Research Article

The Impact of D-limonene on Cell Membrane Barrier of *Pichia kluyveri* Y-11519 from Sichuan Pickles

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Abstract
Sichuan pickles (SCP) are a traditional method of preserving vegetables in China. Through the spontaneous fermentation of microorganisms in brine water, it forms a unique flavor to meet with food industry's requirements. However, the microorganisms in salt water determine the quality of SCP, and the film-forming phenomenon is considered to be the key to the spoilage of SCP, which seriously restricts the industrial development of SCP. We have noticed that in folk, lemon peel is often added to pickles to prevent the appearance of the film. Currently, the extract D-limonene from orange or lemon peel is recognized as a Generally Recognized as Safe (GRAS) food additive and exhibits broad-spectrum antimicrobial properties. However, there have been no reports on the effects of D-limonene on *Pichia kluyveri* (*P. kluyveri*), the microorganism responsible for the "film-forming" phenomenon in SCP. In this study, D-limonene was used to treat *P. kluyveri* Y-11519, a membranous microorganism of SCP, and the cell morphology, surface charge, membrane potential, and intracellular macromolecule leakage before and after treatment were observed. The results showed that the minimum inhibitory concentration of D-limonene against *P. kluyveri* Y-11519 was 20 μL/mL, and the minimum fungicidal concentration was 40 μL/mL. After treatment with this concentration of D-limonene, the growth of *P. kluyveri* Y-11519 cells was delayed, cells exhibited deformation and shrinkage, cell membrane integrity was compromised, permeability increased, intracellular substances leaked, ultimately leading to cell death.

Keywords: Antifungal activity, Cell membrane, D-limonene, Mechanism, *Pichia kluyveri* Y-11519

1 Introduction

Sichuan pickles (SCP) is a fermented food with Chinese regional characteristics. It is fermented by a variety of microorganisms and has a refreshingly crunchy, and form a unique flavor characteristic. It

is widely used in Sichuan cuisine. However, due to the relatively extensive process of traditional natural fermentation production, it is easy to be contaminated by other microorganisms. This leads to the formation of a white film on the surface of the brine, the softening of the tissues, and the production of off-flavors, which are typical signs of the deterioration of SCP [1]–[3]. It reduces the acceptance of consumers, causes potential food safety problems, and seriously restricts the development of industrial kimchi. *Pichia kluyveri*, widely present in nature, easily enters the fermentation environment and has been reported as the main yeast causing film formation in pickles. Kim *et al*., revealed that *P. kluyveri* was associated with the deterioration of Korean kimchi [4]. Cai *et al*., found that *P. kluyveri* SH2 is one of the representative microorganisms of SCP film-forming, and the formation of film in SCP was effectively controlled by Perilla essential oil [5]. However, the specific effects on the membrane barrier have not been elucidated.

Consumer demand is increasing for food products exposed to natural antifungal substances [6]. Natural plant-derived products and spices show potential for addressing antimicrobial reactivity (AMR) due to active ingredients, such as polyketides, alkaloids, and terpenoids [7], [8]. We have noticed that in folk, lemon peel is often added to pickles to prevent the appearance of the film. We tried to solve the film formation problem with limonene reagent. Limonene is a cyclohexanone monoterpene with three tautomeric forms, including D, L, and DL configurations, the most common of which is D-limonene [9]. D-limonene has a pleasant lemon flavor and is a key component in most citrus essential oils and common Chinese spices, such as *cumin*, *Zanthoxylum armatum*, and *Litsea cubeba*. It is recognized by the Food and Drug Administration (FDA) as an edible food additive [10]. Scientific evidence indicates that D-limonene is effective against foodborne bacterial and fungal pathogens, including *Candida tropicalis*, *Penicillium chrysogenum*, *Aspergillus carbonarius*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* with a fairly extensive minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) range [11]–[16]. D-limonene acts as a functional toxin in cell membranes, damaging the phospholipid bilayer, increasing permeability and fluidity, disrupting membrane proteins, and causing function loss. In addition, it displays excellent antiviral activity by inhibiting yeast morphogenesis and adhesion in the human epithelium. Despite the proven antimicrobial effect and inhibitory mechanism, little is known about the impact and role of *P. kluyveri* Y-11519 from SCP and the mechanism behind its inhibition, requiring further elucidation.

