grapes as bio-preservatives, *W. cibaria* 861006 showed excellent ability in preventing fungal spoilage caused by *P. oxalicum*. However, *W. paramesenteroides* 860509 showed limited inhibitory ability, and *W. cibaria* 860106 showed no inhibitory ability in preventing fungal spoilage and were therefore considered not qualified for the bio-preservation application. Furthermore, although *W. cibaria* 860106 has the ability to produce BLIS, the results obtained in this study suggested that either its BLIS or the live cell has no inhibition ability against *P. oxalicum*. In this study, live cell bacterial culture was used in the application test due to its stable antifungal activity, economical cost. However, of concern was that fermentation caused by LAB strains might have a negative effect and cause considerable disquiet. Despite the possibility, this concern did not occur in the grape treatments, judging by its appearance and smell.

The concentrated CFS solutions seem to provide an alternative method to the use of living cells. However, characteristics of the concentrated CFS solutions, such as stability, minimum inhibitory concentration, and effects on foods, remain unclear in the present study. Further studies on this point will be an important task in our future research.

The findings of the present study suggest that *W. cibaria* 861006 has interesting potential for practical application as bio-preservative in the food industry and for agriculture purposes, due to their excellent inhibitory activities against a variety of fungi.

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