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Application of the cell-free supernatant from *Weissella viridescens* to control *Listeria monocytogenes*

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Abstract: *Listeria monocytogenes* can form biofilms on different food contact surfaces, increasing the risk of cross-contamination in food products. *Weissella viridescens* are Gram-positive bacteria and belong to lactic acid bacteria (LAB). The aim of this study was to investigate the antibacterial activity of cell-free culture supernatant (CFS) from *W. viridescens* to control *L. monocytogenes* biofilms as well as their growth on chilled pork. Preliminary results suggest that the antibacterial compounds in *W. viridescens* CFS may be proteinaceous in nature. The minimum inhibitory concentrations (MICs) of most *L. monocytogenes* strains tested was 30 mg mL^{-1}. Biofilm formation of *L. monocytogenes* 10403S on stainless steel and polystyrene was significantly inhibited by the sub-inhibitory concentration of *W. viridescens* CFS (1/8MIC, 1/4MIC, and 1/2MIC). Our results also showed that *W. viridescens* CFS at concentrations higher than MIC (1MIC, 2MIC, and 4MIC) was effective in eradicating the mature biofilms of 10403S strain on various surfaces. Dip applications of *W. viridescens* CFS could inhibit the growth of *L. monocytogenes* on chilled pork. On the whole, *W. viridescens* CFS has the potential to control biofilms as a natural antibiofilm and as an antibacterial agent to inhibit the growth of *L. monocytogenes* on chilled pork.

Keywords: foodborne pathogen; lactic acid bacteria; antibacterial; biofilm; chilled pork

China is a big country of pig breeding and pork consumption in which the main consumption pattern is fresh pork. At present, retail pork in China can be divided into fresh, frozen, and chilled pork according to its temperature. In China, chilled pork has become more and more popular with the increasing attention of consumers to food safety and quality. Chilled pork is a fresh carcass that is rapidly cooled to 0–4 °C within 24 h after being butchered. Compared with fresh pork and frozen pork, chilled pork has better taste, higher nutritional value and microbial safety due to being stored at 0–4 °C during the process of production, circulation and selling (Cui et al. 2021).

Although a low temperature can inhibit microbial growth and extend the shelf life of pork effectively, some psychrotrophic bacteria, such as *Listeria monocytogenes*, can still survive under cold storage conditions. *L. monocytogenes* is an important foodborne pathogen, and it can cause listeriosis which is a rare but life-threatening disease (McLauchlin et al. 2004). The main contamination route for this pathogen is considered to be cross-contamination from food-producing environments to food during processing (Mendonça et al. 2012; Jami et al. 2014). It is challenging to control *L. monocytogenes* in food processing environments because this species is widely distributed

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and can grow under stressful conditions (Ferreira et al. 2014). *L. monocytogenes* is able to adhere and form a biofilm on different food contact surfaces. *L. monocytogenes* growing in biofilms is significantly more resistant to adverse conditions than its free-living counterparts, and biofilms are more difficult to remove (Flemming and Wingender 2010). Therefore, *L. monocytogenes* biofilm poses a great threat to food safety.

Many promising antimicrobials for controlling *L. monocytogenes* and its biofilm have been reported in previous studies (Dong et al. 2021; Li et al. 2021); however, a search for new alternatives is driven by consumer interest in 'natural' products. These 'natural' products are derived from non-chemical sources, including microorganisms. Lactic acid bacteria (LAB) have acquired the 'generally recognised as safe' status, and some of the secondary metabolites produced by LAB have good antibacterial and anti-biofilm activity (Zacharof and Lovitt 2012). *Weissella viridescens*, belonging to LAB, has been found in various food products such as fresh vegetables, meat and fermented animal and plant products (Kameník et al. 2015). The cell-free culture supernatant (CFS) from *W. viridescens* is able to inhibit many Gram-positive and Gram-negative bacteria, such as *L. monocytogenes*, *Clostridium botulinum* and *Escherichia coli* (Patterson et al. 2010). We also found that a strain of *W. viridescens* collected in our laboratory has a good antibacterial activity against *L. monocytogenes* (data not published). However, very little data is available on the effects of *W. viridescens* CFS on the biofilm development of *L. monocytogenes*.