This study explored the effect of D-limonene as a potential antifungal agent against *P. kluyveri* Y-11519 regarding its impact on cell membrane potential, cell membrane permeability, intracellular macromolecular leakage, cell membrane integrity, and cell morphology alteration. This study can provide a theoretical basis for adding D-limonene to Sichuan pickles to inhibit the film formation in the brine, while also offering valuable references for addressing similar film-forming phenomena in other fermented foods.

2 Materials and Methods

2.1 *Reagents*

D-Limonene (HPLC purity ≥ 98%, Beijing Quantitative Spectroscopy Technology Ltd., China) was diluted to concentrations of 0, 5, 10, 20, 40, and 60 μL/mL with phosphate-buffered saline (PBS, 0.01 mol/mL, pH 7.2), sterilized via 0.22 μm filters and stored at –20 °C.

2.2 *Yeast strain and culture conditions*

The *P. kluyveri* Y-11519 strain was obtained from the Sichuan fruit and vegetable preservation laboratory at Xihua University, China (Chengdu, China) via sieving. The *P. kluyveri* Y-11519 was activated in a yeast extract peptone dextrose (YPD) liquid medium at 25 °C, 120 rpm for 24 h. The 6% culture medium was further transferred to 30 mL YPD liquid medium and cultured to the logarithmic phase. Phosphate Buffered Saline buffer (PBS, 0.01 mol/mL, pH 7.2) was used to adjust the cell suspension to a concentration of approximately 10^7 CFU/mL for further studies.

2.3 *Antifungal assays*

2.3.1 The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the D-limonene

The MIC was qualitatively determined via the Oxford

cup diffusion procedure [17], [18]. A 100 μL *P. kluyveri* Y-11519 suspension was spread evenly on a sterile potato dextrose agar plate, after which a sterilized Oxford cup was placed on the plate using forceps. The negative control consisted of 100 μL of D-limonene at concentrations of 5, 10, 20, 40, and 60 μL/mL in an Oxford cup and PBS buffer (0.01 mol/L pH 7.2). Since *Candida spp*. is more sensitive to α-pinene, a 20 μL/mL *α*-pinene solution was set as the positive control [19]. After 24 h static incubation at 25 °C, the lowest D-limonene concentration with an inhibition circle (cm) exceeding 10 around the Oxford cup was used as the MIC. To determine the minimum fungicidal concentration (MFC), D-limonene was prepared at the MIC or higher concentrations and inoculated with 5% of *P. kluyveri* Y-11519 suspension in 5 mL of sterile liquid YPD and incubated for 24 h at 25 °C while constantly shaking. Next, 100 μL of the mixed suspension was evenly spread on sterile potato dextrose agar plates and incubated for 24 h at 25 °C. The minimum concentration at which the colony was completely invisible to the naked eye was considered the MFC of the D-limonene against *P. kluyveri* Y-11519 [20].

2.3.2 The biocidal activity of D-limonene

The YPD medium was prepared at 1/2MIC, MIC, and MFC D-limonene concentrations at a two-fold dilution. The negative control contained no added D-limonene, while different D-limonene concentrations (1/2MIC, MIC, and MFC) without *P. kluyveri* Y-11519 served as the positive control. Then, 100 μL of *P. kluyveri* Y-11519 suspension (adjusted to 10^7 CFU/mL) was inoculated in the logarithmic growth phase. The mixture was vortexed and incubated at 25 °C for 36 h, during which 200 μL samples were collected every 4 h and measured at OD_{600nm} [21]. The D-limonene inhibitory performance against *P. kluyveri* Y-11519 was determined by subtracting the corresponding positive control group from the OD_{600nm} value of the experimental group.

2.4 *Antifungal mechanism*

2.4.1 Cell morphological observation

The effect of D-limonene on the *P. kluyveri* Y-11519 morphology was assessed via scanning electron microscopy (SEM). The log phase *P. kluyveri* Y-11519 was centrifuged for 10 min at 6000 r/min in a freezing centrifuge, washed three times with sterile saline (0.9% NaCl), and The cell density was maintained at 0.6 for all the suspensions. The D-limonene was diluted to a medium via two-fold dilution to final 1/2MIC, MIC, and MFC concentrations. The blank control without D-limonene was added and incubated on a shaking bed at 25 °C for 3 h. The samples were processed as described by Wu *et al*. [22], with slight modifications and observed at 50,000 × magnification via SEM (Thermo Scientific Apreo 2S; Energy Spectrum Ultra Dry Detector, USA).

2.4.2 Cell surface charge determination

The *P. kluyveri* Y-11519 cell surface charge changes were detected using a Zetasizer [23]. After culturing *P. kluyveri* Y-11519 to the logarithmic growth phase, the cells were washed with sterile physiological saline and adjusted to an OD_{600nm} = 2.3. Then, 5% of the bacterial suspension was added to a YPD medium containing different concentrations of D-limonene (0, 1/2 MIC, MIC, MFC). The final concentration of the bacterial suspension in the experimental system was 1×10^7 CFU/mL. The culture was incubated at 25 °C for 3 h, followed by centrifugation at 6000 rpm for 10 min using a cold centrifuge. The cell pellets were washed three times with sterile PBS buffer (0.01 mol/L, pH 7.2) and resuspended to an OD_{600nm} of 0.6 (fungal suspension concentration of 1×107 CFU/mL).

2.4.3 Cell membrane integrity

The effect of D-limonene on the cell membrane integrity of *P. kluyveri* Y-11519 by fluorescence inverted microscopy [16]. The *P. kluyveri* Y-11519 cell were diluted in YPD liquid medium and grown to logarithmic phase, then centrifuged at 6000 rpm for 10 min. The cells were collected, washed three times with PBS (0.01 mol/L pH 7.4), resuspended, diluted, and treated with different D-limonene concentrations (1/2MIC, MIC, and MFC) for 3 h at 25 °C. The staining solution was prepared using a method delineated by Alvarez-Manzo *et al*., with slight modifications [24]. Next, 10 μL of the freshly prepared staining solution was incubated with 10^6–10^7 cells for 25 min at 25 °C while protected from light. The blank control consisted

of cells not treated with D-limonene. Then a few drops on a sterile slide, images were generated using with Nikon Eclipse Ti (Nikon, Tokyo, Japan) with a 10× eyepiece and 20× objective lens (together, 200×).

2.4.4 Cell membrane permeability

The relative *P. kluyveri* Y-11519 cell membrane conductivity was measured to assess the effect of D-limonene on its permeability. The *P. kluyveri* Y-11519 was incubated at 25 °C for 16 h and centrifuged at 6000 rpm for 10 min to obtain sample cells. Referring to a method described by Fikry *et al*. [21], 5% glucose was used as an isotonic solution, to which D-limonene was added at 1/2MIC, MIC, and MFC concentrations and incubated at 25 °C for 0 h, 3 h, and 8 h, respectively. The conductivity was measured using a conductivity meter (DDS-11A, Shanghai Precision Scientific Instruments Co. Ltd., China) (labeled as L_1). The *P. kluyveri* Y-11519 was placed in the isotonic solution and boiled for 5 min, after which the final conductivity was measured and recorded as L_0. The conductivity of the isotonic solution mixtures of the different D-limonene concentrations was measured (marked as L_2). The blank control consisted of cells not treated with D-limonene. The relative conductivity was calculated as follows:

$$\textit{Relative conductivity}(\%) = 100\times[(L_2 - L_1)/L_0] \quad (1)$$

2.4.5 Cell membrane potential

A method delineated by Cai *et al*., [25] was used to analyze the cell membrane potential changes via DiBAC4(3) fluorescent dye. The *P. kluyveri* Y-11519 suspensions (1×10^7 CFU/mL concentrations) were incubated with different D-limonene concentrations (1/2MIC, MIC, and MFC) at 25 °C for 0, 3, and 8 h, respectively. The *P. kluyveri* was collected via centrifugation at 6000 rpm for 10 min, washed with PBS (0.01 mol/L pH 7.2), and resuspended (10^6–10^7 CFU/mL concentrations). Then, 180 μL of the *P. kluyveri* Y-11519 suspension and 20 μL of DiBAC4(3) (PBS buffer 0.01 mol/L pH 7.2 diluted to 10 ug/mL) were pipetted into a black 96-well plate and incubated for 10 min at room temperature. Samples without D-limonene treatment were used as blank controls. The fluorescence intensity (MFI) was measured using an automated zymograph (Tecan Infinite M1000 Pro; Tecan Trading, Mannedorf, Switzerland) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