The objectives of this study were to preliminarily identify the antibacterial compounds of *W. viridescens* CFS, investigate its effect on biofilm formation and mature biofilms by *L. monocytogenes* on abiotic surfaces, and evaluate the application potential of *W. viridescens* CFS for the control of *L. monocytogenes* as surface contaminants on chilled pork.

MATERIAL AND METHODS

Bacterial strains and growth conditions. All *L. monocytogenes* strains used in our study are presented in Table 1. *L. monocytogenes* 10403S and EGD-e were obtained as gifts from Prof. Qin Luo (Central China Normal University, China). The other four *L. monocytogenes* strains (HL09, HL11, HL28, and HL50) came from our laboratory strain collection (Yu and Jiang 2014). *W. viridescens* WV20-15 isolated from a pickles sample was sourced from our laboratory and routinely grown at 30 °C in de Man Rogosa Sharpe (MRS) broth (Huankai Ltd., Guangzhou, Guangdong, China). *L. monocytogenes* strains were grown at 37 °C on brain heart infusion (BHI) agar (Oxoid Ltd., Basingstoke, Hampshire, England) or in BHI broth.

Preparation of *W. viridescens* CFS. *W. viridescens* was inoculated in MRS broth and incubated at 30 °C for 48 h (THZ-82; Tianjing Experimental Instrument Factory, Changzhou, China). Cultures were centrifuged (JIDI-17R; Jidi Instrument Co., Ltd., Guangzhou, China) at 12 000 g for 15 min (4 °C), and the supernatants were sterilised by passing through a 0.22 μm filter (Millipore, Bedford, USA) to obtain CFS.

Antibacterial activity test. The antibacterial activity of *W. viridescens* CFS was tested using the double-layer agar plate method. Cultures of *L. monocytogenes* strains were diluted to 10^7 colony-forming unit (CFU) mL^{-1} with sterile water. The melted MRS agar was cooled to about 50 °C, and then 4.5 mL of the medium was mixed (Vortex-Genie2; Kai Yuan Guo Chuang Technology Co., Ltd., Beijing, China) with 100 μL of diluted bacterial culture. The solidified MRS agar plate was overlaid with the mixture. The oxford cup was placed vertically on the surface of the agar with an aseptic operation, and 150 μL of *W. viridescens* CFS was added into the oxford cup. After incubation at 37 °C for 24 h (DHP-9052; Jiecheng Experimental Apparatus, Shanghai, China), the plates were examined for zones of inhibition.

Identification of antibacterial compounds in *W. viridescens* CFS. Acids produced by LAB can inhibit the growth of other bacteria. In order to eliminate the interference of acids, the *W. viridescens* CFS was adjusted to pH 6.0 with sodium hydroxide (NaOH) solution (1 M) and then assayed for antimicrobial activity. Hydrogen peroxide produced by LAB also had antimicrobial activity. Thus, catalase was added to the *W. viridescens* CFS at a final concentration of 1 mg mL and then incubated (DHP-9052; Jiecheng Experimental Apparatus, Shanghai, China) for 1 h at 30 °C (Luo et al. 2011) to eliminate the influence of hydrogen peroxide. The inhibition activity of the treated *W. viridescens* CFS was determined. The sensitivity of *W. viridescens* CFS to proteolytic enzymes was conducted as described by Ammor et al. (2006). Proteinase K, pepsin and trypsin were separately added to the neutralised *W. viridescens* CFS, with a final concentration of 1.5 mg mL^{-1}. The treated *W. viridescens* CFS was incubated at 37 °C for 1 h, and then its inhibition activity was measured. The neutralised *W. viridescens* CFS without the treatment with proteolytic enzymes was used as a control.