2.4.6 Intracellular macromolecule leakage

Cell membrane integrity is vital for determining bacterial and fungal survival. A log-phase *P. kluyveri* Y-11519 (1×10^7 CFU/mL concentrations) fungal suspension was incubated with various D-limonene concentrations (1/2MIC, MIC, and MFC) at 25 °C for 0, 3, and 8 h, respectively, while shaking (130 rpm). Samples without D-limonene treatment were used as blank controls. The supernatants were collected via centrifugation at 6000 r/min for 10 min, while the nucleic acid and protein content of each supernatant were determined using automated zymography.

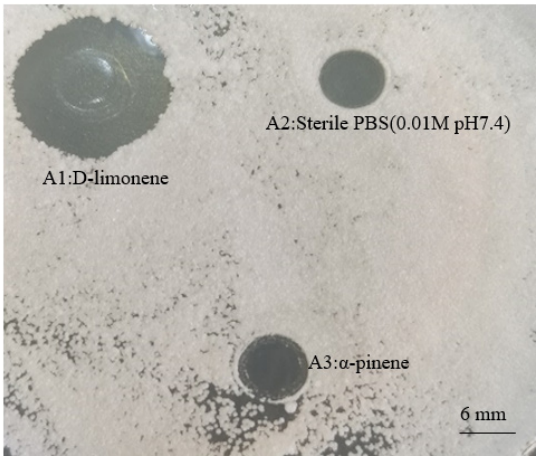
2.5 *Statistical analysis*

The experiment was repeated in triplicate for each sample, and data were expressed as the mean ± standard deviation. Analysis of variance was performed using GraphPad Prism 9 (GraphPad Software, USA), while Origin 2021 (Origin Software, USA) was used for plotting.

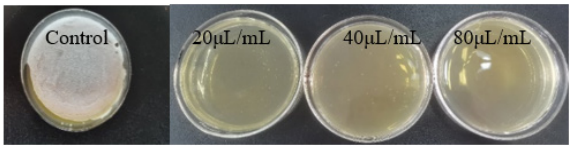
3 Results and Discussion

3.1 *The antifungal activity of D-limonene*

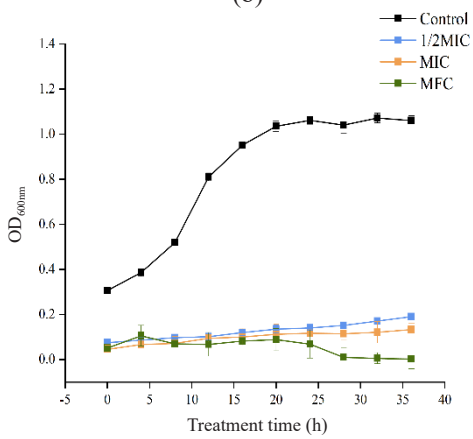
The minimum inhibitory concentration (MIC) of D-limonene against *P. kluyveri* Y-11519 was evaluated using the Oxford cup inhibition method. D-limonene at a concentration of 20 μL/mL significantly inhibited the proliferation of *P. kluyveri* Y-11519, exhibiting an inhibition zone with a diameter of 16.3 ± 0.4 mm. (Figure 1(A1)), showing significantly higher antibacterial activity than α-pinene solutions (Figure 1(A3)). The minimum fungicidal concentration (MFC) results are shown in Figure 1(b). Based on the MIC experiment, the minimum fungicidal concentration of D-limonene against *P. kluyveri* Y-11519 was determined by applying concentrations of 20, 40, and 80 μL/mL of D-limonene. When the concentration of D-limonene was 40 μL/mL, the *P. kluyveri* Y-11519 grew less than 15 colonies on YPD [26], thus confirming 40 μL/mL as the minimum



(a)



(b)



(c)

Figure 1: The D-limonene antibacterial activity against *P. kluyveri* Y-11519: (a) The Oxford cup inhibition circle results, (b) The MFC coating results, and (c) The bacteriostasis curve.

fungicidal concentration of D-limonene against *P. kluyveri* Y-11519. Fungistatic activity is typically evident at an MFC/MIC ratio ≥4, while an MFC/MIC ratio <4 signifies fungicidal activity [25]. The antifungal activity of the D-limonene *P. kluyveri* Y-11519 at 0-36 h is shown in Figure 1(C). The *P. kluyveri* Y-11519 not treated with D-limonene

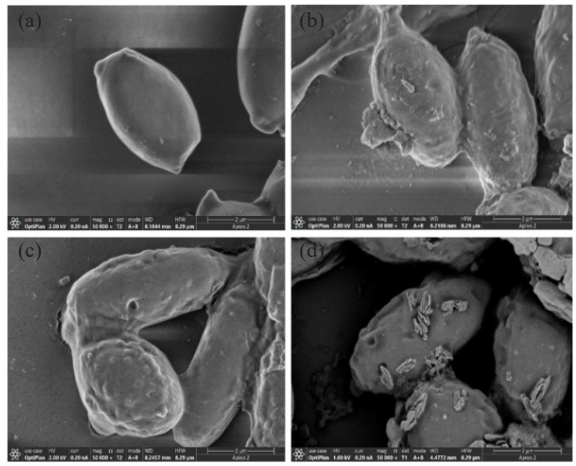


Figure 2: The scanning electron microphotographs of *P. kluyveri* Y-11519: (a) The control, (b) D-limonene treatment at the 1/2MIC level, (c) D-limonene treatment at the MIC level, and (d) D-limonene treatment at the MFC level.

showed a rapid fungal density increase in the negative sample. Contrarily, the fungal density of the susceptible *P. kluyveri* Y-11519 treated with D-limonene at 1/2MIC and MIC declined during the first 12 h of the test, decreasing by 87.4% from 0.810–0.102 OD_{600nm} (p-value < 0.05) and 88.76% from 0.810–0.094 OD_{600nm}, respectively. *P. kluyveri* Y-11519 growth resumed from 14–36 h, presumably due to biofilm formation with extended incubation time, higher stress, and the failure of low D-limonene concentrations to eliminate the *P. kluyveri* Y-11519 The fungal density decreased from 4 h after cultivation, declining by 95.92% to 0.0435 OD_{600nm} over the 36 h incubation period at MFC (p-value < 0.01). These results indicated that the inhibitory effect of different D-limonene concentrations on *P. kluyveri* Y-11519 varied and improved as the incubation time and dosage increased.

3.2 *The effect of D-limonene on cell morphology*

The *P. kluyveri* Y-11519 morphology was observed before and after D-limonene treatment via SEM to further determine its impact on the cell membrane barrier. The *P. kluyveri* Y-11519 morphological changes after D-limonene treatment for 3 h are shown in Figure 2. As shown in Figure 2(a), the untreated *P. kluyveri* cells were structurally intact with smooth,

elliptical surfaces. Contrarily, the *P. kluyveri* cells treated with 1/2MIC D-limonene (Figure 2(b)) displayed rough surfaces with indentations underneath, while significant deformation and pores were evident at the MIC dose (Figure 2(c)), showing contraction and surface adhesion. Furthermore, the D-limonene MFC (Figure 2(d)) severely disrupted the cell morphology, showing cell content leakage. Therefore, D-limonene may damage the cell membrane, leading to cellular component loss and *P. kluyveri* death. Several studies [27], [28] have demonstrated that natural essential oil components penetrate cell walls and disrupt cell membranes, leading to damage and cell permeability changes.