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Table 1. *Listeria monocytogenes* strains used in this study

Strain	Serovar	PFGE pattern	Antibacterial activity of *Weissella viridescens* CFS						MIC of *Weissella viridescens* CFS (mg mL^{-1})
			untreated	NaOH treatment	catalase treatment	proteolytic enzyme treatment			
						proteinase K	pepsin	trypsin	
10403S	1/2a	nd	++	++	++	–	–	–	30
EGD-e	1/2a	nd	++	++	++	–	–	–	30
HL09	4b	P13	++	++	++	–	–	–	30
HL11	1/2a	P3	+	+	+	–	–	–	60
HL28	1/2c	P4	++	++	++	–	–	–	30
HL50	1/2b	P12	++	++	++	–	–	–	30

PFGE – pulsed-field gel electrophoresis; CFS – cell-free culture supernatant; MIC – minimum inhibitory concentration; nd – not detected; antimicrobial activity detected as zones of inhibition with widths of (–) < 10 mm, (+) 10–20 mm, (++) > 20 mm; the diameter of the oxford cup is 6 mm

Determination of minimum inhibitory concentration (MIC). The CFS was lyophilised in a vacuum freeze-drier (LGJ-25C; Sihuan Furui Keyi Technology Development Co., Ltd., Beijing, China) at –70 °C for 48 h. For further experiments, the powdered CFS was dissolved in sterile water to prepare the *W. viridescens* CFS solution. Minimum inhibitory concentrations (MICs) of *W. viridescens* CFS against all *L. monocytogenes* strains used in this study were determined by the broth microdilution method (Romanova et al. 2006). Only *L. monocytogenes* 10403S was used for further experiments because its phenotypic and genotypic characteristics are well documented.

Growth curves. A growth curve analysis of *L. monocytogenes* was performed as described by Pöntinen et al. (2015).

Test surface preparation. Stainless steel chips (type 304, diameter 12.5 mm) and sterile polystyrene coverslips (diameter 14 mm) were used to investigate biofilm formation on abiotic surfaces. Stainless steel chips were immersed in ethanol overnight for degreasing before use, then thoroughly washed with distilled water and autoclaved at 121 °C for 15 min (LDZX; Shenan Medical Instrument Factory, Shanghai, China).

Inhibition of *L. monocytogenes* biofilm formation on abiotic surfaces. Biofilms were assayed using the microplate assay with crystal violet staining as described by Djordjevic et al. (2002). The biofilms were visualised under a DMi1 inverted optical microscope (Leica-Microsystems, Wetzlar, Germany). The effect of *W. viridescens* CFS on *L. monocytogenes* biofilm formation was also investigated. The overnight culture was diluted in BHI broth with different concentrations of *W. viridescens* CFS (1/8MIC, 1/4MIC, and 1/2MIC), and then biofilms were quantified as described above.

Eradication of mature *L. monocytogenes* biofilms on various surfaces. After 48 h of incubation at 37 °C (DHP-9052; Jiecheng Experimental Apparatus, Shanghai, China), the medium was removed, and the biofilm was washed three times with sterile water. Then, 1 mL of sterile water or *W. viridescens* CFS at 1MIC, 2MIC, or 4MIC was added. Plates were incubated at 37 °C for 1 h. After removing the medium, the wells were washed and air-dried. The biofilms were stained with 1% crystal violet and then decolourised with 95% ethanol. The absorbance at optical density $OD_{595\ nm}$ was measured.

Chilled pork surface inoculation and *W. viridescens* CFS application. Chilled pork was purchased from a local supermarket and transported to the laboratory in an icebox at 4 °C. The pork was cut into separate 25 g samples under aseptic conditions. Each 25 g sample was soaked in 500 mL *L. monocytogenes* 10403S suspension (10^5 CFU mL^{-1}) for 5 min and then air-dried for 30 min to facilitate bacterial attachment. After drying, chilled pork samples were completely submerged in different concentrations of *W. viridescens* CFS (1MIC, 2MIC, and 4MIC) for 5 min. Controls were dipped in sterile water. Following dip application, samples were dried and then placed in plastic plates and covered with a fresh-keeping membrane. All samples were stored at 4 °C.

Chilled pork sampling and pathogen enumeration. The following samples were analysed: after *L. monocytogenes* inoculation (day 0) and during storage on days 3, 6, 9, 12, and 15. Three samples from each treatment at each time point were transferred to individual sterile bags containing 225 mL sterile saline and stomached for 1 min at normal speed. The resulting homogenate was serially diluted in sterile saline, and the appropriate dilutions were plated on polymyxin-acriflavin-lithium chlo-

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ride-ceftazidime-aesculin-mannitol (PALCAM) agar (Huankai Ltd., Guangzhou, China). A typical colony of *L. monocytogenes* grown on PALCAM agar plate was small and round with a grey-green colour. The plates were incubated at 37 °C for 24 h (DHP-9052; Jiecheng Experimental Apparatus, Shanghai, China), and typical colonies were counted.