3.3 *The effect of D-limonene on the cell surface properties*

The cell surface charge is determined via the cell surface chemical composition [29]. Most microbial membranes contain phospholipids and proteins, displaying a negative surface charge in physiological conditions, which is mainly caused by various active groups, including -OH, -NH_2, and -COOH on polysaccharides, lipoproteins, and surface proteins [30]. Bruinsma *et al*., found that some bacteria could resist positively charged bactericides (quaternary amines) since they were influenced by their surface charge [29]. As shown in Figure 3, when the treated cells reached the MFC at 3 h, the D-limonene concentration showed a significant cell surface charge change compared to the control. The cell surface charge change became increasingly obvious after 8 h as the D-limonene concentration increased. These results indicated that surface charge of *P. kluyveri* Y-11519 was negative and increased with both incubation time and the dosage under different concentrations of D-limonene. This suggests that D-limonene enhances the potential for cell aggregation and clustering, thereby affecting the normal growth and reproduction of bacteria. The cell membrane surface charge is vital for cell recognition and adhesion, substance synthesis and breakdown, and information transport [31].

3.4 *The effect of D-limonene on cell membrane integrity*

The cell membrane is a barrier that prevents the

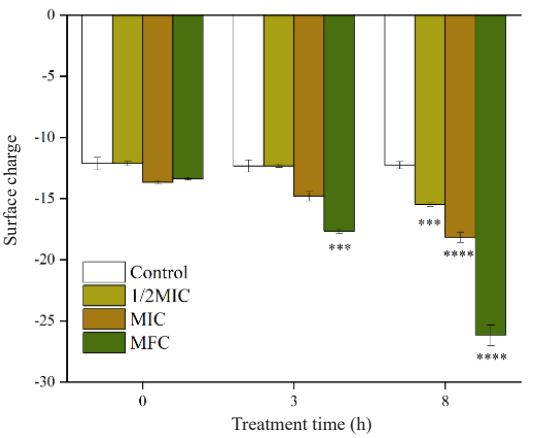


Figure 3: The effect of D-limonene on the *P. kluyveri* Y-11519 cell surface charge. The values represent the means of triplicate measurements. The bars denote the standard deviation ($n = 3$). *** indicates statistical significance at p-value < 0.001 compared with the control. **** indicates statistical significance at p-value < 0.0001 compared with the control.

free entry of extracellular substances into the cell and is essential for maintaining a stable intracellular environment. Cell membrane disruption increases membrane permeability, protoplasm extravasation, intracellular substance leakage, metabolic disorders, and other emergency responses, resulting in the loss of cellular physiological activities [32]. The green fluorescent dye SYB greenI and the red fluorescent dye PI can be used to evaluate cell membrane integrity. SYBR greenI penetrates the cell membrane and binds to double-stranded DNA, showing green to indicate living cells, while PI dye shows red when the cell membrane is damaged and becomes permeable, indicating dead cells [33]. As shown in Figure 4, the blank control sample was almost entirely stained green, indicating untreated cell survival and intact cell membranes (Figure 4(a)). Contrarily, at the same incubation time, more green fluorescence was evident with a small amount of red fluorescence in D-limonene-treated cells at 1/2MIC (Figure 4(b)), while the red fluorescence was more pronounced in the MIC samples (Figure 4(c)). Most of the treated *P. kluyveri* Y-11519 cells emitted yellow or red fluorescence in the MFC samples, with little green fluorescence (Figure 4(d)), suggesting that D-limonene disrupted the *P. kluyveri* Y-11519 cell membrane

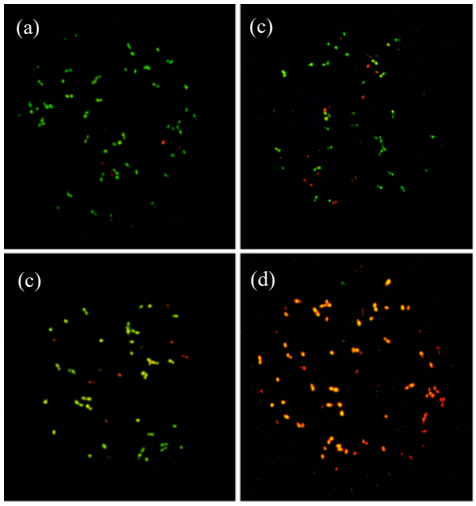


Figure 4: The effect of D-limonene on the *P. kluyveri* Y-11519 cell membrane integrity: (a) The control, (b) D-limonene treatment at the 1/2MIC level, (c) D-limonene treatment at the MIC level, and (d) D-limonene treatment at the MFC level.

integrity in a concentration-dependent manner. Limonene reportedly diffuses across fungal cell membranes due to its lipophilic nature, changing the membrane composition and increasing membrane fluidity, which degrades cell membrane integrity, ultimately causing cell death [34].