Statistical analysis. All the experiments were performed in triplicate. The results are presented as mean ± standard deviation (SD). The statistical significance of the results has been evaluated using the unpaired two-tailed *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Antimicrobial activity of *W. viridescens* CFS against *L. monocytogenes*. As shown in Table 1, neither neutralised treatment nor catalase treatment had an effect on the antibacterial activity of *W. viridescens* CFS against *L. monocytogenes* strains. However, *W. viridescens* CFS did not show any inhibition activities after separate treatments with proteinase K, pepsin, and trypsin. This suggested that the inhibitory compounds in *W. viridescens* CFS are of proteinaceous nature. The MICs of *W. viridescens* CFS against the six tested *L. monocytogenes* strains were also determined (Table 1). Only one strain (HL11) showed MIC of 60 mg mL^{-1}, and the others exhibited the same MICs of 30 mg mL^{-1}.

W. viridescens, belonging to heterofermentative LAB, plays a role in the formation of flavour substances in food fermentation. Some *W. viridescens* strains also exhibit potential antimicrobial properties (Patterson et al. 2010; Li et al. 2019). Li et al. (2019) found that the CFS of *W. viridescens* ZY-6 had antibacterial activity against some foodborne bacteria (*E. coli*, *Salmonella* and *Staphylococcus aureus*). Patterson et al. (2010) reported that *W. viridescens* CFS inhibited the growth of pathogenic bacteria, including *L. monocytogenes*. Our results showed that the CFS from *W. viridescens* WV20-15 could inhibit the growth of *L. monocytogenes* strains. This suggests that *W. viridescens* and its CFS show a good potential application in the control of this pathogen.

Acids and hydrogen peroxide produced by LAB can inhibit the growth of other bacteria. In this study, the antibacterial activity of *W. viridescens* CFS against *L. monocytogenes* strains was not affected by neutralised or catalase treatments, indicating that the antibacterial substances in *W. viridescens* CFS are neither acids nor hydrogen peroxide. However, *W. viridescens* CFS did not show any inhibition activities after separate treatments with proteinase K, pepsin, and trypsin, suggesting that the inhibitory compounds in *W. viridescens* CFS are of proteinaceous nature, probably bacteriocins.

Many researchers have focused on the application of metabolites produced during the fermentation of LAB in food preservation (Jo et al. 2021). Until now, a large number of bacteriocins produced by LAB have been identified that show antibacterial activity against many common foodborne bacteria; however, only a few bacteriocins are approved as food preservatives (Jo et al. 2021). Many studies have reported that strains of *Weissella* could produce bacteriocins with antibacterial activity, such as weissellicin 110 from *Weissella cibaria* (Srionnual et al. 2007) and weissellicin A from *Weissella paramesenteroides* DX (Papagianni and Papamichael 2011). Liu et al. (2018) found that a bacteriocin-producing strain of *W. viridescens* had better suppression performance on the growth, expression of virulence genes and adhesion to Caco-2 cells of *L. monocytogenes* compared to the non-producing strain of *W. viridescens*, indicating that the bacteriocin of *W. viridescens* may play an important role in antibacterial activity against *L. monocytogenes*. In our study, the active compounds in *W. viridescens* CFS that could inhibit the bacterial growth of *L. monocytogenes* are probably bacteriocins. Further studies are required to confirm our presumption, as well as to identify and purify the antibacterial substance from *W. viridescens* CFS.

Growth curve assay. *L. monocytogenes* strain 10403S was selected for further analyses. The effects of *W. viridescens* CFS at different concentrations on the growth of strain 10403S are shown in Figure 1. Our results

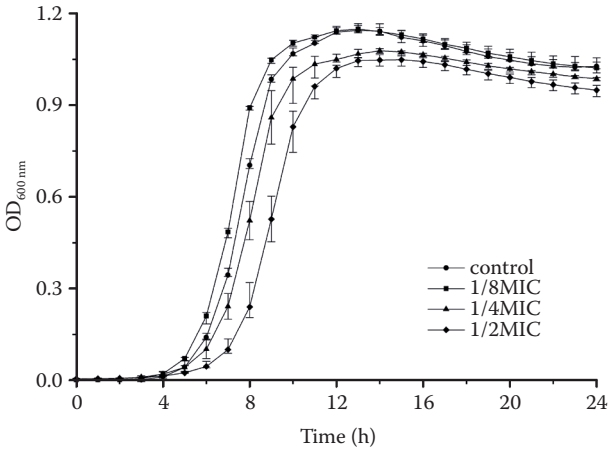

Figure 1. Growth curves of *Listeria monocytogenes* 10403S in the presence of *Weissella viridescens* CFS

OD – optical density; CFS – cell-free culture supernatant; MIC – minimum inhibitory concentration

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showed that the growth of 10403S strain was slightly affected by *W. viridescens* CFS at concentrations of 1/2MIC or below. Therefore, *W. viridescens* CFS concentrations of 1/2MIC, 1/4MIC, and 1/8MIC were chosen to study the effects of *W. viridescens* CFS on the biofilm formation of 10403S strain.

Inhibition of *W. viridescens* CFS on biofilm formation. As shown in Figure 2, the biofilm formation of 10403S strain on various surfaces was significantly inhibited ($P < 0.05$) in the presence of *W. viridescens* CFS. When grown on stainless steel, biofilm biomass of 10403S strain treated with *W. viridescens* CFS at 1/8MIC, 1/4MIC, and 1/2MIC was decreased by 24.9, 31.3, and 37.0%, respectively. When grown on polystyrene, biofilm biomass of 10403S strain treated with *W. viridescens* CFS at 1/8MIC, 1/4MIC, and 1/2MIC was decreased by 22.3, 28.1, and 43.3%, respectively. Results revealed that *W. viridescens* CFS inhibited the biofilm production of 10403S strain in a concentration-dependent manner. Our results also showed that there was no statistically significant difference in biofilm formation between polystyrene and stainless steel.

The morphology of *L. monocytogenes* 10403S biofilms on polystyrene was observed using an inverted microscope after crystal violet staining (Figure 3). The untreated *L. monocytogenes* biofilm was complete and dense. Following the *W. viridescens* CFS treatment, *L. monocytogenes* biofilm showed a decrease in biofilm biomass. With the increase of *W. viridescens* CFS concentration, increasing dispersal and decreased adherence of biofilms were also observed.

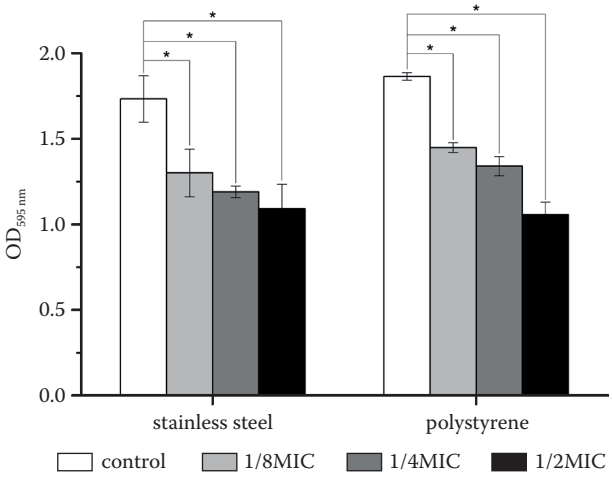

Figure 2. Effect of *Weissella viridescens* CFS on biofilm formation by *Listeria monocytogenes* strain 10403S

*$P < 0.05$ compared to *Listeria monocytogenes* 10403S without *W. viridescens* cell-free culture supernatant (CFS) treatment; OD – optical density; MIC – minimum inhibitory concentration; error bars represent the standard deviation (SD)