3.5 *The effect of D-limonene on cell membrane permeability*

K^+, Na^+, and H^+ ions move across the cell membrane to maintain cell metabolism. The reaction between the inhibitor and the cell membrane destabilizes the ions entering the cell [35]. As shown in Figure 5, the relative *P. kluyveri* Y-11519 cell conductance exceeded that of the control (p-value < 0.01) after 1/2MIC, MIC, and MFC D-limonene treatment, while the relative electrical conductivity values increased at higher D-limonene concentrations. In addition, the relative *P. kluyveri* conductivity increased as the incubation time was extended. Cui [36] found that treating Escherichia coli and Staphylococcus aureus with chrysanthemum essential oil increased the cell membrane permeability and electrolyte leakage, which was a primary cause of death. The fungal cell membrane conductivity measurements in this study

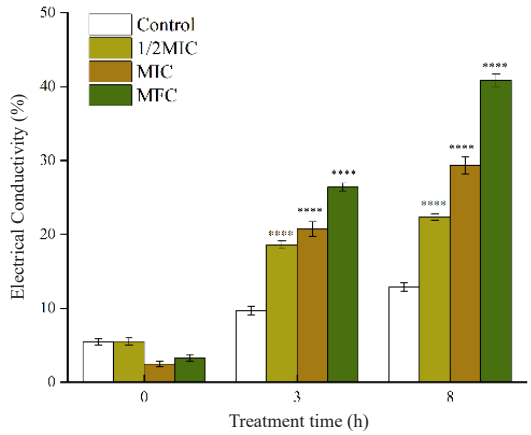


Figure 5: The effect of D-limonene on *P. kluyveri* Y-11519 cell membrane permeability. The values represent the means of triplicate measurements. The bars denote the standard deviation (n = 3). ** indicates the statistical significance at p-value < 0.01 compared with the control. *** indicates the statistical significance at p-value < 0.001 compared with the control. **** indicates the statistical significance at p-value < 0.0001 compared with the control.

showed that treating *P. kluyveri* Y-11519 with D-limonene significantly increased membrane electrolyte leakage, marking cell wall degradation, cytoplasmic material leakage, and cell collapse.

3.6 *The effect of D-limonene on the cell membrane potential*

The reaction between the inhibitor and cell membrane changes the cell membrane potential, while the degree of change responds to the degree of depolarization, which can be assessed by observing the intracellular MFI to reveal the mechanism behind cell membrane damage [37]. Figure 6 shows the variation in the *P. kluyveri* Y-11519 cell membrane potential. according to the diffusion results of DiBAC4(3), the cell membrane potential of *P. kluyveri* Y-11519 changed significantly when exposed to 1/2MIC, MIC and MFC levels of D-limonene for 8 h. The higher MFI indicated that the intra-membrane ATP channels were blocked, leading to cell membrane depolarization and permeabilization. Membrane potential changes interfered with physiological cell activities, such as adhesion, apoptosis, and migration [38]. These results suggested that

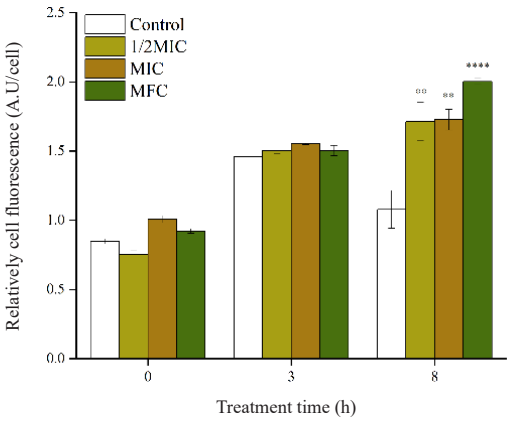


Figure 6: The effect of D-limonene on the *P. kluyveri* Y-11519 cell membrane potential. The values represent the means of triplicate measurements. The bars denote the standard deviation ($n = 3$). ** indicates the statistical significance at p-value < 0.01 compared with the control. **** indicates the statistical significance at p-value < 0.0001 compared with the control.

D-limonene changed the cell membrane potential, severely hindering normal physiological cell functions and leading to cell death, which was consistent with the SEM images.

3.7 *The effect of D-limonene on intracellular macromolecule leakage*

Cell membrane integrity is vital for normal microorganism growth and metabolism. Nucleic acids and proteins, which reside throughout the interior of the cell, and they leakage may lead to the death of the fungi [39]. Compared to the control group, the concentration of D-limonene at the MIC level (after 3 h of treatment) showed a significant increase in the leakage of nucleic acids (Figure 7(a)) and proteins (Figure 7(b)) in the extracellular broth, with a respective increase of 0.29-fold and 0.28-fold. The leakage of nucleic acids and proteins showed a positive correlation with the amount of D-limonene added and increased gradually with the duration of treatment. After 8 hours of treatment with gradient concentrations of D-limonene (1/2 MIC, MIC, and MFC), the measured OD260nm increased by 0.26-fold, 0.53-fold, and 0.48-fold, respectively, while the OD280nm values increased by 0.29-fold, 0.54-fold,

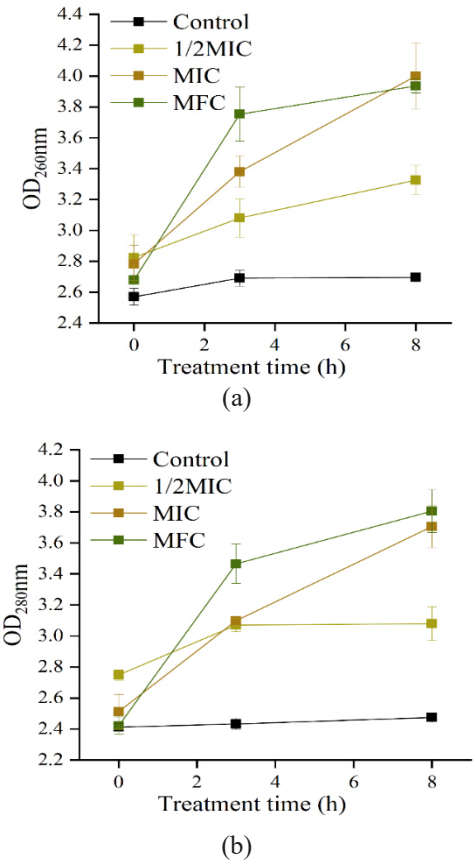


Figure 7: The effect of D-limonene on *P. kluyveri* Y-11519 intracellular macromolecule leakage. The values represent the means of triplicate measurements.

and 0.58-fold. Studies have shown that D-limonene damages cell membranes, leading to macromolecular loss and increasing cell membrane permeability. Zhang *et al*., revealed that plant preservatives caused intracellular macromolecule leakage via damaged cells [40].

4 Conclusions

The film formation is considered a sign of deterioration in the quality of fermented vegetables. It isolates the brine environment, alleviates the pH pressure, and provides favorable conditions for the growth of spoilage microorganisms, accelerating the tissue softening of Sichuan pickles. This poses potential food safety issues and severely hampers the industrial development of Sichuan pickles. Natural compounds offer a new

approach to resolving this problem. The study results clearly establish the anti-yeast nature of D-limonene. The anti-yeast activity is mainly due to cell wall damage, cell deformation, atrophy, and the cell permeability and integrity are disrupted, resulting in significant intracellular material leakage. The results provide a reliable theoretical and scientific foundation for adding D-limonene to Sichuan pickles to inhibit the film formation in the brine, while also offering valuable references for addressing similar film-forming phenomena in other fermented foods.

Author Contributions

C.Z.: conceptualization, investigation, methodology, writing an original draft; research design, data analysis; P.Y., A.T, S.K., T.L., W.K., T.P., S.A.,T.P., A.T., and V.P.: conceptualization, data curation, writing—reviewing and editing; J.T.: funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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