Effect of *W. viridescens* CFS on mature biofilms. As shown in Figure 4, *W. viridescens* CFS was effective in disrupting the mature (preformed) biofilms of 10403S strain on various surfaces. In the present study, the addition of *W. viridescens* CFS at 1MIC, 2MIC, and 4MIC significantly reduced ($P < 0.05$) mature biofilms of 10403S strain on stainless steel by 24.9, 31.3, and 37.0%, respectively. After treatment with *W. viridescens* CFS at 1MIC, 2MIC, and 4MIC, mature biofilms on polystyrene were eradicated by 22.3, 28.1,

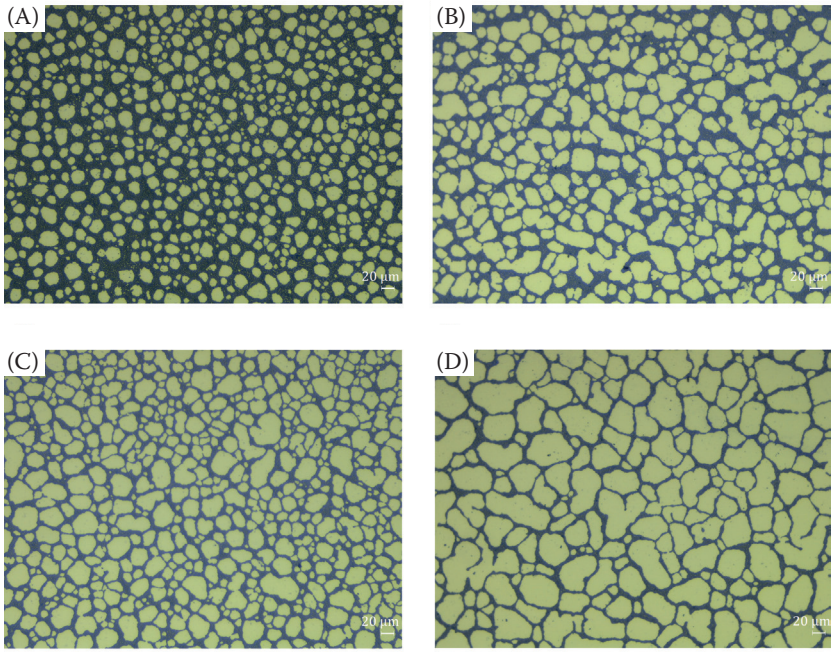

Figure 3. Inverted microscopic analysis of *Listeria monocytogenes* 10403S biofilm on polystyrene: (A) control, (B) 1/8MIC of *Weissella viridescens* CFS, (C) 1/4MIC of *W. viridescens*, (D) 1/2MIC of *W. viridescens*

CFS – cell-free culture supernatant; MIC – minimum inhibitory concentration

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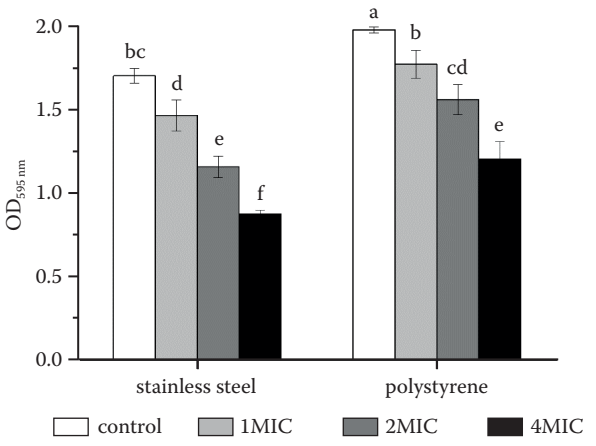

Figure 4. Effect of *Weissella viridescens* CFS on mature biofilms of *Listeria monocytogenes* 10403S

[a–f]Bars labelled with different letters indicate a significant difference at $P < 0.05$; CFS – cell-free culture supernatant; OD – optical density; MIC – minimum inhibitory concentration; error bars represent the standard deviation (SD)

and 43.3%, respectively. *W. viridescens* CFS exhibited the eradication effect on preformed biofilms of 10403S strain in a concentration-dependent manner. After incubation for 48 h, higher biofilm biomass ($P < 0.05$) was observed on polystyrene. Moreover, biofilms on polystyrene were higher than those on stainless steel ($P < 0.05$) after treatment with different concentrations of *W. viridescens* CFS.

In our study, the biofilm formation of *L. monocytogenes* strain 10403S on both stainless steel and polystyrene was significantly weakened by the sub-inhibitory concentration of *W. viridescens* CFS. Our results showed that *W. viridescens* CFS at concentrations higher than MIC was effective in significantly reducing the biomass of the mature biofilms of 10403S strain on stainless steel and polystyrene. Actually, the ability of other antimicrobials to remove the mature *L. monocytogenes* biofilms has been reported in previous studies (Gray et al. 2018; Sun et al. 2021). However, it is difficult to compare results from different studies because the strains used and the conditions for incubating biofilms were different in these studies. Biofilm formation by *L. monocytogenes* is also influenced by various surfaces (Blackman and Frank 1996). Our results showed a higher ability of 10403S strain to form biofilms on polystyrene in the absence of *W. viridescens* CFS, which is in agreement with the previous findings (Sun et al. 2021).

Control of *L. monocytogenes* as surface contaminants on chilled pork. As shown in Figure 5, *L. monocytogenes* counts in control increased gradually during a 15-day storage period at 4 °C. *L. monocytogenes* counts in all treatments were reduced on day 3, and then they increased slowly. On day 15, *L. monocytogenes* populations in control increased to 7.20 log CFU g^{-1}, while counts of all *W. viridescens* CFS dip applications increased to 4.37–5.70 log CFU g^{-1}. Generally, the mean *L. monocytogenes* counts in all treatments were significantly lower than in control at the same time point ($P < 0.05$), suggesting that the application of *W. viridescens* CFS could affect *L. monocytogenes* counts on chilled pork during storage compared to a water dip. Moreover, higher concentrations of *W. viridescens* CFS exhibited better inhibition of the *L. monocytogenes* growth on chilled pork.

In the present study, dip applications of *W. viridescens* CFS could inhibit the growth of *L. monocytogenes* on chilled pork, and the effects were concentration-dependent. *L. monocytogenes* counts in all treatments were significantly lower than in control at the same time point during a 15-day storage period. This suggests a good inhibitory effect of *W. viridescens* CFS on preventing the growth of *L. monocytogenes* on chilled pork. Previous studies have reported that some specific natural antimicrobials in combination are more effective than antimicrobials alone in controlling the *L. monocytogenes* contamination of food (Kozak et al. 2018). Thus, combination with other antimicrobial agents or other physical methods may also improve the efficacy of *W. viridescens* CFS applications.

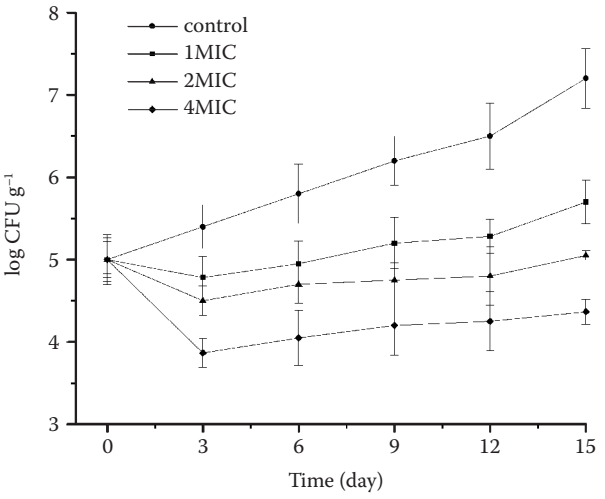

Figure 5. Effect of *Weissella viridescens* CFS on the counts of *Listeria monocytogenes* 10403S on chilled pork

CFS – cell-free culture supernatant; MIC – minimum inhibitory concentration; CFU – colony-forming unit; error bars represent the standard deviation (SD); mean *L. monocytogenes* counts in all treatments were significantly lower than in the control at the same time point ($P < 0.05$)

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CONCLUSION

In summary, the CFS from *W. viridescens* WV20-15 has the potential to control biofilms by *L. monocytogenes* as a natural antibiofilm. Dip application of *W. viridescens* CFS can also inhibit the growth of *L. monocytogenes* on chilled pork. Identification, purification and safety assessment of the antibacterial substance in *W. viridescens* CFS should be performed before its application to chilled pork.

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